OIE

WORLD ORGANISATION FOR ANIMAL HEALTH

MANUAL OF DIAGNOSTIC TESTS AND VACCINES FOR TERRESTRIAL ANIMALS
(mammals, birds and bees)

Sixth Edition
Volume 1

2008

> This *Terrestrial Manual* has been edited by the
> OIE Biological Standards Commission and adopted by
> the International Committee of the OIE

Reference to commercial kits does not mean their endorsement by the OIE. All commercial kits should be validated; tests on the OIE register have already met this condition (the register can be consulted at: www.oie.int).

FOREWORD

The *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*) aims to facilitate international trade in animals and animal products and to contribute to the improvement of animal health services world-wide. The principal target readership is laboratories carrying out veterinary diagnostic tests and surveillance, plus vaccine manufacturers and regulatory authorities in Member Countries. The objective is to provide internationally agreed diagnostic laboratory methods and requirements for the production and control of vaccines and other biological products.

This ambitious task has required the cooperation of highly renowned animal health specialists from many countries. The OIE, the World Organisation for Animal Health, is clearly the most appropriate organisation to undertake this task on a global level. The main activities of the organisation, which was established in 1924 and in 2008 comprised 172 Member Countries and Territories, are as follows:

1. To ensure transparency in the global animal disease and zoonosis situation.

2. To collect, analyse and disseminate scientific veterinary information on animal disease control methods.

3. To provide expertise and encourage international solidarity in the control of animal diseases.

4. Within its mandate under the WTO (World Trade Organization) Agreement on Sanitary and Phytosanitary Measures (SPS Agreement), to safeguard world trade by publishing health standards for international trade in animals and animal products.

5. To improve the legal framework and resources of national Veterinary Services.

6. To provide a better guarantee of the safety of food of animal origin and to promote animal welfare through a science-based approach.

The *Terrestrial Manual*, covering infectious and parasitic diseases of mammals, birds and bees, was first published in 1989. Each successive edition has extended and updated the information provided. This sixth edition includes new chapters on Guidelines for international standards for vaccine banks, Turkey rhinopneumonitis, Small hive beetle infestation (*Aethina tumida*) and camelpox, and *Mycoplasma synoviae* has been added to the chapter on Avian mycoplasmosis (previously the chapter focused on *Mycoplasma gallisepticum*. As a companion volume to the *Terrestrial Animal Health Code*, the *Terrestrial Manual* sets laboratory standards for all OIE listed diseases as well as several other diseases of global importance. In particular it specifies (in blue font) those "Prescribed Tests" that are recommended for use in health screening for international trade or movement of animals. The *Terrestrial Manual* has become widely adopted as a key reference book for veterinary laboratories around the world. Aquatic animal diseases are included in a separate *Aquatic Manual*.

The task of commissioning chapters and compiling the *Terrestrial Manual* was assigned to the OIE Biological Standards Commission by the International Committee of the OIE (General Assembly of national Delegates of Member Countries and Territories). Manuscripts were requested from specialists in each of the diseases or the other topics covered. After initial scrutiny by the Consultant Technical Editor, the chapters were sent to scientific reviewers and to experts at OIE Reference Laboratories. They were also circulated to all OIE Member Countries for review and comment. The Biological Standards Commission and the Consultant Technical Editor took all the resulting comments into consideration, often referring back to the authors for further help, before finalising the chapters. The final text has the approval of the International Committee of the OIE.

A procedure for the official recognition of commercialised diagnostic tests, under the authority of the International Committee, was finalised in September 2004. Data are submitted using a validation template that was developed by the Biological Standards Commission. Submissions are evaluated by appointed experts, who advise the Biological Standards Commission before the final opinion of the OIE International Committee is sought. All information on the submission of applications can be found on the OIE Web site.

The *Terrestrial Manual* continues to expand and to extend its range of topics covered. It is our sincere hope that it will grow in usefulness to veterinary diagnosticians and vaccine manufacturers in all the OIE Member Countries. A new paper edition of the *Terrestrial Manual* is published every 4 years. It is important to note that annual updates to the *Terrestrial Manual* will be published on the OIE website once approved by the International Committee, so readers are advised to check there for the latest information. The *Terrestrial Manual* is published in English, French and Spanish.

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ACKNOWLEDGEMENTS

CONTENTS

VOLUME 1

PART 1   **GENERAL INFORMATION**

PART 2   **OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO
              INTERNATIONAL TRADE**

INTRODUCTION
(How to use this *Terrestrial Manual*)

- ### Arrangement of the *Terrestrial Manual*

Part 1, the beginning of this *Terrestrial Manual,* contains eleven introductory chapters that deal with a variety of general subjects of interest to veterinary laboratory diagnosticians. These chapters are intended to give a brief introduction to their subjects. They are to be regarded as background information rather than standards.

The main part of the *Terrestrial Manual* (Part 2) covers standards for diagnostic tests and vaccines for the diseases listed in the OIE *Terrestrial Animal Health Code.* The diseases are in alphabetical order, subdivided by animal host species. OIE listed diseases are transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders. They have particularly serious socio-economic or public health consequences and are of major importance in the international trade of animals and animal products.

Four of the diseases in Section 2.9 are included in some individual species sections, but these chapters cover several species and thus give a broader description. Some additional diseases that may also be of importance to trade but that do not have a chapter in the *Terrestrial Code* are also included in Section 2.9.

The contributors of all the chapters are listed on pages xxii–xxxv, but the final responsibility for the content of the *Terrestrial Manual* lies with the International Committee of the OIE.

There is an alphabetical index of the diseases at the end of Volume 2.

- ### Format of chapters

Each disease chapter includes a summary intended to provide information for veterinary officials and other readers who need a general overview of the tests and vaccines available for the disease. This is followed by a text giving greater detail for laboratory workers. In each disease chapter, Part A gives a general introduction to the disease, Part B deals with laboratory diagnosis of the disease, and Part C (where appropriate) with the requirements for vaccines or *in vivo* diagnostic biologicals. The information concerning production and control of vaccines or diagnostics is given as an example; it is not always necessary to follow these when there are scientifically justifiable reasons for using alternative approaches. Bibliographic references that provide further information are listed at the end of each chapter.

- ### Explanation of the tests described and of the table on pages xi–xiv

The table on pages xi–xiv lists diagnostic tests in two categories: 'prescribed' and 'alternative'. Prescribed tests are those that are required by the *Terrestrial Animal Health Code* for the testing of animals before they are moved internationally. In the *Terrestrial Manual* these tests are printed in blue. At present it is not possible to have prescribed tests for every listed disease. 'Alternative tests' are those that are suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement. There are often other tests described in the chapters, which may also be of some practical value in local situations or which may still be under development.

- **List of OIE Reference Laboratories**

A list of OIE Reference Laboratories is given in Part 3 of this *Terrestrial Manual*. These laboratories have been designated by the OIE as centres of excellence with expertise in their particular field. They are able to provide advice to other laboratories on methodology. In some cases standard strains of micro-organisms or reference reagents (e.g. antisera, antigens) can also be obtained from the reference laboratories.

The list of OIE Reference Laboratories will be updated by the International Committee of the OIE each year. The revised list is available on the OIE Web site.

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LIST OF TESTS FOR INTERNATIONAL TRADE

The table below lists diagnostic tests in two categories: 'prescribed' and 'alternative'. Prescribed tests are required by the OIE *Terrestrial Animal Health Code* for the international movement of animals and animal products and are considered optimal for determining the health status of animals. In the *Terrestrial Manual* these tests are printed in blue. At present it is not possible to have prescribed tests for every listed disease. Alternative tests are those that are suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement. There are often other tests described in the chapters that may also be of some practical value in local situations or that may still be under development.

Chapter No.	Disease name	Prescribed tests	Alternative tests
2.1.1.	Anthrax	–	–
2.1.2.	Aujeszky's disease	ELISA, VN	–
2.1.3.	Bluetongue	Agent id., AGID, ELISA, PCR	VN
2.1.4.	Echinococcosis/Hydatidosis	–	–
2.1.5.	Foot and mouth disease	ELISA*, VN	CF
2.1.6.	Heartwater	–	ELISA, IFA
2.1.7.	Japanese encephalitis	–	–
2.1.8.	Leishmaniosis	–	Agent id.
2.1.9.	Leptospirosis	–	MAT
2.1.10.	New World screwworm (*Cochliomyia hominivorax*) and Old World screwworm (*Chrysomya bezziana*)	–	Agent id.
2.1.11.	Paratuberculosis (Johne's disease)	–	DTH, ELISA
2.1.12.	Q fever	–	CF
2.1.13.	Rabies	ELISA, VN	–
2.1.14.	Rift Valley fever	VN	ELISA, HI
2.1.15.	Rinderpest	ELISA	VN
2.1.16.	Trichinellosis	Agent id.	ELISA
2.1.17.	*Trypanosoma evansi* infections (including surra)	–	–
2.1.18.	Tularemia	–	Agent id.
2.1.19.	Vesicular stomatitis	CF, ELISA, VN	–
2.1.20.	West Nile fever	–	–
2.2.1.	Acarapisosis of honey bees	–	–
2.2.2.	American foulbrood of honey bees	–	–

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*   Please refer to *Terrestrial Manual* chapters to verify which method is prescribed.

Chapter No.	Disease name	Prescribed tests	Alternative tests
2.2.3.	European foulbrood of honey bees	–	–
2.2.4.	Nosemosis of bees	–	–
2.2.5.	Small hive beetle infestation (*Aethina tumida*)	–	–
2.2.6.	*Tropilaelaps* infestation of honey bees (*Tropilaelaps* spp.)	–	–
2.2.7	Varroosis of honey bees	–	–
2.3.1.	Avian chlamydiosis	–	–
2.3.2.	Avian infectious bronchitis	–	ELISA, HI, VN
2.3.3.	Avian infectious laryngotracheitis	–	AGID, ELISA, VN
2.3.4.	Avian influenza	Virus isolation with pathogenicity testing	AGID, HI
2.3.5.	Avian mycoplasmosis (*Mycoplasma gallisepticum, M. synoviae*)	–	Agg., HI
2.3.6.	Avian tuberculosis	–	Agent id., Tuberculin test
2.3.7.	Duck virus enteritis	–	–
2.3.8.	Duck virus hepatitis	–	–
2.3.9.	Fowl cholera	–	–
2.3.10.	Fowl pox	–	–
2.3.11.	Fowl typhoid and Pullorum disease	–	Agent id., Agg.
2.3.12.	Infectious bursal disease (Gumboro disease)	–	AGID, ELISA
2.3.13.	Marek's disease	–	AGID
2.3.14.	Newcastle disease	–	HI
2.3.15.	Turkey rhinotracheitis (avian metapneumovirus)	–	–
2.4.1.	Bovine anaplasmosis	–	CAT, CF
2.4.2.	Bovine babesiosis	–	CF, ELISA, IFA
2.4.3.	Bovine brucellosis	BBAT, CF, ELISA, FPA	–
2.4.4.	Bovine cysticercosis	–	Agent id.
2.4.5.	Bovine genital campylobacteriosis	Agent id.	–
2.4.6.	Bovine spongiform encephalopathy	–	–
2.4.7.	Bovine tuberculosis	Tuberculin test	Gamma interferon test
2.4.8.	Bovine viral diarrhoea	Agent id.	–
2.4.9.	Contagious bovine pleuropneumonia	CF, ELISA	–
2.4.10.	Dermatophilosis	–	–
2.4.11.	Enzootic bovine leukosis	AGID, ELISA	PCR
2.4.12.	Haemorrhagic septicaemia	–	Agent id.
2.4.13.	Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis	Agent id. (semen only), ELISA, PCR, VN	–
2.4.14.	Lumpy skin disease	–	VN

Chapter No.	Disease name	Prescribed tests	Alternative tests
2.4.15.	Malignant catarrhal fever	–	IFA, PCR, VN
2.4.16.	Theileriosis	Agent id., IFA	–
2.4.17.	Trichomonosis	Agent id.	Mucus agg.
2.4.18.	Trypanosomosis (Tsetse-transmitted)	–	IFA
2.5.1.	African horse sickness	CF, ELISA	Agent id. (real-time PCR), VN
2.5.2.	Contagious equine metritis	Agent id.	–
2.5.3.	Dourine	CF	ELISA, IFA
2.5.4.	Epizootic lymphangitis	–	–
2.5.5.	Equine encephalomyelitis (Eastern and Western)	–	CF, HI, PRN
2.5.6.	Equine infectious anaemia	AGID	ELISA
2.5.7.	Equine influenza	–	HI
2.5.8.	Equine piroplasmosis	ELISA, IFA	CF
2.5.9.	Equine rhinopneumonitis	–	VN
2.5.10.	Equine viral arteritis	Agent id. (semen only), VN	–
2.5.11.	Glanders	CF, Mallein test	–
2.5.12.	Horse mange	–	Agent id.
2.5.13.	Horse pox	–	–
2.5.14.	Venezuelan equine encephalomyelitis	–	CF, HI, PRN
2.6.1.	Myxomatosis	–	AGID, CF, IFA
2.6.2.	Rabbit haemorrhagic disease	–	HI
2.7.1.	Border disease	Agent id.	–
2.7.2.	Caprine and ovine brucellosis (excluding *Brucella ovis*)	BBAT, CF	Brucellin test, FPA
2.7.3/4.	Caprine arthritis/encephalitis & Maedi-visna	AGID, ELISA	–
2.7.5.	Contagious agalactia	–	–
2.7.6.	Contagious caprine pleuropneumonia	CF	–
2.7.7.	Enzootic abortion of ewes (ovine chlamydiosis)	–	CF
2.7.8.	Nairobi sheep disease	–	–
2.7.9.	Ovine epididymitis (*Brucella ovis*)	CF	ELISA
2.7.10.	Ovine pulmonary adenocarcinoma (adenomatosis)	–	–
2.7.11.	Peste des petits ruminants	VN	ELISA
2.7.12.	Salmonellosis (*S.* abortusovis)	–	–
2.7.13.	Scrapie	–	–
2.7.14.	Sheep pox and goat pox	–	VN
2.8.1.	African swine fever	ELISA	IFA
2.8.2.	Atrophic rhinitis of swine	–	–
2.8.3.	Classical swine fever (hog cholera)	ELISA, FAVN, NPLA	–

Chapter No.	Disease name	Prescribed tests	Alternative tests
2.8.4.	Nipah virus encephalitis	–	–
2.8.5.	Porcine brucellosis	ELISA	BBAT, FPA
2.8.6.	Porcine cysticercosis	–	–
2.8.7.	Porcine reproductive and respiratory syndrome	–	ELISA, IFA, IPMA
2.8.8.	Swine influenza	–	–
2.8.9.	Swine vesicular disease	VN	ELISA
2.8.10.	Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or Teschen/Talfan disease)	–	VN
2.8.11.	Transmissible gastroenteritis	–	VN, ELISA
2.9.1.	Bunyaviral diseases of animals (excluding Rift Valley fever)	–	–
2.9.2.	Camelpox	–	–
2.9.3.	*Campylobacter jejuni* and *C. coli*	–	–
2.9.4.	Cryptosporidiosis	–	–
2.9.5.	Cysticercosis	–	Agent id.
2.9.6.	Hendra and Nipah virus diseases	–	–
2.9.8.	*Listeria monocytogenes*	–	–
2.9.8.	Mange	–	Agent id.
2.9.9.	Salmonellosis	–	Agent id.
2.9.10.	Toxoplasmosis	–	–
2.9.11.	Verocytotoxigenic *Escherichia coli*	–	–
2.9.12.	Zoonoses transmissible from non-human primates	–	–

Note: The tests prescribed by the *Terrestrial Animal Health Code* for the purposes of international trade are printed in blue in this *Terrestrial Manual*.

Abbreviations

Agent id.	Agent identification	HI	Haemagglutination inhibition
Agg.	Agglutination test	IFA	Indirect fluorescent antibody
AGID	Agar gel immunodiffusion	IPMA	Immunoperoxidase monolayer assay
BBAT	Buffered *Brucella* antigen test	MAT	Microscopic agglutination test
CAT	Card agglutination test	NPLA	Neutralising peroxidase-linked assay
CF	Complement fixation	PCR	Polymerase chain reaction
DTH	Delayed-type hypersensitivity	PRN	Plaque reduction neutralisation
ELISA	Enzyme-linked immunosorbent assay	VN	Virus neutralisation
FAVN	Fluorescent antibody virus neutralisation	–	No test designated yet
FPA	Fluorescence polarisation assay		

COMMON ABBREVIATIONS USED
IN THIS *TERRESTRIAL MANUAL*

ABTS	2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulphonic acid	FBS	Fetal bovine serum
AGID	Agar gel immunodiffusion	FITC	Fluorescein isothiocyanate
ATCC[1]	American type culture collection	FLK	Fetal lamb kidney (cells)
BBAT	Buffered *Brucella* antigen test	FPA	Fluorescence polarisation assay
BFK	Bovine fetal kidney (cells)	*G*	Relative centrifugal force
BGPS	Beef extract-glucose-peptone-serum (medium)	GIT	Growth inhibition test
BHK	Baby hamster kidney (cell line)	HA	Haemagglutination
BLP	Buffered lactose peptone	HAD	Haemadsorption
BPAT	Buffered plate antigen test	HBSS	Hanks' balanced salt solution
BSA	Bovine serum albumin	HEP	High-egg-passage (virus)
BSF	Bovine serum factors	HEPES	N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid (buffer)
CAM	Chorioallantoic membrane	HI	Haemagglutination inhibition
CEF	Chicken embryo fibroblast	HRPO	Horseradish peroxidase
CF	Complement fixation (test)	IB	Immunoblot test
CFU	Colony-forming unit	ICFTU	International complement fixation test unit
CIEP	Counter immunoelectrophoresis	ICPI	Intracerebral pathogenicity index
CK	Calf kidney (cells)	ID_{50}	Median infectious dose
CNS	Central nervous system	IFA	Indirect fluorescent antibody (test)
CPE	Cytopathic effect	IHA	Indirect haemagglutination
CPLM	Cysteine-peptone-liver infusion maltose (medium)	IPMA	Immunoperoxidase monolayer assay
CSY	Casein-sucrose-yeast (agar)	IU	International units
DEPC	Diethyl pyrocarbonate	IVPI	Intravenous pathogenicity index
DEAE	Diethylaminoethyl	LA	Latex agglutination
DEPC	Diethylpyrocarbonate	LD	Lethal dose
DMEM	Dulbecco's modified Eagle's medium	LEP	Low egg passage (virus)
DMSO	Dimethyl sulphide	LPS	Lipopolysaccharide
DTH	Delayed-type hypersensitivity	MAb	Monoclonal antibody
EDTA	Ethylene diamine tetra-acetic acid	MAT	Microscopic agglutination test
EGTA	Ethylene glycol tetra-acetic acid	MCS	Master cell stock
EID	Egg-infective dose	MDBK	Madin-Darby bovine kidney (cell line)
ELISA	Enzyme-linked immunosorbent assay	MDT	Mean death time
EMTM	Evans' modified Tobie's medium	MEM	Minimal essential medium
EYL	Earle's yeast lactalbumin (balanced salt solution)	MHC	Major histocompatibility complex
FAT	Fluorescent antibody test	MLV	Modified live virus (vaccine)
FAVN	Fluorescent antibody virus neutralisation	m.o.i.	multiplicity of infection

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[1]   American Type Culture Collection, P.O. Box 1549, Manassas, Virginia 20108, United States of America.

MSV	Master seed virus		RBC	Red blood cell
NI	Neutralisation index		RFLP	Restriction fragment length polymorphism
OGP	1-octyl-beta-D-glucopyranoside (buffer)		RK	Rabbit kidney
OPD	Orthophenyldiamine (chromogen)		RPM	Revolutions per minute
OPG	Oxalase-phenol-glycerin (preservative solution)		RSA	Rapid serum agglutination
ORF	Open reading frame		RT-PCR	Reverse-transcription polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis		SAT	Serum agglutination test
PAP	Peroxidase–antiperoxidase (staining procedure)		SDS	Sodium dodecyl sulphate
PAS	Periodic acid-Schiff (reaction)		SPF	Specific pathogen free
PBS	Phosphate buffered saline		SPG	Sucrose phosphate glutamic acid
PCR	Polymerase chain reaction		SRBC	Sheep red blood cells
PD	Protective dose		TCID$_{50}$	Median tissue culture infective dose
PFGE	Pulsed field gel electrophoresis		TMB	Tetramethyl benzidine
PFU	Plaque-forming unit		TSI	Triple sugar iron (medium)
PHA	Passive haemagglutination (test)		VB	Veronal buffer
PPD	Purified protein derivative		VBS	Veronal buffered saline
PPLO	Pleuropneumonia-like organisms		Vero	African green monkey kidney (cells)
PRN	Plaque reduction neutralisation		VN	Virus neutralisation
PSG	Phosphate buffered saline glucose			

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GLOSSARY OF TERMS

The definitions given below have been selected and restricted to those that are likely to be useful to users of this OIE Terrestrial Manual.

- **Absorbance/optical density**

Absorbance and optical density are terms used to indicate the strength of reaction. A spectrophotometer is used to measure the amount of light of a specific wave length that a sample absorbs and the absorbance is proportional to the amount of a particular analyte present.

- **Accuracy**

Nearness of a test value to the expected value for a reference standard reagent of known activity or titre.

- **Assay**

Synonymous with test or test method, e.g. enzyme immunoassay, complement fixation test or polymerase chain reaction tests.

- **Batch**

All vaccine or other reagent, such as antigen or antisera, derived from the same homogeneous bulk and identified by a unique code number.

- **Cell line**

A stably transformed line of cells that has a high capacity for multiplication *in vitro*.

- **Centrifugation**

Throughout the text, the rate of centrifugation has been expressed as the Relative Centrifugal Force, denoted by '***g***'. The formula is:

$$\frac{(RPM \times 0.10472)^2}{980} \times \text{Radius (cm)} = \textit{\textbf{g}}$$

where RPM is the rotor speed in revolutions per minute, and where Radius (cm) is the radius of the rotor arm, to the bottom of the tube, in centimetres.

It may be necessary to calculate the RPM required to achieve a given value of ***g***, with a particular rotor. The formula is:

$$RPM = \frac{\sqrt{g \times 980} \,/\text{Radius (cm)}}{0.10472}$$

- **Cross-reaction**

See 'False-positive reaction'.

- **Cut-off/threshold**

Test result value selected for distinguishing between negative and positive results; may include indeterminate or suspicious zone.

- **Dilutions**

Where dilutions are given for making up liquid reagents, they are expressed as, for example, 1 in 4 or 1/4, meaning one part added to three parts, i.e. a 25% solution of A in B.

- v/v – This is volume to volume (two liquids).

- w/v – This is weight to volume (solid added to a liquid).

- **Dilutions used in virus neutralisation tests**

There are two different conventions used in expressing the dilution used in virus neutralisation (VN) tests. In Europe, it is customary to express the dilution before the addition of the antigen, but in the United States of America and elsewhere, it is usual to express dilutions after the addition of antigen.

These alternative conventions are expressed in the *Terrestrial Manual* as 'initial dilution' or 'final dilution', respectively.

- **Efficacy**

Specific ability of the biological product to produce the result for which it is offered when used under the conditions recommended by the manufacturer.

- **Equivalency testing**

Determination of certain assay performance characteristics of new and/or different test methods by means of an interlaboratory comparison to a standard test method; implied in this definition is that participating laboratories are using their own test methods, reagents and controls and that results are expressed qualitatively.

- **False-negative reaction**

Negative reactivity in an assay of a test sample obtained from an animal exposed to or infected with the organism in question, may be due to lack of analytical sensitivity, restricted analytical specificity or analyte degradation, decreases diagnostic sensitivity.

- **False-positive reaction**

Positive reactivity in an assay that is not attributable to exposure to or infection with the organism in question, maybe due to immunological cross-reactivity, cross-contamination of the test sample or non-specific reactions, decreases diagnostic specificity.

- **Final product (lot)**

All sealed final containers that have been filled from the same homogenous batch of vaccine in one working session, freeze-dried together in one continuous operation (if applicable), sealed in one working session, and identified by a unique code number.

- **Harmonisation**

The result of an agreement between laboratories to calibrate similar test methods, adjust diagnostic thresholds and express test data in such a manner as to allow uniform interpretation of results between laboratories.

- **Incidence**

Estimate of the rate of new infections in a susceptible population over a defined period of time; not to be confused with prevalence.

- **In-house checks**

All quality assurance activities within a laboratory directly related to the monitoring, validation, and maintenance of assay performance and technical proficiency.

- **In-process control**

Test procedures carried out during manufacture of a biological product to ensure that the product will comply with the agreed quality standards.

- **Inter-laboratory comparison (ring test)**

Any evaluation of assay performance and/or laboratory competence in the testing of defined samples by two or more laboratories; one laboratory may act as the reference in defining test sample attributes.

- **Master cell (line, seed, stock)**

Collection of aliquots of cells of defined passage level, for use in the preparation or testing of a biological product, distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

- **Master seed (agent, strain)**

Collection of aliquots of an organism at a specific passage level, from which all other seed passages are derived, which are obtained from a single bulk, distributed into containers in a single operation and processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

- **Performance characteristic**

An attribute of a test method that may include analytical sensitivity and specificity, accuracy and precision, diagnostic sensitivity and specificity and/or repeatability and reproducibility.

- **Potency**

Relative strength of a biological product as determined by appropriate test methods. (Initially the potency is measured using an efficacy test in animals. Later this may be correlated with tests of antigen content, or antibody response, for routine batch potency tests.)

- **Precision**

The degree of dispersion of results for a repeatedly tested sample expressed by statistical methods such as standard deviation or confidence limits.

- **Predictive value (negative)**

The probability that an animal is free from exposure or infection given that it tests negative; predictive values are a function of the DSe (diagnostic sensitivity) and DSp (diagnostic specificity) of the diagnostic assay and the prevalence of infection.

- **Predictive value (positive)**

The probability that an animal has been exposed or infected given that it tests positive; predictive values are a function of the DSe and DSp of the diagnostic assay and the prevalence of infection.

- **Prevalence**

Estimate of the proportion of infected animals in a population at one given point in time; not to be confused with incidence.

- **Primary cells**

A pool of original cells derived from normal tissue up to and including the tenth subculture.

- **Production seed**

An organism at a specified passage level that is used without further propagation for initiating preparation of a production bulk.

- **Proficiency testing**

One measure of laboratory competence derived by means of an interlaboratory comparison; implied in this definition is that participating laboratories are using the same test methods, reagents and controls and that results are expressed qualitatively.

- **Purity**

Quality of a biological product prepared to a final form and:

a) Relatively free from any extraneous microorganisms and extraneous material (organic or inorganic) as determined by test methods appropriate to the product; and

b) Free from extraneous microorganisms or material which could adversely affect the safety, potency or efficacy of the product.

- **Reference animal**

Any animal for which the infection status can be defined in unequivocal terms; may include diseased, infected, vaccinated, immunised or naïve animals.

- **Reference Laboratory**

Laboratory of recognised scientific and diagnostic expertise for a particular animal disease and/or testing methodology; includes capability for characterising and assigning values to reference reagents and samples.

- **Repeatability**

Level of agreement between replicates of a sample both within and between runs of the same test method in a given laboratory.

- **Reproducibility**

Ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories.

- **Room temperature**

The term 'room temperature' is intended to imply the temperature of a comfortable working environment. Precise limits for this cannot be set, but guiding figures are 18–25°C. Where a test specifies room temperature, this should be achieved, with air conditioning if necessary; otherwise the test parameters may be affected.

- **Safety**

Freedom from properties causing undue local or systemic reactions when used as recommended or suggested by the manufacturer and without known hazard to in-contact animals, humans and the environment.

- **Sample**

Material that is derived from a specimen and used for testing purposes.

- **Sensitivity (analytical)**

Synonymous with 'Limit of Detection', smallest detectable amount of analyte that can be measured with a defined certainty; analyte may include antibodies, antigens, nucleic acids or live organisms.

- **Sensitivity (diagnostic)**

Proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results.

- **Sensitivity (relative)**

Proportion of reference animals defined as positive by one or a combination of test methods that also test positive in the assay being compared.

- **Specific pathogen free (SPF)**

Animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also refers to eggs derived from SPF birds.

- **Specificity (analytical)**

Degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false-positives.

- **Specificity (diagnostic)**

Proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.

- **Specificity (relative)**

Proportion of reference animals defined as negative by one or a combination of test methods that also test negative in the assay being compared.

- **Specimen**

Material submitted for testing.

- **Standard Reagents**

- **International Standard Reagents**

  Standard reagents by which all other reagents and assays are calibrated; prepared and distributed by an International Reference Laboratory.

- **National Standard Reagents**

  Standard reagents calibrated by comparison with International Standard Reagents; prepared and distributed by a National Reference Laboratory.

- **Working Standards (reagents)**

  Standard reagents calibrated by comparison with the National Standard Reagent, or, in the absence of a National Standard Reagent, calibrated against a well-characterised in-house standard reagent; included in routine diagnostic tests as a control and/or for normalisation of test results.

- **Sterility**

Freedom from viable contaminating microorganisms, as demonstrated by approved and appropriate tests.

- **Test method**

Specified technical procedure for detection of an analyte (synonymous with assay).

- **Tests**

- **Prescribed**

  Test methods that are required by the OIE *Terrestrial Animal Health Code* for the international movement of animals and animal products and that are considered optimal for determining the health status of animals.

- **Alternative**

  Test methods considered in this *Terrestrial Manual* to be suitable for the diagnosis of disease in a local situation, and that can also be used for import/ export by bilateral agreement.

- **Screening**

  Tests of high diagnostic sensitivity suitable for large-scale application.

- **Confirmatory**

  Test methods of high diagnostic specificity that are used to confirm results, usually positive results, derived from other test methods

- **Working seed**

Organism at a passage level between master seed and production seed.

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2.8.3. Classical swine fever (*hog cholera*)	**Dr T. Drew** VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.

2.8.4. Nipah virus encephalitis (see chapter 2.9.6)

Dr P. Daniels
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2.8.5. Porcine brucellosis

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2.8.6. Porcine cysticercosis (see chapter 2.9.5)

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2.8.7. Porcine reproductive and respiratory syndrome

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2.8.8. Swine influenza

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2.8.9. Swine vesicular disease

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Dr D. Paton
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Dr A.I. Donaldson,
290 London Road, Burpham, Guildford, Surrey GU4 7LB, UK.

2.8.10. Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or Teschen/Talfan disease)

Mr N. Knowles
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2.8.11. Transmissible gastroenteritis	**Dr L.J. Saif** The Ohio State Universtiy, Ohio Agricultural Research and Development Center, Food Animal Health Research Program, 1680 Madison Avenue, Wooster, Ohio 44691-4096, USA.
2.9.1. Bunyaviral diseases of animals (excluding Rift Valley fever)	**Dr G.H. Gerdes** Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa.
2.9.2. Camelpox	**Dr H. Elliott** International Animal Health Division , DEFRA, 1A Page Street, London SW1P 4PQ, UK. **Dr E. Tuppurainen** Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Surrey GU24 0NF, UK.
2.9.3. Campylobacter jejuni *and* C. coli	**Prof. J.A. Wagenaar** Utrecht University, Faculty of Veterinary Medicine, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands. **Dr W.F. Jacobs-Reitsma** RIKILT Institute of Food Safety, Wageningen-UR P.O. Box 230, 6700 AE Wageningen, The Netherlands.
2.9.4. Cryptosporidiosis	**Prof. H. Smith** Scottish Parasite Diagnostic Laboratory, Stobhill General Hospital, Glasgow G21 3UW, UK.
2.9.5. Cysticercosis	**Dr S. Lloyd** Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK.
2.9.6. Hendra and Nipah virus diseases	**Dr P. Daniels** Australian Animal Health Laboratory, CSIRO Livestock Industries, 5 Portarlington Road, Geelong, Victoria 3220, Australia. **Dr M. Narasiman** Veterinary Research Institute, 59, Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia.
2.9.7. Listeria monocytogenes	**Dr J. Lopez** Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, 1015 Arlington Street, Winnipeg, Manitoba R3E 3M4, Canada.
2.9.8. Mange	**Dr J.L. Schlater & Dr J.W. Mertins** Parasitology and Clinical Pathology Section, Pathobiology Laboratory, National Veterinary Services Laboratories, USDA, APHIS, VS, P.O. Box 844, Ames, Iowa 50010, USA.
2.9.9. Salmonellosis	**Dr R. Davies** VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.

2.9.10. Toxoplasmosis	**Dr D. Buxton & Dr S.W. Maley** Moredun Research Institute, Pentlands Science Park, Bush Loan, by Edinburgh EH26 0PZ, Scotland, UK.
2.9.11. Verocytotoxigenic Escherichia coli	**Dr F.A. Clifton-Hadley** VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.
2.9.12. Zoonoses transmissible from non-human primates	**FELASA Working Group on Non-Human Primate Health: H. Weber (Convenor), E. Berge, J. Finch, P. Heidt, F.-J. Kaup, G. Perretta, B. .Verschuere & S. Wolfensohn** FELASA, BCM Box 2989, London WC1N 3XX, UK.

PART 1

GENERAL INFORMATION

INTRODUCTORY CHAPTERS

CHAPTER 1.1.1.

COLLECTION AND SHIPMENT
OF DIAGNOSTIC SPECIMENS

INTRODUCTION

The starting point for the laboratory investigation of an animal disease is the taking of samples. This first introductory chapter considers some of the general principles involved in sample collection, submission and storage. Each of the disease chapters of this Terrestrial Manual *provides specific information on sample collection for that particular disease. Samples may be taken from animals or the environment for a variety of purposes, such as disease diagnosis, disease surveillance, health certification or monitoring the response to treatment or vaccination. The samples collected should be appropriate for the intended purpose, and adequate in number and amount to provide statistically valid results. Diagnostic laboratories require the submission of appropriate samples that arrive at the laboratory in good condition. For disease diagnosis, the tissues sampled should be representative of the condition being investigated and the lesions observed. Samples should be taken with care, to avoid undue stress or injury to the animal or danger to the operator. Where appropriate, samples should be collected aseptically, and care should be taken to avoid cross-contamination between samples.*

The samples should be carefully packaged, labelled, and transmitted to the laboratory by the fastest practicable method, with the appropriate temperature control. There are specific requirements for the packaging and shipping of infectious substances, including diagnostic specimens that must be followed. If material is sent to a laboratory in another country, this laboratory should be consulted in advance to ensure that it is willing to receive the material and to obtain the appropriate import licence. All samples should be accompanied by a letter or submission form, which includes the name and address of the submitter, the origin of the material, the relevant history, animal identification and corresponding specimens, and the tests requested.

A. COLLECTION OF SAMPLES

Before taking samples, careful consideration should be given to the purpose for which they are required. This will determine the type and number of samples needed to provide valid results. When samples are taken from live animals, care should be taken to avoid injury or distress to the animal or danger to the operator and attendants. It may be necessary to use mechanical restraint, tranquillisation or anaesthesia. Whenever handling biological material, from either live or dead animals, the risk of zoonotic disease should be kept in mind and precautions taken to avoid human infection (see also Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). Post-mortem examinations should be carried out under as aseptic conditions as is practicable. Care should be taken to avoid environmental contamination, or risk of spread of disease through insects or fomites. Arrangements should be made for appropriate safe disposal of animals and tissues.

Considerable skill and care are required to decide on the correct samples to be sent to the laboratory. The samples collected should be representative of the condition being investigated and the lesions observed. Also the stage of the disease and lesion development should be considered, as well as the type of test(s) that will be performed. Frequently, a combination of blood samples for serology and tissues from dead or culled animals for

microbiological culture and pathological examination will be required. Recommendations for transport are described later in this chapter.

The disease chapters in this *Terrestrial Manual* provide guidance on samples that should be collected so that information will not be repeated here. In addition, procedures for sample collection and submission have been prepared by national and international authorities (3, 5, 9, 11, 12). These publications provide detailed recommendations of specific samples that should be collected from different species and for a wide variety of suspected diseases. They also provide information on post-mortem procedures, lists of appropriate media, and instructions on submission of samples. The laboratory that is going to perform the assay(s) should be contacted if there are specific questions concerning the type of sample that should be collected.

1. Sample collection from live animals

a) Blood

Blood samples may be taken for haematology or for culture and/or direct examination for bacteria, viruses, or protozoa, in which case it is usual to use anticoagulants, such as ethylene diamine tetra-acetic acid (EDTA) or heparin. They may also be taken for serology, which requires a clotted sample. Blood plasma is also used for some procedures. A blood sample is taken, as cleanly as possible, by venipuncture. In most large mammals, the jugular vein or a caudal vein is selected, but brachial veins and mammary veins are also used. Vena cava veins are also used in pigs. In birds, a wing vein (brachial vein) is usually selected. For techniques for sampling small laboratory animals, see refs 1 and 6. Blood may be taken by syringe and needle or by needle and vacuum tube (not easy in delicate veins but convenient in strong veins). Small quantities of blood are conveniently obtained by pricking with a triangular, solid-pointed needle. Ideally the skin at the site of venipuncture should first be shaved (plucked) and swabbed with 70% alcohol and allowed to dry.

For samples that are collected with anticoagulant, thorough mixing, using gentle agitation only, is necessary as soon as the sample has been taken. It may also be necessary to make a smear of fresh blood on a microscope slide; both thick and thin smears may be prepared. For polymerase chain reactions, EDTA is the preferred anticoagulant. For serum samples, the blood should be left to stand at ambient temperature (but protected from excessive heat or cold) for 1–2 hours until the clot begins to contract. The clot can then be ringed round with a sterile rod and the bottles placed in a refrigerator at 4°C. After several hours, or overnight, the sample can be centrifuged at about 1000 *g* for 10–15 minutes and the serum can be decanted or removed with a pipette. In order to establish the significance of antibody titres, paired serum samples will often need to be collected 7–14 days apart. An alternative method for collecting and transporting blood that is to be used for serology is to place a drop of blood on to filter paper, the blood is dried at room temperature and the sample can then be shipped unrefrigerated. Contact the laboratory to enquire if this method of collection is validated for the required tests.

b) Faeces

At least 10 g of freshly voided faeces should be selected. Faeces for parasitology should fill the container and be sent to arrive at the laboratory within 24 hours. If transport times are likely to be longer than 24 hours, the sample should be sent on ice or refrigerated to prevent the hatching of parasite eggs. Screw top containers or sterile plastic bags should be used for shipment; avoid tubes with rubber stoppers as gas generated can result in blowing the stopper off the tube, ruining the integrity of the sample and contaminating other samples in the package. An alternative and sometimes preferable method is to take swabs from the rectum (or cloaca), taking care to swab the mucosal surface. The swabs should be visibly coated with faecal material; however, samples collected with a swab are inadequate for parasitology. Care should be taken when collecting swabs from small, delicate animals or birds to avoid injury to the animal; small swabs are commercially available that should be used. Swabs should be transported in appropriate transport medium. Faeces are best stored and transported at 4°C.

c) Skin

In diseases producing vesicular lesions, collect, if possible, 2 g of affected epithelial tissue as aseptically as possible and place it in 5 ml phosphate buffered glycerine or Tris-buffered tryptose broth virus transport medium at pH 7.6. Additionally, the vesicular fluid should be sampled where unruptured vesicles are present; if possible, vesicular fluid should be aspirated with a syringe and placed in a separate sterile tube. Plucked hair or wool samples are useful for surface-feeding mites, lice and fungal infections. Deep skin scrapings, using the edge of a scalpel blade, are useful for burrowing mites and, in birds, feather tips can be taken for detection of viral antigen where Marek's disease is suspected.

d) Genital tract and semen

Samples may be taken by vaginal or preputial washing, or by the use of suitable swabs. The cervix or urethra may be sampled by swabbing. Samples of semen are best obtained using an artificial vagina or by

extrusion of the penis and artificial stimulation. The sperm-rich fraction should be present in the sample and contamination by antiseptic washing solutions should be avoided. Specific transport media and conditions are often required.

e)   Eye

A sample from the conjunctiva can be taken by holding the palpebra apart and gently swabbing the surface. The swab is then put into transport medium. Scrapings may also be taken on to a microscope slide. The handles of metal-handled swabs are useful for this, to ensure that sufficient cells are removed for microscopic examination. Mucopurulent nasal and lacrimal discharges are rarely useful.

f)   Nasal discharge (saliva, tears)

Samples may be taken with dacron, cotton or gauze swabs, preferably on wire handles as wood is inflexible and may snap. It may be helpful if the swab is first moistened with transport medium. The swab should be allowed to remain in contact with the secretions for up to 1 minute, then placed in transport medium and sent to the laboratory without delay at 4°C. Long protected nasopharyngeal swabs should be used to collect samples for some suspected viral infections.

g)   Milk

Milk samples should be taken after cleansing and drying the tip of the teat, the use of antiseptics should be avoided. The initial stream of milk should be discarded and a tube filled with the next stream(s), a sample of bulk tank milk can be used for some tests. Milk for serological tests should not have been frozen, heated or subjected to violent shaking. If there is going to be a delay in submitting them to the laboratory, preservatives can be added to milk samples that are being collected for serological testing. If necessary, milk for bacterial examination can be frozen.

2.   Sample collection at post-mortem

Samples of tissue from a variety of organs can be taken at post-mortem. Detailed procedures for conducting a post-mortem examination and collecting samples are described in most pathology text books; a guide to necropsy procedures has been published (10). Post-mortem techniques are also included in some of the national guidelines (3, 5, 9). A summary of these procedures will be provided here.

Animal health personnel should be trained in the correct procedures for post-mortem examination of the species of animals with which they work. The equipment required will depend on the size and species of animal, but a knife, saw and cleaver will be required, and also scalpel, forceps and scissors, including scissors with a rounded tip on one blade, for opening intestines. A plentiful supply of containers and tubes of transport media appropriate to the nature of the sample required should be available, along with labels and report forms. Containers should be fully labelled with the date, tissue and animal identification. Special media may be required for transport of samples from the field. The operator should wear protective clothing: overalls, washable apron, rubber gloves and rubber boots. Additionally, if potential zoonotic diseases are being investigated, the post-mortem examination should be conducted in a biological safety cabinet; if this is not possible, an efficient face mask and eye protection should be worn. If rabies or transmissible spongiform encephalopathies (TSEs) are suspected, it is usual to detach the animal's head.

Tissues may be collected for microbiological culture, parasitology, biochemistry, histopathology and/or immuno-histochemistry, and for detection of proteins or genome nucleic acids. In addition buccal, oropharyngeal or rectal (cloacal) swabs may be collected. The person conducting the post-mortem examination should have sufficient knowledge of anatomy and pathology to select the most promising organs and lesions for sampling. Each piece of tissue should be placed in a fully labelled separate plastic bag or sterile screw-capped jar. Swabs should always be submitted in appropriate transport media. Sterile instruments should be used for collecting specimens for microbiological culture and care should be taken not to contaminate tissues with intestinal contents. Disinfectants should not be used on or near tissues to be sampled for bacterial culture or virus isolation.

The tissues may be sent to the laboratory dry or in bacterial or virus transport medium, depending on the type of specimen and the examinations required; swabs should be sent in transport medium. After collection, the samples for microbiological examination should be refrigerated until shipped. If shipment cannot be made within 48 hours, the samples should be frozen; however, prolonged storage at –20°C may be detrimental to virus isolation. For histopathology, blocks of tissue not more than 0.5 cm thick and 1–2 cm long are cut and placed in neutral buffered 4–10% formalin, which should be at least ten times the volume of the tissue sample. For certain suspected diseases, larger portions of brain are required; the brain is sectioned using a sagittal cut, half is submitted fresh, on ice, and the other half is submitted in 10% buffered formalin. For scrapie, bovine spongiform encephalopathy and other TSEs, details of sample collection are provided in the individual disease chapters in this *Terrestrial Manual*. Store and pack formalin-fixed tissues separately from fresh tissues, blood and smears. Care should be taken to insure that formalin-fixed tissues are not frozen. Once fixed, tissues can be removed from formalin and,

as long as they are kept moist and protected (e.g. by wrapping in formalin-soaked paper towels, then sealed in screw-capped jars), they can be forwarded to the laboratory without formalin.

3. Environmental and feed sampling

Samples may be taken to monitor hygiene or as part of a disease enquiry. Environmental samples are commonly taken from litter or bedding and voided faeces or urine. Swabs may be taken from the surface of ventilation ducts, feed troughs and drains. This kind of sampling is particularly important in hatcheries, artificial insemination centres and slaughterhouses in which specialised equipment is maintained. Samples may also be taken from animal feed, in troughs or bulk containers. Water may be sampled in troughs, drinkers, header tanks or from the natural or artificial supply.

4. Honey bees

Adult bees, either dead or moribund, may be collected in the vicinity of the colonies. Live bees should be killed by freezing. Brood samples are taken by removing a piece of brood comb that shows abnormalities. This should be wrapped in paper and placed in a box for transport to the laboratory. Hive debris may be collected for examination, preferably on a sticky board to trap mobile parasites.

B. SAMPLE SIZE

When investigating a case of clinical disease, the specimens collected should be representative of the condition being investigated and the lesions observed. When developing a programme of surveillance and monitoring for animal health in the absence of clinically evident disease, some general statistical sampling methods should be used. These sampling methods are needed to perform the scientifically based surveys specified in the OIE *Terrestrial Animal Health Code* (14). It is possible to calculate how many animals should be sampled from a herd/flock of a certain size, to achieve a 95% probability of detecting infection or previous exposure assumed to be present in a certain percentage of the animals. The following formulae can give approximate numbers, but a specific sampling programme for the planned surveillance programme should be based on complete formulas available in the references (2, 4) or by the use of a program (FreeCalc) available off the internet: http://www.ausvet.com.au/content.php?page=res_software#freecalc. All calculation examples provided in the following paragraphs can be calculated using FreeCalc. This software also includes "a "pooled prevalence calculator", which describes the calculation of prevalence using pooled samples.

The following formula could be used to calculate the sample size *n* to detect at least one infection with a test that has a 100% sensitivity and specificity; where α is the significance level and 1–α is the level of confidence, *p* is the prevalence in the population. If disease were present in 5% of a herd of 500 animals, it would be necessary to collect specimens from 56 animals to be 95% confident of finding at least one positive, assuming that both the sensitivity and specificity of the test were 100%. In order to make a prediction of disease prevalence, it is critical that the sample be selected from the population by a formal random sampling procedure. As most diagnostic tests do not have specificity and sensitivity of 100%, the number of specimens collected must be adjusted to the sensitivity and specificity of the test that will be used (see also Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases).

$$n = \frac{\ln (\alpha)}{-p)}$$

In the above example α = 0.05, 1– α = 95%, *p* = 0.05 and *n* = 59

If the sensitivity (*Se*) is less than 100%, the above formula should be modified as follows:

$$n = \frac{\ln (\alpha)}{\ln (1-p.Se)}$$

In the above example with α = 0.05, *p* = 0.05, specificity (*Sp*) = 1 and *Se* = 0.95, a minimum of *n* = 62 animals instead of 59 would need to be sampled to have a probability of at least 0.95 of finding a positive animal. The increase in the sample size from 59 to 62 is due to the decrease in the sensitivity of the test from 1 to 0.95. The graph below gives the minimum sample size required for finding at least one positive for several sensitivity and prevalence combinations at α = 0.05 and Sp = 1.

If the test is known to have a specificity of less than 1, the positive results should be confirmed by a test with a higher specificity. If the prevalence is very low and the test used has a specificity of less than 1, it is very likely that a positive test result is a false positive.
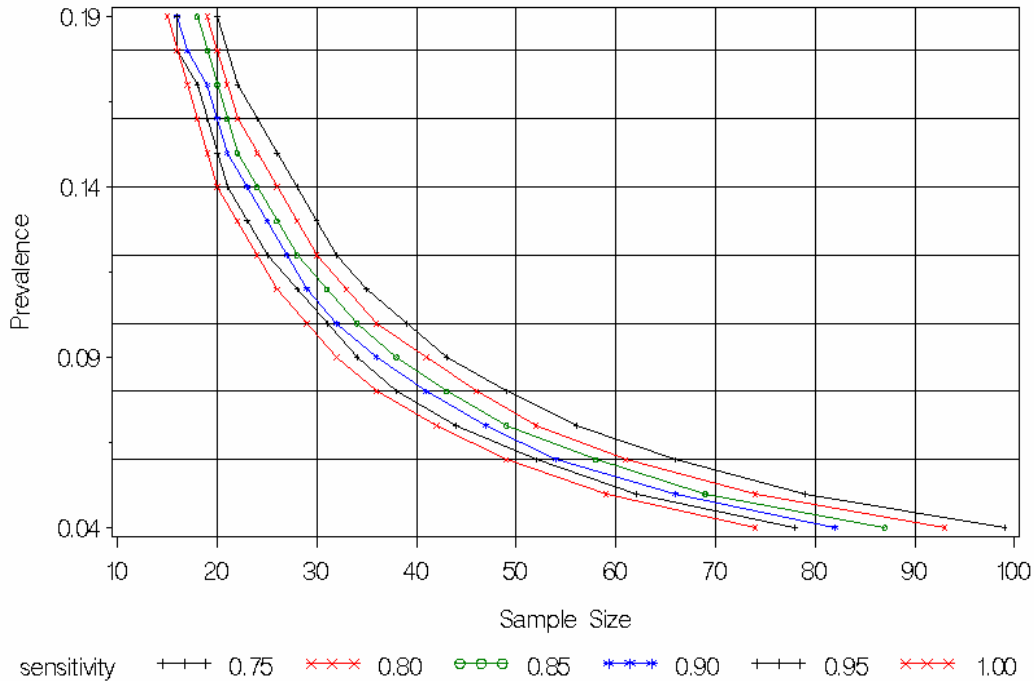
Fig. 1. *Minimum sample size required to be 95% confident of finding infection at various sensitivity and prevalence combinations.*

C. INFORMATION TO BE SENT WITH SAMPLES

It is essential that individual samples be clearly identified using appropriate methods. Marking instruments should be able to withstand the condition of use, i.e. being wet or frozen (use indelible marking pen). Pencil has a tendency to rub off containers and labels attached to plastic will fall off when stored at –70°C. Information and case history should always accompany the samples to the laboratory, and should be placed in a plastic envelope on the outside of the shipping container. As outlined in the following section on transport of samples, this information must also be inside the shipping container. The following are suggested items that should be addressed. It would be advisable to contact the receiving laboratory to determine if it has a submission form that it would like to have submitted with the samples or if it needs other information.

i)   Name and address of owner/occupier and geolocation (latitude and longitude, if available) where disease occurred, with telephone and fax numbers.

ii)   Name, postal and e-mail address, telephone and fax numbers of the sender.

iii)   Diseases suspected and tests requested.

iv)   The species, breed, sex, age and identity of the animals sampled.

v)   Date samples were collected and submitted.

vi)   List of samples submitted with transport media used.

vii)   A complete history would be beneficial for the laboratory and should be included if possible. Some of the components of the history are:

a)   A list and description of the animals examined and the findings of the post-mortem examination.

b)   The length of time sick animals have been on the farm; if they are recent arrivals, from where did they originate.

c)   The date of the first cases and of subsequent cases or losses, with any appropriate previous submission reference numbers.

d)   A description of the spread of infection in the herd or flock.

e)   The number of animals on the farm, the number of animals dead, the number showing clinical signs, and their age, sex and breed.

f)   The clinical signs and their duration including the temperature of sick animals, condition of mouth, eyes and feet, and milk or egg production data.

g)   The type and standard of husbandry, including the type of feed available, possible contact with poison or poisonous plants.

h)   History of foreign travel by owner or of introduction of animals from other countries or regions.

i)   Any medication given to the animals, and when given.

j)   Any vaccines given, and when given.

k)   Other observations about the disease, husbandry practices and other disease conditions present.

D.   PACKAGING AND TRANSPORT OF SAMPLES

1.   Approval to ship specimens

The laboratory that is going to receive the samples should be contacted to ensure that it has the capability to do the testing requested and to see if there are any special packaging or shipping requirements. It is essential to contact the receiving laboratory when material is sent to another country. A special import licence will usually be required for shipment of any biological material to other countries and must be obtained in advance. This licence should be placed in an envelope on the outside of the parcel.

Shipments must be made in accordance with the dangerous goods rules for the particular mode of transport. For air transport it is the International Civil Aviation Organization (ICAO) technical instructions for the safe transport of dangerous goods by air. These are reflected in the International Air Transport Association (IATA) Dangerous Goods Regulations which is the interpretation of ICAO instructions applied to shipments by air (7). These regulations have been described in a United Nations World Health Organization publication (13). The shipper is responsible for checking the variations guidelines to insure that restrictions are met.

2.   Transportation of specimens

The specimens should be forwarded to the laboratory by the fastest method available. If they can reach the laboratory within 48 hours, samples should be sent refrigerated. If dry ice is used, the additional packaging requirements must be met. Infectious substances, which can include diagnostic specimens, are not permitted to be shipped as checked luggage or as carry on luggage and must be shipped as cargo.

3.   Packaging

The shipper should ensure that the specimens are packaged so they arrive at the laboratory in good condition and there is no leakage during shipment. The Dangerous Goods Regulations (DGR) have explicit requirements for packaging and shipment of diagnostic specimens, by all commercial means of air transport (7, 13). In some countries, there are similar requirements for ground shipments and the postal service, but these requirements should be reviewed before shipping. These requirements for air transport are covered in detail in the IATA publication, which are updated every year. The shipper is expected to know and follow the procedures outlined in the current DGR. The following is a summary of the regulations at the time that this revision of the *Terrestrial Manual* was published and it should only be used as a guide for shipping. Shippers must also always check the latest version of the DGR prior to shipping diagnostic specimens. In addition, three of the national guidelines provide explicit directions for packaging and shipping diagnostic specimens and are based on IATA requirements (3, 5, 9).

The DGR outline the procedures for the shipment of infectious substances, which can include diagnostic specimens. Infectious substances are defined in the DGR as substances that are known or are reasonably expected to contain pathogens. Pathogens are defined as micro-organisms (including bacteria, viruses, rickettsiae, parasites, fungi) or recombinant micro-organisms (hybrid or mutant) that are known or reasonably expected to cause disease in humans or animals.

The IATA (7, 13) lists the following exemption from the Dangerous Goods Regulations:

- *3.6.2.2.3.1 Substances which do not contain infectious substances or substances which are unlikely to cause disease in humans or animals are not subject to these Regulations unless they meet the criteria for inclusion in another class.*

- *3.6.2.2.3.2 Substances containing microorganisms which are non-pathogenic to humans or animals are not subject to these Regulations unless they meet the criteria for inclusion in another class.*

- *3.6.2.2.3.3 Substances in a form that any present pathogens have been neutralised or inactivated such that they no longer pose a health risk are not subject to these Regulations unless they meet the criteria for inclusion in another class.*

- *3.6.2.2.3.4 Environmental samples* (*including food and water samples*), *which are not considered to pose a significant risk of infection, are not subject to these Regulations unless they meet the criteria for inclusion in another class.*

- *3.6.2.2.3.5 Dried blood spots, collected by applying a drop of blood on to absorbent material, or faecal occult blood screening tests and blood or blood components that have been collected for the purposes of transfusion or for the preparation of blood products to be used for transfusion or transplantation and any tissues or organs intended for use in transplantation.*

- *3.6.2.2.3.6 Patient specimens for which there is minimal likelihood that pathogens are present are not subject to these Regulations if the specimen is transported in a packaging which will prevent any leakage and which is marked with the words "Exempt human specimen" or "Exempt animal specimen", as appropriate. The packaging should meet the following conditions:*

  (*a*) *The packaging should consist of three components:*
  (*1*) *a leak-proof primary receptacle*(*s*);
  (*2*) *a leak-proof secondary packaging; and*
  (*3*) *an outer packaging of adequate strength for its capacity, mass and intended use, and with at least one surface having minimum dimensions of 100 mm ✗ 100 mm;*

  (*b*) *For liquids, absorbent material in sufficient quantity to absorb the entire contents must be placed between the primary receptacle*(*s*) *and the secondary packaging so that, during transport, any release or leak of a liquid substance will not reach the outer packaging and will not compromise the integrity of the cushioning material;*

  (*c*) *When multiple fragile primary receptacles are placed in a single secondary packaging, they should be either individually wrapped or separated to prevent contact between them.*

*"**Note:** In determining whether a patient specimen has a minimal likelihood that pathogens are present, an element of professional judgment is required to determine if a substance is exempt under this paragraph. That judgment should be based on the known medical history, symptoms and individual circumstances of the source, human or animal, and endemic local conditions. Examples of specimens which may be transported under this paragraph include the blood or urine tests to monitor cholesterol levels, blood glucose levels, hormone levels, or prostate specific antibodies* (*PSA*); *tests required to monitor organ function such as heart, liver or kidney function for humans or animals with non-infectious diseases, or therapeutic drug monitoring; tests conducted for insurance or employment purposes and are intended to determine the presence of drugs or alcohol; pregnancy test; biopsies to detect cancer; and antibody detection in humans or animals. "*

There are also exceptions for some biological products and the shipper of these products is referred to the IATA Regulations for these requirements as not all biological products are exempt. The following is the DGR definition of Biological Products (7, 13):

"Biological products are those products derived from living organisms which are manufactured and distributed in accordance with the requirements of appropriate national authorities, which may have special licensing requirements, and are used either for prevention, treatment, or diagnosis of disease in humans or animals, or for development, experimental or investigational purposes related thereto. They include, but are not limited to, finished or unfinished products such as vaccines."

The DGR state that infectious substances (including diagnostic specimens likely to contain animal or human pathogens) are designated as Category A and B and assigned to UN 2814, UN 2900 or UN 3373.

Category A is defined as an: *"Infectious substance, which is transported in a form that when exposure to it occurs, is capable of causing permanent disability, life threatening or fatal disease in otherwise healthy humans or animals, indicative examples of substances that meet these criteria are given in Table 1 and 2".* Infectious substances meeting this definition that affect humans, including zoonotic agents, are designated UN 2814 and given the shipping name of "***Infectious substance, affecting humans***" those affecting animals only are designated UN 2900 and given the shipping name of "***Infectious substance, affecting animals"***.

Infectious substances shipped for diagnostic purposes that do not meet the criteria for assignment to UN 2814 or UN 2900 are assigned to Category B and must be assigned to UN 3373 and designated as "DIAGNOSTIC SPECIMENS or CLINICAL SPECIMENS or *BIOLOGICAL SUBSTANCES CATEGORY B"*.

The IATA DGR contains an indicative list of pathogens that must be assigned to UN 2814 or UN 2900 (Tables 1 and 2). The pathogens on these lists cannot be assigned to UN 3373 (7, 13).

Table 1. Infectious substances affecting humans that must be designated UN 2814

Bacillus anthracis (cultures only)	Japanese encephalitis virus (cultures only)
Brucella abortus (cultures only)	Junin virus
Brucella melitensis (cultures only)	Kyasanur Forest disease virus
Brucella suis (cultures only)	Lassa virus
Burkholderia mallei – Pseudomonas mallei – Glanders (cultures only)	Machupo virus
Burkholderia pseudomallei – Pseudomonas pseudomallei (cultures only)	Marburg virus
Chlamydia psittaci – avian strains (cultures only)	*Mycobacterium tuberculosis* (cultures only)
Clostridium botulinum (cultures only)	Monkeypox virus
Coccidioides immitis (cultures only)	Nipah virus
Coxiella burnetii (cultures only)	Omsk hemorrhagic fever virus
Crimean-Congo hemorrhagic fever virus	Poliovirus (cultures only)
Dengue virus (cultures only)	Rabies virus (cultures only)
Eastern equine encephalitis virus (cultures only)	*Rickettsia prowazekii* (cultures only)
Escherichia coli, verotoxigenic (cultures only)	*Rickettsia rickettsii* (cultures only)
Ebola virus	Rift Valley fever virus (cultures only)
Flexal virus	Russian spring-summer encephalitis virus (cultures only)
Francisella tularensis (cultures only)	Sabia virus
Guanarito virus	*Shigella dysenteriae* type 1 (cultures only)
Hantaan virus	Tick-borne encephalitis virus (cultures only)
Hantavirus causing haemorrhagic fever with renal syndrome	Variola virus
Hendra virus	Venezuelan equine encephalitis virus (cultures only)
Hepatitis B virus (cultures only)	West Nile virus (cultures only)
Herpes B virus (cultures only)	Yellow fever virus (cultures only)
Human immunodeficiency virus (cultures only)	*Yersinia pestis* (cultures only)
Highly pathogenic avian influenza virus (cultures only)	

Table 2. Indicative examples of animal pathogens forbidden as diagnostic specimens that must
be shipped as infectious substances affecting animals (UN 2900)

African swine fever virus (cultures only)	Peste des petits ruminants virus (cultures only)
Avian paramyxovirus Type 1 – Velogenic Newcastle disease virus (cultures only)	Rinderpest virus (cultures only)
Classical swine fever virus (cultures only)	Sheep-pox virus (cultures only)
Foot and mouth disease virus (cultures only)	Goatpox virus (cultures only)
Lumpy skin disease virus (cultures only)	Swine vesicular disease virus (cultures only)
Mycoplasma mycoides – Contagious bovine pleuropneumonia (cultures only)	Vesicular stomatitis virus (cultures only)

New or emerging pathogens must also be assigned to UN 2814 or UN 2900.

The following is the IATA definition of amplification in culture:

"Cultures are the result of a process by which pathogens are intentionally propagated. This definition does not include patient specimens."

"Patient specimens are those collected directly from humans or animals, including, but not limited to, excreta, secreta, blood and its components, tissue and tissue fluid swabs, and body parts being transported for purposes such as research, diagnosis, investigational activities, disease treatment and prevention."

Note: Cultures of organisms that do not fit into the definition of Category A infectious substance can be transported as Biological Substances, Category B.

The following flow chart summarises the classification of *DIAGNOSTIC SPECIMENS* or *CLINICAL SPECIMENS* or *BIOLOGICAL SUBSTANCES CATEGORY B*.
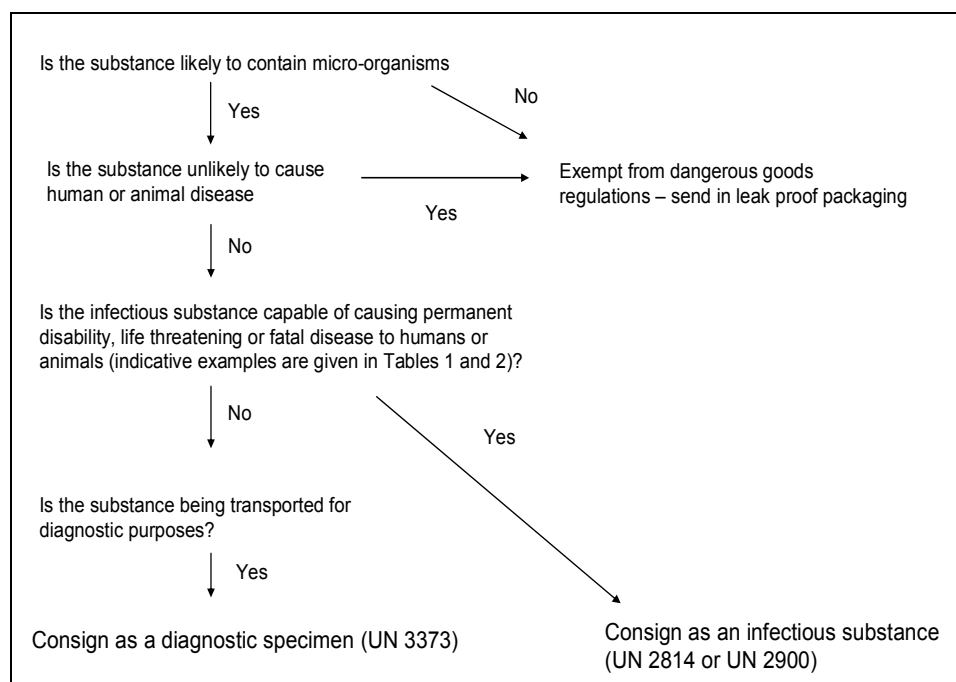
Live animals must not be used to transport infectious substances.

Animal carcasses affected by pathogens of category A or which would be assigned to Category A in cultures only, must be assigned to UN 2814 or UN 2900 as appropriate. Other animal carcasses affected by pathogens included in Category B must be transported in accordance with provisions determined by the Competent Authority.

The packaging of infectious substances and specimens from suspected serious animal diseases, UN 2814 or UN 2900, are outlined in packing instruction 620; a Shippers Declaration of Dangerous Goods must be completed and submitted with these samples. There is also a requirement that the shipper receive training on the IATA-approved shipping procedures for UN 2814 and UN 2900 shipments. Due to the complexity of these guidelines the shipper is referred to the regulations for further information on all UN 2814 or 2900 shipments (7, 13).

The other group, UN 3373, covers 'Diagnostic Specimens or Clinical Specimens or Biological Substances Category B'. This category has a lower risk and packages containing these specimens should be labelled as 'Diagnostic Specimens or Clinical Specimens or Biological Substances Category B'; a Declaration of Dangerous Goods is not needed. IATA packing instruction 650 provides the guidelines for packaging infectious substances assigned to UN 3373 and the following is a summary of these packing instructions. However, the complete procedure, as outlined in the most recent IATA Dangerous Good Regulations, must be followed (7, 13).

i)   Infectious substances assigned to UN 3373 'Diagnostic Specimens' must be packed in good quality packaging, which must be strong enough to withstand the shocks and loadings normally encountered during transport. Packaging must be constructed and closed so as to prevent any loss of contents, which might be caused under normal conditions of transport.

ii) The packaging must consist of three components:

  - a primary receptacle;
  - a secondary packaging; and
  - a rigid outer packaging.

iii) For liquid substances:

  - the primary receptacle(s) must be leak-proof and must not contain more than 1 litre; the secondary packaging must also be leak-proof;
  - adequate adsorbent material must be packed around the primary receptacle(s) to absorb all the fluid in the primary receptacle(s);
  - if multiple primary receptacles are used they should be individually wrapped or separated so as to prevent contact;
  - the primary receptacle or the secondary packaging must be capable of withstanding without leakage an internal pressure of 95 kPa in the range of –40°C to 55°C (–40°F to 130°F);
  - the outer packaging must not contain more than 4 litres. This quantity excludes ice, dry ice, or liquid nitrogen when used to keep specimens cold.

iv) For solid substances:

  - the primary receptacle(s) must be sift-proof and must not exceed the outer packaging weight limit; the secondary packaging must be sift-proof;
  - adequate adsorbent material must be packed around the primary receptacle(s) to absorb all the fluid in the primary receptacle(s);
  - except for packages containing body parts, organs or whole bodies, the outer packaging must not contain more than 4 kg. This quantity excludes ice, dry ice or liquid nitrogen when used to keep specimens cold;
  - if there is any doubt as to whether or not residual liquid may be present in the primary receptacle during transport then packaging suitable for liquids, including absorbent materials, must be used.

v)   An itemised list of contents must be enclosed between the secondary packaging and the outer packaging.

vi) If shipped at ambient temperatures or higher, the primary receptacle must have a positive means of ensuring that it is leak proof, such as a leak proof seal, heat seal or skirted stopper. If screw caps are used they should be sealed with parafilm or tape.

vii) Prefrozen packs or dry ice can be packed around the secondary receptacle. If dry ice is used, there must be an internal support to secure the secondary receptacle in the original position after the dry ice has been dissipated. The outer packaging must permit the release of carbon dioxide. There are additional requirements if liquid nitrogen is used and these are described in the DGR.

viii) Packages containing diagnostic or clinical specimens are not required to have the net quantity marked on the outside of the package. However, where dry ice is used as a refrigerant, the net quantity of dry ice must be shown.

ix) The primary and secondary receptacles must be put into a shipping container with adequate cushioning material.

x) The packaging must be able to withstand a 1.2 metre drop test. (There are additional strength requirements for packaging used for UN 2900 and UN 2814 specimens.)

xi) At least one surface of the outer packaging must have a minimum dimension of 100 mm × 100 mm.

xii) For transport, the label **3373** must be displayed on the external surface of the outer packaging on a background of a contrasting colour and must be clearly visible and legible. The mark must be in the form of a square set an angle of 45° (diamond-shaped) with each side having a length of at least 50 mm, the width of the line must be at least 2 mm, and the letters and numbers must be at least 6 mm high. The proper shipping name "Diagnostic specimen", "Clinical specimen" or "Biological substance category B" in letters at least 6 mm high must be marked on the outer package adjacent to the diamond-shaped mark.

4. Shipping forms

All shipping forms, including the import licence and submission form must be put in an envelope attached to the outside of the shipping container. The forms and labels must be completed as outlined in the DGR and also put on the outside of the container.

E. PRESERVATION OF SAMPLES FOR PROLONGED STORAGE

Establishing a collection of samples for future studies can be very useful. This can include cultures for comparison with future isolates, tissue or serum samples that can be used for the validation of new tests and a collection of fixed tissues, or paraffin blocks, for future histological examination. Possibly the most useful collection is the storage of serum samples. These samples may be useful if a retrospective investigation is carried out to compare the present disease status with that of earlier times.

• Serum banks

Serum samples can provide information about the animals from which the sera were taken. The samples can be tested for a variety of constituents, such as immunoglobulins, trace elements, toxins, hormones and enzymes. If a sufficient number of serum samples have been collected at random from a population, comparisons can be made on the affect of sex, age, breed and geographical location. Results from this comparison can identify high risk groups, vaccination priorities can be established, and patterns and rates of disease determined (8).

A serum bank is a catalogued collection of sera that are stored so as to preserve their immunological and other biochemical properties. Both the catalogue and the storage conditions are essential for a successful serum bank. Each individual sample should be fully documented and identified. The database should contain all relevant information about the origin of the sample and test results obtained. Additional data that may be of interest, such as weather conditions and the animal's productivity may also be included. Accurate records are essential and must be obtained when the blood samples are collected. The first essential is the complete identification of the animal. The amount of detail recorded should be appropriate to the abilities of the operator, accuracy being more important than quantity of information. Although pooling of sera reduces documentation and storage space, it should be avoided as it greatly reduces the usefulness of the material. Care should be taken to collect the blood as aseptically as possible and sterility should be maintained during separation of the serum and all other manipulations. The serum bank catalogue should be well organised and maintained on a computer database with appropriate backup. A suggested methodology has been described in detail (8).

Sera may be stored for periodic use or kept in long-term storage for historical purposes and these two functions should be separated. Storage conditions should minimise loss of immunological and other biochemical properties of the sera. There are three methods: deep freezing, dry storage on paper disks at ambient temperature and lyophilisation (freeze-drying). For long-term storage of sera by deep freezing, a core temperature below –60°C should be maintained. The lower the temperature the better, but lower temperatures are more expensive to maintain. Liquid phase N_2 is at –196°C, vapour phase N_2 is at –100°C and an ultra-low deep freezer will maintain –90°C. Some serum banks have been maintained at –20°C, but the serum may deteriorate and not be suitable for detection of some properties, especially if stored for long periods at this temperature. Deep-freezers should have a system to provide a warning if the temperature rises due to mechanical break down or power failure. A stand-by generator is essential together with alternative cold storage space in case the contents of a freezer must be transferred. Paper disk storage is a simple and inexpensive method, but it allows only a small quantity of serum to be stored and the eluted serum is only suitable for a limited number of tests. The disks should be kept in a cool, dry atmosphere. They can probably provide satisfactory results for up to about 5 years. Lyophilisation is generally regarded as the best method for long-term storage of sera. If freeze-drying conditions are optimised the loss of serum characteristics are minimised. Lyophilisation requires expensive equipment and is a time-consuming process. Lyophilised vials should be stored at 4°C.

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CHAPTER 1.1.2.

BIOSAFETY AND BIOSECURITY IN THE VETERINARY MICROBIOLOGY LABORATORY AND ANIMAL FACILITIES

INTRODUCTION

Laboratory work of the type described in this Terrestrial Manual *should be carried out with a minimum of risk to the health of the staff (*biosafety*) and the environment (*biocontainment*). This requires careful consideration of the risks involved in a particular procedure, followed by appropriate measures to minimise the risk of human disease and of possible release into the environment. This is a complex subject that can only be considered in outline in an introductory chapter. This chapter is concerned almost exclusively with risks from infectious agents, but physical and chemical injuries in microbiology laboratories must also be prevented. Risks from infection are reduced by good laboratory techniques and secure facilities, which aid in the containment of pathogens. It is important to understand that containment of pathogens can be used for two purposes. One is to prevent disease in humans in the laboratory; the other is to prevent the release of the pathogen into the environment and causing disease in animals or humans. Often the same methods of containment are used for both preventing laboratory-acquired infection in humans and for preventing escape of pathogens that could cause an outbreak of animal diseases. Although the methods, techniques and facilities required may be the same, the list of pathogens and categorisation into levels of risk will differ depending on whether it is human or animal diseases control that is the primary objective.*

Existing national and international reference laboratories have considerable experience in the operation of safe working practices and provision of appropriate facilities. When new laboratories are being established, it would be prudent to seek advice from the relevant regulatory authorities and the competent authorities at established institutes. It is important to comply with legislative requirements.

A. ASSESSMENT OF RISK FROM PATHOGENS

It is necessary first to assess the risk from a pathogen, so that it can be assigned to a Risk Group. A further risk assessment can be conducted, based on the proposed work, to determine the appropriate containment level. To assess the risk to humans and animals from a particular pathogen it is necessary to know whether infection with that organism can cause clinical disease and/or mortality in humans and animals, and whether it could then spread to cause disease in the general human and/or animal population. There are additional requirements related to the containment of animal pathogens and the prevention of the spread of infection to animals. To assess these risks it is necessary to know the epidemiological background of the organism and also such attributes of the organism as infectivity for humans and animals, stability in the environment, ability to infect by different routes of exposure, and susceptibility to specific treatments or prophylaxis (1, 2, 5, 6). It is relatively easy to obtain this information when working with a known pathogen, but the problem is more complex in a diagnostic laboratory receiving clinical material that may be infected with a variety of unknown pathogens, some of which could be extremely hazardous to human health or pose a significant threat to animal populations. Some of the considerations to take into account when evaluating risk are:

1. Known occurrence of human and animal infection with the organism or related organisms with similar characteristics, any history of laboratory-acquired infection, infective dose and disease severity; production of toxins or allergens.

2. The volume of culture to be handled and the concentration of the organism likely to be present. (Procedures such as antigen or vaccine production that require large quantities of organisms usually carry a higher risk than attempted isolation procedures.)

3. The origin of the sample, for example samples from wildlife species may contain human or animal pathogens not normally encountered.

4. The history of the isolate being handled. Pathogens on primary isolation or of low passage level are often more dangerous than pathogens of high passage level. In some cases, pathogenicity may be enhanced by passage or subculture using different media.

5. The possibility of aerosol formation should be especially taken into consideration when handling fluid samples or, for example, during grinding, homogenisation and centrifugation.

6. The threat that the organism may pose to food-producing or companion animals or to wildlife, irrespective of the threat to laboratory personnel. Additional precautions for handling and storage are required for animal disease agents from foreign countries.

7. The physical state of the employees. For example, in the case of pregnancy, immunodeficiency or allergy, special precautions may be required. Sometimes certain individuals have to be excluded from particular types of work that would be especially hazardous to them.

8. A higher level of risk may arise when agents such as *Brucella* or *Mycobacterium* are inoculated into animals. To evaluate the impact of animal inoculation, a risk assessment should be conducted and the following factors should be considered:

    i)   Host species versus inoculated species;

    ii)   Strain/treatment and concentration of the inoculum;

    iii) Route of inoculation;

    iv)   Animal housing;

    v)   Types of sampling during the experiment.

9. Some pathogens need to be transmitted by specific vectors or require intermediate hosts to complete their life cycles before they can infect animals and cause disease. In countries where such vectors or intermediate hosts do not occur, or where climatic or environmental factors mitigate against their survival, the pathogen poses a lower risk to animal health than in countries where such vectors or intermediate hosts occur naturally or could survive.

B. GROUPING OF MICROORGANISMS BY HUMAN AND ANIMAL HEALTH RISK

The considerations outlined above have been used by several national authorities to designate microorganisms into four Risk Groups (2, 4) representing increasing risks to human health. Such categorisation of pathogens makes no allowance for people who are particularly susceptible, for example due to pre-existing disease, a compromised immune system or pregnancy. The four Groups may be summarised thus:

*   Group 1 – Organisms that are unlikely to cause human or animal disease and are disease-producing organisms in animals that are enzootic but not subject to official control.

*   Group 2 – Organisms that may cause human or animal disease but are unlikely to be spread in the community or animal population and for which effective prophylaxis and treatment are available; examples of Group 2 animal pathogens:

    i)   They do not depend on vectors or intermediate hosts for transmission.

    ii)   There is very limited or no transmission between different animal species.

    iii) Geographical spread if released from the laboratory is limited.

    iv)   Direct animal to animal transmission is relatively limited.

    v)   Mode of transmission is primarily through ingestion, inoculation or mucus membrane route.

    vi)   The need to confine diseased or infected nondiseased animals is minimal.

    vii) The disease is of limited economic and/or clinical significance.

    viii) Short-term survival in the environment and effective treatment or prevention is available.

    ix)   May be either exotic or enzootic but are subject to official control and have a low risk of spread from the laboratory.

- Group 3 – Organisms that can cause severe human or animal disease and may spread in the community and/or animal population but for which there is usually effective prophylaxis and treatment; examples of Group 3 animal pathogens:

  i) They may depend on vectors or intermediate hosts for transmission.

  ii) Transmission between different animal species may readily occur.

  iii) Geographical spread if released from the laboratory is moderate.

  iv) Direct animal to animal transmission occurs relatively easily.

  v) The statutory confinement of diseased, infected and in-contact animals is necessary.

  vi) The disease is of severe economic and/or clinical significance.

  vii) Prophylactic and/or therapeutic treatments are not readily available or of limited benefit.

  viii) Mode of transmission may be through the airborne route or direct contact.

  ix) Are either exotic or enzootic but are subject to official control and that have a moderate risk of spread from the laboratory.

- Group 4 – Organisms that cause severe human or animal disease, may represent a high risk of spread in the community or animal population and for which there is usually no effective prophylaxis or treatment.

  i) They may depend on vectors or intermediate hosts for transmission.

  ii) Transmission between different animal species may occur very readily.

  iii) Geographical spread if released from the laboratory is widespread.

  iv) Direct animal to animal transmission occurs very easily.

  v) Can be transmitted through casual contact or indirectly.

  vi) The statutory confinement of diseased, infected and in-contact animals is necessary.

  vii) The statutory control of animal movements over a wide area is necessary.

  viii) The disease is of extremely severe economic and/or clinical significance.

  ix) No satisfactory prophylactic and/or therapeutic treatments are available.

  x) Have a high risk of spread from the laboratory into the environment and the national animal population.

Infectious organisms that might be encountered in laboratory work have been assigned to Risk Groups 1–4 by authorities in several countries (2, 4). Some examples of pathogens that may cause disease in humans, and also may be found in a veterinary laboratory, are listed in Table 1 Also, some very serious Group 4 agents, including Hendra and Nipah, have been isolated from diagnostic specimens in veterinary laboratories.

Table 1. *Examples of some of the microorganisms in Risk Groups 2 and 3 that are capable of causing human disease and that may be present in a veterinary laboratory*

Group 2

Viruses: Influenza viruses types A, B, C other than notifiable avian influenza (NAI); Newcastle disease virus; Orf (parapox virus)

Bacteria: *Alcaligenes* spp.; *Arizona* spp.; *Campylobacter* spp.; *Chlamydophila psittaci* (nonavian); *Clostridium tetani; Clostridium botulinum; Corynebacterium* spp.; *Erysipelothrix rhusiopathiae; Escherichia coli; Haemophilus* spp.; *Leptospira* spp.; *Listeria monocytogenes; Moraxella* spp.; *Mycobacterium avium; Pasteurella* spp.; *Proteus* spp.; *Pseudomonas* spp.; *Salmonella* spp.; *Staphylococcus* spp.; *Yersinia enterocolitica; Yersinia pseudotuberculosis*

Fungi: *Aspergillus fumigatus; Microsporum* spp.; *Trichophyton* spp.

Group 3

Viruses: Rabies virus; Equine encephalomyelitis virus (Eastern, Western and Venezuelan); Japanese B encephalitis virus; Louping ill virus

Bacteria: *Bacillus anthracis; Burkholderia mallei* (*Pseudomonas mallei*)*; Brucella* spp.; *Chlamydia psittaci* (avian strains only); *Coxiella burnetti; Mycobacterium bovis*

C. REQUIREMENTS FOR WORK WITH INFECTIOUS AGENTS

1. Known pathogens

Having decided the risk level of certain work it is then possible to decide the appropriate 'containment level' that is needed to minimise the risk of human disease, and the risk of spread of disease to animals and the environment. The containment level is defined by a combination of the physical facilities and working practices employed. Organisms of the four Risk Groups indicated above may be placed into containment levels appropriate for safe working, see below. Laboratories usually appoint a Biological Safety Officer, responsible for ensuring that microorganisms are handled at the appropriate containment level. They should have sufficient expertise and be of sufficient seniority to oversee and advise on all safety matters. In large organisations with a network of laboratories, it is appropriate to appoint a central Safety Officer to advise on and coordinate safety matters of a corporate nature, which are implemented by local laboratory Safety Officers at each site. The working methods for a particular procedure or work station should be written out and readily available. Staff must be fully trained and fully aware of any health risks associated with their work and in procedures for reporting incidents or accidents. Staff should also be given a medical card indicating pathogens to which they might be exposed. In some cases, staff can be specially vaccinated to give additional protection, e.g. when working with the rabies virus; this should also be recorded on the medical card. Such information is useful for a medical practitioner in the event of illness occurring. Regular medical examinations of employees are recommended and, as appropriate, monitoring tests of employees working with the organisms that cause certain serious human diseases, such as brucellosis and tuberculosis.

Much information is available on containment of pathogens, and sophisticated apparatus and buildings may be constructed for containment of the more hazardous organisms as required by the guidelines, standards and regulations of each country. The requirements depend on the containment required, from the most basic to the highest level.

Essential requirements for all laboratory work. The essential requirements for any work with infectious agents, however innocuous they may seem, are as follows:

1. The laboratory should be easy to clean, with surfaces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period;

2. Personnel access to the work area should be restricted; appropriate security measures such as controlled electronic access may be necessary with higher risk agents.

3. Personal protective equipment such as long-sleeved lab coats or gowns, closed-toe footwear, disposable gloves, masks, safety glasses, face shields, and oro-nasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory

4. The laboratory door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.);

5. Food (including chewing gum, candy, throat lozenges and cough drops) and/or drink shall not be stored or consumed in laboratories;

6. Smoking and/or application of cosmetics shall not take place in the laboratory;

7. Pipetting shall not be done by mouth;

8. Care shall be taken to minimise the production of aerosols;

9. Emergency response plans should be developed to deal with the biohazard of spills. Some of the items addressed in the plans should include having effective disinfectant available for cleaning spills, removal of and decontamination of contaminated protective clothing, washing of hands, and cleaning and disinfection of bench tops;

10. Used laboratory glassware and other contaminated material shall be stored safely.. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise decontaminated before disposal. Reusable material shall be decontaminated by appropriate means;

11. No infectious material shall be discarded down laboratory sinks or any other drain;

12. Any accidents or incidents shall be recorded and reported to the Safety Officer.

Containment level for Group 2 pathogens, in addition to the points given above, a Class I, II or III microbiological safety cabinet should be used when there is potential for generating aerosols or when handling large quantities of culture or where there is a real need to protect the biological product (see Section D). Appropriate signs are required at all entry doors to indicate the hazard present and the name and telephone number of the person(s) responsible. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire or other emergency.

Containment level for Group 3 pathogens, it is advisable that the laboratory be in an isolated location; access should be limited to appropriately trained level 3 staff. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire. OIE containment level for Group 3 pathogens surpasses biosafety level-3 (BSL-3) guidelines as outlined by the United States Department of Health and Human Services (DHHS) (16) and the United States Department of Agriculture (USDA) (15).

In addition to the previous requirements, the laboratory shall be under negative pressure and the pressure differentials should be monitored; a procedure should be developed to provide an alarm if there is a problem and personnel to respond to the alarm. A ventilation system is required that removes air from the laboratory through a high efficiency particulate air (HEPA) filter. HEPA filters shall be verified regularly (usually annually); this would include HEPA filters in biosafety cabinets and on room and equipment exhausts. The laboratory should be sealable for fumigation and contain an airlock entry. There is a requirement to treat effluent depending on the pathogen. Biological safety cabinets of Class I, II or III shall be used whenever the process to be undertaken is likely to generate an aerosol (17). It may be necessary for staff to shower on exit from the laboratory and they must wear dedicated laboratory clothing that is left in the laboratory before leaving the building.

Note. Because of the link between bovine spongiform encephalopathy (BSE) and new variant Creutzfeldt-Jakob disease in humans, BSE and related agents are now categorised with the human transmissible spongiform encephalopathies in Risk Group 3. Consequently, veterinarians and laboratory workers conducting necropsies on BSE-suspect animals or handling tissues derived from such animals must conduct the work under appropriately strict containment conditions, sometimes with derogations allowed by the nature of the work and the results of local risk assessment. It is important that appropriate protective clothing be worn and that a strict code of practice be followed to prevent exposure to the agent. Laboratories conducting work on BSE must comply with national biocontainment and biosafety regulations (3).

Containment level for Group 4 pathogens, the most stringent precautions are required, including access to the building through air locks, and the building being maintained under negative air pressure. Inlet air to the laboratory shall be filtered through a single HEPA filter and extracted air through double HEPA filters in series. All work with infective materials shall be conducted in a Class III cabinet or in a Class II cabinet in conjunction with the use of one-piece positive-pressure suits. All sewage from the laboratory, laboratory effluent and autoclave drain effluent shall be treated by appropriate means to ensure that all infectious material is destroyed before entering the sewerage outside the laboratory. Staff shall shower and change their clothing before leaving the building. Other precautions as described for Group 3 would also apply. The use of one-piece positive-pressure suits is now an internationally accepted way of providing additional protection at level 4.

OIE guidelines for the containment level for Group 4 pathogens are generally equal to the USDA's biosafety level 3 Ag guidelines (15). The primary difference between OIE level 4 and BSL-3 Ag is that the BSL-3 Ag guidelines specify that the laboratory will be airtight and shall pass a pressure decay test to confirm that it does not surpass the prescribed maximum leak rate.

2. Diagnostic specimens

Veterinary diagnostic centres readily receive specimens that are submitted because they are suspect for a variety of diseases. The infectious nature of the specimens is usually unknown, but they have the potential to contain biological agents that may cause disease in animals and humans. Practices and procedures need to be in place that will minimise the risk of occupational exposure of employees to such pathogens. Unless suspected of containing a pathogen requiring a higher containment level, it is advisable that initial processing of all unknown specimens should be carried out as though the material contained a Group 2 pathogen. The most important aspects are to prevent percutaneous, mucous membrane exposure, particularly inhalation and ingestion. Biological safety cabinets should be used for all manipulations that may generate aerosols. Class I or II are appropriate depending on the need for protection of the samples from contamination. Additionally, there should be no mouth pipetting, personal protective clothing shall be worn with, in some cases, eye and respiratory protection, depending on the anticipated level of exposure. Although initial diagnostic procedures may be carried out at level 2, once a Group 3 or 4 organism has been isolated (or suspected) further work must be carried out at the higher containment level.

D. MICROBIOLOGICAL SAFETY CABINETS

These are used at the different containment levels, as described in Section C above. They are of three types:

Class I: An open-fronted cabinet designed specifically to provide operator and environmental protection and not to give protection to the work being handled.

Class II: An open-fronted safety cabinet, sometimes referred to as a laminar flow recirculating cabinet. They are designed to give operator, product and environment protection.

Class III: These cabinets are closed, with glove ports at the front, and provide the highest degree of containment by complete separation of work and worker. Some cabinets have a removable glove port and are known as Class III/I cabinets, i.e. they can be used in either mode.

Descriptions of safety cabinets and safe working practices have been published (8, 10, 17).

E. STORAGE OF PATHOGENS

Storage of live pathogens requires appropriate containment and security to avoid risks due to breakage or unauthorised use of material. Storage facilities should be appropriately labelled to indicate the nature of the pathogens (e.g. their Group) and the contact information for the person(s) responsible for them. A complete inventory of the pathogens in storage should be kept up to date and available. Special care must be taken when opening glass vials of freeze-dried pathogens, as these can sometimes shatter. Care must be taken when working with liquid nitrogen or rooms where asphyxiating gasses may be produced.

Many of the considerations given above relate not only to human safety but also to prevention of the spread of infection to animals. In a veterinary laboratory an important responsibility is to minimise any risk of escape of pathogens to animals, either wild or domestic, in the outside community. Close communication must be maintained with the veterinary authorities. There may be national requirements for special licences to work with certain microorganisms.

F. PHYSICAL AND CHEMICAL HAZARDS

Laboratory work involves many manipulations that are potentially dangerous, such as handling glassware and work with needles or other sharp instruments. There shall be appropriate procedures and equipment for the safe and proper disposal of needles and other 'sharps'.

Laboratory staff should be protected from the risk of receiving a burn from hot solids or liquids. Autoclaves shall be fitted with safety devices to prevent accidental opening of doors when under pressure, and be regularly serviced and tested. Heat-protective gloves, apron and face shields with brow and chin guards shall be provided. Extreme cold can also be a risk, for example when working with liquid nitrogen; splashes on exposed skin can be very damaging. Gloves should be worn that provide insulation from cold and that are also waterproof, to prevent penetration of the liquid nitrogen. Face shields with brow and chin guards and boots should also be worn when working with liquid nitrogen. Nitrogen evaporating from liquid nitrogen storage in poorly ventilated rooms can lead to depletion of oxygen with fatal consequences.

Irradiation is a serious health risk that may be present due to the use of X-ray machines, or use of gamma-emitters or other sources. Equipment shall be regularly serviced and tested. All use of radioactive material must be meticulously recorded. All staff must wear a personal radiation-monitoring device and have annual health checks. Local and national regulations must be followed (10).

A wide range of chemicals are used in veterinary laboratories, many of which may be toxic or mutagenic, and some may be carcinogenic. It should be remembered that it is the dose that makes the poison. Vapours are especially hazardous, and some chemicals can be absorbed by penetration of intact skin. Steam sterilisation may make toxic chemicals volatile and endanger personnel who unload the autoclave/pressure steam steriliser. Procedures sufficient to protect pregnant laboratory workers should be followed at all times. A list of hazardous chemicals shall be maintained, and a file record kept of chemicals to which individual staff members could be exposed. This is now a legal requirement in some countries. Chemicals shall be correctly stored in appropriate containers and at the correct temperature. Those that are flammable shall be kept in a fireproof chemical store. A record must be maintained of the purchase and use of hazardous chemicals: how much, when used, by whom and for what purpose. Disposal of some chemicals is subject to official regulation.

Further information on physical and chemical safety precautions can be found in the literature (11, 12).

G. LABORATORY ANIMAL FACILITIES

Work with pathogens in laboratory animals poses special risks. Animal rooms have to be constructed to appropriate standards and containment levels, just as laboratories. Containment in animal houses is very important because of the large amount of infectious agents that they may generate. Similar considerations also apply regarding the training of staff, protective clothing and the recording of working procedures. Special care must be taken to avoid injury to staff, e.g. through animals biting and kicking or self inoculation accidents. Any such incidents must be recorded and wounds appropriately treated. There shall be provision for autoclaving steam sterilisation, incineration or rendering of carcasses and for the thorough cleansing and disinfection of animal rooms. The animal rooms should not only provide a suitable environment for the animals themselves but should be constructed and ventilated in such a way as to ensure comfort for the attending personnel. This is a large subject that can only be referred to briefly here (4, 7). Also, an excellent book on health and safety in laboratory animal facilities is available (18).

H. EMERGENCY PROVISIONS

First-aid equipment should be readily available, but stored in a location that is unlikely to be contaminated by work conducted in the laboratory (for example, in the air-lock or ante-room). This equipment shall be appropriate to the work and properly maintained. It shall be kept ready to hand for immediate emergency use by trained first aid personnel. Bandages and dressings should be available. Some staff shall receive training in safety and first aid from recognised authorities and shall possess a valid certificate as evidence of competence. Personnel working in Containment Level 4 facilities shall have advanced first aid competence. Their names and locations should be known to everyone and posted on notice boards. All staff should be aware of the importance of safety. There must be suitable procedures and equipment for dealing with spillages and decontamination. A record must be kept of all incidents and in some countries there may be a legal obligation to report incidents to the enforcing authority.

There must be written procedures for dealing with emergency failure of all safety and containment systems, for example in biosafety cabinets or biocontainment rooms, which can lead to loss of containment.

Many laboratories have a staff safety committee to increase safety awareness and to discuss safety issues with management. Personnel are responsible for their own safety and those around them. Managers are equally responsible for safety in their area of command and should not allow consideration of speed or cost of work to come before the safety of personnel or containment of animal disease agents.

There must be an emergency procedure for obtaining medical assistance if required, and for hospitalisation in appropriate infectious disease facilities when needed. Fire alarms shall be fitted, and tested regularly. The institute or laboratory must designate a warden to control and communicate in emergency situations and conduct periodic drills to make staff aware of what to do and where to assemble in the event of an emergency. The warden is responsible for checking that everyone is in a safe location. Procedures for natural disasters, such as hurricanes and earthquakes, should be in place where they present a risk. All these procedures should be written down and periodically reviewed.

I. TRANSPORT OF INFECTIOUS MATERIAL

Great care must be taken when preparing and packing diagnostic specimens, infectious materials and pathogens for transport, to ensure that there is no breakage of containers or leakage of contents that could put at risk personnel in the transport system or animals that may come in contact with contamination. Applicable local, national and international regulations for the transportation of dangerous goods (diagnostic or clinical sample and infectious materials) and importation of animal pathogens must be followed. These are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

When categorising animal pathogens into specific Groups, the following criteria should be taken into account:

a) **Group 1 animal pathogens**

   Disease-producing organisms that are enzootic but not subject to official control.

b) **Group 2 animal pathogens**

   Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a low risk of spread from the laboratory.

c)   Group 3 animal pathogens

Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a moderate risk of spread from the laboratory.

d)   Group 4 animal pathogens

Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a high risk of spread from the laboratory into the environment and the national animal population.

J.   CONTAINMENT GROUPS

1.   The principal purpose of containment is to prevent the escape of the pathogen from the laboratory into the national animal population. Some animal pathogens can infect humans. In these instances the risk to human health may demand additional containment than would otherwise be considered necessary from purely animal health considerations. The risk of human to animal transmission of disease must also be considered and controlled. In addition, other animals being used for experimental work on the pathogen should be held in the appropriate containment level.

2.   The level of physical containment and biosafety procedures and practices should be not less than the Group into which the pathogen has been placed and the detailed requirements should be appropriate to the type of organism (i.e. bacterium, virus, fungus or parasite). The lowest containment level will be required for pathogens in Group 1 and the highest level for those in Group 4. Guidance on the containment requirements for Groups 2, 3 and 4 is provided in Section K.

3.   Arthropods may be pathogens or vectors for pathogens. If they are a vector for a pathogen being used in the laboratory, the appropriate containment level for the pathogen will be necessary in addition to the containment facilities for the arthropod.

K.   GUIDANCE ON THE LABORATORY/ANIMAL FACILITY REQUIREMENTS FOR THE DIFFERENT CONTAINMENT GROUPS

	Containment Group		
Requirements of the laboratory/animal facility	**2**	**3**	**4**
A) Laboratory/animal facility setting and structure			
1. It is advisable that the laboratory/animal facility be in an isolated location		Yes	Yes
2. Not next to known fire hazard	Yes	Yes	Yes
3. Workplace separated from other activities	Yes	Yes	Yes
4. Personnel access limited	Yes	Yes	Yes
5. Protected against entry/exit of rodents and insects	Yes	Yes	Yes
6. Liquid effluent must be sterilised and monitored		Yes	Yes
7. Liquid effluent from steam sterilisers shall be sterilised and monitored			Yes
8. Isolated by airlock. Continuous internal airflow		Yes	Yes
9. The laboratory/animal facility shall be under negative pressure and the pressure differentials should be monitored		Yes	Yes
10. Input air to be filtered using HEPA or equivalent such as gas tight damper; exhaust air to be single HEPA filtration for laboratories and double HEPA filtration for animal facilities.		Single on extract	Single for input, double for extract

Requirements of the laboratory/animal facility	Containment Group		
	2	3	4
A) Laboratory/animal facility setting and structure (cont.)			
11. HEPA filters shall be verified regularly (usually annually)		Yes	Yes
12. Mechanical air supply system with fail-safe system and an alarm provided if there is a problem		Yes	Yes
13. Laboratory/animal facility sealable to permit fumigation		Yes	Yes
14. Incinerator, pressure steam steriliser or renderer for disposal of carcasses and waste	Available	Yes	Yes on site
15. The laboratory/animal facility should be easy to clean, with surfaces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period	Yes	Yes	Yes
B) Additional Laboratory facility requirements			
16. Class I or II biological safety cabinet available	Yes	Yes	Yes
17. Class III biological safety cabinet available		Yes	Yes
18 HEPA filters shall be verified regularly (usually annually)	Yes	Yes	Yes
19. Direct access to autoclave/pressure steam steriliser	Yes	Yes with double doors	Yes with double doors
20. Specified pathogens stored in laboratory	Yes	Yes	Yes
21. Double-ended dunk tank required		Preferable	Yes
22. Personal protective clothing and equipment not worn outside laboratory	Yes	Yes	Yes
23. Full body shower and change of clothing required before exiting laboratory		It may be necessary for staff to shower on exit from the laboratory and they must wear dedicated laboratory clothing that is left in the laboratory before leaving the building	Yes
24. Safety Officer responsible for containment	Yes	Yes	Yes
25. Staff receive special training and demonstrate competence in the requirements needed	Yes	Yes	Yes
C) Laboratory discipline			
26. Warning notices for containment area to indicate the hazard present and the name and telephone number of the person(s) responsible	Yes	Yes	Yes
27. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire or other emergency	Yes	Yes	Yes
28. Laboratory must be lockable	Yes	Yes	Yes
29. Authorised entry of personnel	Yes	Yes	Yes
30. Protective clothing, including gloves, masks, eye shields, and oro-nasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory	Yes	Yes	Yes

Requirements of the laboratory/animal facility	Containment Group		
	2	**3**	**4**
C) Laboratory discipline (cont.)			
31. The laboratory door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.)	Yes	Yes	Yes
32. Food and/or drink shall not be stored or consumed in laboratories	Yes	Yes	Yes
33. Smoking and/or application of cosmetics shall not take place in the laboratory	Yes	Yes	Yes
34. Pipetting shall not be done by mouth	Yes	Yes	Yes
35. Care shall be taken to minimise the production of aerosols	Yes	Yes	Yes
36. No infectious material shall be discarded down laboratory sinks or any other drain	Yes	Yes	Yes
37. Used laboratory glassware and other materials shall be stored safely before disinfection. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise made safe before disposal. Reusable material shall be decontaminated by appropriate means	Yes	Yes	Yes
38. Any accidents or incidents shall be recorded and reported to the Safety Officer	Yes	Yes	Yes
39. On entering all clothing removed and clean clothes put on		Yes	Yes
40. On exiting all laboratory clothes removed, individual shall wash and transfer to clean side		Yes	
41. Individual shall shower prior to transfer to clean side			Yes
D) Handling of specimens			
42. Packaging requirements to be advised prior to submission	Yes	Yes	Yes
43. Incoming packages opened by trained staff in appropriately contained reception area	Yes	Yes	Yes
44. Movement of pathogens from an approved laboratory to another requires a licence	Yes	Yes	Yes
45. Standard Operating Procedures covering all areas must be available	Yes	Yes	Yes

1.   Additional requirements for work in animal facilities

1.   The animal facility should be easy to clean, with surfaces that are impervious to water and resistant to chemicals used in the area.

2.   Personnel access to the work area should be restricted; appropriate security measures such as controlled electronic access may be necessary with higher risk agents.

3.   Personal protective equipment such as coveralls, boots, disposable gloves, masks, safety glasses, face shields, and oro-nasal respirators, as appropriate, shall be worn in the animal facility and removed when leaving the animal facility.

4.   The animal facility door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.)

5. Food (including chewing gum, candy, throat lozenges and cough drops) and/or drink shall not be stored or consumed in animal facilities.

6. Smoking and/or application of cosmetics shall not take place in the animal facility.

10. Used laboratory glassware and other materials shall be stored safely before disinfection. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise made safe before disposal. Reusable material shall be decontaminated by appropriate means.

11. No infectious material shall be discarded down animal facility drains without appropriate waste treatment in place.

12. Any accidents or incidents shall be recorded and reported to the Safety Officer.

L. CONCLUSION

High standards of laboratory safety and containment that will ensure healthy working conditions for laboratory staff and protection of the environment must be of the greatest priority. They can only be achieved by careful study of the principles involved followed by practical application to premises, facilities, operating procedures and hygiene. Training of all laboratory personnel must be a high priority and no personnel should be allowed to work until appropriate training and competence has been demonstrated and documented. There is a large published literature on all aspects of the subject, and further reading is recommended (6, 9, 13, 14, 19).

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QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

Valid laboratory results are essential for diagnosis, surveillance, and trade. Such results are achieved by the use of good management practices, valid test and calibration methods, proper technique, quality control, and quality assurance, all working together within a quality management system. These subjects comprise one complex area of critical importance in the conduct of testing and in the interpretation of test results. This subject may be called laboratory quality management, and includes managerial, operational, and technical elements. A quality management programme enables the laboratory to demonstrate that it operates a viable quality system and is able to generate technically valid results. Additionally the quality management programme should enable the laboratory to show that it meets the needs of its clients or customers. The need for the mutual recognition of test results for international trade and the acceptance of international standards such as the ISO/IEC[1] International Standard 17025 (7) for laboratory accreditation also affect the need and requirements for laboratory quality management programmes. The OIE has published a detailed standard on this subject (10). This chapter is not intended to reiterate the requirements of these ISO or OIE documents. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management programme.

KEY CONSIDERATIONS FOR THE DESIGN AND MAINTENANCE OF A LABORATORY QUALITY MANAGEMENT PROGRAMME

In order to ensure that the quality management programme is appropriate and effective, the design must be carefully thought out. The major categories of consideration and the key issues and activities within each of these categories are outlined in the following seven sections of this chapter.

1. The work, responsibilities, and goals of the laboratory

Many factors affect the necessary elements and requirements of a quality management programme. These factors include:

i) The type of testing done;

ii) The use of the test results;

iii) The impact of a questionable or erroneous result;

iv) The tolerance level of risk and liability;

v) Customer needs (e.g. sensitivity and specificity of the test method, costs, turnaround time);

vi) The role of the laboratory in legal work or in regulatory programmes;

vii) The role of the laboratory in assisting with, confirming, and/or overseeing the work of other laboratories; and

viii) The business goals of the laboratory, including the need for any third party recognition and/or accreditation.

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1   International Organization for Standardization/International Electrochemical Commission.

2. Standards, guides, and references

It is recommended that the laboratory choose reputable and accepted standards and guides to assist in designing the quality management programme. The OIE standard on this subject is a useful guideline (10). For laboratories seeking accreditation, the use of ISO/IEC 17025 (7) and/or the OIE standard (10) will be essential. Further information on standards may be obtained from the national standards body of each country, from the International Laboratory Accreditation Cooperation (ILAC), and from accreditation bodies (e.g. the National Association of Testing Authorities [NATA], Australia and the American Association for Laboratory Accreditation [A2LA], United States of America. Technical and international organisations such as the AOAC International (formerly the Association of Official Analytical Chemists) and the ISO publish useful references, guides, and/or standards that supplement the general requirements of ISO/IEC 17025. ISO International Standard 9001 (8), a general standard for quality management systems and one of the many standards in the group commonly termed the 'ISO 9000 series', is not usable for accreditation, as conformity with its requirements does not necessarily ensure or imply technical competence (see Section 3. below). While a laboratory may implement a quality management system meeting the requirements of ISO 9001*, registration* or *certification* is used to indicate conformity with this standard, not *accreditation*, as ISO 9001 is not a competence standard: see Section 3, below.

3. Accreditation

If the laboratory has determined that it needs formal recognition of its quality management programme, then third party verification of its conformity with the selected standard(s) will be necessary. ILAC has published specific requirements and guides for laboratories and accreditation bodies. Under the ILAC system, ISO/IEC 17025 is to be used for accreditation. Definitions regarding laboratory accreditation may be found in ISO/IEC International Standard 17000 (5). Accreditation is tied to *competence* and this is significant as it means much more than having and following documented procedures. Having competence also means that the laboratory:

i)   Has technically valid and validated test methods, procedures, and specifications that are documented in accordance with the requirements of the selected standard(s) and/or guidelines;

ii)   Has adequate qualified and appropriately trained personnel who understand the science behind the procedures;

iii)   Has correct and adequate equipment;

iv)   Has adequate facilities and environmental control;

v)   Has procedures and specifications that ensure accurate and reliable results;

vi)   Can foresee technical needs and problems and implement continual improvements;

vii)   Can cope with and prevent technical problems that may arise;

viii) Can accurately estimate and control the uncertainty in testing; and

ix)   Can demonstrate proficiency to conduct the test methods used.

x)   Has demonstrated competence to generate technically valid results.

4. Selection of an accreditation body

In order for accreditation to facilitate the acceptance of the laboratory's test results for trade, the accreditation must be recognised by the international community. Therefore, the accreditation body should be recognised as competent to accredit laboratories. Programmes for the recognition of accreditation bodies are, in the ILAC scheme, based on the requirements of ISO/IEC International Standard 17011 (6). One may obtain information on recognised accreditation bodies from the organisations that recognise them, such as the Asia-Pacific Laboratory Accreditation Cooperation (APLAC), the Interamerican Accreditation Cooperation (IAAC), and the European Co-operation for Accreditation (EA).

5. Determination of the scope of the quality management programme and/or of the laboratory's accreditation

The quality management programme should ideally cover all areas of activity affecting all testing that is done at the laboratory. However, for the purpose of accreditation, the laboratory should determine the scope of testing to be included in the accreditation. Factors that might affect the laboratory's choice of scope of accreditation include:

i)   The availability and cost of necessary personnel, facilities and equipment;

ii)   The cost of environmental monitoring against the possibility of cross contamination;

iii)   The deadline for accreditation;

iv)   The impact of the test results;

v)   The number of tests done;

vi)   Whether the testing done is routine or non-routine;

vii)   Whether any part of testing is subcontracted out;

viii)   The quality assurance necessary for materials, reagents and media;

ix)   The availability of reference standards (e.g. standardised reagents, internal quality control samples, reference cultures);

x)   The availability of proficiency testing;

xi)   The availability, from reputable sources, of standard and/or fully validated test methods;

xii)   The evaluation and validation of test methods to be done,

xiii)   The technical complexity of the method (s); and

xiv)   The cost of maintaining staff competence to do the testing.

Accreditation bodies also accredit the providers and operators of proficiency testing programmes, and may require the use of an accredited provider, where available and feasible, in order to issue the laboratory a certificate of accreditation. Accreditation against ISO/IEC Guide 43-1 (assessment against ILAC G13:08/2007) is recommended (3, 4).

6.   Test methods

ISO/IEC 17025 requires the use of appropriate test methods and has requirements for selection, development, and validation. The OIE document (10) also provides requirements for selection and validation.

This *Terrestrial Manual* provides recommendations on the selection of test methods for trade and diagnostic purposes in the chapters on specific diseases. In addition, a list of tests for international trade is provided. As stated in the introduction to this list, the prescribed tests that are listed are those that are required by the OIE *Terrestrial Animal Health Code.* These tests are considered to be adequately validated to give reliable results to qualify animals for international movement. Also listed are alternative tests that are suitable for the diagnosis of disease within a local setting, but that may have had limited validation. These tests are generally serological tests.

In the veterinary profession, other standard methods[2] or fully validated methods[3], while preferable to use, may not be available. Many veterinary laboratories develop or modify methods, and most of these laboratories have test programmes that use non-standard methods, or a combination of standard and non-standard methods. In veterinary laboratories, even with the use of standard methods, some in-house evaluation, optimisation, and/or validation generally must be done to ensure valid results.

Customers and laboratory staff must have a clear understanding of the performance characteristics of the test, and customers should be informed if the method is non-standard. Many veterinary testing laboratories will therefore need to demonstrate competence in the development, adaptation, and validation of test methods.

This *Terrestrial Manual* provides more detailed and specific guidance on test selection, optimisation, standardisation, and validation in Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases. The following items discuss test method issues that are of most interest to those involved in the quality management of the laboratory.

a)   Selection of the test method

Valid results begin with the selection of a test method that meets the needs of the laboratory's customers in addressing the diagnostic issues at hand. Considerations for the selection of a test method include:

i)   International acceptance;

ii)   Scientific acceptance;

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2   Standard Methods: Methods published in international, regional, or national standards.
3   Validated Methods: Methods having undergone a full collaborative study and that are published or issued by an authoritative technical body such as the AOAC International.

iii) Method is the current technology or a recent version;

iv) Performance characteristics (e.g. analytical and diagnostic sensitivity and specificity, repeatability, reproducibility, isolation rate, lower limit of detection, precision, trueness, and uncertainty);

v) Behaviour in species and population of interest;

vi) Resources and time available for development, adaptation, and/or evaluation;

vii) Performance time and turnaround time;

viii) Type of sample (e.g. serum, tissue) and its expected quality or state on arrival at the laboratory;

ix) Analyte (e.g. antibody, antigen);

x) Resources and technology of the laboratory;

xi) Nature of the intended use (e.g. export, import, surveillance, screening, confirmatory, individual animal use, herd use);

xii) Customer expectations;

xiii) Safety factors;

xiv) Number of tests to be done;

xv) Cost of test, per sample;

xvi) Existence of reference standards, including reference materials; and

xvi) Availability of proficiency testing schemes.

b) Optimisation and standardisation of the test method

Once the method has been selected, it must be set up at the laboratory. Whether the method was developed in-house or imported from an outside source, generally some additional optimisation is necessary. *Optimisation* is a series of experiments and subsequent data analysis. Optimisation establishes critical specifications and performance standards for the test process and for use in monitoring the correct performance of the test. Optimisation should ensure that a method is brought under statistical control. Optimisation should also determine:

i) Critical specifications for equipment and instruments;

ii) Critical specifications for reagents (e.g. chemicals, biologicals);

iii) Critical specifications for reference standards, reference materials, and internal controls;

iv) Robustness (if applicable);

v) Critical control points and acceptable ranges, attributes or behaviour at critical control points, using statistically acceptable procedures;

vi) The quality control activities necessary to monitor critical control points;

vii) The type, number, range, frequency, and/or arrangement of test run controls needed;

viii) The requirements for control behaviour for the non-subjective acceptance or rejection of test results;

ix) The elements of a fixed, documented test method for use by laboratory staff; and

x) The level of technical competence required of those who carry out and/or interpret the test.

c) Validation of the test method

Validation further evaluates the test for its fitness for a given use. Validation establishes performance characteristics for the test method, such as sensitivity, specificity, and isolation rate; and diagnostic parameters such as positive/negative cut-off, and titre of interest or significance. Validation should be done using an optimised, documented, and fixed procedure. Depending on logistical and risk factors, validation may involve any number of activities and amount of data, with subsequent data analysis using appropriate statistics. Test validation is covered in Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases, and Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

Validation activities might include:

i) Field and/or epidemiological studies;

ii) Comparison with other methods, preferably standard methods;

iii) Comparison with reference standards (if available);

iv) Collaborative studies with other laboratories using the same documented method, and including the exchange of samples, preferably of undisclosed composition or titre. It is preferable that these be issued by a qualified conducting laboratory that organises the study and evaluates the results provided by the participants;

v) Reproduction of data from an accepted standard method, or from a reputable publication;

vi) Experimental infection studies; and

vii) Analysis of internal quality control data.

Validation is always a balance between costs, risks, and technical possibilities. Experienced accreditation bodies know that there are many cases in which quantities such as accuracy and precision can only be given in a simplified way.

It is also important to develop criteria and procedures for the correlation of test results for diagnosis of disease status or regulatory action, including retesting, screening methods, and confirmatory testing.

d) Uncertainty

Laboratories should be able to estimate the uncertainty of the test methods as performed in the laboratory. This includes methods used by the laboratory to calibrate equipment (7).

The determination of measurement uncertainty (MU) is not new to some areas of measurement sciences. However, the application of the principles of MU to laboratories for the life sciences is new. Most of the work to date regarding MU is for areas of testing other than the life sciences, and much of the work has been theoretical. However, as accreditation becomes more important, applications are being developed for the other areas. It is important to note that MU does not imply doubt about the validity of a test result or other measurement, nor is it equivalent to *error*, as it may be applied to all test results derived from a particular procedure. It may be viewed as a quantitative expression of reliability, and is commonly expressed as a number after a +/– sign (i.e. the true value lies within the stated range, as MU is expressed as a range). Standard deviation and confidence interval are examples of the expression of MU. An example of the use of standard deviation to express uncertainty is the allowed limits on the test run controls for an enzyme-linked immunosorbent assay, commonly expressed as +/– n SD.

Although the determination and expression of MU has not been standardised for veterinary testing laboratories (or medical, food, or environmental), some sound guidance exists.

MU must be estimated in the laboratory for each method included in the scope of accreditation. This can be estimated by a series of tests on control samples. MU can also be estimated using published characteristics (9), but the laboratory must first demonstrate acceptable performance with the method. Government agencies may also set goals for MU values for official methods (e.g. Health Canada). Reputable technical organisations and accreditation bodies (e.g. AOAC International, ISO, NATA, A2LA, SCC, UKAS, Eurachem, and the Co-Operation on International Traceability in Analytical Chemistry [CITAC]) teach courses and/or provide guidance on MU for laboratories seeking accreditation. Codex Alimentarius, which specifies standards for food testing, has taken the approach that it is not necessary for a laboratory to take a further estimate of MU if the laboratory complies with Codex principles regarding quality: i.e. that the laboratory is accredited to ISO/IEC 17025, and therefore uses validated methods (e.g. knows applicable parameters such as sensitivity and specificity, as well as the confidence interval around such parameters), participates in proficiency testing programmes and collaborative studies, and uses appropriate internal quality control procedures.

The requirement for "use of appropriate internal quality control procedures" implies that the laboratory must use quality control procedures that cover all major sources of uncertainty. There is no requirement to cover each component separately. Components can be estimated with experiments in the laboratory (Type A estimates), or from other sources (reference materials, calibration certificates, etc.) (Type B estimates). A traditional control sample procedure, run many times by all analysts and over all shifts, usually covers all the major sources of uncertainty except perhaps sample preparation. The variation of the control samples can be used as an estimate of those combined sources of uncertainty.

ISO/IEC 17025 requires the laboratory to identify all major sources of uncertainty, and to obtain reliable estimates of MU. Laboratories may wish to establish acceptable specifications, criteria, and/or ranges at critical control points for each component. Where appropriate, laboratories can implement appropriate quality control at the critical points associated with each source, or seek to reduce the size of a component. Sources of uncertainty include sampling, storage conditions, sample effects, extraction and recovery, reagent quality, reference material purity, volumetric manipulations, environmental conditions, contamination, equipment effects, analyst or operator bias, biological variability, and other unknown or random effects. The laboratory would also be expected to account for any known systematic error (see also

Section 6.b. steps i–vii). Systematic errors (bias) must either be corrected by changes in the method, adjusted mathematically, or have the bias noted in the report statement. If an adjustment is made to the procedure, there may or may not be a need to reassess uncertainty. If there is an adjustment made to correct for bias, then a new source of uncertainty is introduced (the uncertainty of the correction). This must be added to the MU estimate.

There are three principal ways to estimate MU:

1. The components approach (or 'bottom-up' approach), where all the sources of uncertainty are identified, reasonable estimates are made for each component, a mathematical model is developed that links the components, and the variations are combined using rules for the propagation of error (1).

2. The control sample approach (or 'top-down' approach), where measurements on a stable control material are used to estimate the combined variation of many components. Variation from additional sources needs to be added.

3. The method characteristics approach, where performance data from a valid collaborative study are used as combined uncertainties (other sources may need to be added). Laboratories must meet defined criteria for bias and repeatability for the MU estimates to be valid. These should be larger than would be obtained by competent laboratories using their own control samples or components model.

Additional information on the analysis of uncertainty may be found in the Eurachem Guide to Quantifying Uncertainty in Measurement (2).

e) Implementation and use of the test method

Analysts should be able to demonstrate proficiency in using the test method prior to producing reported results, and on an ongoing basis.

The laboratory should ensure, using appropriate and documented project management, records keeping, data management, and archiving procedures, that it can recreate at need all events relating to test selection, development, optimisation, standardisation, validation, implementation, and use. This includes quality control and quality assurance activities.

7. Strategic planning

Continual improvement is essential. It is recommended that the laboratory be knowledgeable of and stay current with the standards and methods used to demonstrate laboratory competence and to establish and maintain technical validity. The methods by which this may be accomplished include:

i) Attendance at conferences;

ii) Participation in local and international organisations;

iii) Participation in writing national and international standards (e.g. participation on ILAC and ISO committees);

iv) Consulting publications;

v) Visits to other laboratories;

vi) Conducting research;

vii) Participation in cooperative programmes (e.g. Inter-American Institute for Cooperation in Agriculture);

viii) Exchange of procedures, methods, reagents, samples, personnel, and ideas;

ix) Wherever possible, accreditation and maintenance thereof by a third party that is itself recognised as competent to issue the accreditation;

x) Preplanned, continual professional development and technical training;

xi) Management reviews;

xii) Analysis of customer feedback; and

xiii) Preventive action implementation

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4   ISO/IEC International Standard 17011 replaces ISO/IEC Guide 58 (1993). Calibration and Testing Laboratory Accreditation Systems – General Requirements for Operation and Recognition.

CHAPTER 1.1.4.

PRINCIPLES OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES

INTRODUCTION

Validation is the evaluation of a process to determine its fitness for a particular use and includes assay optimisation and demonstration of performance characteristics. An assay validated for an infectious disease yields test results that identify the presence of a particular analyte (e.g. components of an infectious agent or antibody induced by it) and allows predictions to be made about the status of the test subjects. Assays applied to individuals or populations have many purposes, such as aiding in: documenting freedom from disease in a country or region, preventing spread of disease through trade, eradicating an infection from a region or country, confirming diagnosis of clinical cases, estimating infection prevalence to facilitate risk analysis, identifying infected animals toward implementation of control measures, and classifying animals for herd health or immune status post-vaccination. A single assay may be validated for one or several intended purposes by optimising its performance characteristics for each purpose (e.g. setting diagnostic sensitivity [DSe] high [such as 99.99%] with associated lower diagnostic specificity [DSp] for a screening assay, or conversely, setting DSp high with associated lower DSe for a confirmatory assay).

The principles of validation discussed in this chapter will focus primarily on methods to detect antibody in sera using an ELISA as an example. However, these same principles are applicable to validation of tests for other analytes in sera or tissues. Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases extends the principles outlined here to a direct method of infectious agent detection, the molecular diagnostic assays.

By considering the variables that affect an assay's performance, the criteria that must be addressed in assay validation become clearer. The variables can be grouped into three categories: (a) the sample – host/organism interactions affecting the analyte composition and concentration in the serum sample; (b) the assay system – physical, chemical, biological and technician-related factors affecting the capacity of the assay to detect a specific analyte in the sample; and (c) the test result – the capacity of a test result, derived from the assay system, to predict accurately the status of the individual or population relative to the analyte in question.

Factors that affect the concentration and composition of analyte in the serum sample are mainly attributable to the host and are either inherent (e.g. age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g. passively acquired antibody, active immunity elicited by vaccination or infection). Nonhost factors, such as contamination or deterioration of the sample, may also affect the analyte in the sample.

Factors that interfere with the analytical accuracy of the assay system include instrumentation, technician error, reagent choice (both chemical and biological) and calibration, accuracy and acceptance limits of controls, reaction vessels, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and error introduced by detection of closely related analytes, such as antibody to cross-reactive organisms, rheumatoid factor, or heterophile antibody.

Measures that influence the capacity of the test result to predict accurately the infection or analyte status of the host[1] are DSe, DSp, and prevalence of the disease in the population targeted by the assay. DSe and DSp are derived from test results on samples obtained from selected reference animals. The methods used to select the reference animals are critical to the accuracy of the estimates (5). The degree to which the reference animals represent all of the host and environmental variables in the population targeted by the assay has a major impact on the accuracy of test-result interpretation. For example, experienced diagnosticians are aware that an assay, validated by using samples from northern European cattle, may not give valid results for the distinctive populations of cattle in Africa.

The capacity of a positive or negative test result to predict accurately the infection and/or exposure status of the animal or population of animals is the most important consideration of assay validation. This capacity is not only dependent on a highly precise and accurate assay (incorporating well-characterised and standardised reagents) and carefully derived estimates of DSe and DSp, but is heavily influenced by prevalence of the infection in the targeted population or the likelihood that an animal is infected based on clinical criteria. Without a current estimate of the disease prevalence in that population or likelihood of infection in an individual animal, the interpretation of a positive or negative test result may be compromised.

Many factors obviously must be addressed before an assay can be considered to be 'validated' (5, 16). However, there is no consensus whether the concept of assay validation is a time-limited process during which only those factors intrinsic to the assay are optimised and standardised, or whether the concept includes an ongoing validation of assay performance for as long as the assay is used. Accordingly, the term 'validated assay' elicits various interpretations among laboratory diagnosticians and veterinary clinicians. Therefore, a working definition of assay validation is offered as a context for the guidelines outlined below. Ideally, all diagnostic assays would be fully validated for one or more purposes, but in practice there are sometimes limitations to the completeness of validation.

DEFINITIONS OF ASSAY VALIDATION

A validated assay consistently provides test results that identify animals as positive or negative for an analyte or process (e.g. antibody, antigen, or induration at skin test site) and, by inference, accurately predicts the infection and/or exposure status of animals with a predetermined degree of statistical certainty[2]. Implicit in this definition is the requirement that the test method was properly developed, optimised, and standardised to achieve performance characteristics that are consistent with the purpose for which the assay is intended.

This chapter will focus on the principles underlying development and maintenance of a validated assay. Previous iterations of this chapter (12) were condensed renditions of a review article (9). At that time, the goal was to provide general principles of assay validation. In this update, the content is reorganised into the parts of assay validation consistent with the format of the OIE Validation Template, and emphasises the absolute necessity of pre-determining the specific purpose(s) for which the assay is intended. In addition to the validation process *per se*, guidance is offered on scientifically sound methods for development, maintenance, and extension of validation criteria for a given assay.

It must be emphasised that an assay, when applied to target populations, will minimise misclassifications of animals as false positive or false negative only to the extent that validity is assured for all elements of the assay validation process (see Section B. Assay Validation – Part I). This assumes that the assay is fit for the purpose for which it is intended (e.g. a confirmatory assay will likely yield many false-negative results if used as a

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1   In this chapter, the terms 'positive' and 'negative' have been reserved for test results and never refer to infection or antibody/antigen status of the host. Whenever reference is made to 'infection' or 'analyte', any method of exposure to an infectious agent that could be detected directly (e.g. antigen) or indirectly (e.g. antibody) by an assay, should be inferred.

2   In this definition, the DSe and DSp are performance characteristics of the assay for a given target population. They determine – together with the disease prevalence in the population – the probability that a given test result reflects the true status of the animal. An assay can be recognised as validated if reliable estimates of DSe and DSp for a given target population are available. This does not imply any minimum threshold values for these parameters. In practical applications, low values of DSe and DSp or diagnostic problems due to low disease prevalence are compensated by the sampling design or by combining multiple diagnostic assays into parallel or serial testing regimens. The selection of assays, the sampling process, the combination of multiple assays into a testing regimen and the interpretation rule for the results define the diagnostic process.

screening assay). It also assumes that a well conceived, designed, and documented test method and proper standardised reagents, in combination with well-trained technicians, will give a stable assay within the laboratory. Furthermore, it assumes a thorough use of the most rigorous experimental design and epidemiological and statistical tools. These are required to reduce bias, random error, and false assumptions about the reference population of animals upon which the assay performance estimates are made (5). Finally, it assumes that when placed in practice, the assay is conducted within the context of a rigorous quality assurance programme.

A. ESSENTIAL PREREQUESITES BEFORE VALIDATION OF AN ASSAY

1. Selection of an assay fit for its intended purpose

The OIE *Standard for Management and Technical Requirements for Laboratories Conducting Tests for Infectious Diseases* (14)[3]. This Standard states that test methods and related procedures must be appropriate for specific diagnostic applications in order for the test results to be of any relevance. In other words, the assay must be 'fit for purpose'.

As outlined in the background information in *Certification of diagnostic assays* on the OIE website (www.oie.int), the first step is selection of an assay type that likely can be validated for a particular use. The intended purpose(s) of an assay have been broadly defined:

1) Demonstrate freedom from infection in a defined population (country/zone/compartment/herd) (prevalence apparently zero):

    1a) 'Free' with and/or without vaccination,

    1b) Historical freedom,

    1c) Re-establishment of freedom after outbreaks.

2) Certify freedom from infection or agent in individual animals or products for trade/movement purposes.

3) Eradication of infection from defined populations.

4) Confirmatory diagnosis of suspect or clinical cases (includes confirmation of positive screening test).

5) Estimate prevalence of infection or exposure to facilitate risk analysis (surveys, herd health status, disease control measures).

6) Determine immune status of individual animals or populations (post-vaccination).

The OIE Standard further states that in order for a test method to be considered appropriate, it must be properly validated and that this validation must respect the principles outlined in the validation chapters of the this *Terrestrial Manual*.

While this chapter deals with validation and fitness for purpose from a scientific perspective, it should also be noted that other factors might impact the relevance of an assay with respect to fitness for purpose. These factors include not only the diagnostic suitability of the assay, but also its acceptability by scientific and regulatory communities, acceptability to the client, and feasibility given available laboratory resources. An inability to meet operational requirements of an assay also may make it unfit for its intended purpose. Such requirements may include running costs, equipment availability, level of technical sophistication and interpretation skills, kit/reagent availability, shelf life, transport requirements, safety, biosecurity, sample throughput, test turn-around times, aspects of quality control and quality assurance.

2. Initial assay development considerations

An indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibody will be used in this chapter to illustrate the principles of assay validation. It is a type of assay that can be difficult to validate because of signal amplification of both specific and nonspecific components (2). This methodology serves to highlight the problems that need to be addressed in any assay validation process. The same basic principles are used in validation of other complex or simple assay formats. However, each unique type of assay, such as an antigen detection ELISA, may have different sample collection and storage requirements. This chapter assumes that the assay developer will have a high level of relevant scientific expertise to achieve proper preparation and use of protocols and reagents, leading to a validated assay that is publishable in peer reviewed journals. Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases describes the principles for validating gene-amplification techniques.

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3   This is a specific interpretation of the more generally stated requirements of the ISO/IEC 17025:2005 international quality standard for testing laboratories (8).

Selection of appropriate samples, calibrated instrumentation and a relevant methodology to achieve the intended purpose are critical elements in assay validation. Continuity in experiments is assured when reagents and samples are chosen, properly prepared, aliquoted, and stored for use in each experiment. This reduces to a minimum the number of variables and guards against failure when the validation process commences. This approach reduces the variability and provides data needed to establish appropriate controls for ensuring each run of the assay is valid.

a) Feasibility studies – selection of samples

Samples are needed for experiments to determine if the proposed assay is feasible. For this preliminary step, it is useful to select four or five sera (in our example) that range from high to low levels of antibodies against the infectious agent in question. In addition, a sample containing no antibody is required. These samples ideally should represent known infected and uninfected animals from the population that eventually will become the target of the assay once it is validated. The samples should have given expected results in one or more serological assay(s) other than the one being validated. The samples are preferably derived from individual animals, but they may represent pools of samples from several animals. These samples can be used in experiments to determine if the assay is able to distinguish between varying quantities of analyte (antibody in our example), and for optimising the reagent concentrations and perfecting the protocol.

A good practice is to prepare a large volume (e.g. 10 ml or more if possible) of each sample and divide it into 0.1 ml aliquots for storage at or below –20°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at +4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of serum with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of serum can denature antibodies so should be avoided). Also, variation between experiments is reduced when the same source of serum is used for all experiments rather than switching among various sera between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples.

Repeated runs using these samples also can provide preliminary repeatability assessments both within and between runs of the assay. When compared with international standards to establish their activity (concentration or titre), one or more of these samples may also serve as secondary standards; such standards provide assurance that runs of the assay are producing accurate data (16).

Finally, these pools of sera may be used as controls in future routine runs of the assay once all steps of the validation process have been completed.

It is highly desirable to include OIE International Standard Sera or other international standard sera (if they are available) at an early stage in assay development. This may facilitate harmonisation between the assay under development and a standard test method in which international standard sera are normally used (15).

b) Selection of method to achieve normalised results

Normalisation adjusts raw test results of all samples relative to values of controls included in each run of the assay (not to be confused with transformation of data to achieve a 'normal' [Gaussian] distribution). The method of normalisation and expression of data should be determined, preferably no later than at the end of the feasibility studies. Comparisons of results from day to day and between laboratories are most accurate when normalised data are used. For example, in ELISA systems, raw optical density (absorbance) values are absolute measurements that are influenced by ambient temperatures, test parameters, and photometric instrumentation. To account for this variability, results are expressed as a function of the reactivity of one or more serum control samples that are included in each run of the assay. Data normalisation is accomplished in the indirect ELISA by expressing absorbance values in one of several ways (16). A simple and useful method is to express all absorbance values as a percentage of a single high-positive serum control that is included in each plate (Sample/Positive or S/P ratio). (This control must yield a result that is in the linear range of measurement.) This method is adequate for most applications. More rigour can be brought to the normalisation procedure by calculating results from a standard curve generated by several serum controls. It requires a more sophisticated algorithm, such as linear regression or log-logit analysis. This approach is more precise because it does not rely on only one high-positive control sample for data normalisation, but rather uses several serum controls, adjusted to expected values, to plot a standard curve from which the sample value is extrapolated. This method also allows for exclusion of a control value that may fall outside expected confidence limits.

For assays that are end-pointed by sample titration, such as serum (viral) neutralisation, each run of the assay is accepted or rejected based on whether control values fall within predetermined limits. Because sample values usually are not adjusted to a control value, the data are not normalised by the strict definition of the term.

Whatever method is used for normalisation of the data, it is essential to include additional controls for any reagent that may introduce variability and thus undermine attempts to achieve a validated assay. The normalised values for those controls need to fall within predetermined limits (e.g. within an appropriate multiple of the standard deviation of the mean of many runs of each control). The chosen limits should reflect a reasonable and tolerable assay run rejection rate and an acceptable risk that some test samples may be misclassified.

B. ASSAY VALIDATION – PART 1

1. Optimisation and standardisation of reagents

Using several well-defined sera, such in-house standards as outlined in Section A.2.a of this chapter, or reference standards from outside sources, the optimal concentrations/dilutions of the antigen adsorbed to the plate, serum, enzyme–antibody conjugate, and substrate solution are determined through 'checkerboard' titrations of each reagent against all other reagents, following confirmation of the best choice of reaction vessels (usually evaluation of two or three types of microtitre plates, each with its different binding characteristics, to minimise background activity while achieving the maximum spread in activity between negative and high-positive samples). Additional experiments determine the optimal temporal, chemical, and physical variables in the protocol, including incubation temperatures and durations; the type, pH, and molarity of diluent, washing and blocking buffers; and equipment used in each step of the assay (for instance pipettes and washers that give the best reproducibility).

The choice of reagents and their characterisation must be carefully addressed, or the assay's performance characteristics likely will be compromised. For example, increased assay specificity can be accomplished through recombinant expression of antigens or by use of monoclonal antibodies in antigen capture or antibody competition assays. Alternatively, the method of reagent production can also lead to reduced specificity and increased variability. For example, if a viral antigen used in the assay is derived from a viral culture system that is also used to produce viral vaccines commonly used in the species targeted by the assay, nonspecific cross reactivity may occur. Absorption of cross reactive antigens that are in both the vaccine and the antigen used in the assay is necessary, or a cell culture control needs to be tested on each serum sample in routine runs of the assay to identify and account for the extent of such cross reactivity. Obviously, anticipation of the negative or positive impacts of reagent choice on the assay under development/validation is a major consideration, and careful experimentation is necessary to establish an optimal assay.

When a reagent such as a serum control sample is nearing depletion, it is essential to prepare and repeatedly test a replacement before such a control is depleted. The prospective control sample is included in 10–20 runs of the assay before depletion of the original control to establish its proportional relationship to the nearly depleted control. If the depleted sample was a positive control in ELISAs where the normalised value is expressed as a per cent of that positive control, the proportional difference in ELISA activity between the original and replacement sera must be factored into the normalisation algorithm to retain the same cut-off, and thus the same DSe and DSp in the assay. When other reagents, such as antigen for capture of antibody, must be replaced, they should be produced using the same criteria as for the original reagents, and tested in at least five runs of the assay using a panel of sera that has been designed for this purpose. Reagent lots (serials) need to be evaluated for consistency so variability is minimised in the assay as new lots are required. Whenever possible, it is important to change only one reagent at a time to avoid the compound problem of evaluating more than one variable at a time. Variability is minimised when reagents are well-characterised using methods other than that of the target assay.

a) Linear operating range of the assay

The range of values that constitute the linear operating range of an assay is best determined by a dilution series in which a high positive serum is serially diluted in a negative serum. Each dilution is then run at the optimal working dilution in buffer, and the results plotted in the form of a 'response-curve'. This curve, sometimes referred to as a 'dose–response curve' as in pharmacological applications, establishes the linear range of assay values that are valid for use in the assay.

b) Calibration against reference reagents

i) *International standards*

Serum standards and other reagents, available from OIE, WHO, FAO, or other international organisations, can be used to harmonise the assay with expected results gained from reference reagents of known activity.

ii) *In-house standards*

The in-house serum controls (used for normalisation of data) and additional secondary serum standards, such as low positive, high positive, and negative sera (used for repeatability estimates in subsequent routine runs of the assay) can be fitted to the response curve to achieve expected values for such sera.

2. Repeatability

Preliminary evidence of repeatability (agreement between replicates within and between runs of the assay) is necessary to warrant further development of the assay. This is accomplished by evaluating results from a minimum of three in-house samples representing activity within the linear range of the assay. Quadruplicates of these samples are tested in at least four runs of the assay to determine within-run (intraplate) variation. Between-run variation is determined by using the same samples in a minimum of 20 runs (total), by two or more operators, preferably on separate days. All runs must be independent of each other.

For reporting purposes, ELISA raw absorbance values are usually used to calculate repeatability during this part of validation because it is uncertain whether the results of the high-positive control serum, which could be used for calculating normalised values, are reproducible in early runs of the assay format. Also, expected values for the controls have not yet been established. Coefficients of variation (CV: standard deviation of replicates ÷ mean of replicates), generally less than 20% for raw absorbance values for most samples (low-titred samples may have larger CVs), indicates adequate repeatability at this stage of assay development. However, if evidence of excessive variation (>30%) is apparent for most samples within and/or between runs of the assay, more preliminary studies should be done to determine whether stabilisation of the assay is possible, or whether the test format should be abandoned. This is important because an assay that is inherently variable has a high probability of not withstanding the rigours of day-to-day testing on samples from the targeted population of animals.

Additional evidence of repeatability is obtained from the many additional runs of the assay that are required later in the validation process to fully validate the assay. This is accomplished by running replicates of each control, standard, and test sample when experiments are conducted to establish other validation parameters (see Section C. Assay Validation – Part 2, below). Such data will lend confidence to repeatability estimates because they will be based on within-run and between-run assay performance using reagents prepared daily, including different lots of reagents that could affect repeatability.

3. Determination of analytical specificity and sensitivity

Analytical specificity of the assay is the degree to which the assay does not cross-react with other analytes and analytical sensitivity is the smallest detectable amount of the analyte in question, i.e., the lowest detection limit of the assay.

Analytical specificity is assessed by use of a panel of samples derived from animals that have been exposed to genetically related organisms that may stimulate cross-reactive antibodies, or sera from animals with similar clinical presentations. This 'near neighbour analysis' is useful in determining the probability of false-positive reactions in the assay. It is also appropriate to document a group specificity criterion that includes detection of the analyte of interest in sera from animals that have experienced infections/exposure to an entire group or serotype of organisms of interest. It is also important to evaluate the analytical specificity of the assay using samples from animals that have been vaccinated. If the assay targets antibody elicited by a virus, vaccination against that virus may produce antibody that interferes with the assay's inferences about infection. Also, if the viral antigen used in the assay is derived from a whole-cell viral culture preparation, containing antigenic reagents (carrier proteins, etc.) in addition to the virus, a vaccinated animal may test falsely positive due to detection of nonviral antibodies.

Analytical sensitivity of an assay can be assessed by quantifying the least amount of analyte that is detectable in the sample. This can be done by limiting dilutions of a standard of known concentration of the analyte. However, such an objective absolute measure is often impossible to achieve due to lack of samples or standards of known concentration or activity. Another approach is to use end-point dilution analysis of samples from known positive animals, to define the penultimate dilution of sample in which the analyte is no longer detectable, or at least, is indistinguishable from the activity of negative sera. When the results for the assay under development are compared with other assay(s) run on the same samples, a relative measure of analytical sensitivity can be estimated.

In addition to analyte standards or samples for which titers have been determined by other assays, it is possible to create samples by spiking a negative sample matrix with known amounts of the analyte in question. In this case, however, spiked samples may be intrinsically different from samples obtained from clinical cases, thus leading to inferences that may not be accurate.

If the intended purpose of the assay is for screening of animals for antibody activity, analytical sensitivity needs to be high to achieve the greatest probability possible for detecting infected animals. If very high analytical sensitivity is not achievable, the assay may not be fit as a screening assay. Alternatively, if confirmation of another independent diagnostic procedure is the purpose for which the assay is intended, analytical specificity is required that minimises the amount of cross-reactivity. If neither of these objectives is obtainable, the reagents need to be recalibrated, replaced, or the assay should be abandoned.

C. ASSAY VALIDATION – PART 2

1. Determining assay performance characteristics after establishment of a standard assay method and reagent criteria

Estimates of DSe and DSp are the primary performance indicators established during validation of an assay. These must be established after the assay and reagents are optimised and standardised; alteration of protocols or reagents may require reestablishment of performance characteristics. They are the basis for calculation of other parameters from which inferences are made about test results. Therefore, it is imperative that estimates of DSe and DSp are as accurate as possible. Ideally, they are derived from testing a series of samples from reference animals of known history and infection status relative to the disease/infection in question and relevant to the country or region in which the test is to be used, but that is not always possible. A sampling design must be chosen that will allow estimation of diagnostic performance characteristics. However this is a difficult process complicated by logistical and financial limitations. It is also limited by the fact that reference populations and gold standards may be lacking. The following are examples of reference populations and methodologies that may aid in determining performance characteristics of the test being validated.

a) Reference animal populations

i) *Infected or exposed and uninfected or nonexposed reference animals*

Selection of reference animals to evaluate performance characteristics requires that the variables attributable to the target population are represented in the infected/exposed and uninfected/unexposed reference animal populations. The variables include but are not limited to species, age, sex, breed, nutritional status, pregnancy, stage of infection, immunological status including vaccination history, and historical, epidemiological, and/or clinical data including herd disease history should be noted and considered.

ii) *Reference animal status determined by other assays*

In serology, the 'standard of comparison' is the results of a method or combination of methods with which the new assay is compared. Although the term 'gold standard' is commonly used to describe any standard of comparison, it should be limited to methods that unequivocally classify animals as infected/exposed or uninfected. Some isolation methods themselves have problems of repeatability and sensitivity. Gold standard methods include unequivocal isolation of the agent or pathognomonic histopathological criteria.

Because a true gold standard may be lacking or is impossible to achieve, relative standards of comparison are often necessary; the most common of these include results from other serological assays. Calculations of DSe and DSp are most reliable when the gold standard of comparison is available. When only relative standards of comparison are available, estimates of DSe and DSp for the new assay may be compromised because the error in the estimates of DSe and DSp for the relative standard is carried over into those estimates for the new assay. Indeed, when using imperfect reference tests without efforts to control for any biases, the DSe and DSp performance estimates of the new test will be flawed and thus unacceptable.

iii) *Experimentally infected or vaccinated reference animals*

Sera obtained sequentially from experimentally infected or vaccinated animals have been used to 'validate' a new assay. Such repeated observations, pre- and post-seroconversion, from the same animals are not acceptable for establishing estimates of DSe and DSp because the statistical requirement of independent observations is violated. Thus, time-point sampling of individual experimental animals is necessary. Also, exposure to organisms under experimental conditions, or vaccination may elicit antibody responses that are not quantitatively and qualitatively typical of natural infection in the target population (9). The strain of organism, dose, and route of administration to experimental animals are examples of variables that may introduce error when extrapolating DSe and DSp estimates to the target population. For these reasons, validation of an assay should not be based solely on experimental animals.

iv) *Reference animals – Status unknown*

When it is not possible to assemble sera from animals of known infection status, it is possible to estimate DSe and DSp by non-gold standard methods or latent class models (3, 7). Because these statistical models are complex, an expert should be consulted to provide assistance on proper ways to conduct and describe the sampling from the target population(s), the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation methods based on peer-reviewed literature.

2. Threshold determination

To achieve performance estimates of DSe and DSp of the new assay, the test results first must be reduced to categorical (positive or negative) status. This is accomplished by insertion of a cut-off point (threshold or decision limit) on the continuous scale of test results. Although many methods have been described for this purpose, three examples will illustrate different approaches, together with their advantages and disadvantages. The first is a cut-off based on the frequency distributions (9) of test results from uninfected and infected reference animals. This cut-off can be established empirically by visual inspection of the frequency distributions, by receiver-operator characteristics (ROC) analysis (6, 17), or by selection that favours either DSe or DSp, depending on the intended use for a given assay (11). A second approach is establishing a cut-off based only on uninfected reference animals, for example the 99th percentile in a frequency distribution of assay values for uninfected reference animals; this provides an estimate of DSp but not DSe. The third method provides an 'intrinsic cut-off' based on test results from sera drawn randomly from within the target population with no prior knowledge of the animals' infection status (4).

If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is difficult to select a cut-off that will accurately classify these animals according to their infection status. Rather than a single cut-off, two cut-offs can be selected that define a high DSe (e.g. inclusion of 99% of the values from infected animals), and a high DSp (e.g. 99% of the values from uninfected animals). The values that fall between these percentiles would then be classified as suspicious or equivocal, and would require testing by a confirmatory assay or retesting for detection of seroconversion.

The selection of the cut-off will typically reflect the intended purpose of the assay. For example, a screening assay designed for high DSe versus a confirmatory assay designed for high DSp will require different cut-offs in the same assay system. Although the intended purpose will dictate the cut-off, a ROC analysis is still desirable, as it will show the potential performance of the assay in other epidemiological settings.

3. Assay performance estimates

a) Number of reference animals required

The number and source of reference samples coupled with the methodologies used to derive DSe and DSp estimates are of paramount importance if the assay is ever to be properly validated for use in the population of animals targeted by the assay. It is possible to calculate the number of reference samples, from animals of known infection/exposure status, required for determinations of DSe and DSp that will have statistically defined limits. Formulae and tables for determining the number of samples required are provided elsewhere (5, 9). Table 1, page 474 of reference 9 reveals how the number of samples tested affects the confidence levels in the calculated estimates of DSe and DSp for the assay. For example, an estimated DSe or DSp of 92% with a confidence level of 75% in that estimate requires 161 analyte-positive (known infected) animals from the population targeted by the assay (with an allowable error of ± 2%). However, to increase confidence in the estimate to a 95% level requires that 542 samples/animals be tested. The number of samples theoretically required to achieve confidence levels ranging from 75% to 99% can be found in this reference table for assays that are anticipated to have DSe or DSp ranging from 80% to 99%.

b) DSe and DSp estimates based on reference animals with defined infection status

The selection of a cut-off allows classification of test results into positive or negative categories. Calculations of DSe and DSp are aided by associating the positive/negative categorical data with the known infection status for each animal using a two-way (2 × 2) table (Table 1). After the cut-off is established, results of tests on standard sera can be classified as true positive (TP) or true negative (TN) if they are in agreement with those of the gold standard (or other standard of comparison). Alternatively, they are classified as false positive (FP) or false negative (FN) if they disagree with the standard. Diagnostic sensitivity is calculated as TP/ (TP + FN) whereas diagnostic specificity is TN/ (TN + FP); the results of both calculations are usually expressed as percentages (Table 1).

Table 1. *Calculations of DSe and DSp aided by a 2 × 2 table that associates infection status with test results from 2000 reference animals*

		Reference animals of known infection status	
		Infected (*n* = 600)	**Uninfected (*n* = 1400)**
Test	**Positive**	570 (TP)	46 (FP)
Result	**Negative**	(FN) 30	(TN) 1354
		Diagnostic sensitivity	**Diagnostic specificity**
		$\dfrac{TP}{TP+FN} = \dfrac{570}{600} = 95.0\%$	$\dfrac{TN}{TN+FP} = \dfrac{1354}{1400} = 96.7\%$

c) DSe and DSp estimates based on animals with infection status not defined

DSe and DSp can be estimated when infection or analyte status of the animals are not defined; however, these latent class statistical models are complex. Expert advice should be sought not only in the design of the evaluation study but the interpretation of the estimates of DSe and DSp as well. It has been recommended to the OIE that an expert group be formed to address the application of latent class models and to draft guidelines for models as they apply to the validation and certification assays by the OIE.

4. Comparison and harmonisation of assays

New assays usually are developed to improve on existing techniques. In order to demonstrate that a new assay is an improvement over an existing technique, there must be some form of comparison that demonstrates the improvement. The comparison may be related to analytical and/or diagnostic performance characteristics. It may also be related to operational characteristics such as cost, ruggedness, turn-around-times, throughput, etc. If the new assay is to be incorporated into a diagnostic regimen involving other test methods, the rationale for its use, interpretation of data and decision-making should be stated.

When an international standard method (15) is available for detection of an analyte, it is possible to harmonise the performance of that method with the one under development. This process requires use of the same serum controls and/or standards in both assays. If OIE Standard Sera or other international standard sera are available, preferably at least three (negative, low positive, and high positive), they should be included in the assay-comparison study. This could lead to a new assay that is indexed to an international standard method and international standard sera (15). Harmonisation of the two assays may then be realised.

It is critical that all samples, test reagents, and the protocol or instructions for running the assay be properly controlled. If the reagents will not be supplied from a common source, the laboratories should produce and characterise the reagents independently. This will allow determination of the adequacy of the protocol for reagent production and characterisation. This provides data needed to determine whether it is necessary to establish a single shared source of well-characterised reagents. Part of the evaluation is the determination that the protocol or instructions are complete, clear and precise. If verbal instructions are required, the developer should consider revision of the protocol to ensure they are comprehensive. If it is determined that the protocol or instructions were interpreted in a different manner, then they should be rewritten and the reproducibility may need to be re-established using the revised protocol or instructions.

D. ASSAY VALIDATION – PART 3

1. Establishing reproducibility and augmenting repeatability estimates of the assay

An assay intended for distribution to many laboratories (such as a commercial kit) must be evaluated for reproducibility, which is defined as the ability of a test method to provide consistent results when applied to aliquots of the same samples tested at different laboratories. This is accomplished by testing a panel of sera in a minimum of three laboratories using the identical test method and serum panels.

A test panel consisting of a minimum of 20 samples is assembled for this purpose. Ideally, these will be individual samples from animals within the target population, representing the range of assay activity anticipated in that population. If such samples are not available, dilution of a high positive with a negative serum to achieve the range of activity is acceptable but not optimal. Replicates of about 20% of the samples are desirable as a check on repeatability within each participating laboratory. Each sample is aliquoted, rendering a series of identical panels for distribution to other laboratories. The sample identity is encoded for blind testing, and each panel is handled, transported to participating laboratories, and stored identically.

The descriptive statistics for test panel data accumulated from the laboratories includes between-laboratory mean, standard deviation, and range of results for each sample as well as controls. Evaluation of precision and accuracy at each laboratory is facilitated by Youden plots. The data will help to inform the legitimacy of the upper and lower control limits of the assay as established by the developer.

In addition, when the panel of samples is tested in each laboratory, it is advisable to run each sample in duplicate or triplicate. This provides a basis for an expanded analysis of repeatability within each laboratory using the assay. Also, when the assay is placed into routine use, repeatability is also monitored by inclusion of at least duplicates for the controls and preferably for each sample as well.

E. ASSAY VALIDATION – PART 4

1. Programme implementation

Ultimate proof of the usefulness of an assay is its successful application(s). These would include international, regional or national programs. As new and improved assays are developed and come on-line, they will ultimately replace existing assays if they prove a better fitness for purpose. However, this will only happen if they are actually put into routine use and their usefulness documented over time. In the natural progression of diagnostic and/or technological improvement, some new assays will become the new standard of comparison. As such, they may progressively achieve national, regional and international recognition. As a recognised standard, these assays will also be used to develop reference reagents for quality control, proficiency and harmonisation purposes. These reference reagents may also become international standards, as well. The last level of validation in the OIE Registry involves documentation related to actual application and levels of recognition for the assay in question. This is intended to provide potential users with an informed and unbiased source of information.

2. Monitoring validity of assay performance

a) Interpretation of test results – factors affecting assay validity

An assay's test results are useful only if the inferences made from them are accurate. A common error is to assume that an assay with 99% DSe and 99% DSp will generate one false-positive and one false-negative result for approximately every 100 tests on animals from the target population. Such an assay may be precise and accurate, but produce test results that do not accurately predict infection status. For example, if the prevalence of disease in a population targeted by the assay is only 1 per 1000 animals, and the false-positive test rate is 1 per 100 animals (99% DSp), for every 1000 tests on that population, ten will be false positive and one will be true positive. Hence, only approximately 9% of positive test results will accurately predict the infection status of the animal; the positive test results will misclassify the animal 91% of the time. This illustrates that the capacity of a positive or negative test result to predict infection status is dependent on the prevalence of the infection in the target population (10). Of course, the prevalence will probably have been determined by use of a serological test with its own inherent misclassification of results.

An estimate of prevalence in the target population is necessary for calculation of the predictive values of positive (PV+) or negative (PV–) test results. When test values are reported without providing estimates of the assay's DSp and DSe, it is not possible to make informed predictions of infection status from test results (9). It is, therefore, highly desirable to provide an interpretation statement with test results accompanied by a small table indicating PV+ and PV– for a range of expected prevalences of infection in the target population. Without provision of such information, test results from the assay may have failed to accurately classify the infection status of animals, and thus do not reflect a fully validated assay.

b) Maintenance of validation criteria

A validated assay needs constant monitoring and maintenance to retain that designation. Once the assay is put into routine use, internal quality control is accomplished by consistently monitoring the assay for assessment of precision and accuracy (1).

Reproducibility between laboratories should be assessed at least twice each year. It is highly desirable to become part of a consortium of laboratories that are interested in evaluating their output. In the near future, good laboratory practice, including implementation of a total quality assurance programme, will become essential for laboratories seeking to meet national and international certification requirements (see Chapter 1.1.3 Quality management in veterinary testing laboratories).

Proficiency testing is a form of external quality control for an assay. It is usually administered by a reference laboratory that distributes panels of samples, receives the results from the laboratories, analyses the data, and reports the results back to the laboratories. If results from an assay at a given laboratory remain within acceptable limits and show evidence of accuracy and reproducibility, the laboratory may be certified by government agencies or reference laboratories as an official laboratory for that assay (13). Panels of sera for proficiency testing should contain a full representation of an analyte's concentration in animals of the target population. If the panels only have high-positive and low-positive sera (with none near the assay's cut-off), the exercise will only give evidence of reproducibility at the extremes of analyte concentration, and will not clarify whether routine test results on the target population properly classify infection status of animals.

c) Enhancement and extension of validation criteria

Because of the extraordinary set of variables that impact on the performance of serodiagnostic assays, it is highly desirable to expand the number of standard sera from animals of known infection status because of the principle that confidence in the estimates of DSe and DSp is enhanced with increasing sample size. Furthermore, when the assay is to be applied in a completely different geographical region, it is essential to re-validate the assay for its new intended use by subjecting it to sera from populations of animals that reside under local conditions. The same is true for establishing DSe and DSp for subpopulations (e.g. age groups, vaccinated/nonvaccinated, etc.).

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CHAPTER 1.1.5.

VALIDATION AND QUALITY CONTROL OF POLYMERASE CHAIN REACTION METHODS USED FOR THE DIAGNOSIS OF INFECTIOUS DISEASES

INTRODUCTION

The diagnosis of infectious diseases is performed by direct and/or indirect detection of infectious agents. By direct methods, the particles of the agents and/or their components, such as nucleic acids, structural or non-structural proteins, enzymes, etc., are detected. The indirect methods demonstrate the antibodies induced by the infections.

The most common direct detection methods are isolation or in-vitro *cultivation, electron microscopy, immunofluorescence, immunohistochemistry, antigen enzyme-linked immunosorbent assay (*antigen-ELISA*), nucleic-acid hybridisation (*NAH*), macro- and microarrays and the various techniques of nucleic acid amplification, such as the polymerase chain reaction (*PCR*) or the isothermal amplification methods, such as nucleic acid sequence based amplification (*NASBA*), Invader or loop-mediated isothermal amplification (*LAMP*). As NAH, macro- and microarrays and the various amplification assays have nucleic acid molecules as targets, they are also termed methods of molecular diagnosis.*

The most common indirect methods of infectious agent detection are serological assays, such as virus neutralisation, antibody-ELISA, haemagglutination inhibition tests, followed by the recently appearing novel methods, such as biosensors, bioluminometry, fluorescence polarisation, chemoluminescence, etc. In general, diagnostic laboratories simultaneously apply both the direct and the indirect methods, in order to provide more certainty in a diagnosis.

The experiences of the last two decades indicate that the PCR techniques will eventually supersede many of the classical direct methods of infectious agent detection. It is clear that the PCR is replacing virus isolation or bacteria cultivation for the detection of agents that are difficult or impossible to culture. There are several reasons for this trend, including that virus isolation requires: i) the presence of replicating organisms (viruses or bacteria); ii) expensive cell culture and maintenance facilities; iii) as long as several weeks to complete the diagnosis in some instances; and iv) special expertise, which is missing or diminishing today in many laboratories. Although PCR assays were initially expensive and cumbersome to use, they have now become relatively inexpensive, safe and user-friendly tools in diagnostic laboratories (2–4, 6, 7, 11–13). The sensitivity and specificity of PCR is generally greater than isolation or antigen capture ELISA procedures. The introduction of various real-time PCR methods, nucleic acid extraction robots, and automated work stations has resulted today in a large arsenal of high throughput, robust, very rapid and reliable assays for molecular diagnosis. In this chapter the diagnostic applicability of the various PCR methods is summarised with special regard to international harmonisation and validation.

A. PCR METHODS USED IN ROUTINE MOLECULAR DIAGNOSTICS

1. The principles of the PCR

Polymerase chain reaction (PCR) implies that there is an enzyme-based amplification reaction in the assay. The term 'chain reaction' refers to several cycles of copying a specified stretch of DNA, in this case from the genome of an infectious agent. The region to be amplified is defined by two (or more) short nucleotide sequences, termed primer sites that flank the target sequence. Primers, short oligonucleotides that are complementary to the primer sites, bind to the DNA strand to be copied. Using a polymerase, which is not denatured during heat cycling, it is

possible to copy the target sequence by joining free nucleotides to the primers. By repeating the heat-cycling regime 20–40 times, the amount of copied target DNA increases exponentially, producing enough for further operations, such as detection, cloning or sequencing. The diagnostic sensitivity of the PCR is very high because several million copies of the selected target are produced. The specificity may also be very high, as determined by the specific nucleotide sequences of the selected target, as well as primer design. The primers can be designed to detect very specific nucleotide sequences in the genomes of the selected target infectious agents, or can be designed to be complementary to more conserved regions, thus enabling detection of members within a family or genus of infectious agent. A more detailed overview of molecular techniques has been published (17).

a)   DNA amplification

If the genome of the infectious agent is DNA, the amplification is performed directly, with or without previous purification of the target DNA. In many cases, use of DNA extracted and purified from the material to be tested (e.g. blood) will result in increased analytical and diagnostic sensitivity.

b)   RNA amplification (reverse-transcription PCR)

The genomes of many infectious agents contain ribonucleic acid (RNA) that cannot be amplified directly by the PCR. For PCR amplification, a single-stranded DNA target is necessary, and this is not available in the case of RNA viruses. This problem can be solved by the addition of a step before the PCR is begun. Using reverse transcriptase, it is possible to transcribe the RNA into complementary DNA (cDNA), which can be used in a PCR assay (the procedure is termed reverse transcriptase PCR). Traditionally, the reverse transcription reaction is performed in a separate reaction vessel and the cDNA produced is then transferred to a new tube for the PCR. However, heat-stable DNA polymerases with reverse transcriptase activity and specific buffers in which RT and DNA polymerases are active are now readily available. Both allow a reverse-transcription PCR amplification to take place in the same tube in direct sequence without any further handling and with less chance of carry-over contamination. In most cases, it will be necessary to extract and purify RNA prior to reverse transcription.

c)   PCR amplicon detection

The PCR product, or amplicon, can be detected using a variety of procedures. The most common include nonspecific detection of the PCR product based on amplicon size using electrophoresis in agarose gel and staining of the DNA with a nonspecific, intercalating dye, such as ethidium bromide (it is now possible to replace the latter with non-carcinogenic dyes, e.g. GelRed), or specific recognition of the amplified target sequence using Southern blot transfer of the DNA followed by hybridisation with oligonucleotide probes complementary to the target sequence. Hybridisation probes can be enzyme, chemiluminescent, or radionucleotide-labelled to allow visual detection of the specific target sequence.

Some examples of PCR methods currently used are given below.

2.   Conventional PCR

'Conventional PCR' (or simply PCR) uses one pair of oligonucleotide primers to amplify a small part of the genome of the infectious agent. Analytical sensitivity is typically high with a minimum number of 100 to 1000 copies of the target DNA detectable. Analytical specificity can be high, dependent on target selection, primer design, and assay optimisation. Both analytical sensitivity and specificity can be further improved by applying nested PCR (see point 3 below). Detection methods, such as Southern blotting followed by hybridisation probes, can further improve sensitivity and specificity, but are time-consuming, require laboratory handling of amplified DNA, and the interpretation of results can be technically subjective. Based on complexity and expense, these detection methods are not generally considered suitable procedures for common practice in diagnostic laboratories today.

3.   Nested PCR

Nested PCR assays use two sets of amplification cycles with four primers, termed external and internal primers. In general, nested PCR assays provide higher analytical sensitivity and specificity compared with conventional PCR assays. However, there is a substantial increased risk of cross contamination as products from the first round of amplification are often used as the starting template in the second round, resulting in the transfer of material between different PCR tubes. The nested PCR has been largely replaced by real-time PCR protocols, which are equally sensitive but have much less risk of contamination. The limit of detection with the nested PCR is typically <10 genomic copies of the target DNA, and analytical specificity is also enhanced because in the nested PCR, four oligonucleotide primers have to bind specifically to the selected targets in order to yield a positive reaction (4).

4.   Real-time PCR

Real-time PCR differs from standard PCR; here the amplified PCR products are detected directly during the amplification cycles, using hybridisation probes, which enhance assay specificity. Various real-time methods,

such as TaqMan, Scorpion primers, fluorescence resonance energy transfer (FRET), Primer-Probe Energy Transfer (PriProET), SybrGreen, Light-Upon-eXtension (LUX) and the Molecular Beacon assays have become popular tools for detection of infectious agents. Real-time PCR has been used for the detection of bacteria, viruses or parasites from a range of animal species (2–4, 14, 17). These assays have several advantages over the 'classical' conventional or nested PCR methods. In general, only one primer pair is used, providing sensitivity often close or equal to traditional nested PCR but with a much lower risk of contamination. Fluorescence, indicating the presence of the amplified product, is measured through the lid or side of the reaction vessel, thus there is no need for post-PCR handling of the amplified DNA. These procedures are considerably less time-consuming compared with traditional post-amplification PCR product detection in agarose gels followed by ethidium bromide or equivalent DNA detection stain and again, the risk of contamination is reduced. The use of a 96-well microtitre plate format, without the need for nested PCR, allows the procedure to be automated and suitable for large-scale testing (10, 17). Diagnosis can be further automated by using robots for DNA/RNA extractions and pipetting. Compared with classical amplification methods, a further advantage of the real-time PCR is that it is possible to perform quantitative assays (6, 7). Using real time PCR, the diagnostic time can be shortened from hours to minutes. Real-time PCR can also be used for reverse-transcription PCR using one-step protocols, thus enabling the RT-step and PCR to take place in the same tube during the same PCR protocol (17).

5. Multiplex PCR

PCR using multiple primers directed at different targets in a single assay are referred to as multiplex PCR assays. In multiplex PCR, various infectious agents can be detected and differentiated in a single reaction vessel at the same time. The different PCR targets amplified in a standard PCR assay are identified based on PCR product size. The use of 'classical' nested PCR methods for the construction of a multiplex assay is complicated by the need for targets of different sizes, as well as primers that may 'compete' with each other in the same reaction mix, both of which can negatively impact PCR efficiency. In contrast, the concept of real-time PCR (single primer pairs) provides excellent possibilities for the construction of highly sensitive multiplex systems (4, 9) based on more uniform target size, uniform amplification conditions, and differential detection of targets using specific hybridisation probes labelled with different fluorophores. It should also be noted that common primers can be used to amplify specific regions of the genome of a group of pathogens and fluorogenic (TaqMan) probes can then be employed to discriminate between members of the group. This is not strictly multiplex PCR although it may mistakenly be described as such.

6. Further methods of molecular diagnosis

Although this chapter focuses on the PCR-based molecular diagnosis, it should be briefly mentioned that besides PCR, there are many other novel methods being introduced for the molecular detection of pathogens, for example the various isothermal amplification methods (such as nucleic acid sequence based amplification [NASBA]*,* Invader or loop-mediated isothermal amplification [LAMP] technologies), various macro- and microarrays using padlock probes, rolling cycle amplification and other molecular approaches. There are also other approaches being assessed to detect and analyse PCR products, such as MALDI and Luminex. The advantage of such approaches, in combination with the multiplex PCR, is that typing of different strains or types by of an organism becomes possible. It is evident that the arsenal of molecular diagnosis is further strengthened by these methods.

B. PRINCIPLES OF ASSAY VALIDATION FOR NUCLEIC ACID DETECTION TESTS

When performing analyses of clinical material it is important to produce data of good quality. For this, some key criteria have to be fulfilled. The establishment of quality assurance (QA) and quality control (QC) systems is required, i.e. a set of quality protocols, including the use of control samples that ensure that the system is working properly and confirms data reproducibility and quality. QA and QC systems, together with trained and competent personnel, have already been established in many laboratories world-wide. Assay validation is another essential factor for assuring that test results reflect the true status of the samples (8).

To predict the diagnostic performance of a diagnostic assay, it is necessary to use a validation methodology to document the expected performance of the assay in question. Validation is the evaluation of a diagnostic assay for the purpose of determining how fit the assay is for a particular use. The general principles of assay validation can be found in Chapter 1.1.4 Principles of validation of diagnostic assays for infectious disease. This chapter extends these validation principles to molecular diagnostic assays. For explanations of terms and definitions please consult Chapter 1.1.4.

C. ASSAY VALIDATION – INTRODUCTION

1. Selection of an assay fit for its intended purpose

The fitness of PCR assays for various purposes is broad. Wherever there is a need for direct detection of an infectious agent, it is generally possible to use PCR. During the first years of PCR diagnostic development, many

laboratories had problems with contamination and performance; thus PCR had a poor reputation as a technique suitable for diagnostic use. Achievements in recent years have reversed that view. New technology (i.e. real-time PCR) has made the technique less prone to producing false positive results caused by contamination and is easier to use. Furthermore, automating the extraction and pipetting procedures using robots has substantially lowered the costs, enhanced repeatability and reduced the required work-load. During the 'early years' many in-house assays were developed, and harmonisation and validation were poor or non-existent. The OIE, National Laboratories and the European Community Reference Laboratories (ECRLs) have an important role to play in driving the validation and harmonisation work forward. It is fair to say that PCR, as it is performed today, is safe (substantially lower risk of false-positive results), usually validated in some form and fit for its intended purposes. Some specific examples of the importance of PCR are given below, definitions of intended purpose(s) can be found in Chapter 1.1.4.

- To diagnose infection when antibody levels are so low that previous exposure cannot be confirmed by an antibody test (e.g. enzyme-linked immunosorbent assay [ELISA] repeatedly in the 'gray zone' during the bovine leukaemia eradication programmes).

- To discriminate between infection and maternal immunity in young animals (e.g. young calves in eradication programmes).

- To detect viral or bacterial nucleic acid when the diagnostic specimen is not suitable for virus isolation due to toxicity (e.g. semen, exam of mummified fetus).

- In the final stage of eradication programmes, when thorough investigation of single cases is necessary (e.g. herpesvirus latency and single reactor animals during the Aujeszky's disease eradication programmes).

- To discriminate vaccine strains from field viruses (DIVA [differentiating infected from vaccinated animals] approaches).

- To determine phylogenetic relationship of viruses and use this information for molecular epizootiology.

- To enable fast and safe first diagnosis in outbreak situations (e.g. the 2006 outbreaks of highly pathogenic avian influenza).

- To determine the viral load (e.g. in porcine circovirus type 2 infections).

- Rapid monitoring of vaccinated animals that appear to have clinical signs.

- Detection of drug resistant mutants of pathogens, etc.

- To demonstrate freedom of infection in live animals or animal products. However, it has to be noted that some infected animals may have no detectable nucleic acid in the tissues being examined.

2. Initial assay development considerations

a) Precautions and controls

Considering the uncertainty about the safety and reliability of the PCR in routine diagnosis, special precautions should be applied in any laboratory using PCR for detecting infectious agents so as to avoid false-positive or false-negative results. These, together with internal controls (e.g. mimics) assure the safe evaluation of the results, free from false-positive results caused by contamination. True internal controls using house-keeping genes also complete with the target PCR but only for reagents that are in vast excess such as the polymerase and nucleotides. Minimal competition implies that the level of the true target is known, which cannot be the case for field samples. Armoured RNAs allow the mimic to be added in the extraction process, which is a step towards knowing that the extraction has worked. A true internal control provides more confidence that the extraction has been performed correctly. The down side of using housekeeping genes as internal controls is that they can be present in greater amounts than target pathogens.

b) Precautions taken to avoid false-positive results

False-positive results (negative samples showing a positive reaction), may arise from either laboratory-related issues, such as cross-contamination, or assay-related factors, such as inefficient optimisation or assay performance. Product carry-over from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments is a possible source of error, and various practices and tools have been applied to prevent false-positive PCR results. Samples and reagents should be

handled in separate laminar air-flow hoods, which are regularly decontaminated using UV light (the use of UV-light demands very careful maintenance to be effective) and bleach. Constructing and using special tube-holders and openers can also help to prevent false-positives (2). In addition, good laboratory practices should be applied, i.e. to perform the basic steps (DNA extraction, mix and primer preparation, sample preparation, agarose gel electrophoresis of amplification products, etc.) in separated laboratory areas or rooms (Figure 1; refs 1, 4, 17). Different sets of pipettes should be used for each of the steps. The use of positive displacement and filtered tips is advisable. It is also, if possible, advisable to have different persons perform the different steps, who are restricted to the respective laboratory areas. Precautions should be taken to prevent the introduction of amplified material from potentially contaminated laboratories into 'clean' laboratory areas by movement restrictions on samples, papers, equipment, persons or any other potential method of contamination. Movement in the opposite direction should only occur after surface decontamination of equipment and tubes etc. and changing of laboratory coats and gloves. If the sample is expected to have a high amount of agent or target nucleic acid, it is preferable to dilute it prior to introducing it into 'clean' laboratory areas.
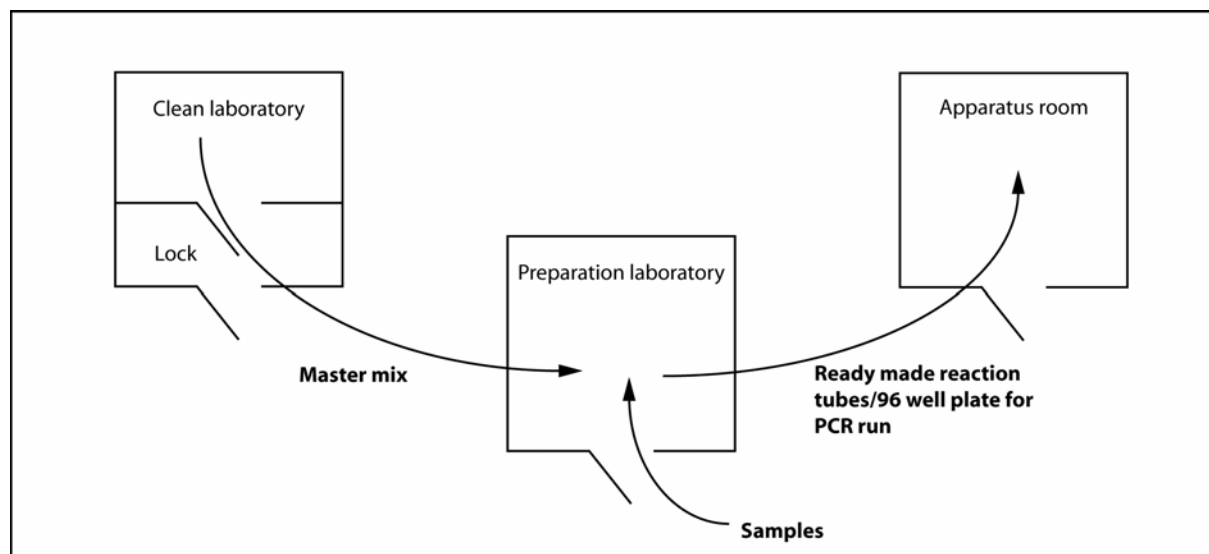
Figure 1. *Recommended laboratory set-up for diagnostic real-time PCR. Samples to be analysed are transferred into the 'Preparation laboratory' for extraction of nucleic acid. PCR master mix is prepared in the 'Clean laboratory' and transferred to the 'Preparation laboratory' for dispensing into PCR plates and adding template. Ready-made reaction tubes/plates are subsequently transferred into the 'Apparatus room' for the PCR run. The 'Clean laboratory' is used only for preparing PCR master mix; no DNA or PCR products are allowed in the room. It is advisable to have an air lock entryway into the 'Clean laboratory' for changing into a lab coat and shoes that are only used herein. The 'Preparation laboratory' is used for processing samples and setting up PCR reactions (with master mix prepared in the 'Clean laboratory'). No PCR products are allowed in this room and nothing from the 'Preparation laboratory' goes back to the 'Clean laboratory'. The 'Apparatus room' contains the PCR machines and nothing from this room goes back to the 'Clean laboratory' or the 'Preparation laboratory'. If the laboratory has a controllable air system, the clean lab should be positive and the other two negative. If nested PCR is going to be performed, two more rooms are recommended. A 'Second PCR laboratory' for setting up the second PCR reaction (which will involve handling PCR products, thus making it impossible to do this in the "Preparation laboratory') and an 'Electrophoreses laboratory' for analysing PCR products in agarose gels.*

It is also very important to include negative controls, i.e. samples that are as similar to the test samples as possible but without having the target. In laboratories experiencing problems with cross-contamination, at least one negative control per five diagnostic samples has been recommended. Both positive and negative control samples should routinely be interspersed with diagnostic samples to assess PCR assay performance.

c) **Precautions taken to avoid false-negative results**

PCR has proven to be a very effective method of detecting nucleic acids, such as viral genomes in clinical specimens. However, an infected animal in the later phases of infection may no longer have viral nucleic acid in the tissues being examined. Consequently, in such cases the negative PCR results should be considered as one part of a complex diagnostic examination.

False-negative results (samples containing the agent of interest but tested as negative) occur mostly due to inhibitory effects and/or pipetting errors; however, issues attributable to sample handling can also yield false-negative results. Therefore, internal controls can be used as indicators of PCR assay efficiency. PCR internal controls may include foreign DNA added to the sample or ubiquitous DNA naturally occurring in the sample. Foreign DNA added to the sample, may include DNA or RNA mimics. DNA mimics, manufactured oligonucleotides, have the same primer-binding sequences as the PCR target, but flank a heterologous DNA fragment of a different size. The identical primer-binding nucleotide sequences allow co-amplification of the target and the mimic in the same tube with minimal competition. The size differences provide easy discrimination by Southern blot analysis. Armored RNA®, an identical concept to DNA mimics, uses a control RNA fragment packaged in bacteriophage coat proteins to protect or stabilise the RNA for control or standardisation of RT-PCR assays (further details on internal controls, see above).

With real-time PCR assays, it is also possible to use internal controls, a naturally occurring housekeeping gene, a selected fragment of the host animal's genome such as beta-actin, GAPDH, or ribosomal RNA. By multiplexing such an intrinsic control with a specifically coloured reporter fluorophore, it is possible to check the sample quality and confirm PCR efficiency, as the target agent and intrinsic DNA are simultaneously detected (14).

Internal controls (for example 'mimics') increase the reliability of diagnostic PCR (1, 4). Caution must be used when designing and validating internal controls. Extensive testing is necessary to ensure that PCR amplification of the added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity. Internal controls are used in concentrations slightly higher than the detection limit of the diagnostic PCR to ensure the test's performance. It should also be remembered that internal controls have a disadvantage, similar to spiked samples, in not being representative of target nucleic acid and can lead to false-negative results.

d) Preparation of standards

Reference laboratories should provide standard samples representative of a given infectious agent. Such samples can be cultivated infectious agents or clinical specimens, etc., which are distributed in such a manner that the infectious agent is well preserved. Thus the samples are distributed frozen, in organic solvents (e.g. Trizol) or other suitable ways. The samples can also be sent as nucleic acids (frozen, freeze-dried or in ethanol). For specific details, see the individual disease chapters. Reference laboratories should also provide the appropriate mimics.

The availability of standard samples is crucial for a successful assay validation. Unfortunately this is often the most problematic issue to solve when planning a validation project. It is not sufficient to use only cultivated agents or spiked samples; true samples from the field may have very different characteristics than these laboratory-generated samples. It might prove very difficult to obtain samples from the field that are truly negative or positive, especially as PCR generally has a higher analytical sensitivity than most 'Gold Standard' methods, thus making it difficult to determine the status of the intended validation samples using already established assays. As mentioned previously, reference laboratories might be one potential source for such standard samples. Alternatively, Bayesian methods offer probabilistic approaches to validate diagnostic tests in the absence of a gold standard (5), but are not further discussed in this chapter.

D. ASSAY VALIDATION – PART 1

1. Optimisation and standardisation of reagents and determination of critical control parameters

Sample collection, preparation and transport (see Chapter 1.1.1 Collection and shipment of diagnostic specimens) and nucleic acid extraction methods (see Chapter 1.1.7 Biotechnology in the diagnosis of infectious diseases and vaccine development) are all critical parameters in test performance and should be optimised for disease diagnosis. Suitable methods vary depending on sample and organism type. In general, blood serum, body tissues and swab samples are suitable samples for easy extraction of target nucleic acids, while faeces, autolysed material and semen samples are more difficult to handle. Extraction of RNA targets differs from extraction of DNA targets, and RNA is more prone to degradation. Both commercial (robotic, spin columns, magnet-based extractions, etc.) and standard chemistry-based methods are used for DNA or RNA extraction. It is crucial to determine the most reproducible and efficient extraction method before further validation of the assay is performed. If the method of extraction is changed, equivalency data should be generated or the entire validation procedure should be repeated.

All equipment used during the process must be properly maintained. Apparatus (heating blocks, refrigerators, freezers, thermocyclers, pipettes, etc.) that require calibration must be calibrated according to the laboratory's quality assurance protocols. It is also important to properly validate the equipment and protocols used. One good

example is the recent implementation of robotic extraction methods for routine diagnostic processing. It is not sufficient to compare the characteristics of this technique with that of the previously used extraction method. The robot and the protocol must be validated to confirm that there is no danger of cross-contamination, e.g. by running a set of mixed positive and negative samples.

When developing 'classical' or real-time PCR assays, all parameters, protocols and reagents need to be optimised. A standardised assay is a method that consistently gives the same result for a given sample when repeated several times and when performed by different analysts in different laboratories.

During the optimisation of the PCR assay, it is also possible to estimate the capacity of the method to remain unaffected by small changes in the main parameters. To achieve an optimised PCR assay, it is essential to evaluate critical parameters in the assay. Examples of such parameters include: incubation times and temperatures, concentrations of buffers, primers, $MgCl_2$, etc., pH, amounts of other components added (e.g. dNTP, bovine serum albumin, etc.). The characterisation of critical control parameters is crucial for identifying critical points that must be properly controlled in the assay. Intentional variations of parameters can lead to a preliminary expression of assay robustness.

2. Repeatability

Agreement between replicates within and between runs of the assay should be accessed at this stage. This gives important information about the assay before further validation is carried out. If excessive variability is encountered, it should be corrected before continuing the validation process.

Repeatability of a PCR assay requires that each replicate be treated as an independent sample. According to assess variation of a replicate (e.g. a triplicate), three individual aliquots of starting analyte are extracted and amplified, and the variation from the mean value detected is determined as an indication of repeatability. Thus, it is not acceptable to assess triplicate amplifications from one extraction. Likewise replicates from multiple runs must be treated as individual samples. This process will result in estimates of intra- and inter-assay variability. In a real-time PCR assay, the Ct-values produced from the replicated samples can be used to determine the inter-run coefficient of variation (CV; see Chapter 1.1.3 Quality management in veterinary testing laboratories, Section 6.d Uncertainty).

It is important that the analyte to be detected in PCR be in the same matrix as test samples destined for use in the assay. For example, if the assay is to be used for demonstrating freedom of an agent in a matrix known for PCR-inhibitory activity (such as semen with extenders), it is particularly important to thoroughly evaluate repeatability.

When new batches from new manufacturers of oligonucleotides or other reagents are introduced into the assay, the repeatability of the assay needs to be re-established on each occasion.

3. Determination of analytical specificity and sensitivity

Analytical specificity is defined as the ability of an assay to distinguish the target agent from other infectious agents. This ability is determined by analysing genetically related pathogens and clinical material obtained from animals with diseases that may mimic that for which the assay is being designed. It is desirable to obtain field samples from infected animals, but this may prove difficult or even impossible. In such cases viruses grown in cell culture can be used. Acceptable cross-reactivity is largely dependent on the intended purpose of the test and must be determined for each case. It is useful to perform '*in silico*' studies as an adjunct to laboratory assessment.

Analytical sensitivity (or limit of detection) is defined as the smallest amount of an agent detected by the assay, and may be represented as number of genome copies, infectious dose, colony-forming units, plaque forming units, etc. of the agent that can be detected and distinguished from a zero result. To determine analytical sensitivity, an end-point dilution is used until the assay can no longer detect the target in question in more than 5% of the replicates. Cloned fragments of the PCR products in question can be used as standard samples, either as DNA or for RNA targets, the RNA being transcribed *in vitro* into DNA. Estimates of analytical sensitivity can vary substantially for the same assay when different sample matrices are used. When setting up a dilution series, it is important to use a diluent that has qualities that are similar to the sample matrix, i.e. dilute positive semen in negative semen and not in buffer.

E. ASSAY VALIDATION – PART 2

Performance characteristics (or assay parameters) give information about how a method functions under specified conditions. Some typical performance characteristics are given in Chapter 1.1.4.

1.   Determining assay performance characteristics

a)   Reference animal populations

i)   *Negative reference animals*

True negative samples, i.e. samples from animals that have had no possible exposure to the agent, can sometimes be difficult to obtain. Often it is possible to collect samples from countries that have eradicated the disease in question. It is important that the negative samples obtained be representative of the samples that will be analysed, i.e. species, age, sex, breed, etc.

ii)   *Positive reference animals*

It is generally problematic to find positive reference animals in sufficient numbers. Naturally infected or experimentally infected animals are needed and their positive status is best demonstrated by isolation of the agent. Before using experimentally infected animals, please see Chapter 1.1.4.

iii)   *Reference animal status determined by other assays*

The term 'gold standard' is commonly used to describe any standard of comparison and should be limited to methods that unequivocally classify animals as infected or uninfected. New PCR assays are generally expected to outperform any already existing 'gold standard' method and thus the established 'gold standard' may not suitable to use as a comparison. This is especially true when demonstrating that a negative reference animal is truly negative. Validation of molecular tests by comparing them to a 'gold standard' test may be complicated by the PCR being more sensitive, resulting in apparent reduced specificity. To an extent this may be resolved by assessing sample derivation, clinical history and sequencing any PCR products to confirm identity.

2.   Threshold determination

Diagnostic sensitivity (DSe; proportion of known infected reference animals that test positive in the assay) and specificity (DSp; proportion of known uninfected reference animals that test negative in the assay). The number of reference samples required to determine estimates and allowable error of both DSe and DSp can be calculated. To do this, a reasonable prediction of both DSe and DSp must be used. Generally, confidence in the estimate is set at 95%. However, no formula can account for the numerous host/organism factors that can affect the outcome of the test. The number of samples to determine estimates of DSe and DSp is outlined in Chapter 1.1.4. For a disease that is not endemic or widespread, it may be difficult initially to obtain the number of samples to achieve a satisfactory confidence interval; but over time, accrual of additional data will enhance confidence in the threshold. The use of spiked samples in PCR is a last resort because such samples might not be representative of naturally infected samples. If samples from naturally infected animals are unavailable, infections induced by means that mimic natural infections may provide samples that are useful. An example is tick-borne infection induced by exposure to infected ticks.

It is not always possible to conform to suggested guidelines (e.g. OIE recommendations on test validation). Faced with low numbers of samples for test evaluation, or for tests with no gold standard, one approach is to introduce molecular tests as 'partially validated' and then add validation data if significant numbers of clinical samples are tested. In this system, positives are confirmed by other means, such as isolation of the pathogen in question or sequencing, and a sample of negatives is also confirmed as suitable (non-inhibitory) for PCR testing using control genes. This principle of 'on-going' validation allows rapid introduction of new tests and reduces the cost of validation. This process must be used under defined conditions. It is only applicable when there is sound evidence from testing an appropriate range of known cultures, spiked samples (to provide analytical data) and some clinical samples (to show that the target is available in particular tissues) that a test may be released as partially validated (15).

F.   ASSAY VALIDATION – PART 3

1.   Establishing reproducibility of the assay

Reproducibility is an important parameter in assay precision. Reproducibility is determined in several laboratories using the identical assay (protocol, reagents and controls). Each of at least three laboratories test the same panel of samples (minimum of 20 samples), with identical aliquots going to each laboratory. This effort will yield estimates of ruggedness of the assay. Reproducibility estimates for the assay are essential before it can be considered valid for deployment to other laboratories.

Currently, reproducibility is rarely completely evaluated in veterinary diagnostic laboratories carrying out PCR assays. Traditionally, many laboratories have used tests developed in-house, probably for practical reasons. When possible, published standardised and validated methods, especially by OIE reference laboratories, ECRLs

or National Laboratories, should be followed. In addition inter-laboratory validation processes should be carried out. This work will help to standardise assays, allowing harmonised diagnostic activity in various countries.

F. ASSAY VALIDATION – PART 4

1. Programme implementation

Reference laboratories play a major role in the implementation of new or validating existing molecular assays. OIE Reference Laboratories ECRLs and National Laboratories are urged to assist in the implementation of promising new assays for their disease of interest. An example is the assistance provided by the OIE and CDRL to implement avian influenza molecular diagnostics in Europe.

2. Monitoring validity of assay performance

a) Interpretation of test results – factors affecting assay validity

A primary factor affecting interpretation of test results is the prevalence of the analyte in the target population. A PCR assay that is highly precise and accurate, with estimates of DSe and DSp approaching 99%, may still provide false inferences (see Chapter 1.1.4). For nucleic acid assays, false-positive results are of particular concern in low prevalence populations. In this instance, it may be necessary to confirm PCR-positive results by sequence analysis of the amplified product to assist in correcting errors due to nonspecific target or primer binding.

b) Maintenance of validation criteria

When the assay is used as a routine test, it is important to maintain the internal QC. The assay needs to be consistently monitored for repeatability and accuracy. Reproducibility between laboratories (proficiency testing) is recommended by the OIE to be completed at least twice a year (16). This testing is usually administered by a reference laboratory that distributes panels of samples, receives the results from the laboratories, analyses the data, and reports the results back to the laboratories. If the assay is to be applied in another geographical region and/or population, it might be necessary to revalidate or document equivalency under the new conditions. Revalidation or equivalency should be determined, if the test is applied to a different sample matrix, e.g. validated on blood and used on another tissue, or validated for cattle tissue and used on another species. Different extraction protocols may be needed if a different species or tissues are tested; which possibly contain different inhibitory factors. This is especially true for PCR assays as it is very common for point mutations to occur in many infectious agents (i.e. RNA viruses). Mutations, which may occur within the primer or probe sites, can affect the efficiency of the assay and, by doing so; the established performance criteria are no longer valid. It is also advisable to regularly confirm the target sequence at the selected genomic regions for national or regional isolates of the infectious agents. This is especially true for the primer sites, to ensure that they remain stable so that the validation of the assay cannot be questioned. Validation and estimation of robustness may need to be repeated when the test is transferred from the developing laboratory to the field as the conditions may be less than optimal and the staff less experienced.

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NB: There is an OIE Collaborating Centre for the Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

LABORATORY METHODOLOGIES FOR BACTERIAL ANTIMICROBIAL SUSCEPTIBILITY TESTING

SUMMARY

Historically, medical practitioners and veterinarians selected antimicrobials to treat bacterial infectious diseases based primarily on past clinical experiences. However, with the increase in bacterial resistance to traditionally used antimicrobials, it has become more difficult for clinicians to empirically select an appropriate antimicrobial agent (15). As a result, in vitro *antimicrobial susceptibility testing (AST) of the relevant bacterial pathogens, from properly collected specimens, should use validated methods. Thus, AST is an important component of prudent antimicrobial use guidelines in animal husbandry worldwide and veterinarians in all countries should have these data available for informed decision-making (1).*

Although a variety of methods exist, the goal of in vitro *antimicrobial susceptibility testing is to provide a reliable predictor of how an organism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, aids in developing antimicrobial use policy, and provides data for epidemiological surveillance. Such epidemiological surveillance data provide a base to choose the appropriate empirical treatment (first-line therapy) and to detect the emergence and/or the dissemination of resistant bacterial strains or resistance determinants in different bacterial species. The selection of a particular AST method is based on many factors such as validation data, practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preference.*

The use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the speed and accuracy of susceptibility testing. Numerous DNA-based assays are being developed to detect bacterial antibiotic resistance at the genetic level. These methods, when used in conjunction with phenotypic analysis, offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes and can be used in tandem with traditional laboratory AST methods.

INTRODUCTION

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the World Organisation for Animal Health (OIE), the Food and Agriculture Organisation (FAO) and the World Health Organization (WHO) as a serious global human and animal health problem. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is, however, an increasingly troublesome situation due to the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and even commensal organisms.

Historically, many infections could be treated successfully based on the clinician's past clinical experience (i.e. empirical therapy). However, this is becoming more the exception than the rule. Resistance has been observed to essentially all of the antimicrobial agents currently approved for use in human and veterinary clinical medicine. This, combined with the variety of antimicrobial agents currently available, makes the selection of an appropriate agent an increasingly more challenging task. This situation has made clinicians more dependent on data from *in vitro* antimicrobial susceptibility testing, and highlights the importance of the diagnostic laboratory in clinical practice.

There is a number of antimicrobial susceptibility testing (AST) methods available to determine bacterial susceptibility to antimicrobials. The selection of a method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preference. Standardisation and harmonisation of AST methodologies, used in epidemiological surveillance of antimicrobial drug resistance, are critical if data are to be

compared among national or international surveillance/monitoring programmes of OIE Member Countries. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method. In the absence of standardised methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared. The method used to select samples for inclusion in antimicrobial resistance surveillance programmes, as well as the methods use for primary bacterial isolation, are also important factors that should be standardised or harmonised to allow direct comparison of data between different regions; consideration of these issues is addressed in an OIE document (17).

As the science of AST has progressed, a greater understanding of the multiple factors that could affect the overall outcome of susceptibility testing has become clearer. This chapter provides guidelines and standardisation for AST methodologies, and interpretation of antimicrobial susceptibility test results.

1.   Test requirements

In order to achieve standardisation of AST methods and comparability of AST results, the following requirements apply:

i)   the use of standardised AST methods and the harmonisation of AST test parameters (including choice of antimicrobial agents and subsequent interpretive criteria) are essential,

ii)   standardised AST methods, including all critical specifications and interpretive criteria, should be clearly defined, documented in detail and used by all participating laboratories,

iii)   all AST methods should generate accurate and reproducible data,

iv)   all data should be reported quantitatively,

v)   establishment of national or regional designated laboratories is essential for the coordination of AST methodologies, interpretations and appropriate operational techniques used to ensure accuracy and reproducibility (e.g. quality controls),

vi)   microbiological laboratories should implement and maintain a formal quality management programme (see Chapter 1.1.3 Quality management in veterinary testing laboratories),

vii)   laboratories should have acquired a third party accreditation that includes the AST methodologies to be used within the scope of that accreditation. The accreditation body should meet accepted international Laboratory Accreditation Cooperation [ILAC]) standards and guidelines regarding the standards used for the accreditation process. The accreditation standards used should include the requirement for participation in proficiency testing programmes,

viii) specific bacterial reference/quality control strains are essential for determining intra- and inter-laboratory quality control, quality assurance and proficiency testing.

2.   Selection of antimicrobials for testing and reporting

Selecting the appropriate antimicrobials for susceptibility testing can be difficult given the vast numbers of agents available. The following guidelines should be noted:

i)   the FAO/OIE/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance recommends creating a list of veterinary and human critically important antimicrobials for susceptibility testing and reporting,

ii)   selection of the most appropriate antimicrobials is a decision best made by each OIE Member Country in consultation with the appropriate bodies and organisations,

iii)   antimicrobials in the same class may have similar *in-vitro* activities against select bacterial pathogens. In these cases, a representative antimicrobial should be selected that predicts susceptibility to other members of the same class,

iv)   certain microorganisms can be intrinsically resistant to particular antimicrobial classes; therefore it is unnecessary and misleading to test certain agents for activity *in vitro.* The type of intrinsic resistance has to be determined for these organisms via either the scientific literature or through testing,

v)   the number of antimicrobials to be tested should be limited in order to ensure the relevance and practicality of AST.

Periodic review of microorganisms that are currently predictably susceptible to certain antimicrobial agents is recommended to ensure that emergent, unexpected resistance is detected. Emerging resistance may also be suspected following poor response to a standard antimicrobial treatment regime.

3. Antimicrobial susceptibility testing methodologies

The following requirements should be respected:

i)   bacteria subjected to AST must be isolated in pure culture from the submitted sample,

ii) standard reference methods should be used for identification so that the subject bacteria are consistently and correctly identified to the genus and/or species level,

iii) bacterial isolates considered to be the most important and a sampling of other isolates, should be stored for future analysis (either lyophilisation or cryogenic preservation at –70°C to –80°C).

The following factors influencing AST methods should be determined, optimised, and documented in a detailed standard operating procedure:

i)   once the bacterium has been isolated in pure culture, the optimum concentration of the inocula must be determined to obtain accurate susceptibility results. Bacteria or other organisms used in AST testing should be from a fresh culture,

ii) the composition and preparation of the agar and broth media used (e.g. pH, cations, thymidine or thymine, use of supplemented media). Performance and sterility testing of media lots should also be determined and documented as well as employed procedures,

iii) the content of antimicrobial in the carrier (antibiotics used in microtitre plates, disk, strip, tablet),

iv) composition of solvents and diluents for preparation of antimicrobial stock solutions,

v)   growth and incubation conditions (time, temperature, atmosphere e.g. CO_2),

vi) agar depth,

vii) number of concentrations tested per broth and agar dilution,

viii) the test controls to be used, including the reference organisms used,

ix) the subsequent interpretive criteria.

For these reasons, special emphasis has to be placed on the use of documented procedures and validated, well documented methods, as sufficient reproducibility can be attained only through the use of such methodology.

4. Selection of antimicrobial susceptibility testing methodology

The selection of an AST methodology may be based on the following factors:

i)   ease of performance,

ii) flexibility,

iii) adaptability to automated or semi-automated systems,

iv) cost,

v)   reproducibility,

vi) reliability,

vii) accuracy,

viii) the organisms and the antimicrobials of interest in that particular OIE Member Country,

ix) availability of suitable validation data for the range of organisms to be susceptibility tested.

5. Antimicrobial susceptibility testing methods

The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly (14, 15):

i)   disk diffusion,

ii) broth dilution,

iii) agar dilution.

a) Disk diffusion method

Disk diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from disks, tablets or strips, into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture (see section 3.i). Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk.

The diffusion of the antimicrobial agent into the seeded culture media results in a gradient of the antimicrobial. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. The diameter of this zone of inhibition around the antimicrobial disk is related to minimum inhibitory concentration (MIC) for that particular bacterium/antimicrobial combination; the zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antibiotic in the disk and its diffusibility.

Note: Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone of inhibition are not acceptable AST methodology.

- **Considerations for the use of the disk diffusion methodology**

Disk diffusion is straightforward to perform, reproducible, and does not require expensive equipment. Its main advantages are:

i)   low cost,

ii)   ease in modifying test antimicrobial disks when required,

iii)   can be used as a screening test against large numbers of isolates,

iv)   can identify a subset of isolates for further testing by other methods, such as determination of MICs.

Manual measurement of zones of inhibition may be time-consuming. Automated zone-reading devices are available that can be integrated with laboratory reporting and data-handling systems. The disks should be distributed evenly so that the zones of inhibition around antimicrobial discs in the disc diffusion test do not overlap to such a degree that the zone of inhibition cannot be determined. Generally this can be accomplished if the discs are no closer than 24 mm from centre to centre, though this is dependent on disk concentration and the ability of the antimicrobial to diffuse in agar.

b) Broth and agar dilution methods

The aim of the broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial that inhibits the growth of the bacterium being tested (MIC, usually expressed in mg/ml or mg/litre). However, the MIC does not always represent an absolute value. The 'true' MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Therefore, MIC determinations performed using a dilution series may be considered to have an inherent variation of one dilution.

Antimicrobial ranges should encompass both the interpretive criteria (susceptible, intermediate and resistant) for a specific bacterium/antibiotic combination and appropriate quality control reference organisms.

Antimicrobial susceptibility dilution methods appear to be more reproducible and quantitative than agar disk diffusion. However, antibiotics are usually tested in doubling dilutions, which can produce inexact MIC data.

Any laboratory that intends to use a dilution method and set up its own reagents and antibiotic dilutions should have the ability to obtain, prepare and maintain appropriate stock solutions of reagent-grade antimicrobials and to generate working dilutions on a regular basis. It is then essential that such laboratories use quality control organisms (see below) to assure accuracy and standardisation of their procedures.

- **Broth dilution**

Broth dilution is a technique in which a suspension of bacterium of a predetermined optimal or appropriate concentration is tested against varying concentrations of an antimicrobial agent (usually serial twofold dilutions) in a liquid medium of predetermined, documented formulation. The broth dilution method can be performed either in tubes containing a minimum volume of 2 ml (macrodilution) or in smaller volumes using microtitration plates (microdilution). Numerous microtitre plates containing prediluted antibiotics within the

wells are commercially available. The use of identical lots in microdilution plates may assist in the minimisation of variation that may arise due to the preparation and dilution of the antimicrobials from different laboratories. The use of these plates, with a documented test protocol, including specification of appropriate reference organisms, will facilitate the comparability of results among laboratories.

Due to the fact that most broth microdilution antimicrobial test panels are prepared commercially, this method is less flexible than agar dilution or disk diffusion in adjusting to the changing needs of the surveillance/monitoring programme.

Because the purchase of antimicrobial plates and associated equipment may be costly, this methodology may not be feasible for some laboratories.

- **Agar dilution**

Agar dilution involves the incorporation of varying concentrations of antimicrobial agent into an agar medium, usually using serial twofold dilutions, followed by the application of a defined bacterial inoculum to the agar surface of the plate. These results are often considered as the most reliable for the determination of an MIC for the test bacterium/antimicrobial combination.

The advantages of agar dilution methods include:

i) the ability to test multiple bacteria, except bacteria that swarm, on the same set of agar plates at the same time,

ii) the potential to improve the identification of MIC endpoints and extend the antibiotic concentration range,

iii) the possibility to semi-automate the method using an inoculum-replicating apparatus. Commercially produced inoculum replicators are available and these can transfer between 32 and 36 different bacterial inocula to each agar plate,

Agar dilution methods also have certain disadvantages, for example:

i) if not automated, they are very laborious and require substantial economic and technical resources,

ii) once the plates have been prepared, they normally should be used within a week,

iii) the endpoints are not always easy to read nor is the purity of the inoculum easy to verify.

Agar dilution is often recommended as a standardised AST method for fastidious organisms (8), such as anaerobes, *Campylobacter* and *Helicobacter* species.

c)   Other bacterial AST and specific antimicrobial resistance tests

Bacterial antimicrobial MICs can also be obtained using commercially available gradient strips that diffuse a predetermined antibiotic concentration. However, the use of gradient strips can be very expensive and MIC discrepancies can be found when testing certain bacteria/antimicrobial combinations compared with agar dilution results (2, 5).

Regardless of the AST method used, the procedures should be documented in detail to ensure accurate and reproducible results, and appropriate reference organisms should always be tested every time AST is performed in order to ensure accuracy and validity of the data.

The appropriate AST choice will ultimately depend on the growth characteristics of the bacterium in question. In special circumstances, novel test methods and assays may be more appropriate for detection of particular resistance phenotypes. For example, chromogenic cephalosporin-based tests (8) (e.g. nitrocefin) may provide more reliable and rapid results for beta-lactamase determination in certain bacteria, whereas inducible clindamycin resistance in *Staphylococcus* spp. may be detected using a disk diffusion method employing standard erythromycin and clindamycin disks in adjacent positions and measuring the resultant zones of inhibition (e.g. D-zone) (18).

Similarly, extended-spectrum beta-lactamase (ESBL) (8) activity in certain bacteria can also be detected by using standard disk diffusion susceptibility test methods incorporating specific cephalosporins (cefotaxime and ceftazidime) in combination with a beta-lactamase inhibitor (clavulanic acid) and measuring the resulting zones of inhibition. Additionally, chloramphenicol resistance attributed to production of chloramphenicol acetyl transferase can be detected in some bacteria via rapid tube or filter paper tests within 1–2 hours (8). Also penicillin-binding protein 2a (PBP 2a) can be detected in methicillin resistant staphylococci with a latex agglutination test (13).

d) Future directions in antimicrobial susceptibility/resistance detection

The use of genotypic approaches for detection of antimicrobial resistance genes has been promoted as a way to increase the rapidity and accuracy of susceptibility testing (3). Numerous DNA-based assays are being developed to detect bacterial antibiotic resistance at the genetic level. The newest and perhaps most state-of-the-art approach is to predict antimicrobial resistance phenotypes via identification and characterisation of the known genes that encode specific resistance mechanisms.

Methods that employ the use of comparative genomics, genetic probes, microarrays, nucleic acid amplification techniques (e.g. polymerase chain reaction [PCR]), and DNA sequencing offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes (3, 4, 10). Genotypic methods have been successfully applied to supplement traditional AST phenotypic methods for other organisms including methicillin-resistant staphylococci, vancomycin-resistant enterococci, and detection of fluoroquinolone resistance mutations (3, 4, 10). PCR methods have also been described for beta-lactamases, aminoglycoside inactivating enzymes, and tetracycline efflux genes (4, 10).

Technological innovations in DNA-based diagnostics should allow for the detection of multiple resistance genes and/or variants during the same test. The development of rapid diagnostic identification methods and genotypic resistance testing should help reduce the emergence of antimicrobial resistance, by enabling the use of the most appropriate antimicrobial when therapy is initiated. However, DNA techniques have to be demonstrated to be complimentary to AST methods and results.

Additionally, new technological advances may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data for surveillance and monitoring programmes. However, despite the new influx of genotypic tests, documented and agreed upon phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens.

6. Antimicrobial susceptibility breakpoints and zone of inhibition criteria

The objective of *in-vitro* AST is to predict how a bacterial pathogen may respond to the antimicrobial agent *in vivo*. The results generated by bacterial *in-vitro* antimicrobial susceptibility tests, regardless of whether disk diffusion or dilution methods are used, are generally interpreted and reported as resistant, susceptible or intermediate to the action of a particular antimicrobial. No single formula for selection of optimal breakpoints has been established. The process involves a review of existing data and is influenced by the subjectivity of individuals tasked with selecting the appropriate breakpoints.

Generally, antimicrobial susceptibility breakpoints are established by national standards organisations, professional societies or regulatory agencies. The relevant documents should be consulted. However, there can be notable differences in breakpoints for the same antimicrobial agent within and among countries due to differences between standards setting organisations and regulatory agencies and because of regional or national decisions on dosing regimens (6).

As mentioned previously, antimicrobial susceptibility testing results should be recorded quantitatively:

i)   as distribution of MICs in milligrams per litre or mg/ml,

ii)   or as inhibition zone diameters in millimetres.

The following two primary factors enable a bacterium to be interpreted as susceptible or resistant to an antimicrobial agent:

i)   The development and establishment of quality control ranges (8), using diffusion when possible and dilution testing, for quality control reference microorganisms.

        Establishment of quality control ranges is essential for validating test results obtained using a specific AST method. The allowable interpretive category ranges for the reference organisms should be established prior to determining breakpoints for susceptibility or resistance. The use of reference organisms is a quality control and quality assurance activity. However, it is only necessary to require the used of reference organisms.

ii)   The determination of the appropriate interpretive criteria regarding establishment of breakpoints (8).

        This involves the generation of three distinct types of data:

        •   MIC population distributions of the relevant microorganisms,

        •   pharmacokinetic parameters and pharmacodynamic indices of the antimicrobial agent,

        •   results of clinical trials and experience.

The interpretation of the data involves creating a scattergram from the bacterial population distribution (representative bacterial species), by plotting the zone of inhibition against the logarithm to the base 2 of the MIC for each bacterial pathogen. The selection of breakpoints is then based on multiple factors, including regression line analysis that correlates MICs and zone diameters of inhibition, bacterial population distributions, error rate bounding, pharmacokinetics, and ultimately, clinical verification.

The development of a concept known as 'microbiological breakpoints', or 'epidemiological cut-off points', which is based on the population distributions of the specific bacterial species tested, may be more appropriate for some antimicrobial surveillance programmes. In this case, bacterial isolates that deviate from the normal wild-type susceptible population would be designated as resistant, and shifts in susceptibility to the specific antimicrobial/bacterium combination could be monitored (12). There is a great advantage in the recording of quantitative susceptibility data in that such data may be analysed according to clinical breakpoints as well as by using epidemiological cut-off values.

7. Antimicrobial susceptibility testing guidelines

A number of standards and guidelines are currently available for antimicrobial susceptibility testing and subsequent interpretive criteria throughout the world (6). Amongst others, these include standards and guidelines published by:

Clinical Laboratory and Standards Institute (CLSI/NCCLS, USA),
British Society for Antimicrobial Chemotherapy (BSAC, UK),
Comité de l'Antibiogramme de la Société française de Microbiologie (CASFM, France),
Swedish Reference Group for Antibiotics (SIR, Sweden),
Deutsches Institut für Normung (DIN, Germany),
Japanese Society for Chemotherapy (JSC, Japan),
Commissie richtlijnen gevoeligheidsbepalingen (CRG, the Netherlands).

At this time, only the CLSI/NCCLS has developed protocols for susceptibility testing of bacteria of animal origin and determination of interpretive criteria (8). However, protocols and guidelines are available from a number of standards organisations and professional societies (i.e. Clinical and Laboratory Standards Institute, British Society for Antimicrobial Chemotherapy, Japan Society for Chemotherapy [JSC], Swedish Reference Group for Antibiotics [SIR], Deutshes Institute für Normung, Comité de L'Antibiogramme de la Société Française de Microbiologie, Werkgroep richtlijnen gevoeligheidsbepalingen, and others) for susceptibility testing for similar bacterial species that cause infections in humans. It is possible that such guidelines can be adopted for susceptibility testing for bacteria of animal origin, but each country must evaluate its own AST standards and guidelines. Additionally, efforts focusing on both standardisation and harmonisation of susceptibility/resistance breakpoints on an international scale are progressing. These efforts have primarily focused on the adoption of the standards and guidelines of the CLSI, which provide laboratories with methods and quality control values enabling comparisons of AST methods and generated data (8, 16). For those OIE Member Countries that do not have standardised AST methods in place, the adoption of CLSI standards would be an appropriate initial step towards acceptable methods and harmonisation.

As a first step towards comparability of monitoring and surveillance data, Member Countries should be encouraged to strive for harmonised and standardised programme design (14). Data from countries using different methods and programme design may otherwise not be directly comparable (7, 14). Notwithstanding this, data collected over time in a given country may at least allow the detection of emergence of antimicrobial resistance or trends in prevalence of susceptibility/resistance in that particular country (11). However, if results achieved with different AST methods are to be presented side by side, then comparability of results must be demonstrated and consensus on interpretation achieved.

Note: This will be best accomplished by the use of accurate and reliable documented AST methods used in conjunction with monitoring of AST performance while using well characterised reference microorganisms among participating laboratories.

8. Comparability of results

To determine the comparability of results originating from different surveillance systems, results should be reported quantitatively including information on the performance of the methods, the reference organisms and the antimicrobial.

AST data, consisting of cumulative and ongoing summary of susceptibility patterns (antibiograms) among clinically important and surveillance microorganisms should be created, recorded and analysed periodically at regular intervals (9). Data must also be presented in a clear and consistent manner so that both new patterns of resistance can be identified and atypical findings confirmed or refuted. This data should be available on a central data bank and published yearly.

Cumulative AST data will be useful in monitoring susceptibility/resistance trends in a region over time and assessing the effects of interventions to reduce antimicrobial resistance.

9. Quality control and quality assurance

Adequate quality control/quality assurance systems should be established in AST performing laboratories:

i)   quality control refers to the operational techniques that are used to ensure accuracy and reproducibility of AST,

ii) quality assurance.

The following components should be determined and monitored:

i)   precision of the AST procedure,

ii) accuracy of the AST procedure,

iii) qualifications, competence, and proficiency of the laboratory personnel, as well as the personnel that interpret the results and those that are involved in monitoring of antimicrobial resistance,

iv) performance of the appropriate reagents.

The following requirements should be respected:

i)   Strict adherence to specified and documented techniques in conjunction with quality control (i.e. assurance of performance and other critical criteria) of media and reagents.

ii) Record keeping of:

• lot numbers of all appropriate materials and reagents,

• expiration dates of all appropriate materials and reagents,

• equipment calibration and monitoring,

• critical specifications for AST performance (reference results, time, temperature etc.).

iii) The appropriate reference microorganism(s) should always be used regardless of the AST method employed.

iv) Reference microorganisms are to be obtained from a reliable source for example, from the American Type Culture Collection (ATCC[®]), reliable commercial sources, or institutions with demonstrated reliability to store and use the organisms correctly.

v)   Reference microorganisms should be catalogued and well characterised, including stable defined antimicrobial susceptibility phenotypes. Records regarding these reference organisms should include the established resistant and susceptible ranges of the antimicrobials to be assayed, and the reference to the method(s) by which these were determined.

vi) Laboratories involved in AST should use the appropriate reference microorganisms in all AST testing.

vii) Reference strains should be kept as stock cultures from which working cultures are derived and should be obtained from national or international culture collections. Reference bacterial strains should be stored at designated centralised or regional laboratories. Working cultures should not be subcultured from day to day as this introduces contamination and the method of producing working cultures should ensure that stock cultures are rarely used. This may be accomplished with the production of an intermediate stock of cultures derived from the original cultures that are used to crate day-to-day working cultures.

viii) The preferred method for analysing the overall performance of each laboratory should test the working stock of the appropriate reference microorganisms on each day that susceptibility tests are performed.

Because this may not always be practical or economical, the frequency of such tests may be reduced if the laboratory can demonstrate that the results of testing reference microorganisms using the selected method are reproducible. If a laboratory can document the reproducibility of the susceptibility testing methods used, testing may be performed on a weekly basis. If concerns regarding accuracy, reproducibility, or method validity emerge, the laboratory has a responsibility to determine the cause(s) and repeat the tests using the reference materials. Depending on the cause(s), daily reference material use and any other corrective action may be re-initiated.

ix) Reference microorganisms should be tested each time a new batch of medium or plate lot is used and on a regular basis in parallel with the microorganisms to be assayed.

x)   Appropriate biosecurity issues should be addressed in obtaining and dispersing microorganisms to participating laboratories.

10. External proficiency testing

To ensure that reported antimicrobial susceptibility data are accurate; OIE Member Countries should initiate an inter-laboratory proficiency testing programme. External proficiency testing can be carried out on a national basis. Laboratories in Member Countries are also encouraged to participate in international inter-laboratory comparisons (e.g. Enter-Net) (6). All bacterial species subjected to AST should be included.

Countries should appoint or establish designated reference or national laboratories that are responsible for:

i)   monitoring the quality assurance programmes of laboratories participating in surveillance and monitoring of antimicrobial resistance,

ii)   characterising and supplying to those laboratories a set of reference microorganisms,

iii)   creating, managing, and distributing samples to be used in external proficiency testing,

iv)   creating a central database available on the internet (e.g. European Antimicrobial Resistance Surveillance System [EARSS]) that contains the different susceptibility/resistance profiles for each bacterial species under surveillance.

11. Conclusion

Although a variety of methods exist, the goal of *in-vitro* antimicrobial susceptibility testing is the same: to provide a reliable predictor of how a microorganism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, provides data for surveillance, and aids in developing antimicrobial use policies.

In-vitro antimicrobial susceptibility testing can be performed using a variety of formats, the most common being disk diffusion, agar dilution, broth macrodilution, broth microdilution, and a concentration gradient test (e.g. E test®). Each of these procedures requires the use of specific testing conditions and methods, including media, incubation conditions and times, and the identification of appropriate quality control organisms along with their specific QC ranges. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method. In the absence of standardised methods or reference procedures, antimicrobial susceptibility/resistance results from different laboratories cannot be reliably compared.

The use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the rapidity and accuracy of susceptibility testing. Additionally, new technological advances may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data into surveillance and monitoring programs. Despite the new influx of genotypic tests however, standardised phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens.

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NB: There is an OIE Reference Laboratory for Antimicrobial resistance (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

BIOTECHNOLOGY IN THE DIAGNOSIS OF INFECTIOUS DISEASES AND VACCINE DEVELOPMENT

INTRODUCTION

Molecular biological methods have become increasingly applicable to the diagnosis of infectious diseases and vaccine development. To become widely used the methods need to be easy, safe, sensitive, reproducible and eventually automated to facilitate the evaluation of large numbers of samples.

The purpose of this chapter is to provide general background information for the nonspecialist. Two issues of the OIE Scientific and Technical Review *are concerned with biotechnology and the diagnosis of animal diseases, and may be consulted for a more detailed review (*103, 104*). The following is an outline of the topics briefly reviewed in this chapter.*

A.   Detection of nucleic acids
*1.   Polymerase chain reaction (*PCR*) and real-time PCR*
2.   Diagnosis by restriction fragment length polymorphisms and related DNA-based approaches
3.   Diagnosis by DNA probes and DNA microarray technology
4.   Nucleic acid extraction

B.   Detection of protein
1.   Immunohistochemistry
2.   Immunoblotting
*3.   Antigen-capture enzyme-linked immunosorbent assay (*ELISA*)*
4.   Proteomics

C.   Antibody detection
*1.   Competitive ELISA (*C-ELISA*)*
2.   Production of antigens by recombinant DNA technology

D.   Vaccines
1.   Gene deletion vaccines – bacteria
2.   Marker vaccines and companion diagnostic tests
3.   Virus-vectored vaccines
4.   DNA vaccines
5.   Other developments in vaccine technology

A. DETECTION OF NUCLEIC ACIDS

1.   Polymerase chain reaction (PCR) and real-time PCR

The PCR exploits natural DNA replication mechanisms and results in the *in-vitro* production of large quantities of a desired sequence of DNA from a complex mixture of heterogeneous sequences (134). PCR can amplify a selected region of 50 to several thousand base pairs into billions of copies. A detailed discussion on the methodology and applications of PCR is given in Mullis *et al.* (93).

The amplification of DNA by the PCR is accomplished via a cyclic succession of incubation steps at different temperatures. The target DNA is first heat-denatured to separate the two complementary strands to provide a single-stranded template. Specific primers (short synthetic molecules of DNA complementary to both strands and

flanking the target sequences) are then annealed to the single-stranded template at low temperature and extended with DNA polymerase at an intermediate temperature. Once the polymerase has synthesised a new strand of DNA, the product is separated from the template by heating to a higher temperature. These steps, referred to as cycles, are repeated 20–40 times, resulting in amplification of target DNA sequences. The key to the geometric amplification of target DNA sequences by the PCR is the selection of paired primers that, when extended, will create additional reciprocal primer-annealing sites for primer extension in subsequent cycles. To detect RNA (e.g. RNA viruses), a cDNA copy of the RNA must first be made using reverse transcriptase (RT). The cDNA then acts as the template for amplification by the PCR. This technique is referred to as RT-PCR.

Any PCR product generated has, by definition, a characteristic size. Its identity is generally confirmed using DNA probes (see below), or restriction digests, which can be used to provide RFLPs (see above). More commonly, since the advent of automated cycle sequencing techniques, identification is via direct sequencing of the PCR product. For example, sequencing has been used in the virulence typing of avian influenza A virus, in which virulence-associated structural motifs at the haemagglutinin gene cleavage site are reliable indicators of high pathogenicity in chickens (63). The sensitivity of a PCR may be enhanced by the use of a second set of primers to amplify a sub-fragment from the PCR product of the first reaction. This technique is commonly referred to as 'nested PCR' and has been used to detect low levels of *Anaplasma marginale* in persistently infected cattle (158). However, the use of nested PCR can increase the rate of false-positive results.

PCR is a highly sensitive procedure for detecting infectious agents in host tissues and vectors, even when only a small number of host cells are infected. PCR can target and amplify a gene sequence that has become integrated into the DNA of infected host cells. It can also target and amplify unintegrated viral gene sequences. It is clear that PCR has a role in the testing of vaccines to detect contamination. However, it does not differentiate between viable and nonviable organisms or incomplete pieces of genomic DNA, and this may complicate interpretation of results and affect the applicability of PCR in this role.

PCR may prove to be very useful in the diagnosis of chronic-persistent infections, such as those caused by retroviruses (bovine leukaemia virus, caprine arthritis/encephalitis virus, etc.). These diseases present serious problems in terms of diagnosis and prevention since infected animals are a constant potential source for transmission.

When PCR is used for diagnosis, a great deal of care is required to avoid contamination of the samples because the exquisite sensitivity of the technique can easily lead to false-positive results. Multicentre studies have shown that positive samples are detected consistently between laboratories, but that false positives are frequently obtained with known negative samples, indicating the continuing presence of contamination problems (139). Systems have been developed to deal with this problem, for example the dUTP-UNG system (d-uracil triphosphate and uracil-N-glycosylase). This system uses an enzymatic reaction to specifically degrade PCR products from previous PCR amplification (in which dUTP has been incorporated) without degrading native nucleic acid templates (25). This, of course, does not exclude contamination of the sample with extraneous virus. A new generation of robotic workstations is now available where PCR reactions may be set up with only a single tube open at any one time. This greatly reduces the risk of contamination. It is also important to control for potential 'negative' results caused by the presence of interfering substances in the PCR reaction mixture or patient's sample by the inclusion of a template known to produce a PCR product (25). Use of these precautions allows the PCR to become a realistic option for the diagnostician.

To expand its utility in veterinary diagnostics and pathogen identifications, PCR has been extensively modified in the past years. PCR using broadly conserved primers is designed for identification of classes of pathogens. The best example is the use of sequences of the 16s rRNA gene, an evolutionarily conserved gene in bacterial species (52). Using PCR primers that are complementary to these conserved sequence regions, one can determine the presence of any bacteria of a desired class from the sample. It must be noted that a positive PCR result needs to be further characterised by hybridisation with species-specific probes, analysis by restriction enzyme digestion, or by sequencing. Similarly, consensus PCR is designed to use degenerate primers targeting conserved sequence regions or motifs of a group of related pathogens (169). Use of degenerate primers targeting the sequence regions of the herpesviral DNA polymerase gene has led to identification of many previously unrecognised herpesviruses in various animal species (40, 76). On the other hand, multiplex PCR is designed to use two or more primer pairs directed at pathogen-specific unique sequences within a single reaction for simultaneous detection of multiple pathogens that are of interest (41). Multiplex PCR has the advantage of a high degree of sensitivity and specificity. However, there have been reports that multiplexing can reduce sensitivity compared with single reactions, because of competition. If it is important to have a very sensitive assay, this should be considered.

Classical PCR methods for diagnosis of pathogens, both bacterial and viral, are now being complemented and in some cases replaced with real-time PCR assays. Real-time PCR monitors the accumulation of PCR product during the amplification reaction, thus enabling identification of the cycles during which near-logarithmic PCR product generation occurs. In other words, the assay can be used to reliably quantify the DNA or RNA content in a given sample. In contrast to conventional PCR, real-time PCR requires less manipulation, is more rapid than conventional PCR techniques, has a closed-tube format therefore decreasing risk of cross-contamination, is

highly sensitive and specific, thus retaining qualitative efficiency, and provides quantitative information. In many cases, the real-time PCR assays have proved to be more sensitive than existing reference methods (58, 175). The recent development of portable real-time PCR machines and assays (128) raises the exciting prospect of these techniques being used for rapid (less than 2 hours) diagnosis of disease outbreaks in the field.

Validation of PCR techniques is covered in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases. This chapter also discusses internal controls to insure the validity of PCR results.

2. Diagnosis by restriction fragment length polymorphisms and related DNA-based approaches

Serological tests that are commonly used to identify microorganisms may not distinguish between isolates of closely related pathogens, whether they be viruses, bacteria, fungi or parasites. A DNA-based procedure will offer the better discrimination that is often required and an appropriate starting point may be analyses for restriction fragment length polymorphisms (RFLP).

The RFLP approach is based on the fact that the genomes of even closely related pathogens are defined by variation in sequence. For example, the linear order of adjacent nucleotides comprising the recognition sequence of a specific restriction enzyme in one genome may be absent in the genome of a closely related strain or isolate.

In practice the RFLP procedure consists of isolating the target pathogen, extracting DNA or RNA (with subsequent reverse transcription to DNA) and then digesting the nucleic acid with one of a panel of restriction enzymes. The individual fragments within the digested DNA are then separated within a gel by electrophoresis and visualised by staining with ethidium bromide. Ideally each strain will reveal a unique pattern, or fingerprint. Many different restriction enzymes may be considered at the outset of a new piece of work, so that analyses of many molecular fingerprints from digestions with several individual restriction enzymes may be undertaken and combination of the best set of results will allow a comprehensive differentiation between strains or isolates. A good example of the application of this technique is the differentiation between rabies virus biotypes from dog or vampire bat origin in Latin America (83).

Of greater utility to the study of pathogens is a modification to the basic RFLP technique whereby the polymerase chain reaction (PCR) is incorporated as a preliminary step. The PCR method (described in more detail in Section 1 of this chapter) is used to amplify a specific region of the genome (known by the investigator to be variable in sequence between pathogens), which then serves as the template DNA for the RFLP technique. This new combination (PCR-RFLP) offers a much greater sensitivity for the identification of pathogens and is especially useful when the pathogen occurs in small numbers or is difficult to culture, two features that characterise the intestinal protozoan parasite *Cryptosporidium* spp. Both RFLP and, more especially, PCR-RFLP are immensely useful for the genotyping of strains of *Cryptosporidium* as they can identify sources of human infection and provide a commentary on their epidemiology and occurrence (16, 145). The involvement of specific strains or types in a disease outbreak can be thus defined and the epidemiological tracing of isolates within a country or between countries should be possible.

There are many other examples in which the RFLP/PCR-RFLP techniques are proving useful for discriminating between genotypes; for example, the fungus *Candida* (32), the porcine reproductive and respiratory syndrome virus (162) and the bacterium *Helicobacter pylori* (54).

The human pathogen *Candida krusei* provides a good illustration of the general application of a range of molecular techniques for differentiating isolates. Dassanayake *et al.* (32) investigated the genetic diversity of eleven oral isolates of *C. krusei* and identified five different genotypes by pulsed field gel electrophoresis (PFGE), nine genotypes by RFLP using the enzyme *Hin*fI, while DNA fingerprinting by the randomly amplified polymorphic DNA approach (RAPD-PCR) revealed three, eight or eleven genotypes depending on the primers used.

The incorporation of PFGE facilitates the separation of large (up to megabase size) fragments of DNA and can be a useful adjunct to the basic RFLP analysis. Jager *et al.* (66) used a combination of the rare-cutting restriction enzyme *Not*I and PFGE to characterise 80 isolates of *Coxiella burnetii* derived from animals and humans in Europe, USA, Africa and Asia. They distinguished 20 different restriction patterns and phylogenetic analysis of the different RFLP patterns revealed evolutionary relationships among groups that corresponded to the geographical origin of the isolates. No correlation between restriction group and the virulence of an isolate was detected in this study, but similar approaches on some other pathogens have made such a connection. Grigg and Boothroyd (53), for example, identified three restriction sites within the 35-fold-repetitive B1 locus that were capable of discriminating type I (mouse-virulent) from type II or III (mouse-avirulent) strains of *Toxoplasma gondii*.

RFLPs have clear value for use in epidemiological studies but more critical interpretation of RFLP data involves the construction of databases to determine whether the RFLP profiles are linked to factors such as virulence,

host range and clinical significance. In practice, it is usual not to rely on one restriction site but to use sites from several locations within the genome to classify the isolate. A continuing issue for veterinary diagnosticians is the correct assessment of any molecular differences found between isolates of a pathogen as the loss or acquisition of restriction endonuclease site(s) may not be associated with differences in the ability of the pathogen to cause disease, i.e. an RFLP difference may not be functionally significant, except as a distinguishing feature.

Polymorphic RAPD markers that define individual strains, etc. may be sequenced and thence used as a sequence-confirmed amplified region (SCAR). Thus conversion of an anonymous polymorphic marker to a SCAR means that a single PCR may be done to more simply identify a specific genome. Lewin *et al.* (75) used the approach to identify 19 unique multilocus genotypes among 29 strains of the protozoan, *Leishmania donovani*.

The techniques by which DNA from a pathogen may be detected and characterised continue to improve and evolve. The ultimate discriminatory procedure is that of genome sequencing. Sequencing of a well characterised portion of the genome is playing an important role in pathogen characterisation and epidemiological studies. Sequencing the products amplified by PCR using degenerate primers targeting a gene common to the viruses in the same family has become an important diagnostic tool, especially for identification of previously unrecognised members in the family. Sequencing of degenerate PCR amplicons from the herpesviral DNA polymerase gene is a good example (169). In a few cases the whole viral genome has been sequenced. For example, the outbreak of severe acute respiratory syndrome (SARS) and the sequencing of the 29, 751-base genome of the associated coronavirus (86) usefully revealed that the virus was only moderately related to other known coronaviruses, including two human coronaviruses and did not closely resemble any of the three previously known groups of coronaviruses. This degree of interrogation at the level of nucleic acid will not be available to study the majority of pathogens. Techniques such as RFLP, PCR-RFLP, RAPD-PCR and SCAR analyses will continue to play a central role in the identification of, and discrimination between, isolates of most pathogens.

3. Diagnosis by DNA probes and DNA microarray technology

Conventional DNA probing and microarray analysis are two sides of the same coin. Fundamental to both processes is the binding (hybridisation) of DNA, derived from a sample suspected of containing a pathogen (the 'unknown'), with highly characterised DNA derived in advance from a pathogen of interest (the 'known' DNA).

In conventional DNA probing the unknown DNA (or RNA), the target, is immobilised on a solid surface e.g. a filter. The known DNA, made into a probe by labelling or tagging it in some way, is in the liquid phase and is applied to the target. In microarray diagnosis it is the known DNA (large oligonucleotides or complementary DNA) that is the target, immobilised on a glass slide, and the unknown DNA, in the liquid phase, that is labelled to make a probe.

In conventional DNA probing the target can be nucleic acids extracted from clinical material or cultured cells and either (a) added to filters (a dot or slot blot) or (b), less conveniently in a diagnostic context, transferred to a filter after gel electrophoresis. The amount of pathogen in a clinical sample might be too low for detection. Consequently one might amplify the nucleic acid by PCR or reverse transcription PCR (RT-PCR), the PCR product being applied to a filter. In order to visualise a probe bound to its target, the probe can be labelled with a radioactive nuclide or, more commonly and safely, 'tagged' non-radioactively. For example, biotin or psoralen–biotin may be incorporated into the probe, bound probe being detected by addition of streptavidin linked to an enzyme for subsequent generation of colour or light (chemiluminescence).

A microarray is so-called because it can comprise 20,000 or more different known DNAs, each DNA being spotted onto glass slides, to form the array. Each spot is only around 10 µm in diameter. DNAs complementary to parts of selected genes of pathogens can be used to make the arrays (17). However, if large numbers of pathogens are to be investigated then it would be logistically easier to use large oligonucleotides. The microarray that was used to identify the SARS virus as being a coronavirus had oligonucleotides comprising 70 nucleotides (70-mer) (174). In microarray probing it is the sample from which a probe is made. Essentially nucleic acid is extracted from a sample and an (RT-) PCR performed using random oligonucleotide primers. In this way part of all the nucleic acids in the sample – both of host and pathogen origin – are amplified. These PCR products, representative of every nucleic acid in the sample, are labelled with a fluorescent dye and applied to the microarray. Under optimised conditions only the DNA derived from the pathogen will bind to the DNA on the glass slide. If one is interested in detecting only a particular pathogen or group of related pathogens then pathogen-specific oligonucleotides can be used to amplify these within the sample for probe production.

Microarrays for detecting pathogens can be designed for several levels of differentiation. In the case of oligonucleotide target DNAs one might initially design oligonucleotides to be able to detect and differentiate pathogens at the genus level. One would choose a number, perhaps 10 or so, of oligonucleotides with a high degree of sequence conservation (consensus oligonucleotides) within a given genus, such that a probe made from a field sample containing a member of that genus would be likely to hybridise to at least some of the oligonucleotides, whilst not hybridising (or hybridising to a lesser degree) to those corresponding to related genera, e.g. to differentiate *Apthovirus* (foot and mouth disease, FMDV) isolates from *Enterovirus* strains in the

Picornaviridae family. One could then select other sets of oligonucleotides, placed on the same array slide, able to characterise a pathogen more specifically, e.g. to differentiate the seven types of FMDV, and potentially to even further refinement at subtype level.

In conventional DNA probing the detection of a pathogen is limited by the number of probes used, whereas in microarray analysis one is limited only by the number of target DNAs on the array. If a microarray has 1000 different oligonucleotides, then to achieve the same resolving power by conventional probing would require 1000 probes, 1000 separate probing reactions. The great advantage of microarray analysis in searching for pathogens is that hundreds of pathogens can be looked for simultaneously when probing a single microarray slide. Clearly microarray analysis has great potential when one is investigating diseases of unknown aetiology, diseases where more than one pathogen might be present, and when subtyping is required. To enhance sensitivity in pathogen detection, microarrays can be coupled with PCR amplifications. These PCRs are usually designed to amplify one or more conserved genes, or multiple sequences, such as PCR using broadly conserved primers, consensus PCR and multiplex PCR as mentioned in the above section. When one has a particular pathogen in mind, then the use of a microarray would be less justifiable, since the production and hybridisation of slides is relatively expensive. Instead, for these more simple cases, one might use pathogen/subtype specific PCRs, followed by sequencing or restriction fragment analysis for confirmation.

If previous experience of biotechnology is indicative of the future, then one would expect microarray equipment and reagents to become less expensive, leading to greater application of this technology in animal disease diagnosis. It will assist in the search for hitherto undiscovered viruses or the characterisation of bacterial strains in terms of virulence, anti-microbial sensitivity or other important markers. One of the main challenges faced when using array-based approaches is the handling and analysis of the vast data sets that are generated.

4. Nucleic acid extraction

The main variants of molecular diagnostic assays described later in this section are wholly dependent upon the availability of 'clean' nucleic acid mixtures to act as a template for the reactions. While it is relatively easy to extract DNA from bacterial cultures or blood, it is technically more challenging to prepare suitable material from fecal samples or abortion material. This stage is critical because if the target material has not been purified of contaminants in the clinical sample, the assay stage is compromised and may yield false results (Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases) There are a number of specialised methods for particular types of samples and tissues, some of which are now commercially available either as manual or automated systems for robotic workstations. These are destined to improve the reliability of nucleic acid extraction from different samples, but it remains a challenging area.

B. DETECTION OF PROTEIN

1. Immunohistochemistry

As an adjunct to the isolation of causative organisms from tissue, immunohistochemistry is rapidly becoming a standard tool in diagnostic laboratories for the identification of antigens associated with viral, bacterial and protozoal microorganisms and transmissible spongiform encephalopathy (124). The *in situ* detection of antigens in fixed tissues offers a number of advantages over other diagnostic techniques. These advantages are: (a) convenience of sample submission; (b) safe handling of potential human pathogens; (c) retrospective studies of stored specimens; (d) rapidity; and (e) the detection of nonviable organisms (55). Immunohistochemistry is also used for the detection of abnormal prion protein (PrP^{Sc}) in brain tissue to confirm scrapie, bovine spongiform encephalopathy and other transmissible spongiform encephalopathies, and has proved to be more sensitive than the standard histopathological examination for diagnosis of these diseases (156). Demonstration of PrP^{Sc} in lymphoid tissue biopsies, e.g. nictitating membrane, can also be used for the preclinical diagnosis of scrapie (101). As the number of monoclonal antibodies (MAbs) to defined antigens increases, the use of immunohistochemistry for the identification of organisms and other specific markers for autoimmunity and neoplasia will increase. The limiting step in the process of immunohistochemistry is identifying a MAb/antigen combination that will bind in formalin-fixed tissues. This may be overcome by using frozen sections or employing antigen retrieval techniques (e.g. proteolytic enzyme digestion, microwaving) before immunostaining.

2. Immunoblotting

Immunoblotting combines the high resolution of gel electrophoresis with the specificity of immunochemical detection and offers a means of identifying immunodominant epitopes recognised by antibodies from infected animals or MAbs directed against the target agent. The immunoblotting procedure can be divided into six steps:

a)   Preparation of the sample

b)   Resolution of the antigen by gel electrophoresis

c)   Transfer of the separated polypeptides to a membrane support (nitro-cellulose membrane)

d)   Blocking nonspecific binding sites on the membrane

e)   Incubation with detecting antibody

f)   Detection of bound antibody

The choice of detecting antibody is critical. Polyclonal sera are composed of a range of antibodies reflecting the full repertoire of the immune response to a particular complex antigen. They will therefore detect a number of distinct polypeptides giving a characteristic 'profile' of reactivity. MAbs bind to only one epitope, therefore they are useful in identifying highly specific polypeptides. After incubation with the detecting antibody, any antibodies bound to specific protein bands are visualised using enzyme-labelled conjugated anti-species antisera and a suitable substrate/chromogen.

Immunoblotting is performed chiefly in diagnostic laboratories to identify and/or characterise infectious agents based on antigen specificity or to use known antigens to look for a specific serological response. False-positive and false-negative results in other diagnostic assays can often be resolved by immunoblotting (89). As an example of antigen detection, immunobloting is a major screening method for BSE and scrapie; it has been used on millions of brain stem samples in Europe and elsewhere for the detection of prion protein (138). Revised immunobloting techniques (e.g. SAF-Western blot) are used as confirmatory test methods (8) and to differentiate between scrapie and BSE (146). Immunoblotting is also often used to determine the specificity of individual MAbs. Individual purified polypeptides (or recombinant expressed proteins) may also be transferred to nitrocellulose membranes by immunoblotting to examine the reactivity of test sera to individual proteins. This characteristic profile of reactivity may be used to help distinguish between animals that have been vaccinated or infected, such as the enzyme-linked immunoelectrotransfer blot (EITB), a western blot for FMD, which is widely used in South America (10). The major factor affecting the success of an immunoblotting technique is the nature of the epitopes recognised by the antibodies. Most high resolution gel techniques involve some form of denaturation of the antigen. This destroys conformational determinants and allows only the detection of linear or non-conformational epitopes. Most polyclonal antisera contain antibodies to both linear and conformational epitopes, but MAbs are directed at single epitopes; thus if they target conformational epitopes, they will not react with denatured protein.

To detect pathogens in tissue samples, which contain a large amount of protein, selective concentration of antigen and highly sensitive detection methods are required. Good examples of these techniques have been used for the detection of the scrapie prion protein (PrP^{Sc}) (91). Following the elimination of the normal prion protein by proteninase digestion, the PrP^{Sc} is concentrated by sodium phosphotungstate precipitation or alcohol precipitation, and the antigen-antibody complex on the filter or ELISA plate is detected by using a chemical luminescence substrate and the use of a CCD camera or X-ray film to eliminate background emission (12, 133).

3.   Antigen-capture enzyme-linked immunosorbent assay (ELISA)

The antigen-capture enzyme-linked immunosorbent assay (ELISA) facilitates detection of antigen from pathogens directly from an animal prior to or during clinical disease. The ELISA commonly follows a sandwich assay format using capture and detecting antibodies (either specific MAbs or polyclonal antibodies). Antigen from the test sample is first captured by a specific MAb or polyclonal antibody bound to a solid-phase support and its presence is detected through use of a second MAb or polyclonal antibody, which may either be radio- or more generally, enzyme-labelled (conjugated). If the detecting antibody is not conjugated then an anti-species conjugate (reactive to the detector antibody) is used. The capture antibody selects the target antigen from other competing protein in sample suspensions and ensures that it is semi-concentrated to increase the chances of its detection. The desired characteristics of the capture MAb are strong binding to the pathogen, recognition of a conserved epitope highly specific for the target agent, and the ability to attach to an ELISA plate without loss of reactivity. In addition, a second MAb recognising an epitope other than that recognised by the capture MAb that is bound to the ELISA plate is often used as part of the indicator system. However, it may be difficult to identify MAbs of comprehensive intra-typic reactivity and polyclonal antisera may be preferred to increase the likelihood of reaction against all antigenic variants. Examples of antigen-capture ELISAs are the system for detection of *Anaplasma marginale* in the blood of preclinical cattle (160), the use of antigen-capture ELISA on cattle blood samples for the detection of bovine viral diarrhoea virus (88, 136), and the rapid detection of rinderpest and peste des petits ruminants virus antigens in clinical samples (79). Respiratory syncytial antigen in nasal secretions was captured using ELISA with MAbs directed against epitopes of the viral capsid (102). Several capture ELISAs for the detection of prion protein have been extensively validated and are used as rapid screening methods worldwide (42, 44, 91). Capture ELISAs are also widely used for chronic wasting disease testing in deer60) and scrapie testing in sheep and goats (43). A related antigen capture method, the Priostrip test, which is a dipstick method, is also used as a rapid BSE screening test (44). Related antigen-capture methods using immunomagnetic beads are now important and well accepted methods for detecting certain bacterial infections, including *Listeria, Salmonella* and *Escherichia coli*. The principle of this technology relies on immunomagnetic separation, i.e. using small super-paramagnetic particles or beads coated with antibodies against surface

antigens of cells. Both intact bacteria and their soluble antigenic determinants can be detected after magnetic extraction from the test sample, using a second antibody in a sandwich format. As solid surface-free binding, the antigen-capture assays using immunomagnetic beads can enhance the kinetics of an antigen-antibody reaction. As a result, both the nonspecific binding and the incubation time are reduced.

Validation of tests to detect antibody is addressed in Chapter 1.1.4 Principals of validation of diagnostic assays for infectious disease.

4. Immunochromatography

Immunochromatograhy provides a convenient method for detection of antigens in several minutes without special apparatus (3, 84). The sample is applied on one end of the filter and the microbeads (such as colloidal gold) conjugated with antibody is applied. The antibody binds to microbead-antigen complex, is trapped on the second antibody that is on the filter, and is easily visualised at the site where the second antibody was fixed.

5. Proteomics

The proteome is the total complement of proteins expressed within a cell, a tissue or an organism and proteomics is the study of proteins, including their expression level, post-translational modification and interaction with other proteins, on a large scale. Since not all proteins are expressed at all times, but are dependent on physiological and environmental factors, proteomics can provide an excellent global view of disease processes at the protein level. Because the application of proteomics to novel drug discovery promises huge economic returns, companies all over the world have rapidly poured resources into this new research field (22).

Many methods used in proteomics, including two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) were established years ago. However recent advances in MS techniques, together with whole genome sequencing and the development of powerful bioinformatics and robotics platforms, have revolutionised protein identification. The general principle of proteomics is that proteins are separated, usually by 2DGE on polyacrylamide gels, then protein spots are excised, digested with trypsin, and the resultant peptides analysed by MS. The masses of these peptides are then compared to the predicted masses of peptides derived by computational analyses of genome databases, resulting in gene identification. MS can also be used to deduce the amino acid sequence of peptides and to characterise post-translational modifications such as glycosylation or phosphorylation. 2DGE shows some drawbacks, particularly for the separation of hydrophobic proteins, and other separation techniques based on liquid chromatography are now finding favour for some applications. Nevertheless, 2DGE is the method of choice for creating quantitative maps of protein expression and many thousands of proteins can be analysed in a short space of time.

Alterations in the proteome of body tissues or of fluids such as serum, urine or cerebro-spinal fluid can be measured directly so changes that occur in a disease state can be accurately pinpointed. As well as identifying molecules that may be targets for novel therapies, this approach is a very powerful tool for early-stage diagnosis of disease. The best-established clinical applications of proteomics are so far in the identification of markers for the early diagnosis of cancers, such as bladder cancers in urine (127). However, considerable research efforts are also ongoing on other areas such as heart disease (68), Alzheimer's disease (27) and insulin-dependent diabetes (1).

The use of proteomics for the diagnosis of infectious disease is in its infancy but may prove to be of considerable importance. For example, definitive diagnosis of chronic hepatitis B virus (HBV) infection still relies on liver biopsy, but proteomic analysis of serum samples shows that the expression of at least seven serum proteins is changed significantly in chronic HBV patients (57). Similarly, the ante-mortem differential diagnosis of Creutzfeldt-Jakob disease (CJD) may be aided by proteomics since preliminary data show that seven proteins in cerebro-spinal fluid (CSF) are differentially expressed between patients with variant or sporadic CJD (28).

An extremely useful application of proteomics to the diagnosis of infectious disease is in the identification of novel diagnostic antigens by screening serum from infected and uninfected individuals against immunoblotted, 2DGE mapped proteomes of infectious agents. Using this type of approach with human sera, nine new potential immunodiagnostic antigens were identified in *Helicobacter pylori* (50), over 80 antigens in *Borrelia burgdorferi* that could potentially differentiate between patients with early or late symptoms of lyme disease (68) and seven antigens of *Toxoplasma gondii* that could potentially differentiate between acute and latent toxoplasmosis (68).

Within the veterinary field, proteomics-based research projects are now underway and these will undoubtedly yield novel diagnostic tools for the future. Proteome maps are being derived for a range of veterinary pathogens including bacteria such as *Brucella melitensis* (92) and *Streptococcus agalactiae* (65), protozoa such as *Toxoplasma gondii* (29), *Eimeria tenella* (21) and *Trypanosoma brucei* (130) and nematodes such as *Haemonchus contortus* (179).

C. ANTIBODY DETECTION

1. Competitive enzyme-linked immunosorbent assay (C-ELISA)

Competitive ELISA (C-ELISA) is an immunoassay that can be used to detect or quantify antibody or antigen using a competitive method. The C-ELISA for detetion of specific antibodies has largely replaced the indirect ELISA for large-scale screening and sero-surveillance. The C-ELISA offers significant advantages over the indirect assay since samples from many species may be tested without the need for species-specific enzyme-labelled conjugates for each species under test. Many antigens are extremely difficult or time consuming to purify. If used in an indirect assay, they would result in high background values due to nonspecific binding. However, relatively crude antigens may be used in the C-ELISA provided the 'detecting antibody" has the desired specificity. The principle of a competitive assay for the detection of antibodies is competition between the test serum and the detecting antibody. Specific binding of the detecting antibody is detected using an appropriate anti-species conjugate. A reduction in the expected colour obtained is due to binding of antibodies in the test serum, which prevent binding of the detecting antibody.

The detecting antibody may be polyclonal or monoclonal depending on the required specificity. MAbs directed against highly conserved epitopes will give broadly reactive assays whereas those directed against highly specific epitopes will result in a highly specific test. One of the early reports on the use of the C-ELISA was its use in detecting anti-bluetongue virus antibody (4). This used an MAb against a highly conserved epitope on bluetongue virus (BTV) P7 and allowed detection of antibodies to all 24 serotypes of BTV. The epitope was not shared in any of the other closely related Orbivirus serogroups, therefore the test was also BTV-specific. The specificity of the assay can therefore be tailored depending on the specificity of the detecting antibody. Sensitivity of C-ELISA is improved using detecting antibody directly conjugated with an enzyme (77).

The C-ELISA format has been successfully used in the screening of large numbers of pig sera for classical swine fever antibodies (177), the detection of antibody to malignant catarrhal fever virus in inapparently infected sheep, deer and bison (77, 78) and antibodies to *Babesia equi* and *B. caballi* in persistently infected horses (71, 72). A competitive ELISA for brucellosis, based on the immunodominent *Brucella* smooth lipopolysaccaride, has been widely used as a screening test for brucellosis in bovine (98), caprine and ovine (96), porcine (97) and sea mammals (99). More recently, a solid-phase C-ELISA was used for the large-scale serological surveillance during the UK FMD outbreak in 2001 (107). This facilitated the testing of some 3 million sera over a period of less than one year.

2. Production of antigens by recombinant DNA technology

Advances in molecular biology and genetics in the 1970s initiated the development of recombinant DNA technology. Since then the impact of this technology is such that it plays a vital role in scientific research as well as in the diagnosis and treatment of disease. Recombinant DNA technology simply refers to the transfer of a gene from one organism into another – literally, the recombination of DNA from different sources. The objectives of recombinant DNA technology include identifying genes, isolating genes, modifying genes, and re-expressing genes in other hosts or organisms. These steps permit scientists and clinicians to identify new genes and the proteins they encode, to correct endogenous genetic defects, and to manufacture large quantities of specific gene products such as hormones, antigens for use in vaccines, and other proteins produced by biological agents of interest. Of particular importance is the degree of specificity in diagnostic tests attainable by the use of recombinant protein. One example is the use of ESAT-6/CFO-10, (immunogenic secreted antigens) present in virulent *Mycbacterium bovis* and *M. tuberculosis* but not in avirulent BCG or most environmental mycobacteria, for the diagnosis of tuberculosis in cattle and humans (24, 161). Overlapping peptides of *M. tuberculosis* antigens ESAT-6 and CFP-10 increased specificity when they were used in the ELISPOT assay for gamma interferon detection for the diagnosis of *M. tuberculosis* infection (61). This has the potential for providing a degree of specificity in diagnosis not achievable with purified protein derivative (PPD), the bacterial extract currently used.

Native proteins are perhaps the ideal antigens, providing sequence-specific and surface structural epitopes. Many current diagnostic tests require test antigens that need to be continuously produced from cell culture or harvested from an infected animal. These antigen preparations are expensive and often have a short shelf-life, with each new batch of antigen requiring standardisation. Natural proteins are rarely available in a completely pure form, and antibodies often develop against contaminating polypeptides that can lead to false-positive results. Recombinant DNA technology produces antigens that offer many advantages over antigens isolated from other biological sources. These advantages include a high purity, high specific activity and since the protein is synthesised in genetically modified laboratory-grown cells, each preparation of the protein product is identical to the previous preparation, ensuring batch-to-batch consistency. When recombinant antigens are used in combination with the C-ELISA format, purification of the recombinant antigen from the lysate may not be necessary as the specificity of the C-ELISA resides mainly in the MAb used. An example of the procedure is the cloning of the envelope genes of caprine arthritis/encephalitis lentivirus in a vaccinia expression vector (80). Synthetic peptides can also be used as valuable antigens for veterinary laboratory diagnosis. The peptide-based diagnostic tests rely on the selection of short fragments containing the most potent antigenic (linear) epitopes

that are recognised by specific antibodies induced by the whole viral proteins. In recent years, synthetic peptides that mimic specific epitopes of infectious agents' proteins have been used in diagnostic systems for various human and animal diseases. Both recombinant proteins and synthetic peptides as antigens are useful for the companion diagnostic tests in DIVA, differentiating infected from vaccinated animals. Marker vaccines carry at least one less antigenic protein than the corresponding wild-type virus, which allows the serological tracing of wild type strains in vaccinated individuals (59).

An outline of the procedure for the production of an antigen by recombinant DNA technology is as follows. The identification of an antigen of potential diagnostic or scientific significance is achieved through the study of the antibody response of the host to the proteins of the organism in question. Immunodominant antigens, defined proteins of the organism against which the host responds with the highest potential diagnostic titre, are of particular interest as they are major stimulants of cellular and humoral immunity against the disease of interest. Antigen discovery studies are widely used to identify biologically relevant, immunodominant antigens for use in generating MAbs as well as in vaccine development. Once a protein of interest has been identified, the gene encoding the protein is generated using messenger RNA (mRNA) from the organism as a template for making cDNA. This method of cloning the gene encoding the protein of interest requires a prior knowledge about the gene sequence, either directly from the organism of interest or through the use of gene sequences from closely related species. An alternative method, when gene sequence data is not available, is the generation of recombinant libraries from the genomic DNA of the organism or from cDNA synthesised from mRNA. Fragments of the recombinant libraries can be cloned into an expression system, which may be prokaryotic or eukaryotic, and the gene library screened for expression of the protein.

There is a wide choice of expression systems. Protein may be expressed in bacteria, usually *E. coli* (121), yeast (26), insect cells using baculovirus (147), or in eukaryotic cells by infection with appropriate viral vectors (143) or by permanent transfection. Differences in glycosylation when prepared in bacterial, insect or mammalian cell cultures can modify protein structure and its reactivity with antibody. Antigen may need to be extracted from the cell or may be secreted. Purification is often, but not always, necessary. An upcoming trend in the production of antigens for use in assays is in the development of synthetic peptide antigens. This allows antigens to be tested as diagnostic reagents based on the gene sequence, without expression of the whole protein being necessary, thus shortening the process. An example is the production of peptide antigens from two immunodominant antigens, reported to be promising candidates as diagnostic reagents for the detection of *M. bovis* infection in cattle (172). In recent years, the use of plants for a protein expression system has shown promise. For the expression of candidate antigens in plants, plant viruses offer the advantages of speed of product development, flexibility, and high levels of gene expression among others (48).

Genome sequences of hundreds of bacteria and thousands of viruses have already been determined. The antigen gene can easily be cloned with PCR technology, using primers designed from the nucleotide sequence of closely related species (126). The gene can also be expressed and its product can be purified using tag peptide. The antigenicity of the gene products can then be determined. Systematic screening of the antigen gene *in silico*, from genome sequence data, accelerates the development of diagnostic kits and vaccine (159).

D. VACCINES

1. Gene deletion vaccines – bacteria

During the past decade, recombinant DNA technology has made it possible to construct safer live vaccines. Live attenuated bacterial vaccines confer better protection against challenge than killed vaccines (64). The reasons for this improved protection are not yet clear, but one could be that live vaccines are able to express antigens *in vivo* necessary for protection that killed vaccines preparations do not contain. Another reason could be that live vaccines are able to stimulate antigen presenting cells (APC) in a manner in which killed vaccine preparations are unable to. Most likely it is a combination of both, novel antigen expression and interaction with APC. Generation of live attenuated bacterial vaccines relied mainly on the generation and selection of mutants by serial passage in alternate animal hosts, prolonged culture *in vitro*, changes in temperature growth or chemical modification, which resulted in undefined attenuations based on the accumulation of numerous genetic mutations. In some cases, for unknown reasons, these mutants reverted to wild-type phenotype and therefore could not be used as vaccines (144). In 1981 Hosieth & Stocker (62), using transposon technology, developed *Salmonella typhimurium* strains with defined genetic mutations auxotrophic for aromatic aminoacids (*Aro*) that were unable to survive in the immunocompetent host. These strains were able to confer protection against virulent challenge in the murine model of salmonellosis and in several domestic species, although for unknown reasons, not all the mutants were able to confer protection in the domestic species (141). In 1992, Jones *et al.* (67) developed a live attenuated salmonella mutant using precise genomic excision of two genes involved in the aromatic amino acid pathway, which resulted in an even lower probability of the strain reverting to wild-type phenotype. This mutant proved to be a vaccine with relatively mild clinical secondary effects and able to confer protection in cattle against virulent challenge at the age in which the host is more susceptible. This vaccine strain has also been used as a delivery vector for guest antigens, which brings closer to reality the ideal single dose

multivaccine (170). Developments in molecular biology and a greater understanding of the host pathogen interaction will permit the rational design of safer and more efficient vaccines with markers that will allow the distinction between vaccinated and infected hosts. Although most of the developments described here focus on salmonella, similar technologies are being applied to other bacterial pathogens. The technology has been used in bovine tuberculosis; if cattle are vaccinated with BCG, the ESAT-6/CFO-10 peptide cocktail detects infected, not vaccinated, animals in the IF gamma assay.

2. Marker vaccines and companion diagnostic tests

In animal health, one can either vaccinate animals in order to prevent a disease or try to eliminate the infection through strict application of sanitary measures such as slaughtering of infected and in-contact animals. For certain diseases for which no vaccine exists (e.g. African swine fever) and particularly for zoonotic infections (e.g. Nipah virus infection of pigs), the systematic slaughtering of infected animals is the only available solution. Diagnosis of infection is of paramount importance whatever the measures taken to fight the disease. Diagnosis can be direct, through the detection and identification of the infectious agent using immunological or molecular technologies, or indirect, based upon the detection of specific antibodies against the suspected infectious agent. The latter methods have a major drawback in that one must wait until antibodies are synthesised by the animal after infection and generally they do not allow distinction between a humoral immune response resulting from an infection or a vaccination.

This problem can be overcome by adopting new approaches to vaccine development (105) using molecular technologies that allow the production of marker vaccines associated with companion diagnostic tests. There are currently two types, either based on the detection of a serological response against a protein whose gene has been deleted in the vaccine strain (either used as a replicating vaccine or as an inactivated vaccine derived from such a deleted virus vaccine strain), or on the detection of the serological response to virus nonstructural proteins (purified inactivated vaccines). In the case of the deletion vaccines the gene coding for a non-essential protein, the marker characteristic, is always linked with the detection test while in the case of subunit vaccines (e.g. protein E2 of classical swine fever virus expressed in baculovirus) the choice of the marker test assay may be linked to several other of the virus proteins. For harmonisation purposes, an agreed protein should be chosen for the test (e.g. protein gE of pseudorabies virus). In the first type of marker vaccines, the marker must always be negative since a positive marker, for instance provided through the insertion of a gene coding for a foreign protein, is not suitable; such a vaccine will only show if the animal has been vaccinated but will not indicate if the animal was also infected with the wild virus. Marker vaccine used with the intention of distinguishing a serological response resulting from either vaccination or infection must always be associated with a companion diagnostic test that can be used during a prophylactic campaign with the aim of eliminating the infectious agent. Previous veterinary vaccines were mainly designed to prevent clinical signs in animals following an infection without taking too much account of the epidemiological impact of vaccination on the excretion of wild virus following infection and on its dissemination/circulation. If marker vaccines are used with the aim of eliminating a virus they must have a clear impact on the epidemiology of the infection.

There can be problems with this approach, for example if wild virus multiplication is inhibited to the point that it does not induce the synthesis of specific antibodies in all vaccinated animals. Therefore, most of the available marker vaccines can only be used for herd certification and not for individual animal certification.

a) Marker vaccines with one gene deletion: the examples of pseudorabies and infectious bovine rhinotracheitis

Pseudorabies in pigs and infectious bovine rhinotracheitis are two infections caused by herpesviruses that become latent in an animal, even when it has already been vaccinated (111, 115, 116). The first marker vaccine became available to prevent pseudorabies infection in pigs (165) following the development of an attenuated strain of pseudorabies virus by Bartha in Hungary (7) that had a spontaneous deletion in the gE glycoprotein. Analogous vaccines were later developed for infectious bovine rhinotracheitis.

As mentioned above, the herpesvirus responsible for infectious bovine rhinotracheitis becomes latent after infection, whether or not the animal has been vaccinated. It does not matter if the vaccine is an inactivated or an attenuated one, either way the animal becomes a latent carrier after infection with a wild virus. Moreover, all the attenuated vaccine strains establish latency after vaccination, including gE deleted strains. It should be borne in mind that attenuated vaccines produced with identical strains, deleted or not, are generally more efficacious than their inactivated counterparts (18, 69, 70).

In an area where vaccination is prohibited, all animals serologically positive with regard to infectious bovine rhinotracheitis virus must be considered as potentially infected and latent carriers of a wild virus. Similarly, in an area where animals are vaccinated with a conventional (non-deleted) vaccine, either attenuated or inactivated, it is impossible to distinguish between vaccinated and infected cattle and so if an elimination programme is in place, all the seropositive animals must also be eliminated from the herd.

A solution is the use of a marked/deleted vaccine that allows the differentiation of antibody produced from vaccine versus that produced due to infection. The deleted protein in the vaccine strain must have the following characteristics:

1) Be a structural protein, in order to be able to produce inactivated vaccines;

2) Be non-essential in order to be able to produce the vaccine;

3) Not be an essential protective immunogen in order to still have an efficacious vaccine;

4) Induce a significant and long lasting humoral immune response when present in order to be used (when deleted) as a marker;

5) Be present in all the wild virus strain;

6) Induce a humoral or cellular immune response by a pathogen in an already vaccinated animals.

If such a marker vaccine is used, whenever an animal is seropositive towards the deleted protein, it must be seen as infected and eliminated. The gD protein of herpesviruses, being a major protective immunogen, cannot be deleted but contrarily may be used to develop subunit vaccines. The main problem encountered with the use of marker vaccines against infectious bovine rhinotracheitis is their inability to completely prevent wild virus circulation when used within the framework of an elimination programme.

No available vaccine is able to induce sterile immunity for these diseases. As a consequence the vaccination schedule must be more stringent than a conventional one designed merely to protect against clinical signs in the herd. Vaccination must be repeated according to a strict schedule to reduce the possibility of wild virus excretion and must, in addition, be associated with strict sanitary measures (79). Within the framework of a coordinated virus elimination campaign, vaccination must prevent the excretion of wild virus by naïve animals and prevent re-excretion by latently infected ones.

The efficacy of repeated vaccination using an inactivated gE negative vaccine administered intramuscularly has been investigated under field conditions in the Netherlands. This study showed a significantly reduced incidence of seroconversion against wild virus in the vaccinated group compared with the placebo injected control animals. In addition, wild virus circulation, while not completely restricted, was nevertheless significantly reduced (18) and in some circumstances even prevented (166).

b) **Vaccination against classical swine fever with subunit vaccines**

An elimination programme for classical swine fever has been set up within the European Union. Vaccination using conventional vaccines is now prohibited and a slaughter policy is in place. This policy is challenged by the existence of a strong antigenic relationship with other pestiviruses, such as the virus responsible for bovine viral diarrhoea (BVD/MD), that impede serological diagnosis, the insidious circulation of hypovirulent strains (14) and, last but not least, the presence of a wild reservoir in wild boar (*Sus scrofa*) in continental Europe (5).

The classical, conventional, vaccines have a well proven efficacy (123) and even prevented the emergence of asymptomatic carriers when they were of sufficient potency (15, 74). Live attenuated vaccines were more efficacious than their inactivated counterparts in this respect (31) and they contributed greatly to the elimination of the disease. Their one disadvantage was the creation of a population of serologically positive animals, which is not acceptable if a slaughter policy is in place. Subunit vaccines have recently been developed by expressing the E2 protein, a major immunogen of classical swine fever virus, either in a baculovirus system (van Rijn, 1999 129 /id}) or in vaccinia or pseudorabies viruses (E1) (132, 168). The baculovirus expressed E2 protein vaccine allows distinction between infected or vaccinated animals when used with reliable companion diagnostic tests to detect the presence of specific antibodies directed against other major immunogens of classical swine fever virus not present in the subunit vaccine, such as NS2 protein, a conserved virus protein. Unfortunately, inactivated vaccines are not sufficiently efficacious from an epidemiological standpoint (37) when compared to the former conventional vaccines (35, 163). Moreover, the companion diagnostic tests currently available are not fully reliable and therefore limit the use of these subunit vaccines in the field.

c) **Vaccination against foot and mouth disease using highly purified vaccines**

Preventive vaccination has been prohibited in the European Union since 1991. This prohibition ended a 30-year period of vaccination and consequently completely naïve cattle herds now exist in Europe (149). This situation is particularly detrimental when the disease is accidentally reintroduced (39). The contingency plan that has evolved to deal with unexpected outbreaks is mainly based on information and training of the concerned partners in the European Union. In order to overcome the risks associated with the complete susceptibility of European livestock, concentrated, highly purified virus antigen vaccine banks have been established (135) and there is the possibility of using these as marker vaccines in case of an emergency outbreak (34).

This is possible since, when highly purified vaccines are used, whenever an animal is found that is seropositive to the nonstructural proteins (NSP) coded by the virus using an ELISA diagnostic test kit (33), it must have been infected by a wild virus. The NSP are produced during the virus replication in the infected animal and in cell culture. The NSP should be removed from FMDV antigen by purification during vaccine production and appropriate testing of the antigens should be conducted to demonstrate absence of seroconversion to NSP in vaccinated animals. The NSP are synthesised at the same level as the structural proteins during infection and so produce a good humoral immune response. Infected animals become seropositive to NSP and the antibodies to NSP are usually detected using ELISA diagnostic test kits or the EITB. Unfortunately, the companion diagnostic tests currently available only permit certification of freedom from FMD at the herd level and not the individual animal level. When virus multiplication occurs and are not present in the extracellular virions used to produce purified inactivated vaccines.

d) Equine influenza as a special case

A similar approach as that used for FMD has been applied, in a different context, to equine influenza (110). When carrying out studies on the duration of protective immunity with equine influenza inactivated vaccines, it is useful to have a diagnostic tool that allows the exclusion of antibodies due to intercurrent infection of the experimental animals by a wild influenza virus. A diagnostic test has been developed (13) based on the serological response to a nonstructural protein coded by the virus.

3. Virus-vectored vaccines

Many virus species, including adenoviruses, herpesviruses and poxviruses, have been used as delivery systems (vectors) for foreign antigens. The virus can be used simply as a vector, for example the vaccinia-rabies recombinant virus, or as both a vector and a vaccine against the infection by the wild vector itself. An example of a virus acting both as a vector and a self vaccine is the recombinant capripox virus expressing a peste des petits ruminants virus antigen (11). A vector virus may undergo full multiplication cycle leading to the production of progeny virus or abortive multiplication cycle without the production of progeny virus, such as in the case of the avipoxvirus vector in mammalian species.

The most commonly used vectors are poxviruses and this chapter will therefore focus on the use of poxviruses as vaccine vectors (117).

A number of features make poxvirus recombinants suitable as vaccines:

i) the stability of freeze-dried vaccine (28), its low cost, ease of manufacture and administration;

ii) the vaccine can be administrated by several routes (46) and in the case of vaccinia virus it has even been shown that the virus can be administrated *per os* (this feature has been used for vaccinating wildlife) (113);

iii) the ability to induce both antibody and cytotoxic T cell responses against the foreign antigen with long lasting immunity after a single inoculation (142, 143);

iv) the packing flexibility of the genome, which allows large amounts of the genome to be lost or deleted and foreign DNA to be inserted in its place (at least 25 kb), thus enabling multivalent vaccines to be created (118, 119, 142, 143);

v) the use of recombinant poxviruses as vaccines allows discrimination between naturally infected versus vaccinated animal since the recombinant vaccine displays a defined subset of the antigens of the pathogens concerned.

Within each genus of the Poxviridae family the members are antigenically related (90). This antigenic relationship has raised an important question concerning the use of poxvirus-derived vectors as live vaccines, as pre-existing immunity against the vector could reduce the success of a subsequent vaccination performed with a homologous poxvirus vector (30, 73). To circumvent this problem, the use of different combinations of vectors and/or routes of immunisation has been implemented (45, 125).

a) Vaccinia virus as a vector

The first recombinant vaccinia to be used in the field is the recombinant vaccinia-rabies vaccine (VRG) used for oral vaccination of foxes against rabies. This was developed using the Copenhagen strain and tested in many potential target species under laboratory conditions (20, 112, 155) before it was eventually used under field conditions in 1987 (114) and proved to be safe and efficacious (20). It has been used on a large-scale in several European countries that were, as a consequence, freed from rabies (19) as well as in North America.

The safety of vaccinia virus can be enhanced by multiple gene deletions. This has been demonstrated by the engineering of the NYVAC strain of vaccinia virus (152). The Copenhagen strain of vaccinia virus was chosen as the vaccine substrate and based on the entire DNA sequence (49), on extensive knowledge of virulence-related genes and on genes determining host range replication competency, unwanted genetic information was deleted from the viral genome in a very precise manner. The resulting virus, named NYVAC, has 18 open reading frames deleted compared to the parental strain. NYVAC is highly attenuated as demonstrated in many animal studies. Intracranial inoculation of newborn and young adult mice demonstrated a very favourable dose range compared with either the parental or other vaccinia strains. Most significantly there is no dissemination of the virus in immunocompromised hosts. NYVAC has dramatically reduced the ability to replicate in a variety of human tissue culture cells and is unable to produce infectious particles in humans. Several animal and human trials have demonstrated the safety of the NYVAC strain-derived vectors (108, 151, 176).

b) Avipoxvirus vectors

When considering the development of avipox-derived vectors for the production of vaccines for birds, the use of attenuated strains is recommended in order to reduce the safety risk and the potential consequences arising from environmental spread to other avian species. Attenuated derivatives of fowlpox virus, like TROVAC, and canarypox virus, like ALVAC, have been extensively tested and their safety demonstrated in a variety of species, including immunocompromised animals and human volunteers. These viruses can be used under laboratory safety conditions level 1, the lowest category for recombinant organisms (109).

Despite the fact that their multiplication is restricted to avian species, attenuated strains of avipoxviruses have been demonstrated to be efficacious and extremely safe vectors for mammals. Inoculation of avipox based recombinants in mammalian cells results in expression of the foreign gene and inoculation into mammalian species induces protective immunity without producing progeny viruses (153, 154). This observation demonstrates that they have a significant safety advantage for human and animal use. As immunisation can be achieved in the absence of productive replication, it eliminates the potential for dissemination of the vector within the vaccinates and, therefore, the spread of the vector to non-vaccinated contacts or to the general environment. Moreover, the use of this vector in species that are not a reservoir of avipoxviruses renders the likelihood of recombination *in vivo* nil. Additionally, these vectors can be used for vaccination of individuals with pre-existing immunity to vaccinia virus.

In the past decade, a great number of recombinant viruses have been produced using the attenuated canarypox ALVAC strain as the parental strain. An impressive number of trials, both in humans and animals, have demonstrated the safety and protective efficacy of vaccines using this vector.

4. DNA vaccines

DNA vaccination is the direct introduction into host cells of a bacterial plasmid DNA that expresses an antigenic protein under the control of a eukaryotic cell promoter (129). As a consequence, the foreign antigen is expressed within the host cell and can stimulate the induction of both humoral and cell-mediated immune responses. This approach to vaccination has been effective against a wide-range of viruses, bacteria and parasites and not only has many of the benefits of live vaccines but also has several advantages over more conventional approaches to vaccination. For example, DNA vaccines encoding foreign genes are inexpensive and easy to produce; they obviate the need for complex carrier organisms; the risks associated with live vaccines are absent; and the impact of pre-existing immunity to the organism or vector on vaccine efficacy is circumvented. However, a disadvantage of DNA vaccination is that, as the plasmid persists for a long time, there is a potential for chromosomal integration with resulting cell transformation.

The immune response of DNA vaccines can be further improved by simultaneous inoculation of immunostimulators, such as CpG motif sequences (122), plasmids expressing cytokines (178), plasmids expressing co-stimulatory molecules (85), or even conventional adjuvants (167). Immunogenicity can also be improved by first priming with a plasmid DNA vaccine expressing an immunogenic protein followed by subsequent boosting with the protein or with a recombinant virus vector expressing the protein, the so-called 'prime-boost' approach (171).

Several DNA vaccines for veterinary use are currently being developed in cattle, pigs and poultry (100, 106, 167). West Nile-Innovator® DNA is a novel vaccine for horses to aid in the prevention of viremia caused by the potentially deadly West Nile virus; it represents a tremendous milestone in DNA science and technology. Delivery of the DNA is either by intramuscular, intradermal or intranasal inoculation, particle-mediated intradermal delivery using a gene gun in which the DNA is precipitated onto gold microspheres (82), or it can be accomplished using attenuated intracellular bacteria, such as *Shigella flexneri* or *Salmonella typhimurium* (38). Live attenuated bacterial vaccines allow vaccination via the mucosal surfaces and specific targeting to antigen presenting cells located at the inductive sites of the immune system. While this latter approach has several advantages, there are a number of safety issues that need to be addressed before this method of delivery is accepted. The disadvantages of DNA vaccination should also be borne in mind: these include potential chromosomal

reintegration with resulting cell transformation as the plasmid persists in the host for a long time, although this risk is low.

Another DNA vaccine strategy is based on the use of a DNA vector consisting of recombinant Semliki Forest virus (SFV) cDNA under the control of a eukaryotic promoter and expressing a foreign gene (9). Unlike conventional DNA vectors, the promoter is not directly driving the expression of the foreign antigen, but directs the synthesis of a recombinant SFV replicon RNA transcript. Translation of this RNA molecule produces a SFV replicase complex that allows replication of the RNA in the cell cytoplasm and results in high-level production of the mRNA for the encoded foreign antigen. Since expression mediated by the SFV vector is transient and lytic, there is less risk from possible chromosomal integration.

Applications of bacterial artificial chromosome (BAC) technology have opened new avenues for manipulation of large DNA virus genomes, such as herpesviruses (2, 23). The use of BAC clones of herpesviruses is not only a powerful tool for studying viral gene functions and pathogenesis (173), but also has great potential in herpesviral vaccine development (94). Experimental studies using BAC clones as vaccines for herpesvirus infections have delivered on their promise (120, 150, 157). This technology for the generation of novel herpesvirus vaccines will have significant impact and application in veterinary medicine.

5. Other developments in vaccine technology

Subunit vaccines, which contain purified protein or glycoprotein components of a pathogen that have been identified as carrying critical epitopes involved in inducing a protective immune response (6) have distinct safety advantages and recent improvements in their production using recombinant DNA technology may facilitate their more widespread use (36). Synthetic peptide vaccines have also been engineered (87), however, thus far they have not been shown to be very effective in inducing protection against infectious diseases. There may be many reasons why synthetic peptides may not induce protective immunity. For example, even so-called linear peptides exhibit a degree of conformational flexibility so that they adopt a different structure from that of the parent molecule and therefore induce antibodies of low avidity for the pathogen in question. A potential disadvantage of using peptides that represent single antigenic sites to stimulate a protective antibody response is the possibility of selecting for antigenic mutations in the pathogen.

A number of strategies have been developed for inducing cytotoxic T cell (CTL) responses using peptides, such as coupling CTL epitopes to toxins that are able to invade eukaryotic cells or constructing virus-like particles carrying foreign CTL epitopes (137, 140). However, the utility of this approach in outbred populations is limited by the polymorphism of the major histocompatability complex molecules. Other virus-like particle vaccines that involve self-assembling proteins that can be used to carry foreign antigens have been made from particles produced from the TYA gene of the yeast retrotransposon Ty (47). A vaccine composed of empty virus-like particles produced by expressing the four main structural proteins of bluetongue virus in baculovirus has been shown to protect against challenge with bluetongue virus (131).

Another interesting approach is the development of 'edible vaccines'. Plants can be engineered to express a number of foreign proteins and can express multiple transgenes at one time (148). The oral delivery of subunit vaccines expressed in plants would be particularly suited to protect against intestinal pathogens. A disadvantage would be that antigens delivered orally would be susceptible to proteolytic degradation. Moreover, oral delivery of antigens tends to induce tolerance rather than active immunity. However, tolerance can be circumvented by expression of a fusion protein composed of the antigen with the B subunit of the heat labile enterotoxin (LT-B) of E. coli (56).

Cloning the whole viral genome has been made possible by reverse genetics, especially of negative-stranded RNA viruses. By reverse genetics, the function of various NSPs, as well as the hidden function of virion proteins, can be elucidated, thus enabling the construction of an RNA virus by recombinant technology. This also allows the identification of the escape strategy the virus uses to avoid the host defence mechanisms, such as interferon (51, 164). Reverse genetics also provides a novel approach for the attenuation of viruses by deleting anti-IFN or cytokine functions of the virus (195). Reverse genetics can be applied to RNA viruses that have a relatively simple genome structure, such as avian influenza virus. It has been observed that the presence of basic amino acid residues at the cleavage site of the haemagglutinin gene assists the avian influenza virus to replicate within the animal. Conversely, highly pathogenic avian influenza viruses, having this type of genetic structure, can be attenuated by removing the basic amino acid residue from the site (81, 95).

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PRINCIPLES OF VETERINARY VACCINE PRODUCTION

SUMMARY

A reliable supply of pure, safe, potent, and effective vaccines is essential for maintenance of animal health and the successful operation of animal health programmes. Immunisation of animals with high quality vaccines is the primary means of control for many animal diseases. In other cases, vaccines are used in conjunction with national disease control or eradication programmes.

The requirements and procedures described here are intended to be general in nature and to be consistent with published standards that are generally available for guidance in the production of veterinary vaccines. The approach to ensuring the purity, safety, potency, and efficacy of veterinary vaccines may vary from country to country depending on local needs. However, proper standards and production controls are essential to ensure the availability of consistent, high quality products for use in animal health programmes.

As the pathogenesis and epidemiology of each disease varies, the role and efficacy of vaccination as a means of control also varies from one disease to another. Some vaccines may be highly efficacious, inducing an immunity that not only prevents clinical signs of the disease, but may also prevent infection and reduce multiplication and shedding of the disease-causing agent. Other vaccines may prevent clinical disease, but not prevent infection and/or the development of the carrier state. In other cases, immunisation may be completely ineffective or only able to reduce the severity of the disease. Thus the decision whether to recommend vaccination as part of an animal disease control strategy requires a thorough knowledge of the characteristics of the disease agent and its epidemiology, as well as the characteristics and capabilities of the various available vaccines. There is also growing public interest in the beneficial implications for animal welfare of the use of veterinary vaccines as a means of disease control. In any case, if vaccines are used, successful performance requires that they be produced in a manner that ensures a uniform and consistent product of high quality.

NOMENCLATURE

The nomenclature for veterinary biological products varies from country to country. For example, in the United States of America (USA) the term 'vaccine' is used for products containing live or inactivated viruses or protozoa, live bacteria, or nucleic acids. Products containing killed bacteria and other microorganisms are identified as bacterins, bacterial extracts, conventional or recombinant subunits, bacterintoxoids, or toxoids, depending on the type of antigen they contain. For example, products containing antigenic or immunising components of microorganisms may be called 'subunits' or 'bacterial extracts', and those produced from the inactivation of toxins are called 'toxoids'. In the European Union (EU), Immunological Veterinary Medicinal Products are defined as 'products administered to animals in order to produce active or passive immunity or to diagnose the state of immunity', see Directive 2001/82/EC, as amended by Directive 2004/28/EC. For this chapter, however, the term 'vaccine' will include all products designed to stimulate active immunisation of animals against disease, without regard to the type of microorganism or microbial toxin from which they may be derived or that they contain. This use is more consistent with international nomenclature. 'Vaccine' will not be used in this discussion in reference to biological products recommended for passive immunisation, immunomodulation, treatment of allergies, or diagnosis.

VACCINE TYPES OR FORMS

Vaccines may be prepared as live or inactivated (killed) products. Some live vaccines are prepared from low virulence, mild, field isolates of a disease-causing agent that have been found to be safe and effective when

administered by an unnatural route or under other conditions where exposure to the microorganism will immunise rather than cause disease. Other live vaccines are prepared from isolates of disease-causing agents that have been modified by passage through laboratory animals, culture media, cell cultures, or avian embryos to select a variant of reduced virulence. The development of recombinant DNA (rDNA) procedures has provided some unique opportunities for vaccine production. Modified live vaccines may now be specifically produced by deletion of virulence-related genes from a microorganism. Others are produced by the insertion of genes that code for specific immunising antigens from a disease-causing microorganism into a nonvirulent vector microorganism. Nucleic-acid-mediated vaccines containing plasmid DNA are being developed. The DNA is usually in plasmid form and codes for immunising antigens from disease-causing microorganisms.

Killed products may contain: 1) Cultures of microorganisms that have been inactivated by chemical or other means; 2) Inactivated toxins; or 3) Subunits (antigenic parts of microorganisms) that have been extracted from cultures or that have been produced through rDNA procedures.

Both live and inactivated vaccines may be formulated with adjuvants designed to enhance their efficacy. Frequently used adjuvants are typically water-in-oil emulsions (either single or double), made with mineral or vegetable oil and an emulsifying agent. Other adjuvants, such as aluminium hydroxide gel or saponin, are also used. In addition to these traditional adjuvants, vaccines are being developed that include additional ingredients that induce immunomodulatory effects in the host animal and serve to enhance the efficacy of the product. These ingredients may include immunogenic components of microorganisms such as killed bacteria, which stimulate the immune response to other fractions contained in the vaccine, or cytokines, which may be used to regulate specific aspects of the immune system and are included in rDNA constructs used in products manufactured through biotechnology.

QUALITY ASSURANCE

The consistent production of pure, safe, potent, and efficacious vaccines requires quality assurance procedures to ensure the uniformity and consistency of the production process. As production processes for vaccines provide a great opportunity for variability, care must be taken to control variability to the greatest extent possible, preferably using validated procedures, and to protect the product from contamination through all stages of production.

Vaccine purity, safety, potency, and efficacy must be ensured by consistency in the production process. Consistent product quality (batch-to-batch uniformity) must be built in at each stage. Final product testing is used as a check to verify that the controls on the production procedures have remained intact and that the released product meets the specification previously agreed with the licensing authority.

Regulatory authorities in different countries have developed various approaches to ensuring the quality of vaccines. Although alike in their ultimate goal, these systems may vary in the emphasis given to control of the production process (process standards) in comparison with control through testing of the final product (performance standards). The control procedures selected should be those that best fit the conditions under which vaccines are being produced and, where possible, comply with good manufacturing practice.

The control standards and procedures established for a product define the risk or possibility of producing and releasing a product that is worthless, contaminated, dangerous, or harmful. The acceptable degree of risk may depend on the benefits to be gained by having the product available to prevent disease losses. Thus standards may justifiably vary from country to country or product to product, depending on local animal health conditions. However, control authorities should strive to establish control standards and procedures that ensure a finished product of the highest purity, safety, potency and efficacy possible.

The optimal quality assurance system should address both production procedures and final product testing in proper balance. An absolutely fail-safe system that would result in no risk of releasing an unsatisfactory product would probably be too expensive with regard to cost of production as well as control. Thus regulatory officials and manufacturers of vaccines must select control procedures that are capable of ensuring an acceptable low level of risk in relation to hazard. Such procedures, however, must not be burdensome to the extent that they inhibit the development and availability of the products needed to provide proper preventative medical care at a cost that is acceptable to the consumer.

PRODUCTION FACILITIES

Facilities used for the production of vaccines should be designed to protect the purity of the product throughout the production process and to safeguard the health of the personnel. They must be constructed so that: 1) they can be readily and thoroughly cleaned; 2) they provide adequate separation of preparation rooms; 3) they have adequate ventilation; 4) they have ample clean hot and cold water and efficient drainage and plumbing; and 5) they have dressing rooms and other facilities for personnel that are accessible without passing through

biological product preparation areas. Facilities must be adequate to provide for all applicable production functions, such as: storage of master seeds, ingredients, and other production materials; preparation of growth media and cell cultures; preparation of glassware and production equipment; inoculation, incubation, and harvest of cultures; storage of in-process materials; inactivation, centrifugation, addition of adjuvant, and formulation of product; filling, desiccation, sealing of containers, labelling and storage of final product; quality control testing of in-process materials and final product; and research and development.

Separate areas are generally required for different activities. All rooms and air-handling systems must be constructed so as to prevent cross-contamination from other products and to prevent contamination by people or equipment. Virulent or dangerous microorganisms must be prepared and stored in rooms separate from the remainder of the establishment. In particular, challenge organisms must be completely separated from vaccine strains. All equipment that comes into contact with product must be sterilised using validated procedures.

Production facilities have to be designed in such a way that contamination of the external environment is prevented. Any material used during production has to be made safe before leaving the facility. If highly contagious microorganisms are propagated, the exhaust air must be treated to prevent escape of infectious agents. Personnel must follow safety procedures such as showering, and avoid contact with susceptible animals after leaving the production facilities.

Although the quality and design of production facilities may vary significantly, they must always meet standards considered to be appropriate for the vaccines that are to be produced. For example, the requirements for facilities for the production of chicken embryo vaccines administered by oral, intranasal or intraocular routes in chickens may not need to be quite as demanding as those for the production of cell culture vaccines administered subcutaneously or intramuscularly.

FACILITIES PLAN

For each vaccine made in a facility, there should be a detailed production plan that describes where each step in the production process will occur. This plan should be documented in a detailed standard operating procedure (SOP) or by providing a building blueprint and accompanying blueprint legend. Each room in the establishment should be uniquely identified, and all functions performed and all microorganisms involved should be specified for each room. Disinfection procedures, monitoring of equipment and other procedures used in the operation of the facilities to prevent contamination or errors during production should also be documented. This plan should be updated as new products or microorganisms are added to the facility, or other changes or improvements in procedures are developed.

DOCUMENTATION OF THE MANUFACTURING PROCESS

A detailed Outline of Production, a series of SOPs, or other documents should also be prepared to describe the protocol for the manufacture and testing of each product produced in an establishment. Criteria and standards for source materials should be clearly and accurately documented. Documentation should also address such things as: the source, isolation, and passage (subculturing) history of each strain of microorganism; the source and sequence of nucleic acid elements, or peptides included in products derived from biotechnology, including plasmids or other vectors used in the construction of genetically modified microorganisms for use as master seeds; methods for identifying the microorganisms and determining their virulence and purity; the medium or cell culture system used for seed and production cultures, including the methods used to demonstrate that media are free from contamination; the source of ingredients of animal origin; methods of media sterilisation; storage conditions of cell lines and seed cultures; size and types of containers used for growth of cultures; methods for preparing seed cultures and inoculating production cultures; time and conditions for incubation; observations during growth; criteria and specifications for satisfactory harvest material; and harvest techniques. There should be documentation on measures implemented by the firm to minimise the risk of transmissible spongiform encephalopathies (TSE) agent contamination in ingredients of animal origin and procedures to insure that fetal bovine serum is free of pestiviruses. It should also include: a description of all tests conducted to assess the purity and quality of the product as it proceeds through the production process; each step in the formulation of the final product; the tests used for assessing the purity, safety, potency, and other requirements of each batch/serial of completed product; the specifications for finishing, including packaging and labelling with complete indications and recommendations for use; and the expiry date established for the product.

Guidelines for the preparation of such documents for veterinary vaccines are published by competent control authorities. This documentation is intended to define the product and to establish its specifications and standards. It should serve along with the blueprints and blueprint legends (or production plan and SOPs) as a uniform and consistent method of producing the product that should be followed in the preparation of each batch/serial.

RECORD KEEPING

The producer should establish a detailed record-keeping system capable of tracking the performance of successive steps in the preparation of each biological product. Records kept should indicate the date that each essential step was taken, the name of the person who carried out the task, the identity and quantity of ingredients added or removed at each step, and any loss or gain in quantity in the course of the preparation. Records should be maintained of all tests conducted on each batch/serial. All records relevant to a batch/serial of product should be retained for at least 2 years after the expiry date on the label, or in line with the requirements of the competent control authority. In addition, a record should be maintained of all labels used on all products, with each label identified as to its name, product number, product licence number, package size, and label identification number. All labels printed should be accounted for. Records must be kept concerning sterilisation and pasteurisation procedures. These are usually made by means of automatic recording devices. The manufacturer must also keep complete records for all animals at the establishment, including health prior to being used for any tests, results of tests performed, treatment administered, maintenance, necropsy, and disposal.

MASTER SEED

The objective of testing the master seed is to ensure vaccine safety, quality and efficacy. Safety should be tested in an early stage. A master seed should be established for each microorganism used in the production of a product to serve as the source of seed for inoculation of all production cultures. Working seeds and production seeds may be prepared from the master seed by subculturing; generally the final production cultures should not be more than five (sometimes ten) passages from the master seed. The number of passages should be determined by data and designated in each case. Using a master seed and limiting the number of passages of seed microorganism in this manner assists in maintaining uniformity and consistency in production. Records of the source of the master seed should be maintained. For genetically modified microorganisms, the source of the gene(s) for the immunogenic antigens and the vector microorganism should be identified. Furthermore, the gene sequences introduced into the seed microorganism genome during construction of the modified seed should be provided. The master seed should consist of a single uniform batch/serial of seed that has been mixed and filled into containers as one batch/serial. Master seed should be frozen or desiccated and stored at low temperatures such as –40°C or –70°C, or under other conditions found to be optimal for maintaining viability. Each master seed should be tested to ensure its identity, safety and efficacy. Genetically modified seeds should also be tested to ensure stability and safety of the inserted gene sequences. Purity should also be established by testing to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses.

MASTER CELL STOCKS

When cell cultures are used to prepare a product, a master cell stock (MCS) should be established for each type of cell to be used. Records of the source of the master cell stock should be maintained. For each product, the highest and lowest passage levels of cells that may be used for production should be established and specified in the Outline of Production or SOP. Some control authorities do not permit more than 20–40 subcultivations. Each MCS should be characterised to ensure its identity, and its genetic stability should be demonstrated when subcultured from the lowest to the highest passage used for production. The karyotype of the MCS should be shown to be stable with a low level of polyploidy. Freedom from oncogenicity or tumorogenicity should be demonstrated by *in-vivo* studies in appropriate species using the highest cell passage that may be used for production. Purity of MCSs should be established by testing to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses.

- **Primary cells**

Primary cells are defined as a pool of original cells derived from normal tissue up to and including the tenth subculture used in the production of biologicals. In the case of products for use in poultry, these cells are usually obtained from specific pathogen free embryonating chicken eggs that have originated in an unvaccinated flock subjected to intensive microbiological monitoring. Other primary cells are derived from normal tissue of healthy animals and are tested for contamination with a wide variety of microorganisms as appropriate, including bacteria, fungi, mycoplasmas, and cytopathic and/or haemadsorbing-inducing agents or other extraneous viruses. The use of primary cells has an inherently higher risk of introducing extraneous agents compared with the use of cell lines and should be avoided where alternative methods of producing effective vaccines exist. Indeed, some control authorities only allow the use of primary cells in exceptional cases.

- **Embyronating eggs**

Embyronating eggs are also commonly used in the production of biologicals. In almost all cases they should be derived from specific pathogen free chicken flocks that have been intensively monitored for infectious agents and

have not been vaccinated. The route of inoculation of the egg and the choice of egg material to be harvested are dependent on the particular organism that is being propagated.

INGREDIENTS

The specifications and source of all product ingredients should be defined in the Outline of Production, SOP, or other appropriate documents. The Outline of Production must be approved by the National licensing agency. All ingredients of animal origin that are not subject to a validated sterilisation procedure should also be tested to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses. Their country of origin should be known. Measures should be implemented by the firm to avoid the risk of TSE agent contamination by ingredients of animal origin. Some control authorities discourage the use of preservatives or (more importantly) antibiotics as a means of controlling adventitious contamination during production and prefer the use of strict aseptic techniques to ensure purity. However, they sometimes allow the use of preservatives in multidose containers to protect the product during use. These control authorities usually limit any addition of antibiotics in the manufacture of the product to cell culture fluids and other media, egg inocula, and material harvested from skin or possibly other tissues. They normally permit the use of no more than three antibiotics in the same product. Some control authorities prohibit the use of penicillin or streptomycin in vaccines administered by aerosol or parenterally. If the antibiotics used are not recommended for use in the target species, they should be shown to have no harmful effects in the vaccinated animals and not result in the contamination of food derived from vaccinated animals.

SAFETY TESTS

The intrinsic safety of vaccines should be demonstrated early in the development stage and documented as part of the licensing dossier. Safety studies during development and licensing for all products should include the safety of a single dose, of an overdose and of repeated single doses. Additional data are derived for live vaccines from the increase in virulence tests and by assessing risk to the environment and in-contact animals, as discussed below. Safety should be demonstrated in each species for which the product is indicated. As a general rule, overdose studies are required for all vaccines: ×10 for live and ×2 for inactivated vaccines (if this is not practical, an indication of safety may be obtained from the results of the potency tests). For inactivated virus or bacterial products, where host animals are used for potency testing, safety may be determined by measuring local and systemic responses following vaccination and before challenge in the potency tests. Further evidence concerning the safety of products is derived from field safety trials (discussed below). Vaccines derived through biotechnology should be evaluated as discussed in the classification of biotechnology-derived vaccines and release of live rDNA vaccines below.

INCREASE IN VIRULENCE TESTS

With live vaccines, there is concern that the organism might be shed from the host and transmitted to contact animals, causing disease if it retains residual virulence or reverts to virulence. Therefore, all live vaccines should be tested for virulence by means of passage studies. Vaccine organisms are propagated *in vivo* by inoculating a group of target animals with master seed, in principle; this inoculation uses the natural route of infection for that organism that is most likely to result in infection and reversion and, if possible, that represents a recommended route of administration of the vaccine manufactured from this master seed. The vaccine organism is recovered from tissues or excretions and is used directly to inoculate a further group of animals, and so on. After not less than four passages, i.e. use of a total of five groups of animals (more for poultry products), the isolate must be fully characterised, using the same procedures used to characterise the master seed. Regulatory authorities opinion varies in whether or not it is acceptable to propagate *in vitro* between passages organisms that otherwise cannot be passaged five times because of their degree of attenuation. The vaccine organism must retain an acceptable level of attenuation after propagation in this way.

ASSESSING RISK TO THE ENVIRONMENT

The ability of each live vaccine to shed, to spread to contact target and non-target animals, and to persist in the environment must be evaluated to provide information for assessing the risk of the vaccine to the environment, taking into account human health. In some cases this may be done in conjunction with the increase in virulence tests. These and additional considerations are especially important in the case of products based on biotechnology or recombinant DNA techniques; more information about such products is provided in the sections at the end of this chapter.

EFFICACY TESTS

The efficacy of veterinary vaccines should be demonstrated by statistically valid vaccination–challenge studies in the host animal, using the most sensitive, usually the youngest, animals for which the product is to be recommended. Data should support the efficacy of the vaccine in each animal species by each vaccination regimen that is described in the product label recommendation, including studies on the onset of protection when claims for onset are made in the product labelling and for the duration of immunity. The tests should be performed under controlled conditions starting, wherever possible, with seronegative animals. Where validated potency tests are available, target species vaccination–challenge studies may not be required if predictive serological test results are available. The application of procedures to replace, reduce, and refine animal tests (the 'three Rs rule') should be encouraged whenever possible.

Efficacy studies should be conducted with final product vaccine that has been produced at the highest passage level from the master seed that is permitted in the Outline of Production, or other documentation of the manufacturing process. This will have specified the minimum amount of antigen per dose that must be in the final product throughout the entire authorised shelf-life. Where a range of antigen level per dose is permitted, the antigen level per dose in the vaccine tested for efficacy must be at or below the minimum permitted amount. The precise challenge method and the criteria for determining protection vary with the immunising agent and should be standardised whenever possible.

Field efficacy studies may be used to confirm the results of laboratory studies or to demonstrate efficacy when meaningful vaccination–challenge studies are not feasible. However, it is generally more difficult to obtain statistically significant data to demonstrate efficacy under field conditions. Protocols for field studies are more complex, and care must be given to establish proper controls to ensure the validity of the data. Even when properly designed, field efficacy studies may be inconclusive because of uncontrollable outside influences. Some problems include: a highly variable level of challenge; a low incidence of disease in nonvaccinated controls; and exposure to other organisms causing a similar disease. Therefore, efficacy data from both laboratory and field studies may be required to establish the efficacy of some products, as well as 'posteriory' field trials linked to vaccinovigilance.

INTERFERENCE TESTS

For products with two or more antigenic components, tests must confirm that there is no interference between individual components, that is, one component causing a decrease in the protective immunological response to another component. Interference testing should be conducted for each combination product prior to approval.

A loss of potency may also result when residual inactivating agent in a killed liquid product used as a diluent for a desiccated live fraction reduces the viability of the live organisms because of viricidal or bacteriocidal activity. Each batch/serial of liquid killed vaccine that is to be used as a diluent for live vaccines must, therefore, be tested for viricidal or bacteriocidal activity prior to release.

Consideration must also be given to possible interference between two different vaccines from the same manufacturer recommended to be given to the same animal within a 2-week period.

CONSISTENCY OF PRODUCTION

Prior to marketing approval of any new product, each establishment should produce in its facilities three consecutive production batches/serials of completed product to evaluate the consistency of production. These batches/serials should be prepared according to the procedures described in the Outline of Production and blueprints and legends, SOPs or other documentation of the manufacturing process and should therefore be 'typical of production'. Some authorities require that the size of each of the three batches/serials should be at least one-third the size of the average batch/serial that will be produced once the product is in production.

The manufacturer should test each of these batches/serials for purity, safety, and potency as provided in the Outline of Production or other documentation of the manufacturing process. Applicable Standard Requirements and test procedures, for example those described in CFR (Code of Federal Regulations) Title 9 part 113, in the Annex to EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or as described in this *Terrestrial Manual* may be used. Satisfactory test results should be demonstrated for all three batches/serials prior to approving the production of the product in the facilities and its release for marketing. Each subsequent batch/serial should be tested in the same manner with satisfactory results prior to release for marketing.

STABILITY TESTS

Stability studies (based on an acceptable potency test) are required to establish the validity of the expiry date that appears on the product package. Some authorities allow the use of accelerated stability tests to determine a provisional expiry date for products, e.g. incubating at 37°C for 1 week for each year of dating. Such estimates must be confirmed by periodic real-time potency tests on at least three different batches/serials through the period of time indicated by the expiry date, and 3–6 months beyond. For products containing viable organisms, testing should be done at release and at the approximate expiry date until a statistically valid record has been established. For non-viable products, each batch/serial presented for licensing is tested at release and at periodic intervals through, or past, the requested expiry date. If at the end of the dating period (shelf life) specified, the product is tested and found still to be above the release quality, consideration can be given to extending the designated shelf life, by request to the control authority. Stability testing also provides the opportunity to test for residual moisture and for other important parameters, such as the stability of adjuvant emulsions.

BATCH/SERIAL RELEASE FOR DISTRIBUTION

Prior to release, the manufacturer must test each batch/serial for purity, safety, and potency, as well as perform any other tests described in the firm's Outline of Production or other documentation of the manufacturing process for that product. In countries that have national regulatory programmes that include official control authority re-testing (check testing) of final products, samples of each batch/serial should also be submitted for testing in government laboratories by competent authorities. If unsatisfactory results are obtained for tests conducted either by the manufacturer or by competent authorities, the batch/serial should not be released. In such cases, subsequent batches/serials of the product should be given priority for check testing by competent authorities.

1. Batch/serial purity test

Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on: master seeds, primary cells, MCSs, ingredients of animal origin if not subjected to sterilisation (e.g. fetal bovine serum, bovine albumin, or trypsin), and each batch/serial of final product prior to release.

Purity test procedures have been published, for example in CFR Title 9 part 113, in the annex to EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this *Terrestrial Manual,* for the detection of extraneous viruses, bacteria, mycoplasma and fungi, including for example: *Salmonella*, *Brucella,* chlamydial agents, haemagglutinating viruses, avian lymphoid leukosis, pathogens detected by a chicken inoculation test, chicken embryo inoculation test, lymphocytic choriomeningitis, cytopathic and haemadsorbing agents, and pathogens detected by enzyme-linked immunosorbent assay, polymerase chain reaction, or the fluorescent antibody technique. Procedures used to ensure that fetal or calf serum and other ingredients of bovine origin are free of pestiviruses should be of high concern and well documented. Tests to be used to ensure purity vary with the nature of the product, and should be prescribed in the Outline of Production or other documentation of the manufacturing process. As tests for the detection of TSE agents in ingredients of animal origin have not been developed, vaccine manufacturers should document in their Outlines of Production or SOPs the measures they have implemented to minimise the risk of such contamination in ingredients of animal origin. This relies on three principles: first, verification that sources of all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy; second, that the tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents; third, where relevant, that the processes applied to the material have been validated for inactivation of TSE agents. Methods of production should also document the measures taken to prevent cross contamination of low risk materials by higher risk materials during processing.

2. Batch/serial safety test

Batch/serial safety tests are required for the release of each batch/serial and typical tests are described in CFR Title 9 part 113, in the European Pharmacopoeia, in this *Terrestrial Manual* and elsewhere. Standard procedures are given for safety tests in mice, guinea-pigs, cats, dogs, horses, pigs, and sheep and are generally conducted using fewer animals than are used in the safety tests required for licensing. Batches/serials are considered satisfactory if local and systemic reactions to vaccination with the batch/serial to be released are in line with those described in the registration dossier and product literature. Some authorities do not permit batch/serial safety testing in laboratory animals, requiring a test in one of the target species for the product.

3. Batch/serial potency test

Batch/serial potency tests, required for each batch/serial prior to release, are designed to correlate with the host animal vaccination–challenge efficacy studies. For inactivated viral or bacterial products, potency tests may be

conducted in laboratory or host animals, or by means of quantitative *in-vitro* methods that have been validated reliably to correlate *in vitro* quantification of important antigen(s) with *in vivo* efficacy. The potency of live vaccines is generally measured by means of bacterial counts or virus titration. Recombinant DNA or biotechnology-based vaccines should also be tested. Live genetically modified organisms can be quantified like any other live vaccine by titration, and expressed products of recombinant technology are quantified by *in vitro* tests, which can be easier to perform compared with tests on naturally grown antigens because of the in-process purification of the desired product.

When testing a live bacterial vaccine for release for marketing, the bacterial count must be sufficiently greater than that shown to be protective in the master seed immunogenicity (efficacy) test to ensure that at any time prior to the expiry date, the count will be at least equal to that used in the immunogenicity test. When testing a live viral vaccine for release, the virus titre must, as a rule, be sufficiently greater than that shown to be protective in the master seed immunogenicity test in order to ensure that at any time prior to the expiry date, the titre will be at least equal to that used in the immunogenicity test. Some control authorities specify higher bacterial or viral content than these. It is evident that the appropriate release titre is primarily dependent on the required potency and secondarily dependent on the rate of decay of the bacteria or viruses in the vaccine, as indicated by the stability test.

Standard Requirements have been developed and published by competent authorities for potency testing several vaccines. These tests can be found in CFR Title 9 part 113, in the European Pharmacopoeia, and in this *Terrestrial Manual*.

OTHER TESTS

Depending on the form of vaccine being produced, certain tests may be indicated and should be provided as appropriate in the Outline of Production or other documentation of the manufacturing process. These tests may concern: the level of moisture contained in desiccated products, the level of residual inactivant in killed products, the complete inactivation of killed products, pH, the level of preservatives and permitted antibiotics, physical stability of adjuvants, retention of vacuum in desiccated products, and a general physical examination of the final vaccine. Tests for these purposes may also be found in CFR Title 9 part 113, in EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this *Terrestrial Manual*.

SAMPLING

Samples should be selected from each batch/serial of product. The selector should pick representative final containers from each batch/serial and store these samples at the storage temperature recommended on the label. The producer should keep these reserve samples at the recommended storage temperature for a minimum of 6 months after the expiry date shown on the label, so that they are available to assist in evaluating the cause of any field problems reported from the use of the vaccine. The samples should be stored in a secure storage area and be tamper-evident.

LABELLING

Standards for labelling products will vary from country to country; however, the label indications and all claims that are made on the label should be supported by appropriate data that have been reviewed and approved by competent authorities. It is recommended that all labels for veterinary vaccines be water-proof and contain the following information, although for very small containers, the label may instead refer to the carton label or to an enclosed package insert for some of the less prominent information:

1.   The true name of the product, prominently lettered and with equal emphasis on each word;

2.   The name and address of the producer (and also the importer for imported products);

3.   The recommended storage temperature;

4.   A statement that the product is 'for veterinary (or animal) use only'. Full instructions for use, including all required warnings;

5.   For food animals, a statement indicating that the animals should not be vaccinated within a specified number of days before slaughter. This will depend on the vaccine (e.g. type of adjuvant) and is not required for all products;

6.   The expiry date;

7.   The batch/serial number by which to identify the product in the producer's record of preparation;

8.   The licence number for the product; in some countries this is replaced by the licence number of the establishment/manufacturer;

9.   The recoverable quantity and number of doses;

10. A statement that the entire contents of a multidose container should be used when the container is first opened (or with appropriate holding time for certain products, as supported by data) and that any unused portions should be disposed of in a proper manner;

11. A safety warning to the operator, if appropriate, e.g. accidental self-injection with oil emulsion vaccines.

12. Where it is allowed for an antibiotic to be added to a vaccine during the production process, the statement "Contains (antibiotic name) as a preservative" or an equivalent statement indicating the antibiotic added should appear on the carton or enclosures if used. If cartons are not used, such information should appear on the final container label.

Labels may also include other factual statements that are not false or misleading. Special restrictions concerning the use or handling of the product, when applicable, should also be indicated.

Similar information should also be given in a Product Data Sheet that is provided as a package insert. This will also contain much more detail about method of use and possible adverse reactions.

FIELD TESTS (SAFETY AND EFFICACY)

All veterinary biological products administered to animals should be tested for safety and, if possible, for efficacy in the field, using good clinical practice, before being authorised for general use. Field studies are designed to demonstrate efficacy under working conditions and to detect unexpected reactions, including mortality, that may not have been observed during the development of the product. Under field conditions there are many uncontrollable variables that make it difficult to obtain good efficacy data, but demonstration of safety is more reliable. The tests should be done on the host animal, at a variety of geographical locations, using appropriate numbers of susceptible animals. The test animals should represent all the ages and husbandry practices for which the product is indicated; unvaccinated controls must be included. The product tested should be one or more production batches/serials. A protocol should be developed indicating the observation methods and the recording methods.

INSPECTION OF PRODUCTION FACILITIES

Establishments that are approved to produce veterinary biologicals should be subject to in-depth inspections of the entire premises by national competent authorities to ensure compliance with the Outline of Production and blueprints and legends, SOPs, or other documentation of the manufacturing process. These inspections may include such items as: personnel qualifications; record keeping; general sanitation and laboratory standards; research activities on products being developed; production procedures; operation of sterilisers, pasteurisers, incubators, and refrigerators; filling, desiccating, and finishing procedures; care and control of animals; testing procedures; distribution and marketing; and product destruction. It is desirable to have good manufacturing practice (for manufacturing) and good laboratory practice (for quality assurance testing). (See chapter 1.1.3 Quality management in veterinary testing laboratories, for guidelines.)

The inspectors should prepare a comprehensive report documenting the findings of the inspection and stating the actions that the establishment must take to improve its production processes. The establishment should receive a copy of the report. When necessary a follow-up inspection should be conducted to determine whether appropriate action has been taken to correct deficiencies. Continued reassessment in this manner is needed to ensure that production facilities continue to be operated in an acceptable manner.

UPDATING THE OUTLINE OF PRODUCTION

Before production procedures are changed, the corresponding Outline of Production or other documentation of the manufacturing process should be changed. Establishments should have internal review procedures to evaluate all changes in production before they are initiated. Changes should also be reviewed and approved by competent authorities prior to their implementation. In cases where a significant production step is altered, revisions may require additional data to support the purity, safety, potency, and/or efficacy of the product. In countries with regulatory programmes that include check testing the final product at national laboratories, revisions should entail testing of the new product by competent authorities.

PERFORMANCE MONITORING

Manufacturers are required to maintain an adverse reaction notification system and an effective mechanism for rapid product recall. These should both be subject to audit by regulatory bodies. In many countries, the manufacturer must notify all adverse reactions immediately to the regulatory authority, along with any remedial action taken. An alternative used in some countries is that if at any time, there are indications that raise questions regarding the purity, safety potency, or efficacy of a product, or if it appears that there may be a problem regarding the preparation, testing or distribution of a product, the manufacturer must immediately notify the regulatory authorities concerning the circumstances and the action taken.

After release of a product, its performance under field conditions should continue to be monitored by competent authorities. Consumer complaints may serve as one source of information; however, such information needs to be investigated to determine whether or not the reported observations are related to the use of the product. Users of veterinary vaccines should be informed of the proper procedures for making their complaints. The manufacturer of the product should be informed of all complaints received by competent authorities. Competent authorities should also ascertain whether they have received other similar complaints for this product and, if so, whether the manufacturer has taken appropriate action. Control laboratories may test samples of the batch/serial of product involved, if necessary.

When the investigation is complete, a final report should be prepared and a summary of the findings sent to the complainant and to the manufacturer. When it is determined that a product is causing serious problems, immediate action should be taken to remove the product from the market and to notify animal health authorities.

ENFORCEMENT

National programmes established to ensure the purity, safety, potency, and efficacy of veterinary vaccines must have adequate legal authority to ensure compliance with product registration conditions and other programme requirements. The goal should be to obtain voluntary compliance with established regulatory requirements. However, when violations occur, competent authorities must have adequate legal authority to protect animal and human health. Authority for detention, seizure, and condemnation of products found to be worthless, contaminated, dangerous, or harmful may be valuable for this purpose. Under such authority, product may be detained for a period of time, and if during that time compliance cannot be achieved, competent authorities may seek a court order or decree for seizure and condemnation.

The authority to remove or suspend establishment and/or product licenses, obtain injunctions, and stop the sale of product is also needed. Civil penalties or criminal prosecution may also be necessary for serious or deliberate violations.

LICENSING OF PRODUCTS DERIVED THROUGH BIOTECHNOLOGY

Recent advances in biotechnology have made possible the development and commercialisation of new biological products with useful antigenic and diagnostic properties. Many such products have now been licensed or approved, and more are being developed. Products of rDNA technology do not differ fundamentally from conventional products. Therefore, existing laws and regulations are fully applicable to these new products.

CLASSIFICATION OF BIOTECHNOLOGY-DERIVED VACCINES

Each competent authority with power to regulate organisms and products derived from recombinant techniques should ensure that the public health and the environment are protected from any potentially harmful effect. For the purpose of evaluating licence applications, veterinary vaccines derived through rDNA technology may be divided into three broad categories. The division is based on the products' biological properties and on the safety concerns they present.

Category I consists of nonviable or killed products that pose no risk to the environment and present no new or unusual safety concerns. Such products include inactivated microorganisms, either whole or as subunits, created by using rDNA techniques.

Category II products contain live microorganisms modified by adding or deleting one or more gene(s). Added genes may code for marker antigens, enzymes, or other biochemical by-products. Deleted genes may code for virulence, oncogenicity, marker antigens, enzymes, or other biochemical by-products. The licence application must include a characterisation of the DNA segments added or deleted, as well as a phenotypic characterisation of the altered organism. The genetic modifications must not result in any increase in virulence, pathogenicity, or

survivability of the altered organism in comparison with the wild-type form. It is important that the genetic modification does not cause a deterioration in the safety characteristics of the organism.

Category III products make use of live vectors to carry recombinant-derived foreign genes that code for immunising antigens. Live vectors may carry one or more foreign gene(s) that have been shown to be effective for immunising target host animals. The use of DNA vaccines containing recombinant-derived foreign genes that code for immunising antigens (plasmid DNA vaccines) constitutes a new approach to vaccine development. The proper categorisation of this type of rDNA-derived product will be established as biological properties and safety characteristics are determined. These new vaccines may find application in a wide variety of situations much as conventional products have. Guidelines for the development, production, characterisation, and control of these new products are still preliminary and subject to change as new data and knowledge are developed. Information concerning the current thinking on regulatory guidelines may be found on the Internet at the following addresses: http://www.cba.unige.it/VL/bio-info.html; http://www.aphis.usda.gov/vs/cvb; http://www.orcbs.msu.edu/biological/biolsaf.htm; http://www.pestlaw.com/index.html; http://www.emea.europa.eu/pdfs/vet/iwp/000798en.pdf

RELEASE OF LIVE rDNA PRODUCTS

The release of live rDNA microorganisms (Categories II and III) for field testing or general distributions as an approved or licensed product may have a significant effect on the quality of the human and animal environment. Before release is authorised, the manufacturers of the vaccine should conduct a risk assessment to evaluate the impact on the human and animal environment. In the USA, for example, a procedure is adopted that could be used as a model system in other countries. The European Union has adopted a similar system. It is performed as follows:

A risk assessment is carried out that should contain the following information: the purpose and need for the proposed action; the alternatives considered; a list of the government agencies, organisations, and persons consulted; and the affected environment and the potential environmental consequences. The topics discussed should include: the characteristics of the vaccine organism, human health risks, animal health risks for both target and nontarget animals, persistence in the environment, and increase in virulence.

If the risk assessment results in a finding by competent authorities that the proposed release of the recombinant vaccine into the environment for field trials or general distribution would not have a significant impact on the environment, a notice should be published and distributed to the public announcing this and that the risk assessment and findings are available for public review and comment. If no substantive comments are received to refute the findings, competent authorities may authorise the field testing or grant the license or approval for general distribution.

The preparation of a risk assessment and the findings made from the assessment may also include the scheduling of one or more public meetings if a proposed action has ecological or public health significance. Such meetings should be announced through a public notice. Interested persons should be invited to make presentations, along with presentations by the producer of the product, and government personnel. The transcripts of such meetings should become part of the public record.

If, in the course of preparing a risk assessment, competent authorities conclude that the proposed action may have a significant effect on the human environment, an Environmental Impact Statement (EIS) should be prepared. The EIS provides a full and fair discussion of the significant environmental impacts, and informs decision-makers and the public of any reasonable alternatives that would avoid or minimise the adverse impacts. (Environmental documents are considered in CFR Title 40 part 1508.) See also EU Directive 2001/18/EC and http://www.emea.europa.eu/pdfs/vet/iwp/000404en.pdf

FURTHER READING

The following are some suggested texts that contain guidelines on aspects of vaccine production.

A.	COUNCIL OF EUROPE (2005). European Pharmacopoeia, Fifth Edition. Editions of the Council of Europe, Strasbourg, France.

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D.   EUROPEAN COMMISSION (2006). The Rules Governing Medicinal Products in the European Union. Eudralex. Volumes 1–9. European Commission Enterprise and Industry DG; Directorate F – Consumer goods. Latest versions only available at http://pharmacos.eudra.org/F2/eudralex/index.htm.

E.   GAY C.G. & ROTH H.J. (1994). Confirming the safety characteristics of recombinant vectors used in veterinary medicine: a regulatory perspective. Recombinant vectors in vaccine development. *Dev. Biol. Stand.*, **82**, 93–105.

F.   ROTH H.J. & GAY C.G. (1996). Specific safety requirements for products derived from biotechnology. *In:* Veterinary Vaccinology, Pastoret P.-P., Blancou J., Vannier P. & Verschueren C., eds. Elseviers Science Publishers B.V. Amsterdam, The Netherlands.

G.   PASTORET P.P., BLANCOU J., VANNIER P. & VERSCHUEREN C., EDS (1997). Veterinary Vaccinology. Elsevier Science, Amsterdam, The Netherlands.

H.   UNITED STATES DEPARTMENT OF AGRICULTURE (USDA) (2000). Code of Federal Regulations, Title 9, Parts 1–199. US Government Printing Office, Washington DC, USA.

I.   USDA-APHIS[1]-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1999). Categories of Inspection for Licensed Veterinary Biologics Establishments. Veterinary Services Memorandum No. 800.91. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

J.   USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1999). Veterinary Biological Product Samples. Veterinary Services Memorandum No. 800.59. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

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L.   USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Veterinary Biologics General Licensing Considerations No. 800.200, Efficacy Studies. USDA-APHIS-Veterinary Biologics, 4700 River Road, Riverdale, Maryland 20737, USA.

M.   USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Veterinary Biologics General Licensing Considerations No. 800.201, Back Passage Studies. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

N.   USDA-APHIS-VETERINARY SERVICES (1964–1994). Standard Assay Methods, Series 100–900. National Veterinary Services Laboratories, Ames, Iowa 50010, USA.

O.   USDA-APHIS- VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1984). Basic License Requirements for Applicants. Veterinary Biologics Memorandum No. 800.50. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA

P.   USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1988). Guidelines for the Preparation and Review of Labeling Materials. Veterinary Services Memorandum No. 800.54. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

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[1]   United States Department of Agriculture (USDA), Animal and Plant Health Inspection Services (APHIS). USDA-APHIS-CENTER FOR VETERINARY BIOLOGICS HOME PAGE: http://www.aphis.usda.gov/vs/cvb/index.html

RISK ANALYSIS FOR BIOLOGICALS FOR VETERINARY USE

GENERAL CONSIDERATIONS

All products, including biologicals for veterinary use, derived from animals have some capacity to transmit animal disease. The level of this capacity depends on the inherent nature of the products, their source, the treatment that they might have undergone, and the purpose for which they are intended. Biologicals for *in vivo* use in particular will have the highest probability of exposure to animals and as such present the highest risk. Products used for *in vitro* purposes can introduce disease into animal populations through deliberate or inadvertent use *in vivo*, contamination of other biologicals, or spread by other means. Even products for diagnosis and research have the potential for close contact with animals. Exotic micro-organisms, some highly pathogenic, which may be held for research and diagnostic purposes in countries free from infection or the diseases they cause, could possibly contaminate other biological products.

Veterinary Authorities of importing countries shall make available specific procedural requirements for approval or licensing of biologicals for veterinary use. They may limit supply to registered institutions or *in vitro* use or for non-veterinary purposes where such assurance cannot be provided.

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RISK ANALYSIS FOR VETERINARY VACCINES

INTRODUCTION

Risk analysis for veterinary vaccines has to be founded on the principles of quality assurance, which includes quality control, in the production of veterinary vaccines. These recommendations are focused mainly on the risk related to the contamination of vaccines by infectious agents particularly in regard to the risk of importing exotic diseases. The major risk of introducing a disease into a country is through importation of live animals or animal products and rarely through veterinary vaccines. Veterinary vaccines can however be contaminated by disease agents if master seeds, strains, cell cultures, animals or ingredients of animal origin such as fetal calf serum used in production are contaminated or if cross contamination occurs during the production process.

PRINCIPLES

Exporting countries and importing countries should agree on a system of classification of risks associated with veterinary vaccines taking into account factors such as purification procedures which have been applied.

Exporting countries and importing countries should agree on risk analysis models to address specific issues and products. Such risk analysis models should include a scientific risk assessment and formalised procedures for making risk management recommendations and communicating risk. The regulation of veterinary vaccines should include the use of either qualitative or quantitative models.

Risk analysis should be as objective and transparent as possible. Step risk and scenario tree methods should be used in risk assessment whenever appropriate, as they identify the critical steps in the production and use of the products where risks arise and help to characterise those risks.

The same conclusions about risk analysis may be reached by differing methods. Where methods may differ in countries, the concept of equivalence should apply wherever possible and the methods should be validated to ensure they are of comparable sensitivity.

MANUFACTURING PRACTICES

The manufacture of veterinary vaccines has special characteristics which should be taken into consideration when implementing and assessing the quality assurance system. Due to the large number of animal species and related pathogenic agents, the variety of products manufactured is very wide and the volume of manufacture is often low; hence, work on a group basis is common. Moreover, because of the very nature of this manufacture (cultivation steps, lack of terminal sterilisation, etc.), the products must be particularly well protected against contamination and cross contamination. The environment must also be protected especially when the manufacture involves the use of pathogenic or exotic biological agents and the worker must be particularly well-protected when the manufacture involves the use of biological agents pathogenic to man.

These factors, together with the inherent variability of immunological products, means that the role of the quality assurance system is of the utmost importance. It is important that vaccines should be manufactured in accordance with a recognised codified system that includes specifications regarding equipment, premises, qualification of personnel as well as quality assurance and regular inspections.

A commonly agreed system of facility inspection carried out by qualified and specialised inspectors must be in place to assure confidence.

INFORMATION TO BE SUBMITTED WHEN APPLYING FOR REGISTRATION IN THE IMPORTING COUNTRY

The manufacturer or Veterinary Authority of the exporting country should make available to the importing country the pharmacopoeia it uses. For the importing country it is necessary to have documented both the quality control methods used and the source of each batch of starting materials. The key steps of the manufacturing process of veterinary vaccines should be described in detail to help risk analysis. Risk analysis has to be focused on the quality and safety parts of the application file. Laboratory safety testing should cover target and non-target organisms to obtain sufficient biological data. All test procedures used should correspond with the state of scientific knowledge at the time and should be validated.

The description of the method of preparation of the finished product should include an adequate characterisation of the substances needed to prepare the working seeds, the description of the treatments applied to starting materials to prevent contamination, and a statement of the stages of manufacture at which sampling is carried out for process control tests.

The results of control tests during production and on finished product, as well as the sensitivity of these tests, have to be available for risk analysis. The stepwise procedures of the control tests should also be available.

CATEGORISATION OF VETERINARY VACCINES

To assist in risk analysis, countries should establish a system of categorisation of veterinary vaccines taking into account criteria such as pathogens used as active ingredients, their inherent characteristics and the risk they pose.

In case of live vectored vaccines, the safety of the vector to the targeted and non-targeted species and to human beings must be assessed. Special attention should be paid to potential tissue tropism or host range modification of the recombinant.

VACCINOVIGILANCE

Exporting countries and importing countries should ensure that a reliable system of vaccinovigilance (post licensing monitoring) is established to identify, at the earliest stage, any serious problems encountered from the use of veterinary vaccines. Vaccinovigilance should be ongoing and an integral part of all regulatory programmes for veterinary vaccines, especially live vaccines.

RISK COMMUNICATION

Reliable data in support of applications submitted in importing countries should be provided by the manufacturer or the Veterinary Authority of the exporting country. Relevant data on risk analysis, changes in animal health situations and vaccinovigilance should be shared by Veterinary Authorities on a continuous basis.

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TESTS FOR STERILITY AND FREEDOM FROM CONTAMINATION OF BIOLOGICAL MATERIALS

INTRODUCTION

Sterility is defined as the absence of living organisms. It is achieved by heating, by filtration, by treatment with ethylene oxide or by ionising irradiation, and by conducting any subsequent processes aseptically. Freedom from contamination is defined as the absence of specified living organisms. This may be achieved by selecting materials from sources shown to be free from the specified organisms and by conducting subsequent procedures aseptically. Adequate assurance of sterility and freedom from contamination can only be achieved by proper control of the primary materials used and their subsequent processing and storage. Tests on the product are necessary to check that this control has been achieved.

A. GENERAL PROCEDURES

1. Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply.

2. Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method effective for the materials concerned. The method must reduce the level of contamination to be undetectable, as determined by an appropriate sterility test (see paragraph B.3 below).

3. If a sterilisation process is used, it shall be validated to demonstrate its suitability and adequately controlled to show that it has functioned properly on each occasion.

4. Materials that are not sterilised and those that are to be processed further after sterilisation must be handled aseptically.

5. The environment in which any aseptic handling is carried out must be maintained in a clean state and protected from external sources of contamination, and must be controlled to prevent internal contamination.

B. LIVING VIRAL VACCINES FOR ADMINISTRATION BY INJECTION

1. Materials of animal origin shall be (a) sterilised, or (b) obtained from healthy animals that, in so far as is possible, should be shown to be free from pathogens that can be transmitted from the species of origin to the species to be vaccinated, or any species in contact with them, or (c) the material shall be shown to be free from such pathogens.

2. Seed lots of virus and of any continuous cell line used for virus growth shall be shown to be free from bacteria, fungi, mycoplasmas, extraneous viruses and other pathogens that can be transmitted from the species of origin to the species to be vaccinated or any species in contact with them. For the production of avian vaccines and the quality control procedures for these vaccines, it is recommended that specific pathogen-free embryonated chicken eggs be used.

3. Each batch of vaccine shall pass a test for sterility that is similar to published methods (1–3, 6).

4. Each batch of vaccine shall pass tests appropriate to prove that the vaccine is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be vaccinated, tests in embryonated eggs, and, where necessary, tests in animals.)

5. Some countries require that each batch of vaccine pass a test for freedom from mycoplasma. Suitable test methods have been published (1, 2, 6).

6. Tests for freedom from certain specific bacteria may be required, e.g. tests for *Salmonella*, *Mycobacterium tuberculosis* and *M. paratuberculosis*, *Brucella* spp. and *Leptospira* spp. (1, 2).

C. LIVING VIRAL VACCINES FOR ADMINISTRATION THROUGH DRINKING WATER, SPRAY, OR SKIN SCARIFICATION

1. Paragraphs B.1, 2, 4, 5, and 6 apply.

2. A limited number of contaminating, nonpathogenic bacteria and fungi may be permitted (see Section J.2.5 below).

D. INACTIVATED VIRAL VACCINES

1. Paragraphs B.1, 2 and 3 apply.

2. Each batch of vaccine shall pass a test for inactivation of the vaccinal virus. This is done before addition of preservative. The inactivation process and the tests used to detect live virus after inactivation must be validated and shown to be suitable for their intended purpose.

3. Demonstration that the method of inactivation also inactivates representative pathogens may be required unless the vaccine satisfies the conditions of paragraphs B.4 and B.5.

E. LIVING BACTERIAL VACCINES

1. Paragraphs B.1 applies.

2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas.

3. Each batch of vaccine shall pass a test for purity carried out using solid media and ignoring the growth of the vaccinal bacterium.

4. Some countries require that each batch of bacterial vaccine passes a test for freedom from mycoplasmas. Suitable test methods have been published (ref. 6, and for avian mycoplasmas ref. 2).

F. INACTIVATED BACTERIAL VACCINES

1. Paragraphs B.1, B.3, and E.2 apply.

2. Each batch of vaccine shall pass a test for inactivation of the vaccinal bacterium. If appropriate, the test for sterility may be used for this purpose.

G. SERA FOR ADMINISTRATION TO ANIMALS

1. Paragraph B.1 applies. Some countries require quarantine, health certification, and specific disease tests be completed for all serum donor animals (1).

2. Paragraph B.2 or E.2 applies, as appropriate, if a virus or a bacterium is used in serum production.

3. Each batch of serum shall pass a test for sterility. Suitable test methods have been published (1, 2).

4. Each batch of serum shall pass tests appropriate to prove that the serum is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be treated, tests in embryonated eggs and, where necessary, tests in animals.)

5. Some countries require that each batch of serum passes a test for freedom from mycoplasmas. Suitable test methods have been published (ref. 6, and for avian mycoplasmas ref. 2).

H. DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

1. Paragraphs B.1 and 3 apply.

2. Paragraphs B.2 and D.2 apply if a virus is used in the production of the diagnostic agent; E.2 and F.2 apply if a bacterium is used.

I. EMBRYOS, OVA, AND SEMEN

Special precautions must be taken with relation to the use of embryos, ova and semen (4).

J. PROTOCOL EXAMPLES

1. General procedures

Materials used in the production of biological products should be sterilised and/or tested to ensure freedom from contaminants before being used. Samples of the finished biological product should also be tested for bacterial, fungal, or mycoplasmal contaminants.

The assays for bacteria, mycoplasma, fungi, and viruses described here are derived from various sources and they are given as examples of methods that can be used with confidence.

2. Detection of bacteria and fungi

These assays describe the materials and methods that are used for the detection of bacteria and fungi by either the membrane filtration method, or the direct inoculation of fluid media method used for materials that are unsuitable for membrane filtration.

2.1. General procedure for detecting viable bacteria and fungi

Standard tests for detecting extraneous bacteria and fungi in raw materials, seed stocks, or final product are: the membrane filtration test or the direct inoculation sterility test.

For the membrane filtration technique, a filter having a nominal pore size not greater than 0.45 µm and a diameter of at least 47 mm should be used. Cellulose nitrate filters should be used if the material is aqueous or oily; cellulose acetate filters should be used if the material is strongly alcoholic, oily or oil-adjuvanted. Immediately before the contents of the container or containers to be tested are filtered, the filter is moistened with 20–25 ml of Diluent A or B.

Diluent A – for aqueous products or materials: Dissolve 1 g peptic digest of animal tissue in water to make 1 litre, filter or centrifuge to clarify, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

Diluent B – for oil-adjuvanted products or materials: Add 1 ml polysorbate 80 to 1 litre Diluent A, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

If the biological being tested has antimicrobial properties, the membrane is washed three times after sample application with approximately 100 ml of the appropriate diluent (A or B). The membrane is then transferred whole to culture media, aseptically cut into equal parts and placed in media, or the media is transferred to the membrane in the filter apparatus. If the test sample contains merthiolate as a preservative, fluid thioglycollate medium (FTM) is used and the membranes are incubated at both 30–35°C and 20–25°C. If the test sample is a killed biological without merthiolate preservative, FTM is used at 30–35°C and soybean casein digest medium (SCDM) at 20–25°C. If the sample tested is a live viral biological, SCDM is used at both incubation temperatures. Recently, it has been suggested that sulfite-polymyxin-sulfadiazine agar be used to enhance the detection of *Clostridium* spp. when the membrane filtration technique is used (5).

If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to aseptically transfer the biological material directly into liquid media. If the biological being tested has antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be determined before the test is started. To determine the correct medium volume to negate antimicrobial activity, 100 colony-forming units (CFU) of the control microorganisms listed in Table 1 are used. If the test sample contains merthiolate as a preservative, FTM is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible after an appropriate incubation time (see Section J.2.2). If the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at 30–35°C and SCDM at 20–25°C. If the test sample is a live viral biological, SCDM is used at both incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a clostridial component, the use of FTM with 0.5% added beef extract (FTMB) in place of FTM is preferred. It may also be desirable to use both FTM and SCDM for all tests.

Table 1. *Some American type culture collection*[1] *strains with their respective medium and incubation conditions*

		Incubation	
Medium	**Test microorganism**	**Temperature (°C)**	**Conditions**
FTM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
FTM	*Candida krusei* ATCC # 6258	20–25	Aerobic
SCDM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
SCDM	*Candida kursei* ATCC # 6258	20–25	Aerobic
FTMB	*Clostridium sporogenes* ATCC # 11437	30–35	Anaerobic
FTMB	*Staphylococcus aureus* ATCC #6538	30-35	Aerobic

For both membrane filtration and direct inoculation sterility tests, all media are incubated for no fewer than 14 days. At intervals during incubation, and after 14 days' incubation, the test vessels are examined for evidence of microbial growth. Microbial growth should be confirmed by subculture and Gram stain.

2.2. Growth promotion and test interference

The sterility of the media should be confirmed by incubating representative containers at the appropriate temperature for the length of time specified for each test.

The ability of the culture media to support growth in the presence and absence of product, product components, cells, seeds, or other test material should be validated for each product to be tested, and for each new batch or lot of culture media.

To test for ability to support growth in the absence of the test material, media should be inoculated with 10–100 viable control organisms of the suggested American Type Culture Collection (ATCC) strains listed in Table 1 and incubated according to the conditions specified.

To test for ability of the culture media to support growth in the presence of the test material, containers should be inoculated simultaneously with both the test material (see Section J.2.3) and 10–100 viable control organisms. The number of containers used should be at least one-half the number used to test the product or product component. The test media are satisfactory if clear evidence of growth of the control organisms appears in all inoculated media containers within 7 days. In the event that growth is evident, the organism should be identified to confirm that it is the organism originally added to the medium. The sterility test is considered invalid if any of the media show inadequate growth response, or if the organism recovered is not the organism used to inoculate the material.

2.3. Number of items to be tested

The number of items in a batch determines the number of containers that should be tested for sterility. If the batch size is not more than 100, then 10% or four containers, whichever is the greater, should be tested. If the batch contains between 100 and 500 containers, then ten containers should be tested. If the batch has more than 500 containers, then 2% or 20 containers, whichever is the lesser, should be tested. An alternative is to test a maximum of 10 containers for all serials other than autogenous products.

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1   American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.

The amount of sterility test inoculum is dependent on the quantity of biological in each container. If the quantity is less than 1 ml, then the entire contents are used for each medium. If the quantity is from 1 to 4 ml, then half the contents are used in each medium. If the quantity is from 4 to 20 ml, then 2 ml inoculum per medium is used. If the quantity in each container is from 20 to 100 ml, then 10% of the contents are used per medium. If the quantity per container is greater than 100 ml, then 10% or 50 ml, whichever is the greater, is used to inoculate each medium.

2.4. Interpretation of sterility test results

If growth is found in any medium but it can be demonstrated by controls that the media or technique were faulty, then the first test is declared invalid and may be repeated. If microbial growth is found in any of the test vessels of the first test but there is no evidence invalidating it, then a retest may be conducted. The minimum number of biological containers, test vessels, and membrane filters in a retest is double the number used in the first test. If no growth is found in the first test or retest, the biological meets the requirements of the test and is considered satisfactory for sterility. If microbial growth is found in any of the retest vessels, the biological is considered unsatisfactory for sterility. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest may be repeated.

2.5. General procedure for testing live viral vaccines produced in eggs and administered through drinking water, spray, or skin scarification for the presence of bacteria and fungi

Each batch of final container biological should have an average contamination of not more than one bacterial or fungal colony per dose for vaccines recommended for poultry, or ten colonies per dose for other animals (see Section J.2.3 above to determine the number of samples to test). From each container sample, each of two Petri dishes are inoculated with vaccine equal to ten doses if the vaccine is recommended for poultry, or one dose if recommended for other animals. To each plate add 20 ml of brain–heart infusion agar containing 0.007 IU (International Units) of penicillinase per ml. One plate should be incubated at 30–35°C for 7 days and the other at 20–25°C for 14 days. Colony counts are made at the end of each incubation period. An average colony count of all the plates representing a batch should be made for each incubation condition. If the average count at either incubation condition exceeds one colony per dose for vaccines recommended for poultry, or ten colonies per dose for vaccines recommended for other animals in the initial test, one retest to rule out faulty technique may be conducted using double the number of unopened final containers. If the average count at either incubation condition of the final test for a batch exceeds one colony per dose for vaccines recommended for poultry, or ten colonies per dose for vaccines recommended for other animals, the batch of vaccine should be considered unsatisfactory.

2.6. General procedure for testing seed lots of bacteria and live bacterial biologicals for purity

Each seed lot of bacteria or batch of live bacterial biological should be tested for purity by inoculation of SCDM, which is incubated at 20–25°C for 14 days, and FTM, which is incubated at 30–35°C for 14 days (see Section J.2.3 above to determine the number of samples to be tested and the amount of test inoculum to be used). A sterile pipette or syringe and needle is used to aseptically transfer the quantity of biological directly into the two types of culture medium. The minimum ratio of inoculum to culture medium is 1/15.

If the inoculum or growth of the bacterial vaccine renders the medium turbid so that the absence of atypical microbial growth cannot be determined by visual examination, subcultures should be made from all turbid tubes on day 3 through to day 11. Subculturing is done by transferring 0.1–1.0 ml to differential broths and agar and incubating for the balance of the 14-day period. Microscopic examination by Gram stain should also be done.

If no atypical growth is found in any of the test vessels when compared with a positive control included in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found but it can be demonstrated by control that the media or technique were faulty, then the first test may be repeated. If atypical growth is found but there is no evidence invalidating the test, then a retest may be conducted. Twice the number of biological containers and test vessels of the first test are used in the retest. If no atypical growth is found in the retest, the biological is considered to be satisfactory for purity. If atypical growth is found in any of the retest vessels, the biological is considered to be unsatisfactory for purity. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest may be repeated.

3. Detection of *Mycoplasma* contamination

3.1. General procedure for detecting *Mycoplasma* contamination

Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master cell stock (MCS), and all ingredients of animal origin not steam sterilised should be tested for the absence of mycoplasmas. Solid and liquid media that will support the growth of small numbers of test organisms, such as typical contaminating organisms *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. fermentans*,

M. hyorhinis, *M. orale*, and *M. synoviae* should be used. The nutritive properties of the solid medium should be such that no fewer than 100 CFU should occur with each test organism when approximately 100–200 CFUs are inoculated per plate. An appropriate colour change should occur in the liquid media when approximately 20–40 CFUs of each test organism are inoculated. The ability of the culture media to support growth in the presence of product should be validated for each product to be tested, and for each new batch or lot of culture media.

One sample of each lot of vaccine, MSV, etc., should be tested. Inoculate each of four plates of solid medium with 0.25 ml of the sample being tested, and inoculate 100 ml of the liquid medium with 10 ml of the sample. An alternative is to inoculate each of the plates with 0.1 ml and to inoculate 100 ml of liquid medium with 1 ml of the sample being tested. Incubate two plates at 35–37°C aerobically (an atmosphere of air containing 5–10% CO_2 and adequate humidity) and two plates anaerobically (an atmosphere of nitrogen containing 5–10% CO_2 and adequate humidity) for 28 days. On day 3 or day 4 after inoculation, subculture 0.25 ml from the liquid media on to two plates of solid media. Incubate one plate aerobically and the second anaerobically at 35–37°C until day 28 of the test. Repeat the subculture procedure on day 6, 7, or 8 and again on day 13 or 14. An alternative method is to subculture on days 3, 5, 10, and 14 on to a plate of solid medium. All the subculture plates are incubated for 10 days except for the 14-day subculture, which is incubated for 14 days. Observe the liquid media every 2–3 days and, if any colour change occurs, subculture immediately.

3.2. Interpretation of *Mycoplasma* test results

At the end of the incubation period (day 28), examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The test sample passes the test if the growth of mycoplasma colonies has occurred on the positive controls, and if growth has not occurred on any of the solid media inoculated with the test material. If at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or is broken, the test is invalid and should be repeated. If mycoplasma colonies are found on any agar plate, the test should be repeated once to confirm the mycoplasma contamination. Twice the volume (0.5 ml) of biological material being tested may be used in the retest. If mycoplasma colonies are found on any of the agar plates of the retest, the test sample should be considered unsatisfactory because of mycoplasma contamination. Some mycoplasmas cannot be cultivated, in which case the MSV and MCS have to be tested using an indicator cell line (Vero cells), DNA staining, or polymerase chain reaction (PCR) methods.

4. Detection of *Salmonella* contamination

Each batch of live virus biological made in eggs should be free from contamination with *Salmonella*. This testing must be done before bacteriostatatic or bactericidal agents are added. Five samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the lesser, of the sample should be used to inoculate 100 ml of tryptose broth and tetrathionate broth. The inoculated broths should be incubated for 18–24 hours at 35–37°C. Transfers from these broths should be made on to MacConkey and Salmonella–Shigella agar, incubated for 18–24 hours, and examined. If no growth typical of *Salmonella* is noted, the agar plates should be incubated an additional 18–24 hours and again examined. If colonies typical of *Salmonella* are observed, further subculture on to suitable differential media should be made for positive identification. If *Salmonella* is found, the batch of biological is unsatisfactory.

5. Detection of viruses in biological materials

Biological materials subject to viral contamination that cannot be sterilised before use, such as ingredients of animal origin (for example, serum), primary cells, line cells or viral seed stocks, should be tested before they are used. Assays are described to detect viral contaminants by cytopathic effects (CPE), haemadsorption, haemagglutination, fluorescent antibody techniques and other suitable methods, e.g. PCR and enzyme-linked immunosorbent assay. All biological materials should be specifically tested for pestiviruses. Avian materials and vaccines should be inoculated on to primary avian cell cultures, eggs and/or chicks for the detection of avian viruses. In addition to examining for CPE and cellular abnormalities in these inoculated cells/eggs/chicks, tests for haemadsorbing and haemagglutinating viruses should also be included.

Cells shall be tested in the following manner. On day 0, primary or frozen cells to be tested are seeded on 75 cm^2 (or similar) flasks; 7 days later, at least two 75 cm^2 flasks are prepared. On day 14, one flask is used to test the cells for cytopathology, haemadsorption, and fluorescent antibody staining (procedures follow). The other flask is passaged a second time, and on day 21 is subjected to three freeze–thaw cycles. An alternative method is to freeze–thaw the cells at 26 days instead of 21 days. After the third freeze–thaw cycle, the cells are centrifuged at 2000 *g* for 10 minutes, and the supernatant is used to inoculate appropriate virus-sensitive cells, i.e. cells susceptible to viruses that may be present in the species of animal from which the cells were obtained, cells susceptible to viruses that may occur in the animals in which the material is going to be used and cells susceptible to pestiviruses. These cells are then passed twice at 7-day intervals, and tested for cytopathology, haemadsorption and by fluorescent antibody staining.

Ingredients of animal origin are tested on both African green monkey kidney (Vero) cells and on a cell line or primary cells derived from the same species as the ingredient under test. Cells are inoculated using 75 cm^2 flasks with 3.75 ml of test material in 25 ml of media or 15% of the test material, whichever is the lesser. The cells are passaged two or three times at 7-day intervals, and tested for cytopathology, haemadsorption and fluorescent antibody staining. The cells should be observed for cytopathology every 2 to 3 days, and prior to each subculture, throughout the incubation period.

MSV are tested on Vero cells, cell lines or primary cells of the species for which the product is intended, and cell lines or primary cells of the species in which the product is prepared (if different from the intended species).

For each cell type required for testing, 1 ml of the test MSV is thawed or reconstituted and neutralised with the addition of 1 ml monospecific antiserum. The serum must be shown to be free from antibodies against any of the contaminants for which the test is intended. Antisera should also be tested for nonspecific inhibiting affects At least two cell types are always required, so a minimum of 2 ml of MSV and 2 ml of antiserum are required. The antiserum is allowed to neutralise the MSV at room temperature for 1 hour. Of the MSV/antiserum mixture, 2 ml is then inoculated on to a 75 cm^2 flask of the appropriate cells. If the MSV is known to be high-titred or is a difficult agent to neutralise, or if the blocking serum is known to be low-titred, the blocking antiserum can be added to the growth medium at a final concentration of 1–5%. The cells should be passaged at least twice over a 14-day period, and the final culture is examined for cytopathology, haemadsorption and by fluorescent antibody staining.

The May–Grünwald–Giemsa staining procedure is usually used to detect cytopathology caused by extraneous viruses. Monolayers are usually prepared on two-chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides are removed leaving the rubber gasket attached to the slide. The slides are rinsed in warm Dulbecco's phosphate buffered saline (PBS), fixed in alcohol and placed on a staining rack. The slides are stained for 15 minutes at room temperature with May–Grünwald stain diluted 1/5 with absolute methanol. The May–Grünwald stain is removed by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain diluted 1/15 in deionised water. The Giemsa stain is removed by inverting the slides and rinsing them in deionised water for 10–20 seconds. The slides are air-dried, and paraffin oil and a cover-slip are applied. The May–Grünwald–Giemsa stain will differentially stain DNA and RNA nucleoproteins. DNA nucleoproteins stain red-purple, while RNA nucleoproteins stain blue. The monolayers are examined with a conventional microscope for the presence of inclusion bodies, an abnormal number of giant cells, or other cytopathology attributable to a viral contaminant. The inoculated monolayers are compared with the noninoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, the test material should be considered unsatisfactory.

Testing to detect extraneous viruses that produce haemadsorption in infected cells is usually carried out on monolayers of the second passage of test-material-inoculated cell cultures and noninoculated cell cultures. The monolayers are usually on 75 cm^2 plastic flasks. Guinea-pig, chicken, and any other blood for use in this assay is collected in an equal volume of Alsever's solution. The blood may be stored at 4°C for up to 7 days if it is washed several times in Alsever's solution before storage in an equal volume of Alsever's. Just prior to use, the stored erythrocytes are again washed by adding 5 ml of blood in Alsever's solution to 45 ml of calcium- and magnesium-free PBS and centrifuging in a 50 ml centrifuge tube at 500 *g* for 10 minutes. The supernatant is removed by suction and the erythrocytes are suspended in PBS and recentrifuged. This washing procedure is repeated at least twice until the supernatant is clear. Erythrocytes from each species are combined by adding 0.1 ml of each type of packed blood cells to 100 ml of PBS. The erythrocytes from different species may be kept separate or combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate the flasks at 4°C for 30 minutes. The monolayers are washed twice with PBS and examined for haemadsorption. If no haemadsorption is apparent, 5 ml of the erythrocyte suspension is added to each flask, the flasks are incubated at 20–25°C for 30 minutes, rinsed as before, and examined for haemadsorption. Separate flasks may be used for each incubation temperature if desired. Monolayers are examined for the presence of haemadsorbtion both grossly (using an illuminated glovebox) and microscopically. It is important to compare the noninoculated monolayers with the test monolayers to detect nonspecific haemadsorption that may occur with some cell types. The use of calcium- and magnesium-free PBS and fresh erythrocytes should prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an extraneous agent is found, the test material should be considered to be unsatisfactory.

Tests to detect extraneous viruses by fluorescent antibody usually use monolayers of the second passage of test-material-inoculated cell cultures and noninoculated cell cultures. The monolayers are usually on eight-chamber tissue culture slides. One positive control slide (consisting of eight monolayers) is made for each antiviral conjugate by inoculating each monolayer with approximately 100 TCID$_{50}$ (50% tissue culture infective dose) of the appropriate virus. Three groups of monolayers are stained with each antiviral conjugate. They are Group 1 – the second passage of test-material-inoculated cell cultures; Group 2 – the second passage of the noninoculated cell cultures; and Group 3 – the second passage of noninoculated cell cultures (for the production of positive control cell cultures). At the time of staining, the plastic walls of the slides are removed, leaving the rubber gasket attached to the slide. The slides are rinsed in Dulbecco's PBS, fixed for at least 10 minutes in acetone at 4°C, and dried. Approximately 0.1 ml of each conjugate is placed on each well of one slide from Groups 1, 2, and the corresponding positive control slide from Group 3. The slides are incubated in a humidified

chamber at 37°C for 30 minutes, rinsed once in Dulbecco's PBS, and placed in a container of Dulbecco's PBS for 10 minutes. The slides are rinsed thoroughly in deionised water and dried. All slides are examined for fluorescence attributable to each specific virus. The three slides from each group with the same conjugate are compared. If the slide prepared from cells inoculated with test material shows any evidence of specific viral fluorescence, the MSV should be considered unsatisfactory.

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FURTHER READING

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APPENDIX 1.1.9.1.

RISK ANALYSIS FOR BIOLOGICALS FOR VETERINARY USE OTHER THAN VACCINES

INTRODUCTION

For the purpose of this chapter, the term 'biologicals' means 'biologicals for veterinary use other than veterinary vaccines'.

CATEGORISATION OF BIOLOGICALS

Categorisation provides a means of facilitating risk analysis for the international trade in biologicals.

The categorisation system should take into account the source, the nature and the stated purpose of the biologicals. By conducting generic risk analyses, and by developing generic certification and quality assurance, continued supply of products can be made available without the need for repeated risk assessments that are expensive and consume significant resources. Once made, the risk assessment can be linked to appropriate manufacturing and testing parameters. Categories of biologicals for veterinary use into which generic risk assessments could apply may include (not in order of risk):

1. synthetic material;

2. amino acids, alcohols, esters, sugars and vitamins;

3. cosmetics;

4. plant extracts and processed biochemicals of plant origin;

5. products derived by microbial fermentation;

6. diagnostic, analytical and immunochemical kits for *in-vitro* use;

7. material of human origin;

8. therapeutics;

9. implantables of animal origin;

10. antibodies and immunoglobulins;

11. deoxyribonucleic acid (DNA), ribonucleic acid (RNA), restriction enzymes and other products of molecular biology;

12. cell-lines and hybridomas;

13. animal proteins, hormones, enzymes, albumins, tissue extracts and culture media containing animal material;

14. animal serum;

15. micro-organisms (conventional or genetically modified);

16. probiotics;

17. preserved specimens, microscope slides and smears.

All of these materials may contain pathogens depending on their source and processing procedures.

INFORMATION TO BE SUBMITTED WHEN APPLYING FOR AN IMPORT LICENCE

When undertaking risk analysis for biologicals, Veterinary Authorities should follow the *Terrestrial Manual*. The manufacturer or the Veterinary Authority of the exporting country should make available detailed information, in confidence if necessary, on the source of the materials used in the manufacture of the product (e.g. substrates). They should make available details of the method of manufacture (and where appropriate inactivation) of the substrates and component materials, the quality assurance procedures for each step in the process, final product testing regimes, and the pharmacopoeia with which the product must conform in the country of origin. They should also make available challenge organisms, their biotypes and reference sera, and other means of appropriate product testing.

RISK ANALYSIS PROCESS

Risk analysis should be as objective and transparent as possible and should be performed in accordance with Section 2 of the *Terrestrial Code*, and certification in line with Section 5 of the *Terrestrial Code*. Of necessity, assessment of the country and commodity factors and risk reduction measures will be based largely on manufacturers' data. These data depend on quality assurance at all stages of manufacture, rather than on testing of the final product alone.

Domestic exposure may be influenced by the approved usage of the product. Veterinary Authorities may place limits on usage of some products (e.g. restricting usage to institutions of appropriate biosecurity).

BIOCONTAINMENT

Suitable biocontainment may be necessary for many forms of biologicals. In particular, the importation of exotic micro-organisms should be carried out in accordance with Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.

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CHAPTER 1.1.10.

GUIDELINES FOR INTERNATIONAL STANDARDS FOR VACCINE BANKS

INTRODUCTION

Emergency vaccination is one of several measures that may be deployed to control outbreaks of disease as it provides a valuable adjunct to the application of the essential zoosanitary measures. These include rapid diagnosis, tracing, movement control and disinfection, and may also include slaughter of infected and in-contact animals.

The terms 'emergency vaccine' and 'emergency vaccination' can have different connotations, but are usually applied to differentiate between routine, prophylactic (preventive) vaccination and emergency vaccination, the latter being applied as an immediate response to an outbreak of disease. Emergency vaccination may be applied in a number of different circumstances and in a number of different ways, including the following:

a) *Against an outbreak of disease in a country that is normally free of this disease and that does not normally vaccinate against this disease. It may be applied as **Ring Vaccination** or **Barrier Vaccination**, outside of and around a focus of the disease to inhibit outward spread.*

b) *Against an outbreak of disease in a neighbouring country or region when emergency **Barrier Vaccination** may be applied along the border in the country or region that is at risk.*

c) *As a complimentary measure in a stamping-out policy, when emergency vaccination is applied to the animal population around an outbreak location, usually <u>within</u> the protection zone in which outbreaks have occurred, by so-called **Suppressive** or **Dampening Down Vaccination**. This is a form of ring vaccination that is followed by killing of the vaccinated animals.*

d) *Against an outbreak of disease in a country that does normally vaccinate but where emergency vaccine is applied to boost existing immunity.*

e) *Against an outbreak of disease in a country that <u>does</u> normally practice preventative vaccination, but where the vaccine(s) employed do not provide protection against the strain involved in the outbreak.*

Criteria that determine the successful application of emergency vaccination include rapid access to vaccine(s) that (i) contain virus strain(s) of sufficient antigenic relatedness to the outbreak strain(s) (ii) are of the required type of vaccine formulation (iii) have acceptable safety and potency (iv) have appropriate availability, including quantity and immediacy of supply and (v) meet considerations of cost. The evident need to hold strategic reserves, or banks, of such valuable commodities is best exemplified by foot and mouth disease (FMD) vaccines. They are specified in contingency plans for use in an FMD outbreak and have led to an escalation in the establishment of national and international FMD vaccine reserves for use all over the world (3), providing assurance that vaccine would be readily available and at the disposal of the country requiring it.

Emergency FMD vaccines are normally formulated to a higher potency than its conventional counterpart and there are banks who stipulate a requirement of at least 6 PD_{50} (50% protective dose) per dose for cattle in contrast to the minimal statutory requirement of $3PD_{50}$. Higher potency can be achieved by simply increasing the antigen payload per dose and its benefits can include rapidity, magnitude and duration of the protective response (1, 4). However, conventional vaccines may also be used in an emergency, particularly when vaccine of appropriate strain composition is immediately available or where revaccination might be desired in an already pre-immune population.

The concept of vaccine banks, exemplified by FMD, and the increased reliance on such banks is indicative of it being a very practical adjunct to other control measures that could usefully be adopted for a number of other diseases such as bluetongue, classical swine fever and avian influenza.

DEFINITION OF A VACCINE BANK

Strategic reserves, or banks as they are more commonly referred, are of two types. They may hold the final end product, a ready-to-use formulated vaccine, and/or the antigen component, which can be stored for a very long time for subsequent formulation into vaccine as and when required. The latter has been more commonly adopted for FMD because of the economic benefits, and this avoids constantly replacing vaccines that exceed their shelf-life. Stockpiles of antigens, or ready-to-use vaccine, will be referred to as vaccine banks in this chapter.

TYPES OF VACCINE BANKS

A country may hold its own national bank and/or it may be part of a larger group of countries that have drawing rights and share a bank such as exemplified by the North American or European Union FMD vaccine banks. Such consortiums may share a common geographical region, or have similar disease status and approach to control. The bank may be held on the territory of one or several of its members or be retained by the manufacturer, and, if held as antigen, would be formulated for use either by the manufacturer, or in a dedicated facility maintained by the bank members. However, in the latter case, the recent increasing demands by licensing authorities to require the same standards of independent manufacturing facilities as those of the commercial sector with a marketable product, is making this option very difficult. In the case of an antigen bank, a contract between the authorities and the vaccine manufacturer (formulation and filling) has to clearly define the details of formulation of the vaccine, e.g. time between reception of order and delivery, availability of buffers and vials, etc.

The location of stored antigens is of vital importance since the need to formulate vaccine may require antigen to be returned to the original manufacturer, incurring a delay in supply. Even if the antigens are held by the commercial sector, delay following a request for the supply of emergency vaccine might still occur if the manufacturer is currently in the middle of production of a product. The time to produce the vaccine should be about 48–72 hours. Delays in the production and despatch of emergency vaccine to control an outbreak may lead to wider spread of the disease and further difficulty in its control. Formulated vaccine would of course allow for immediate access. However, beside the wasteful and uneconomic implications resulting from regular replacement of the vaccine, it may not always contain the most suitable strain to deal with an outbreak.

The economic benefits of sharing a bank are obvious, but they also provide potential to stockpile greater doses and a wider number of vaccine strains, and reduce the problem of deciding on the introduction of narrow spectrum vaccine strains. Collaboration between vaccine banks would also be an economic way of increasing the amount of emergency vaccine available. Care would be required to ensure that collaborating banks operate to the same standards that drawing rights were clearly defined and that regular contact was maintained between banks to confirm the safety, efficacy and availability of the vaccines. Issues related to regulatory compliance would also need to be addressed at an early stage to ensure that vaccine produced from the bank would be authorised for use in any of the participating countries.

SELECTION OF VACCINES FOR A BANK

Depending on the disease and the likely contingency requirements, a range of vaccine strains may be required. Disease control authorities in consultation with the vaccine bank administrators must decide upon the vaccine strains that should be held and on what basis they should be stored (i.e. as a separate antigen component for subsequent formulation, or as a ready to use formulation). The value of any vaccine bank is very much dependant upon the appropriateness of what it holds for field application, particularly in respect to diseases that are made up of several serotypes and have wider strain variation. The potential of an outbreak not adequately covered by a banked vaccine must be alleviated by continuous monitoring of the global disease situation and recognition that additional vaccine strains may need to be included in the banks portfolio or, in the case where no suitable vaccine strain is available, developed speedily for subsequent inclusion.

The world as an interdependent community that encompasses rapid and extensive movement of people, animals and animal products and the increasing awareness of the potential to deliberately introduce disease through bio-terrorism heightens the risk of an incursion and makes prediction of specific threat difficult. To improve the process of vaccine selection, a continuous exchange of information and increased co-operation and collaboration between different international, regional and national laboratories, vaccine manufacturers and the

vaccine/antigen banks authorities should be encouraged. Risk analysis studies should be done to classify the virus strains to be stored with the priority level of high, medium and low. Close liaison with national and international reference laboratories is therefore recommended as some laboratories already provide periodic recommendations on strains that should be included in FMD vaccine banks. In the context of the risk of bio-terrorism, disease control authorities may consider it pertinent to restrict the information released relating to the storage of specific stockpiles of antigens and/or vaccines.

QUANTITIES OF VACCINE REQUIRED IN A BANK

The decision as to how many doses of vaccine are required is complex and problematic, embracing questions of serotypes, strains, use of mono or polyvalent vaccines, and type of formulation. Factors bearing on the decision include the type of disease, the different circumstances and ways of applying emergency vaccination (items a to e) described in the introduction), number, species and location of livestock that are to be protected, geographical considerations, knowledge of the current and predicted global epidemiological situation, and the analyses of risks of introduction and spread of disease, together with cost–benefit studies. In determining the supply of emergency vaccines, decisions on the quantity of the product inevitably involve a compromise between the cost of purchase and the likely number of doses required. The minimum vaccine requirement might therefore be based on the number of doses that could be distributed and applied in the first week of vaccination, the expectation would be that additional supplies could by then have been procured, either from other banks or from commercial sources. For example, 500,000 bovine doses of different FMD vaccine strains were routinely maintained by the International FMD Vaccine Bank (IVB), and drawing rights by member countries varied from 100,000 to 500,000 bovine doses. Nevertheless, this would soon be exhausted if used in an area of high livestock density.

ACQUISITION OF VACCINES FOR A BANK

According to both the type of vaccine bank and the disease concerned, the acquisition of the appropriate vaccine(s) or antigen(s) will depend on whether they are available from the commercial sector or government institutions or produced in-house.

Regulatory concerns on existing, or potential, immunological veterinary medicinal products (IVMPs) and the advisability to use approved, authorised medicines, will predispose a bank to acquire, or maintain, its vaccines and antigens selectively. It is recommended that appropriately licensed manufacturers that have the necessary Marketing Authorisation (MA) and internationally accepted standards of Good Manufacturing Practice (GMP), modern quality assurance (QA) and Qualified Person (QP) product release should be used as authorised sources.

This has certainly been exemplified in recent years by FMD vaccine banks in which there has been a strong preference for purchasing and holding antigens/vaccines within the commercial manufacturing sector and thus avoiding the expense and difficulties of maintaining a dedicated 'licensed' facility compliant with GMP for the purpose of formulation in an emergency.

Disease control authorities should consider the option of requesting a tender for antigens/vaccine from more than one supplier, particularly where regulatory considerations are of paramount importance, and they may wish to seek advice from appropriate licensing authorities on the necessary standards required. Request for tenders can then ensure not only a competitive price but a veterinary medicinal product manufactured to an acceptable level of quality. It should also establish suppliers that can produce the desired vaccines/antigens and dose amounts within a specified time period that meet necessary, or indeed mandatory, tests of compliance such as safety and efficacy.

Where the requirement is to hold antigens/vaccines at a site other than at the principle site of manufacture, disease control authorities may wish to consider only accepting them after they have been shown to have passed the necessary acceptance testing procedures such as safety and/or efficacy. Alternatively, if the antigen/vaccine has to be located in the bank prior to completion of any acceptance testing, then the antigen/vaccine should be stored apart and labelled as quarantine material until the testing shows full compliance to the vaccine banks requirements.

REGULATORY STANDARDS – SAFETY, EFFICACY AND QUALITY

Regulatory requirements for a veterinary medicinal product must be considered by any country wishing to have the necessary authorisation to use emergency vaccine in an outbreak situation. For example, all veterinary medicinal products that are placed on the market in the European Union (EU) must hold a marketing authorisation and the EU lays down the requirements for such authorisations. The EU also has emergency

provisions under Articles 7 and 8 that permit release of a vaccine without an authorisation in the country requiring it. However, a more recent EU Directive 2003/85/EC on current and future policy on control of FMD places more emphasis on the use of vaccines as part of a vaccinate-to-live policy. This makes the issue of an authorised product even more essential, particularly where vaccinated animals are intended for the food chain and require the support of agencies responsible for human health. Therefore, it is important that licensed products be used; unlicensed products are very much a last resort.

Quality, safety and efficacy are therefore all the more important and these will vary depending on the disease. Where particular immunologicals are covered by individual monographs in official Pharmacopoeias (e.g. FMD vaccine in the European Pharmacopoeia – Monograph 63) then the standards for Safety, Efficacy, Sterility and Quality are laid down. For the other case where the immunological comes under the Pharmacopoeia general section on Vaccines for Veterinary Use then those minimum standards should apply, and disease control authorities may wish to add, to the minimum standards, other individual requirements. These standards might include antigen strain identity, freedom from adventitious agents, innocuity, absence of toxicity, quantity of antigen payload per dose, safety, potency and sterility, and manufacture in officially approved quality assured (QA) good manufacturing practice (GMP) premises.

Any adjuvant or pharmacologically active ingredient used in a formulation must also conform to the necessary guideline requirements including residues in food-producing species.

Differentiating between animals that have been vaccinated and animals that have either recovered from infection or that have acquired sub-clinical infection post-vaccination may also be an important issue, as is the case for FMD. The detection of antibodies to non-structural polyproteins (NSPs) such as 3ABC of FMDV has been shown to be a sensitive and specific method to differentiate between infection and vaccination. This relies on manufacturing methods whereby the NSP component can be reduced to a level that will not cause detectable sero-conversion following vaccination making purity of vaccine an important consideration.

STORAGE OF VACCINES/ANTIGENS IN A BANK

It is important that the areas of storage chosen to hold emergency antigens/vaccines are suitable in the context of the required national or internationally accepted standards of GMP. This is usually covered when a bank is held in a 'licensed' and routinely inspected vaccine plant. However, if the bank is located outside a nominated vaccine formulation facility, regulatory considerations again may be of paramount importance and Disease Control Authorities may wish to seek advice from appropriate licensing authorities on the necessary standards required.

If the vaccine bank is associated with a laboratory, or other facility, where pathogens are handled, this should be completely independent of the bank storage facilities, and maintenance and monitoring personnel should obey a quarantine period before entering the bank.

Appropriate storage of antigens/vaccines in an emergency reserve will be very much dependent on the disease to which they are targeted to. The antigen may be a chemically inactivated or killed virus, for example such as that used in FMD vaccines, or it may be an attenuated vaccine such as that exemplified by Bluetongue vaccines. The antigens themselves may be concentrated and held at ultra-low temperature, over liquid nitrogen for example, or may be a freeze dried commodity where low temperature is not necessarily important. Whatever the method of storage, it is vitally important that they are optimally maintained and routinely monitored in order to have some assurance that they will be efficacious when needed. Managers of vaccine banks should therefore ensure that the necessary arrangements are in place to monitor their reserves on a routine basis and to include, where necessary and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product. For example, 24-hour storage temperature may be recorded as well as periodic inspection of the bottles containing the antigen for cracks or leakage. In this context, managers may wish to also consider the possibility of independent testing, or of greater reliance on overseeing/auditing of the manufacturer's test procedures.

The need for routine testing of stocks for stability is evident, and therefore depositories of antigens/vaccines should include a large number of small samples that are representative of the larger stock for such purposes stored side by side with it.

Whilst not directly related to the establishment, storage and operation of vaccine or antigen banks, Countries should nevertheless recognise the importance of contingency planning to ensure that the stored vaccine, if required, is distributed and administered in an efficient and prompt manner. They should make certain that the necessary cold-chain facilities are available, that vaccination protocols are defined in advance, that vaccination teams are established and trained appropriately and that all the other necessary documentation, equipment, reagents and clothing is stockpiled to sufficient levels to support any potential vaccination campaign. Therein the benefits of also performing periodic exercises and simulations should not be overlooked.

It would be advisable for member countries to monitor the literature published on important advances that are being made in subjects relating to vaccine bank technology. Ongoing research does lead to improvements of product, equipment, manufacture and distribution and therein more efficient and practical use of Banks. In this context there has been a recent study examining methods of prolonging the storage of fully formulated vaccine by a novel formulation procedure (2).

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THE ROLE OF OFFICIAL BODIES IN THE INTERNATIONAL REGULATION OF VETERINARY BIOLOGICALS

SUMMARY

The official control of veterinary biologicals is vested in various national and regional organisations that differ in their approach to ensuring the quality, safety and efficacy of the products. International harmonisation of regulations concerning biological products did not begin until well after those concerning chemically defined products. The first biological products for veterinary use were not manufactured and distributed until the end of the nineteenth century. They were often produced under unsophisticated conditions, and distributed or sold without any control other than those of their manufacturers. Later, each manufacturer developed its own standards. In Europe, these were subject to State controls as early as 1895 for certain diagnostic products (e.g. mallein, tuberculin) or vaccines. Gradually the conditions for international harmonisation of standards evolved, beginning with the comparative testing of products being issued by different European laboratories. It was only in the second half of the twentieth century that national laws covering veterinary biologicals were imposed. These demanded that precisely defined techniques be followed before biological products for veterinary use could be licensed. This was followed by considerable efforts to harmonise these national regulations, first at the regional level (notably in Europe and the Americas) then at the global level, notably by the Office International des Épizooties (OIE) with the publication of the first edition of the OIE Manual of Standards for Diagnostic Tests and Vaccines in 1989.

World-wide harmonisation of standards for veterinary biologicals will be of help to Chief Veterinary Officers who must follow the instructions given in the OIE International Animal Health Code, as they apply to all biological products for use in international trade. It will also be of assistance to vaccine producers, who have expressed their wish for world-wide harmonisation of registration rules so as to simplify and facilitate marketing of their products. Evidently, it will also be of interest to farmers and to consumers, who would benefit from the fact that the safety and efficacy of the products that they use would have been assured to a uniformly high level.

The different sections of this chapter will review and compare regulations from the regions of the world that have made most progress in this field and will describe current attempts at harmonising these regulations on an international scale.

__Note:__ In this chapter the term 'veterinary biological' will be taken to include vaccines for use in animals, antisera for use in animals, and in-vivo diagnostic preparations.

A. REGULATION OF VETERINARY BIOLOGICALS: PRESENT SITUATION

1. In Japan

1.1. Introduction

Medicinal products that are exclusively used for animals, including veterinary biologicals, are under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries, and securing their quality, efficacy and safety is stipulated in the Pharmaceutical Affairs Law (1). Since 1972, registration procedures have been developed with the aim of rationalising the examination procedure and facilitating the acquisition of approval. These procedures are stipulated in the Pharmaceutical Affairs Law and other related regulations. Consequently, a speedy and

simple examination procedure has been achieved with emphasis on the assurance of quality, safety and efficacy. The Food Safety Commission was established in the Cabinet Office, Government of Japan, in July 2003. In the case of approval examination, re-examination and re-evaluation, all veterinary vaccines, except products of dogs and cats, must comply with the Food Safety Basic Law.

1.2. Regulations governing the approval and quality assurance of veterinary biologicals

a)   Application for approval and licence

A person intending to release veterinary biologicals on the market shall obtain the license for marketing approval holders and the marketing approval for each biological from the Minister of Agriculture, Forestry and Fisheries. The application for the marketing approval should be submitted with designated appended documents, such as those on clinical studies. Of the latter, the safety studies and clinical studies using the target animal species should have been performed in compliance with GLP (Good Laboratory Practice) and GCP (Good Clinical Practice). A marketing approval holder shall comply with the standard of GQP (Good Quality Practice) and the GVP (Good Vigilance Practice).

A licence to manufacture veterinary biologicals is issued by the Minister of Agriculture, Forestry and Fisheries and must be renewed every 5 years. Conformity to GMP (Good Manufacturing Practice) is stipulated as one of the conditions for obtaining or renewing the licence to manufacture.

b)   National assay

After receiving a licence, each batch of the veterinary biological must be examined by the National Veterinary Assay Laboratory according to the procedures of the Assay Standard for Veterinary Biological Products (3, 9). A marketing approval holder must apply it to the national assay. Each product for marketing must include an official identification stamp on the container or the package as a seal.

c)   Re-examination and re-evaluation

Re-examination is performed on newly approved veterinary biologicals. Usually a field assessment of the veterinary vaccines is conducted over a period of 6 years following initial approval of the veterinary vaccines. During this investigation, the efficacy and the safety are re-examined.

Re-evaluation is performed on availability of approved products after marketing by order of the Minister of Agriculture, Forestry and Fisheries. This may happen when it is suspected that a veterinary biological does not conform to the latest standards of veterinary biological products.

d)   Minimum requirement of veterinary biological products

The examinations provide information about the consistency of the manufacturing process and the quality of the product: manufacturing methods, properties of strains used for manufacturing, methods of quality control, methods of storage and shelf life, according to the standards given in the 'Minimum Requirement of Veterinary Biological Products' (2). Any product that does not conform to these product standards cannot be manufactured, imported or marketed.

e)   Cases of rejection of approval

When the quality of the veterinary biological that has been submitted for approval is found to be unsatisfactory, or its adverse effects are marked as compared with its indications, the product is judged to be of little value and approval is not given.

f)   Cancellation of approvals

At the time of granting approval to market, the quality, safety and efficacy of the product are carefully examined with reference to the latest available technology. However, if scientific knowledge acquired since the granting of approval indicates that there could be a health hazard associated with the product, re-examination and re-evaluation are performed and an order of 'cancellation of approval' may be made.

1.3. Procedure for marketing approval

When a person intends to market veterinary biologicals, an application for approval to market the veterinary drug must be submitted on a designated form to an official in charge of veterinary biologicals at the Department of Animal Hygiene of each Prefecture. If the documentation is satisfactory, the application for approval to market, together with appended documents, are sent to and reviewed by the Secretariat of the Ministry of Agriculture, Forestry and Fisheries. At that time, a hearing may be conducted if necessary. The application is then discussed in the Pharmaceutical Affairs Sub-council, Pharmaceutical Affairs and Food Sanitation Council, and if any problems are not found, notice of approval to market the veterinary product is sent to the applicant.

2. In the European Union

2.1. Introduction

The pharmaceutical legislation of the European Union (EU), which has evolved over a 30-year period, covers both medicinal products for human and veterinary use. Harmonisation of requirements in the area of veterinary medicines began in 1981 with the adoption of Directives 81/851/EEC and 81/852/EEC, laying down common requirements for manufacturing and marketing authorisations, based on the evaluation of the quality, safety, and efficacy of the product. These Directives, and subsequent veterinary and human pharmaceutical legislation, were consolidated into Directive 2001/82/EC and 2001/83/EC for veterinary and human products, respectively. A series of detailed guidelines were first published in 1994 entitled 'Rules Governing Medicinal Products in the EU' (7). These have since been updated and describe in detail the legal basis for obtaining marketing authorisations, how dossiers should be compiled and how they should be assessed. These rules serve as extremely useful reference publications for any authority that is setting up a system for authorisation of veterinary biologicals. The rules were formally adopted and applied specifically to veterinary biologicals from 1993. Many additional measures were taken to further harmonise the procedures and the criteria for the evaluation of veterinary medicinal products, such as framework requirements and interpretive guidelines for their testing, principles and guidelines of GMP, and a Community procedure for the evaluation of high-technology products. However, granting of authorisations remained at the national level. As a consequence, although applications were evaluated on the basis of these harmonised criteria and procedures, and in some cases simultaneously by the authorities of the Member States, there were differences in the decisions reached by the Member States on individual products. This was why in 1990 the Commission proposed a new system for marketing authorisation for medicinal products, which was adopted by the Council of Ministers in 1993 and entered into force on 1 January 1995.

One of the first consequences was the creation of the European Medicines Evaluation Agency (EMEA) in London, United Kingdom (UK).

New legislation for veterinary products (Regulation 726/2004 and Directive 2004/28/EC) was published in May 2004. This legislation, for the main part, came into force in 2005 and resulted in a number of changes aimed at strengthening public and animal health, and environment protection by reinforcing requirements and controls. Directive 2001/82/EC already stated that the competent authorities cannot grant a marketing authorisation (MA) without having conducted a benefit–risk analysis. The document in the MA dossier must "demonstrate that the benefit bound to efficacy outweighs potential risk". But the relation between benefit and risk was not defined in that Directive. The new Directive gives the definition of the "risk–benefit balance": an evaluation of the positive therapeutic effects of the veterinary medicinal product in relation to the risk".

The risks concern:
- The animal;
- The user of medicinal product;
- The consumer liable to ingest animal food containing medicinal products residues;
- The environment.

With the revision of the Directive, if no medicinal product is available for 3 consecutive years, its MA is secluded. Before the revision, the MA was renewed every 5 years. Now, a single renewal is required. The pharmacovigilance is reinforced.

Title IV of regulation 726/2004 related to responsibilities and administrative structure of the European Agency has come into force in April 2004 in order to face the consequences of the enlargement of the EU.

2.2. The role of the European Medicines Evaluation Agency

In 1995, a new European system for the authorisation of medicinal products came into force. After 10 years of cooperation between national registration authorities at the EU level and 4 years of negotiations, the Council of the EU adopted, in June 1993, three directives and one regulation, which together form the legal basis of the system (5).

The EMEA was established by Council Regulation 2309/93/EEC of 22 July 1993. (OJ No. L 214, 24.8.1993), and London, UK, was chosen as its location by decision made by the Heads of State and Government on 29 October 1993. This agency formulates opinions and, apart from the administrative staff and the management board, is composed of two scientific committees, the CHMP (Committee for Human Medicinal Products) in charge of medicinal products for humans and the CVMP (Committee for Veterinary Medicinal Products) in charge of medicinal products for use in animals.

The CVMP is responsible for the evaluation of applications for marketing authorisation for products derived from biotechnology, for productivity enhancers, new chemical entities and other innovative new products. In addition,

the CVMP makes recommendations regarding MRLs (maximum residue limits) for substances used in food-producing animals. To support its activities, the CVMP relies on a pool of experts put at the disposal of the agency by the EU Member States. These experts may participate in any of the CVMP working parties. Among the working parties, the Immunologicals Working Party (CVMP/IWP) advises the CVMP on general policy issues such as the elaboration and revision of guidelines on immunological products. A scientific advice working party foreseen in regulation 726/2004 has been created. The aim of this working party is to advise applicants during the development phase of a veterinary medicinal product. The guidelines for the testing of veterinary medicinal products are contained within 'The Rules Governing Medicinal Products in the EU', last published by the European Union in 1999 (7). New guidelines, and revisions of old guidelines, are no longer produced in hardcopy form and may be accessed on the EMEA web site at www.emea.eu.int and/or the DG Enterprise web site at pharmacos.eudra.org.

2.3. Present European procedures for marketing authorisation

Four registration procedures for human and veterinary medicinal products have become available through this new legislation:

1. The centralised procedure allows a unique marketing authorisation (MA) to be obtained and made available in all the Member States. This applies to high technology products defined in the annex to the Regulation. It is optional for innovative medicinal products. This procedure was extended to veterinary vaccines covering animal diseases that are subject to Community prophylactic measures.

2. The national procedure allows an MA to be obtained for a medicinal product in a single country or in a country that will be the origin of a mutual recognition procedure.

3. The mutual recognition procedure: applications for authorisation of a product may still be obtained in a single Member State (the 'Reference Member State') by means of a national procedure. The same 'Rules Governing Medicinal Products in the EU' apply. Following approval in the Reference Member State, applications may be made, if desired, to other 'Concerned' Member States for identical authorisations to be granted on the basis of '*mutual recognition*'.

4. The new decentralised procedure is the addition of the national one and the mutual recognition procedure, i.e. it is based upon the principle of mutual recognition of national authorisations. At the beginning of this procedure, all Member States are associated; this is immediately followed by a mutual recognition procedure.

The most important change is the compulsory aspect of *arbitrage* in the case of a disagreement between Member States during the mutual recognition or the decentralised procedures. If a Member State considers that there is a serious risk to public health, a pre-arbitrage procedure must be carried out. In such a situation, an MA holder cannot remove his/her demand. The arbitrage allows a decision to be made on whether there is a "serious risk" with the use of the medicinal product. Finally, the decision (to grant or refuse the MA) is harmonised throughout the community.

2.4. Manufacturing authorisation and batch release control

In accordance with Directive 2001/82/EC, authorisation is also required for the manufacture of veterinary medicinal products, including immunologicals. This directive provides for regular inspections and stipulates that manufacture must be supervised by a 'qualified person', who certifies that each batch is in conformity with the approved specifications for the product. For the implementation of these requirements, the Commission has adopted Directive 91/412/EEC relating to the principle and guidelines of GMP, and published a detailed guide on GMP developed by a group of pharmaceutical inspectors from the Member States.

The new directive also establishes GMP for active starting materials for medicinal products. This provision is reinforced through the provision of the opportunity for Member States to carry out inspections of active materials destined for the manufacturers of veterinary medicinal products.

Manufacturers are required to have the services of a qualified person at their disposal to certify that each batch of product has been manufactured and checked in accordance with the conditions for marketing authorisation. This is a basic requirement of the pharmaceutical legislation. In the case of batches imported from third countries, each batch has to undergo a full qualitative and a quantitative analysis of at least the active ingredients in the first Member State of import into the EU, under the supervision of a qualified person. Not until this control by the qualified person has been carried out can a batch circulate within the EU without further control. In the special case of immunological veterinary medicinal products, an additional step may be introduced. Directive 90/677/EEC allows those Member States that consider it necessary to ask for the submission of samples of each production batch of the bulk and/or finished product for examination by a control laboratory before that batch is placed on the market. This official batch release does not relinquish the requirement of batch control by the qualified person. Except in specially justified circumstances, batch release

carried out by one national control laboratory must be recognised without repetition by the other Member States. To ensure the smooth operation of this provision, an administrative information exchange procedure has been agreed between the Competent Authorities. Although all Member States do not require official batch release for veterinary immunologicals, it was felt by all that they had to be involved in this information exchange scheme. Discussions are in progress to establish a harmonised system of batch release in all Member States of the EU.

2.5. The role of the European Pharmacopoeia

The past 30 years have seen profound changes in the organisation and regulation of medicinal products in European countries (4). Thirty years ago, each country had its own regulations, and among them the European countries had two-thirds of the world's pharmacopoeias. The European Pharmacopoeia Convention has now been signed by 35 Member States[1]; moreover 17 European and non-European countries[2], and the World Health Organisation (WHO) have observer status. Close relations are maintained with the licensing authorities of the European Economic Area, where integration is developing through contact with the EMEA and the implementation of common directives and guidelines on medicines for human and veterinary use. In 1990, the European Pharmacopoeia co-founded, with the Japanese Pharmacopoeia and the United States (US) Pharmacopoeia, the Pharmacopoeial Discussion Group (PDG); this group is working assiduously for harmonisation at the world level, and it participates in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) programme. A parallel programme for veterinary medicinal products, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), has recently turned its attention to harmonising the requirements for certain types of tests routinely performed on extraneous agents (see Section C.4 below). The European Pharmacopoeia defines the minimum acceptable standards for products to be authorised within the European Union because compliance with monographs is a mandatory requirement within Directive 2001/82/EC. This requires that products must comply with the relevant specific monograph where one exists or with the general monographs where one does not.

The European Pharmacopoeia also contains a department called the 'European Department for the Quality of Medicines' (EDQM). This creates, maintains and distributes the international standard reagents referred to in monographs of the European Pharmacopoeia. To date there are few standards for veterinary biologicals, but the EDQM is becoming increasingly active in this area and it is anticipated that several more will be available in the near future.

3. In the United States of America

3.1. Introduction

In the United States of America (USA), veterinary biologics or veterinary biological products are defined as all viruses, sera, toxins (excluding substances that are selectively toxic to microorganisms, e.g. antibiotics), or analogous products at any stage of production, shipment, distribution, or sale, that are intended for use in the treatment (prevention, diagnosis, management, or cure) of diseases of animals and that act primarily through the direct stimulation, supplementation, enhancement, or modulation of the immune system or immune response. The term biological products includes, but is not limited to, vaccines, bacterins, allergens, antibodies, antitoxins, toxoids, immunostimulants, certain cytokines, antigenic or immunising components of live organisms, and diagnostic components that are of natural or synthetic origin or that are derived from synthesising or altering various substances or components of substances such as microorganisms, genes or genetic sequences, carbohydrates, proteins, antigens, allergens, or antibodies.

3.2. Legal basis

The Virus/Serum/Toxin Act of 1913 (the 'VST Act'), as amended, 21 U.S.C. Sections 151 to 159, provides the legal authority for the regulation of immunologicals and biologicals for animal use in the USA. The regulatory programme implementing the requirements of the VST Act is administered by the Center for Veterinary Biologics (CVB), Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). Administrative regulations, duly promulgated and with effect of law, are published in Title 9, Code of Federal Regulations, Parts 101 to 118 (6). In addition, APHIS has issued programme guidance in CVB Notices, Veterinary Services Memoranda, Veterinary Biologics General Licensing Considerations, and the Veterinary Biologics Program Manual. These may be accessed on the CVB web site at www.aphis.usda.gov/vs/cvb.

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1   Austria, Belgium, Bosnia Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugolsav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Portugal, Romania, Serbia and Montenegro, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, Turkey, United Kingdom and the European Union; Member States must apply the standards of the European Pharmacopoeia.

2   Albania, Algeria, Australia, Brazil, Canada, the People's Republic of China, Georgia, Israel, Poland, Madagascar, Malaysia, Morocco, Senegal, Syria, Tunisia, Ukraine, United States of America; Observer States do not have to apply the European Pharmacopoeia standards. Some of them apply the standards on a voluntary basis.

The VST Act requires that products governed by the Act that enter channels of commerce be 'not worthless, contaminated, dangerous or harmful'. The regulatory scheme implementing these standards is structured to require manufacturers of these products to apply for licences prior to marketing, and to place certain evidentiary responsibilities on those applicants, i.e. manufacturers are required to demonstrate through the submission of certain information, research data, and test results that their products are 'pure, safe, potent and efficacious'. The APHIS programme for immunologicals and biologicals for animal use regulates the manufacture and release of products on to the market through a system of licensing, inspection, testing and post-marketing surveillance that ensures that the statutory and regulatory standards are met.

3.3. Licensing and initial inspection

Any person or firm seeking to manufacture in the USA an immunological or biological for animal use must obtain from APHIS both a licence to manufacture at a specified facility (Establishment Licence), and a licence for every particular product to be manufactured (Product Licence). These licence requirements apply whether the product is to be released on to the US market or is to be exported to markets abroad. Typically, an applicant will request a facility licence at the same time as the licence for the first product. Once the facility licence and one product licence have been obtained, a firm that seeks to manufacture and market new products needs only to apply for additional product licences. A person or firm located overseas that seeks to market its product in the USA must also apply for marketing authorisation. In the case of an imported product, however, the authorisation is termed a 'permit' rather than a 'licence'.

To obtain a facility licence, the applicant must submit for approval the blueprint (that is, the architect's plan of the buildings) and blueprint legends for the facility. APHIS reviews these blueprints and legends to ensure that the facility will operate in a manner consistent with GMP. If the applicant subsequently makes any physical or operational changes to the facility, revised blueprints and legends must be submitted immediately.

To obtain a product licence, the applicant must establish the purity and identity of all master seeds and master cell stocks that will be used in the manufacture of the product, and must submit for approval a detailed outline of production. The outline of production includes not only the details of the method of product manufacture, but also a description of the procedures for collecting and submitting samples and for releasing batches. The applicant must also provide information regarding the professional and technical credentials of company personnel, and must identify a qualified individual (termed under US regulations as the 'government liaison') who acts as the official contact with CVB during the licensing process, and who is subsequently responsible for the submission of the firm's test reports in conjunction with the release of the product on to the market. The applicant is required to submit test data that demonstrate that the product produced in accordance with the outline is pure, safe, potent and efficacious. The applicant must submit to CVB laboratories samples of three consecutive batches of the product so that the results of the applicant's tests of the product can be confirmed.

Finally, before the facility or product licences are issued, the applicant's premises are subject to a comprehensive inspection by APHIS examiners. The inspection ensures that the facility is operating in a manner consistent with GMP by confirming that the establishment is configured in the manner set out in the approved blueprints and legends, that the production line is set up and operating in accordance with the approved outline of production, and that records are adequately kept and maintained for each step in production. The inspection also confirms that the applicant follows procedures consistent with GLPs, that the in-process and final product testing programme is conducted properly and appropriately documented, that the sampling is conducted properly, and that adequate procedures for determining and documenting the release of the product on to the market are in place.

3.4. Post-licensing inspection

Once a firm has been issued facility and product licences, APHIS will routinely conduct thorough follow-up inspections of the facility to ensure that the licensee continues to operate the establishment in accordance with the programme regulations and in the manner represented at the time of licensing. Post-licensing inspections are conducted unannounced periodically. If the licensee proposes any significant changes to the facility or to the method of production of a licensed product, APHIS retains the right to conduct a special inspection prior to approving the changes.

3.5. Testing

Each licensee is responsible for thoroughly testing all of its production processes and each serial (or lot) of every product prior to release on to the market. The type and amount of testing required depends on the particular product, but is determined and approved by the regulatory authority prior to the issuing of the product licence. A qualified individual employed by the licensee ('government liaison') is responsible for selecting the samples to be tested, for monitoring the licensee's testing programme, and for certifying the test results to the regulatory authority.

At the same time that the firm selects its samples for its own in-house testing, it also selects samples to be submitted to the CVB laboratories. The CVB retains the right to conduct confirmatory testing. CVB then selects a

percentage of the samples submitted for confirmatory testing to verify the accuracy and proficiency of the manufacturer's tests. The testing is conducted prior to marketing authorisation for each serial. By regulation, CVB policy stipulates that it is required to put its selected samples on test within 14 days of the date on which the samples are received; ordinarily, samples are put on test sooner than the 14-day limit so that the testing of production by the firm and the CVB proficiency testing programme are effectively being conducted at the same time.

When the firm receives the results of its own tests, the government liaison submits those results to the regulatory authority along with a batch release form, initiating the release procedure. If the batch has not been selected as part of the proficiency testing programme, or if it has been selected but the CVB tests confirm the company's test results, the release form is counter-signed by the regulatory authority completing the release procedure. If either the company tests or the proficiency tests indicate that the batch may be unsatisfactory, the batch is not eligible for release.

If the licensee makes a proposal to modify its facility or its operation in any way that could affect the purity, safety, potency or efficacy of the product, the regulatory authority may require the licensee to provide data demonstrating the purity, safety, potency and efficacy of the product as well as to submit samples of the product to CVB's laboratories for confirmatory testing.

3.6. Post-marketing surveillance

CVB operates a post-marketing surveillance programme to monitor the performance of products on the market. Under this programme, CVB typically learns of any problems relating to product quality through consumer reports or complaints, although the programme regulations also place an obligation on the licensee to inform CVB of any problem that comes to its attention regarding the purity, safety or potency of the product. CVB has the legal authority to intervene in the marketplace where there are serious concerns with respect to the purity, safety, potency or efficacy of the product.

B. COMPARISON OF EUROPEAN UNION AND UNITED STATES REGULATIONS

Veterinary biologicals must meet certain basic criteria, regardless of the Regulatory Agency overseeing their production. These criteria include:

- Safety: the product must be safe in the target species and, if live, in species exposed to shed organisms;
- Efficacy: the product should be effective according to claims indicated on the label;
- Quality: includes purity, potency and consistency;
- Purity: the product must be free from contaminating agents;
- Potency: each batch of product should be formulated, and tested, to ensure effectiveness and reproducibility of activity as demonstrated in the registration data.

Although, on a global basis, agencies and regulations differ, all strive to ensure that products offered to the end-consumer conform to these basic standards.

The EU uses a complete system that is a combination of GMP, including validated processes and specifications of materials, together with production methods that ensure the quality of the final product. In-process and batch controls (tests) constitute additional guarantees of the quality of IVMPs. The safety tests are conducted under GLP and the field efficacy tests under GCP. The USA defines acceptable manufacturing processes in the outline of production and detailed facility description (blueprints and blueprint legends), and relies on inspection and confirmatory testing to achieve the same goal. Although different, both systems are designed to allow only pure, safe, potent, and effective biologicals to be released to the consumer.

In addition to the data provided by the applicant, expert reports have to be included in the EU marketing authorisation application file (dossier). Each main section of the EU dossier, including analytical, safety and efficacy, must be reviewed by an independent expert. The assessment of the expert is included in the marketing authorisation file. No such system of third-party review exists under the USDA registration system with the exception of certain biotechnologically derived products.

There are many procedural differences between the EU and the USA. Harmonisation between the two systems should be established where possible, on the recognition of equivalence for tests and procedures that are performed to assess a vaccine and that ensure quality, safety and efficacy of the product. Mutual recognition agreements (MRAs) covering veterinary biologicals have been signed between the EU and Australia and between the EU and New Zealand. These MRAs are now at an operational stage. Progress on MRAs between the EU and the USA, regarding veterinary biologicals, is likely to take longer to achieve.

C. THE ROLE OF INTERNATIONAL ORGANISATIONS

Most nations have a range of official acts that regulate the sale and use of veterinary biologicals. Almost all of these acts stipulate 'minimum requirements' for quality, safety and efficacy of veterinary biologicals (mostly vaccines), to be tested at independent laboratories, usually under State supervision. These acts and tests may differ from one country to another, and they involve costs and restrictions for producers, users and testers.

Many of the vaccines described in this *Terrestrial Manual* are produced and/or used in countries that do not currently apply regimens of authorisation and testing as stringent as those described in this chapter. Nevertheless, it is useful to be aware of the regulations operating in different regions and, therefore, the testing and inspection that is likely to have been carried out there on a veterinary biological.

The idea of harmonising this testing to simplify and reduce costs on a regional, or even world scale, is not new, and much has been accomplished during the past 20 years. The purpose of this section is to review the current situation by describing the role of international organisations in the regulation of veterinary vaccines.

In this section the term 'international organisation' refers to those concerned with animal health on a world-wide scale (OIE, the Food and Agriculture Organization of the United Nations [FAO] and the WHO).

1.   The role of the OIE (World Organisation for Animal Health)

The OIE was founded in Paris in 1924 as the world organisation for animal health, and comprises 172 Member Countries in the year 2008. The principal aims of the OIE are: to ensure transparency in the global animal disease and zoonosis situation, to collect, analyse and disseminate scientific veterinary information, to provide expertise and encourage international solidarity in the control of animal diseases, within its mandate under the Agreement on Sanitary and Phytosanitary Measures (SPS Agreement) of the World Trade Organization (WTO), to safeguard world trade by publishing health standards for international trade in animals and animal products, to improve the legal framework and resources of national Veterinary Services and to provide a better guarantee of the safety of food of animal origin and to promote animal welfare through a science-based approach (13).

Within the OIE there are four Specialist Commissions: the Terrestrial Animal Health Standards Commission, which deals with the *Terrestrial Animal Health Code,* the Biological Standards Commission, the Scientific Commission for Animal Diseases and the Aquatic Animal Health Standards Commission (including diseases of molluscs and crustaceans). In addition, there are three Working Groups: the Working Group on Wildlife Diseases, the Working Group on Animal Production Food Safety and the Working Group on Animal Welfare.

Among the Specialist Commissions, the one most closely connected with standardisation is the Biological Standards Commission. This Commission establishes standards for diagnostic methods (including diagnostic preparations) and for vaccines. Its terms of reference reflect the Commission's obligation to participate in the standardisation of biological products, including vaccines used for prophylactic purposes. The Biological Standards Commission is responsible for the preparation of the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (11), and the organisation of Reference Laboratories for many of the diseases on the OIE List.

However, full standardisation of vaccine testing can be achieved only when the necessary standards have been devised. It is hoped to reach the goal of standardisation and wide availability of standards through the participation of OIE Reference Laboratories. The functions and responsibilities of experts at the over 170 OIE Reference Laboratories include the provision of a centre of excellence in a designated activity; standardisation of methods; preparation, storage and distribution of standard antisera, antigens and other reagents.

Among the OIE Collaborating Centres, three may be involved at some stage in veterinary vaccine control and/or harmonisation: the Collaborating Centre for Veterinary Medicinal Products in Fougères (France), the Collaborating Centre for ELISA (enzyme-linked immunosorbent assay) and Molecular Techniques in Animal Disease Diagnosis in Vienna (Austria), and the Collaborating Centre for the Diagnosis of Animal Diseases and Vaccine Evaluation in the Americas in Ames (USA).

In 1994, following discussions with the International Technical Consultation on Veterinary Drug Registration (ITCVDR), the OIE set up an Ad hoc Group on the Harmonisation of Veterinary Medicines, which was the first step towards the creation of the VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products) (see Section C.4 below).

In May 2003, the OIE International Committee adopted a resolution entitled OIE Procedure for Validation and Certification of Diagnostic Assays (Test Methods) for Infectious Animal Diseases. This resolution requires the OIE Director General to make provisions to establish an OIE registry for assays with levels of validation specified. Fitness for purpose should be used as a criterion for validation.

2. The role of the Food and Agriculture Organization of the United Nations

FAO, established in 1945, is responsible for agricultural development and food production. The Animal Production and Health Division ('AGA') within the Agriculture Department is concerned with livestock development, and it includes the Animal Health Service ('AGAH'), the main role of which is to assist Member Countries in the control of animal diseases, with the aim of improving livestock production as an integral component of general social, economic and agricultural development. FAO's involvement in testing veterinary biologicals is primarily through its technical assistance system to Member Countries to set up and even execute independent quality control of vaccines and other biologicals. One example is FAO's assistance to the AU (African Union) in setting up a system for continental testing of veterinary vaccines, especially against rinderpest and contagious bovine pleuropneumonia, by the Pan African Veterinary Vaccine Center (PANVAC). FAO also commissions, at the request of Member Countries, initiatives for either quality assurance of vaccines and other biologicals or expert consultations on this subject, or publication of manuals on the production and quality control of vaccines. Furthermore, two auxiliary services can be asked to intervene on matters concerning these products, namely Codex Alimentarius and the Division of Nuclear Techniques in Food and Agriculture. The latter is operated jointly by FAO and the International Atomic Energy Agency (IAEA) based in Vienna (Austria). It has an Animal Production and Health Section, which assists veterinary services and research institutes in developing countries to establish radio-immunoassay (RIA) and ELISA techniques. Linked to this activity is a quality assurance scheme under which laboratories in receipt of FAO/IAEA ELISA kits are required to routinely monitor internal quality controls and to periodically (once or twice a year) test a batch of unknown samples, and to forward the results to IAEA. The overall aim is to provide assurance to all concerned that the results being generated through the use of such internationally standardised and validated kits can be relied upon as correct.

3. The role of the World Health Organization

Currently WHO is not directly involved in preparing international reference preparations (i.e. antigens or antibodies) for purely veterinary use, but has developed and still retains in the National Institute for Biological Standards and Control, Potter's Bar (UK) some materials related to purely veterinary diseases (e.g. Newcastle disease live vaccine, classical swine fever serum). WHO wishes to retain a role in this area in instances where the veterinary reference preparations and guidance documents have a direct relevance to human health (8, 10, 15, 16). This involves zoonotic and potentially zoonotic agents and other infectious agents of animal origin that are potential contaminants of biological products, whether these are vaccines produced in cell cultures or organs for xenotransplantation. At the meeting of the Expert Committee on Biological Standardization in October 1998, a review of currently retained international standards and reference preparations for veterinary medicine was carried out and a list of candidates for discontinuation, replacement and revision was suggested (8). The Expert Committee however decided to defer taking action on preparation of veterinary reference materials pending an evaluation by WHO with its partners in the veterinary field of the need for these various biological products. In addition, the present day topicality of certain preparations, especially veterinary vaccines against known zoonoses (e.g. anthrax, brucellosis) adopted and/or revised in the 1960s and 1970s, also needs to be evaluated.

The format of the list of Requirements for Biological Substances published as an Appendix to each report of the Expert Committee on Biological Standardization was revised in 1998 and should facilitate the retrieval of information and achieve the aim of improved transparency.

4. The role of VICH

4.1. What is VICH?

a) Short description of VICH

VICH is a trilateral (EU-Japan-USA) programme aimed at harmonising technical requirements for veterinary product registration. Its full title is the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products. VICH was officially launched in April 1996.

b) Background and history

The initiative to begin the harmonisation process came in 1983 when the first International Technical Consultation on Veterinary Drug Registration (ITCVDR) was held. Since then a series of government and industry initiatives have been developed, culminating in the formation of the VICH.

The Codex Alimentarius formed a Committee on Residues of Veterinary Drugs in Foods in 1985. Standard requirements for veterinary product registration were adopted in Europe in 1981.

The US Food and Drug Administration and the European Commission have held regular bilateral meetings for the last decade to discuss common areas of interest. This has involved mutual exchange of guidelines for consultation.

The first International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) was held in Brussels in November 1991. The meeting brought together regulators and industry representatives from the USA, the EU and Japan to address quality, safety and efficacy requirements in the three regions.

Meetings on harmonisation of veterinary biologicals were held in Ploufragan, France, in January 1992, in Arlington, USA, in 1994 and in Singapore in 1995.

In January 1993 the GHOST (Global harmonisation of standards) discussion document was published by FEDESA[3]. It set out a programme for the international harmonisation of registration requirements for veterinary pharmaceuticals and biologicals.

Following discussions at ITCVDR and the OIE conferences, the OIE set up an ad hoc group on harmonisation of veterinary medicinal products in 1994.

c) The creation and scope of VICH

Preparatory work for the establishment of VICH was carried out by this OIE ad hoc group. During 1994 and 1995, two meetings were held at which the scope of veterinary harmonisation was discussed and the membership and objectives of the VICH proposed.

On the subject of food safety standards, it was decided that the VICH should complement the work of Codex and JECFA[4]. Issues related to GLP and GMP that are already the subject of mutual agreements will not normally come within the remit of the VICH. Issues related to biologicals were considered appropriate to fall within the scope of VICH.

Fundamental to the selection of priority topics for consideration by the VICH was the discussion document prepared by COMISA for the Steering Committee. This report:

• assesses those ICH guidelines which could be adapted to the VICH programme;

• defines in detail areas of non-harmonisation between the EU, the US and Japan and provides a series of 'concept papers' on key topics; and

• puts forward preliminary suggestions for priority topics.

With all the ground-breaking work completed, the Steering Committee of the VICH held its first meeting in April 1996, at which the membership and the working procedures were agreed and a work programme established.

d) The objectives of VICH

The objectives of the VICH are along the same lines as those of the ICH.

• Establish and implement harmonised regulatory requirements for veterinary medicinal products in the VICH regions, which meet high quality, safety and efficacy standards and minimise the use of test animals and costs of product development.

• Provide a basis for wider international harmonisation of registration requirements.

• Monitor and maintain existing VICH guidelines, taking particular note of the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) work programme and, where necessary, update these VICH Guidelines.

• Ensure efficient processes for maintaining and monitoring consistent interpretation of data requirements following the implementation of VICH guidelines.

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3   FEDESA: Fédération Européenne de la Santé Animale. In 2002, FEDESA became the International Federation for Animal Health (IFAH).
4   JECFA: Joint FAO/WHO Expert Committee on Food Additives.

- By means of a constructive dialogue between regulatory authorities and industry provide technical guidance enabling response to significant emerging global issues and science that impact on regulatory requirements within the VICH regions.

e)   Progress toward achieving the VICH objectives

- For veterinary immunologicals there is an ongoing programme of harmonisation in a number of areas including target animal safety studies, reversion to virulence and tests for the presence of *Mycoplasma*. To date only a relatively small number of VICH guidelines have been developed for veterinary biologicals and it is worth emphasising the difficulties in reaching agreement on veterinary biologicals between the three regions.

- The only two adopted VICH Guidelines for veterinary biologicals are testing of residual formaldehyde adopted May 2003 and testing of residual moisture also adopted in May 2003. Several other Guidelines that apply to veterinary biologicals and all other veterinary medicinal products have also been adopted.

Following an in-depth reflection held by all parties concerned by VICH under the auspice of OIE, the second phase of VICH for 2006–2010 was publicly launched during a public conference "VICH3" held in Washington in May 2005.

More information is available at: http://vich.eudra.org.

CONCLUSION

At the moment, there is a clear intention to achieve greater international harmonisation of regulatory requirements for veterinary biologicals (14). Progress has already been achieved through international organisations to allow fair competition in the marketing of veterinary products. Although past efforts by international organisations have not resulted in a level of harmonisation sufficient to facilitate international trade, they have laid the groundwork for current efforts. National authorities recognise the advantages of harmonisation and are now committed to working toward this goal.

The efforts of international organisations have made the goal of harmonisation possible and have resulted in an organisation and process for proceeding toward this goal. Success in achieving this goal will depend on the willingness of participating national authorities to work together and accept the compromises that will be necessary to resolve the difficult scientific and policy issues.

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PART 2

OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO INTERNATIONAL TRADE

CHAPTER 2.1.1.

ANTHRAX

SUMMARY

Definition of the disease: *Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans, and some avian species can contract it. Mortality can be very high, especially in herbivores. The aetiological agent is the spore-forming, Gram-positive rod-shaped* Bacillus anthracis*. The disease has world-wide distribution and is a zoonosis.*

Description of the disease: *The disease is mediated by exotoxins. Peracute, acute, subacute and, rarely, chronic forms of the disease are reported. Ante-mortem clinical signs may be virtually absent in peracute and acute forms of the disease. Subacute disease may be accompanied by progressive fever, depression, inappetence, weakness, prostration and death. Acute, subacute, and chronic disease may show localised swelling, fever; and in chronic disease the only sign may be enlarged lymph glands.*

Identification of the agent: Bacillus anthracis *is readily isolated in relatively high numbers from blood or tissues of a recently dead animal that died of anthrax, and colony morphology of* B. anthracis *is quite characteristic after overnight incubation on blood agar. The colony is relatively large, measuring approximately 0.3–0.5 cm in diameter. It is grey-white to grey, non-haemolytic with a rough, ground-glass appearance and has a very tacky, butyrous consistency. The vegetative cells of* B. anthracis *are large, measuring 3–5 µm in length and approximately 1 µm in width. Ellipsoidal central spores, which do not swell the sporangium, are formed at the end of the exponential cell growth phase. The cells stain strongly Gram positive, and long chains are often seen* in vitro*, while paired or short chains are seen* in vivo*. Visualisation of the encapsulated bacilli, usually in large numbers, in a blood smear stained with polychrome methylene blue (*M'Fadyean reaction*) is fully diagnostic.*

Serological tests: *Antibody detection in serum from infected animals is rarely used for diagnostic purposes and is essentially a research tool. The predominant procedure today is the enzyme-linked immunosorbent assay (*ELISA*).*

Requirements for vaccines and diagnostic biologicals: *The most widely used livestock anthrax vaccine developed by Max Sterne in 1937, is a live, non-encapsulated, spore former held in suspension. In Russia and Eastern Bloc countries, an equivalent type of vaccine is used (*strain 55*). The Pasteur vaccine is no longer used in Italy. A new vaccine, Carbosap, has been developed that retains both plasmids and exhibits very low virulence. A list of producers is given in the World Health Organization anthrax guidelines.*

A. INTRODUCTION

Anthrax, an acute bacterial disease of primarily herbivores, is transmissible to humans. The aetiological agent, *Bacillus anthracis*, is a Gram-positive spore-forming rod-shaped bacterium. Anthrax is known by many names around the world including charbon, woolsorters disease, ragpickers disease, malignant carbuncle, and malignant pustule.

Animals become infected by ingesting spores or possibly by being bitten by flies that have fed on an infected animal or carcass. Infected animals are usually found dead as death can occur within 24 hours. A careful post-mortem examination of recently dead animals may show any number of lesions, none of which is pathognomonic or entirely consistent. To avoid environmental contamination, post-mortem examinations of carcasses of animals suspected to have died of anthrax is discouraged. Lesions most commonly seen are those of a generalised septicaemia often accompanied by an enlarged spleen having a 'blackberry jam' consistency and poorly clotted blood. Haemorrhage from the nose, mouth, vagina and/or anus at death is not a common sign.

Gram-positive rod-shaped *Bacillus anthracis* is the only obligate pathogen within the genus *Bacillus.* Most of the other species of *Bacillus* are common ubiquitous environmental saprophytes, although a number, notably *B. cereus, B. licheniformis* and *B. subtilis*, are occasionally associated with food poisoning in humans and with other clinical manifestations in both humans and animals.

More than 95% of human anthrax cases take the cutaneous form and result from handling infected carcasses or hides, hair, meat or bones from such carcasses. *Bacillus anthracis* is not invasive and requires a lesion to infect. Protection for veterinarians and other animal handlers involves wearing gloves, and other protective clothing when handling specimens from suspected anthrax carcasses and never rubbing the face or eyes. The risk of gastrointestinal anthrax may arise if individuals eat meat from animals infected with anthrax.

The risk of inhaling infectious doses becomes significant in occupations involving the processing of animal by-products for manufacturing goods (industrial anthrax). These include the tanning, woollen, carpet, bone processing, and other such industries, where the potential for aerosolisation of substantial numbers of spores increases the risk of exposure to infectious doses.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Demonstration of encapsulated *B. anthracis* in smears of blood or tissues from fresh anthrax-infected carcasses and growth of the organism on blood agar plates is relatively uncomplicated and within the capability of most bacteriology laboratories. Difficulty may be encountered in the case of pigs and carnivores in which the terminal bacteraemia is frequently not marked, or in animals that received antibiotics before death.

Recovery of *B. anthracis* from old decomposed carcasses, processed specimens (bone meal, hides), or environmental samples (contaminated soil) is also often difficult, requiring demanding and labour-intensive procedures.

a) Culture and identification of *Bacillus anthracis*

i) Fresh specimens

Bacillus anthracis grows readily on most types of nutrient agar, however, 5–7% horse or sheep blood agar is the diagnostic medium of choice. Blood is the primary clinical material to examine. Swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be spread over blood agar plates. After overnight incubation at 37°C, *B. anthracis* colonies are grey-white to grey, 0.3–0.5 mm in diameter, non-haemolytic, with a ground-glass moist surface, and very tacky when teased with an inoculating loop. Tailing and prominent wisps of growth trailing back toward the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a 'medusa head' appearance. Confirmation of *B. anthracis* should be accomplished by the demonstration of a capsulated, spore-forming, Gram-positive rod in blood culture. Absence of motility is an additional test that can be done.

The susceptibility of *B. anthracis* to the gamma bacteriophage was first described by Brown & Cherry in 1955 (3). The phage is available from various national central veterinary laboratories and anthrax reference laboratories. The procedure for the test is simply to streak a lawn on a blood or nutrient agar plate, or portion of a plate (several tests can be done on one plate) with the suspect organism and place a 10–15 µl drop of the phage suspension on one side of the streaked area and place a 10-unit penicillin disk to the other side. Allow the drop of phage suspension to soak in and incubate the plate at 37°C. A control culture should be included; the Sterne vaccine or the NCTC strain 10340 can be used for this. If the culture is *B. anthracis*, the area under the phage will be devoid of bacterial growth, due to lysis, and a clear zone will be seen around the penicillin disk after overnight incubation. (*Note: phage-resistant* B. anthracis *isolates are encountered occasionally; similarly, there are a few reports in the literature of penicillin-resistance*.)

ii) Capsule visualisation

Virulent encapsulated *B. anthracis* is present in tissues and blood and other body fluids from animals that have died from anthrax. The bacteria should be looked for in smears of these specimens that have been dried, fixed and stained with polychrome methylene blue (M'Fadyean's reaction). The capsule stains pink,

whereas the bacillus cells stain dark blue. The cells are found in pairs or short chains and are often square-ended (the chains are sometimes likened to a set of railway carriages – so-called 'box-car' appearance). Gram and Giemsa stains do not reveal the capsule. The capsule is not present on *B. anthracis* grown aerobically on nutrient agar or in nutrient broths, but can be seen when the virulent bacterium is cultured for a few hours in a few millilitres of blood (defibrinated horse or sheep blood seems to work best). Alternatively, the capsule is produced when the virulent *B. anthracis* is cultured on nutrient agar containing 0.7% sodium bicarbonate and incubated in the presence of CO_2 (20% is optimal, but a candle jar works well). The agar is prepared by reconstituting enough nutrient agar base powder for 100 ml of agar in 90 ml of water. Autoclave and cool to 50°C in a water bath. Add 10 ml of a filter-sterilised (0.22–0.45 µm filter) 7% solution of sodium bicarbonate. Mix and pour into Petri dishes. The encapsulated *B. anthracis* will form mucoid colonies and the capsule can be visualised by making thin smears on microscope slides, fixing, and staining with polychrome methylene blue.

iii) Other specimens

Identification of *B. anthracis* from old, decomposed specimens, processed materials, and environmental samples, including soil, is possible but these samples often have saprophytic contaminants that outgrow and obscure *B. anthracis* on nonselective agars. The following procedure is suggested:

a) The sample is blended in two volumes of sterile distilled or deionised water and placed in a water bath at 62.5 ± 0.5°C for 30–60 minutes.

b) Tenfold dilutions to 10^{-2} or 10^{-3} are then prepared. From each dilution, 10–100 µl are plated on to blood agar and optionally 250–300 µl on to PLET agar (polymyxin, lysozyme, EDTA [ethylene diamine tetra-acetic acid], thallous acetate) (7, 11). All plates are incubated at 37°C.

c) Blood agar plates are examined for typical colonies as previously described after overnight incubation, and the PLET plates are examined after 40–48 hours. Confirmation of the identity of suspect colonies as *B. anthracis* is done as described above.

PLET medium (7, 11) is prepared by using heart-infusion agar base (DIFCO) made up to the manufacturer's instructions with the addition of 0.25–0.3 g/litre EDTA and 0.04 g/litre thallous acetate. (*NOTE: thallous acetate is poisonous and should be handled with care.*) The mixture is autoclaved and uniformly cooled to 50°C before adding the polymyxin at 30,000 units/litre and lysozyme at 300,000 units/litre. After mixing thoroughly, the agar is dispensed into Petri dishes.

Reports of procedures for direct detection of *B. anthracis* in soils and other environmental specimens using the polymerase chain reaction (PCR) are emerging. None of these has become routinely applicable at the present time.

Animal inoculation may be considered for recovery of *B. anthracis* if all other methods fail. Examples of when this might occur are specimens from animals that received antibiotic therapy before death or environmental samples containing sporostatic chemicals. Due to the increasing concern to eliminate the use of animals for biological testing, this approach should be used as a last resort and only if justified. Adult mice or guinea-pigs are the animals of choice. If the samples involved are soils, the animals should be pretreated, the day before testing, with both tetanus and gas gangrene antiserum. The samples are prepared as described for culturing, including heat-shocking at 62.5°C for 15 minutes. Mice are injected subcutaneously with 0.05–0.1 ml; guinea pigs are inoculated intramuscularly with up to 0.4 ml (0.2 ml in each thigh muscle). Any *B. anthracis* present will result in death in 48–72 hours and the organism can be cultured from the blood as described above.

b) Immunological detection and diagnosis

It needs to be borne in mind that *B. anthracis* is antigenically very closely related to *B. cereus,* which is considered a ubiquitous component of the environmental microflora. The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin antigens, produced during the exponential phase of growth, and the capsule of *B. anthracis*. This places considerable constraints on the extent to which immunological methods can be used in routine detection methodology.

i) Ascoli test

In 1911, Ascoli (1) published a procedure for the detection of thermostable anthrax antigen in animal tissue being used for by-products. This uses antiserum raised in rabbits to produce a precipitin reaction. The test lacks high specificity, in that the thermostable antigens of *B. anthracis* are shared by other *Bacillus* spp., and is dependent on the probability that only *B. anthracis* would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction. Nowadays, it appears to be used in Eastern Europe only.

To perform the Ascoli test, put approximately 2 g of sample in 5 ml of saline containing 1/100 final concentration of acetic acid and boil for 5 minutes. The resultant solution is cooled and filtered through filter paper. A few drops of rabbit antiserum (see preparation below) are placed in a small test tube. The filtrate from the previous step is gently layered over the top of the antiserum. A positive test is the formation of a visible precipitin band in under 15 minutes. Positive and negative control specimen suspensions should be included.

Antiserum is prepared in rabbits by the subcutaneous inoculation of Sterne anthrax vaccine on days 1 and 14. On days 28 and 35, the rabbits receive 0.5 ml of a mixture of several strains of virulent *B. anthracis* not exceeding 10^5 colony-forming units (CFU)/ml suspended in saline. Alternatively, the live virulent bacteria can be inactivated by prolonged suspension in 0.2% formalised saline, but the antigen mass needs to be increased to 10^8–10^9 CFU/ml. The suspension should be checked for inactivation of the *B. anthracis* before animal inoculation by culture of 0.1 ml into 100 ml of nutrient broth containing 0.1% histidine and, after incubation at 37°C for 7 days, subculture on to blood or nutrient agar. The dose regimen for the formalised suspension after initial vaccination on days 1 and 14 is increasing doses of 0.1, 0.5, 1, and 2 ml given intravenously at intervals of 4–5 days. Following either procedure, a test bleed at 10 days after the last injection should determine whether additional 2 ml doses should be administered to boost the precipitin titre.

ii) Immunofluorescence

While some success has been achieved with immunofluorescence for capsule observation in the research situation (4), it does not lend itself to routine diagnosis.

c) Confirmation of virulence with the polymerase chain reaction

Full confirmation of virulence can be carried out using the PCR. The following instructions are taken from ref. 11. Template DNA for PCR can be prepared from a fresh colony of *B. anthracis* on nutrient agar by suspension of a loop of growth in 25 µl sterile deionised (or distilled) water and heating to 95°C for 20 minutes. Following cooling to approximately 4°C, and brief centrifugation, the supernatant can be used for the PCR reaction.

Suitable primers (2, 5) for confirming the presence of the pX01 and pX02 plasmids are given in the table below.

Target	Primer ID	Sequence 5'–3'	Product size	Concentration
Protective antigen (PA)	PA 5 3048–3029	TCC-TAA-CAC-TAA-CGA-AGT-CG	596 bp	1 mM
	PA 8 2452–2471	GAG-GTA-GAA-GGA-TAT-ACG-GT		
Capsule	1234 1411–1430	CTG-AGC-CAT-TAA-TCG-ATA-TG	846 bp	0.2 mM
	1301 2257–2238	TCC-CAC-TTA-CGT-AAT-CTG-AG		

PCR can be carried out in 50 µl volumes using the above primers, 200 µM each of dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$ and 2.5 units of ampli Taq DNA polymerase™[1], all in NH_4 buffer, followed by the addition of 5 µl of template DNA. A 2% agarose gel has been found to work best with these small fragments.

Alternatively, 'Ready-To-Go™' beads are available from Pharmacia Biotech[2]. These are premixed, predispensed, dried beads, stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 µl PCR reactions. The template can be added in a 2.5 µl volume.

The following PCR cycle can be used: 1 × 95°C for 5 minutes; 30 × 95°C for 0.5 minute followed by 55°C for 0.5 minute followed by 72°C for 0.5 minute; 1 × 72°C for 5 minutes; cool to 4°C.

It should be noted that, in use for some years now in an anthrax reference facility, the primers given in the table above have proved successful for confirming the presence or absence of pXO1 and/or pXO2 in pure cultures of isolates from animal (including human) or environmental specimens or samples. They are unsuitable, however, for direct detection of *B. anthracis* in such specimens or samples. A choice of

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[1] This product is available from Applied Biosystems;
(https://products.applied biosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601622
[2] Uppsala, Sweden, product number 27-9555-01

alternatives can be found in reference 6 and 9. For the rare possibility that an isolate may lack both pXO1 and pXO2, a chromosomal marker should also be run; primers for these are also supplied in references 6 and 9.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The most widely used vaccine for prevention of anthrax in animals was developed by Sterne in 1937 (10). He derived a rough variant of virulent *B. anthracis* from culture on serum agar in an elevated CO_2 atmosphere. This variant, named 34F2, was incapable of forming a capsule and was subsequently found to have lost the pX02 plasmid, which codes for capsule formation. It has become the most widely used strain world-wide for animal anthrax vaccine production. In Central and Eastern Europe, an equivalent pX02⁻ derivative, Strain 55, is the active ingredient of the current livestock vaccine. A list of manufacturers of anthrax vaccine for use in animals is given in Appendix V of reference 11.

The following information concerning preparation of the anthrax vaccine for use in animals is based on references 8 and 12. Generalised procedures are given; national regulatory authorities should be consulted in relation to Standard Operating Procedures that may pertain locally.

1. Seed management

a) Characteristics of the seed

Anthrax vaccine production is based on the seed-lot system. A seed lot is a quantity of spores of uniform composition processed at one time and maintained for the purpose of vaccine preparation. Each seed lot is no more than three passages from the parent culture and must produce a vaccine that is efficacious and safe for use in animals. It is recommended that a large seed lot be prepared from the parent strain and preserved by lyophilisation for future production lots. The parent culture can be purchased[3]. The seed lot is acceptable for anthrax vaccine if a vaccine prepared from the seed lot or a suspension harvested from a culture derived from a seed lot meets the requirements for control of final bulk with respect to freedom from bacterial contamination, safety and efficacy (immunogenicity).

b) Preparation of the master seed

Seed lots are cultured on solid media formulated to promote sporulation of the organism (see Section C.2 below). The solid medium formula given in reference 8 is: 50 g tryptic digest of casein; 10 g yeast extract; 0.1 g $CaCl_2.6H_2O$; 0.01 g $FeSO_4.7H_2O$; 0.05 g $MgSO_4.7H_2O$; 0.03 g $MnSO_4.4H_2O$; 5.0 g K_2HPO_4; 1.0 g KH_2PO_4; 22 g agar; 1000 ml deionised or distilled water. The ingredients are dissolved in the water with the appropriate amount of heating; the solution is adjusted to pH 7.4, distributed into Roux bottles (120 ml per bottle) or other appropriate container, sterilised by autoclaving and cooled in the horizontal position. After the agar has solidified, excess liquid should be removed aseptically and the bottles left in an incubator (37°C) for at least 2 days to dry and to check the sterility.

Volumes of 2 ml of vaccine seed from a reference laboratory should be spread across the agar in Roux bottles, which should be incubated at 37°C until at least 80% sporulation is apparent by microscopic examination of aseptically extracted loopfuls (at least 72 hours). The growth is harvested with 10 ml per bottle of sterile deionised or distilled water and checked for purity. After washing three times in sterile deionised or distilled water with final suspension, also in sterile deionised or distilled water, sterilised lyophilisation stabiliser is added and the suspension is dispensed into lyophilisation vials and freeze-dried.

c) Preparation and testing of the working seed

Reconstitute a vial of seed stock and inoculate several slants (approximately 10 ml) of sporulation (casein digest) agar. Incubate at 37°C for 72 hours and store in a refrigerator. Test the slants for purity by culture on to nutrient agar plates and in nutrient broth (0.1 ml in 100 ml of nutrient broth). The latter should be subcultured on to nutrient agar after incubation at 37°C for 7 days and should be a pure culture of *B. anthracis*. A sample of the broth culture should also be checked for lack of motility.

Volumes of seed needed for a production run should be calculated on the basis of harvesting the spores from each slant with 10 ml of sterile deionised or distilled water and using this to inoculate five Roux bottles.

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3   National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom.

d) Safety of the seed lot

Not less than 5×10^9 culturable spores should be injected subcutaneously into each of three healthy, 1–2-year-old, unvaccinated sheep, which must survive an observation period of at least 10 days.

e) Immunogenicity of the seed lot

At least 10 healthy guinea-pigs, 300–500 g in weight should be inoculated with 5×10^6 viable spores and observed for 21 days. At least 80% of the animals should survive. The immunised animals, together with three unimmunised controls, should then be challenged with 10 median lethal doses (LD_{50}) of the strain 17 JB of *B. anthracis*. During a 10-day observation period, none of the immunised animals should succumb to the challenge while all the controls should die from anthrax. The test should be repeated if one of the immunised animals dies.

2. Method of production

a) Preparation of vaccine concentrate

Roux bottles with casein digest agar are prepared as for the master seed in Section C.1.b above. One Roux bottle can be expected to yield about 2000 doses of vaccine. Each Roux bottle is inoculated with 2 ml of working seed suspension and incubated at 37°C with porous plugs for several days until small loopfuls of culture from randomly selected bottles show at least 90% of the organisms to be in sporulated forms when examined in wet mounts by phase contrast (phase bright spores) or following staining for spores. The growth from each bottle is then harvested with 20 ml of physiological saline. Tests for contaminants should be carried out by subculture to nutrient agar plates and inoculation of 100 ml nutrient broth with 0.1 ml of harvested spores followed by subculture to nutrient agar after 7 days at 37°C and by tests for motility. Acceptable harvests (i.e. those showing no evidence of contaminants) are pooled.

b) Glycerination

Twice the volume of sterile, pure, neutral glycerol should be added to the bulk pool. Saponin (0.1% final concentration) may also be added at this point if it is to be included as an adjuvant. Mix thoroughly (the inclusion of sterilised glass beads may be helpful). Carry out a purity test as before and hold for 3 weeks at ambient temperature to allow lysis of any vegetative bacteria, determine the viable spore count and store under refrigeration thereafter.

c) Determining titre and dilution for use

The number of culturable spores in the product is then calculated by spreading tenfold dilutions on nutrient agar plates. The suspension is diluted so that the final bulk contains the number of culturable spores desired. The diluent should contain the same proportions of saline, glycerol and (if being included) saponin as present in the vaccine concentrate. The vaccine should contain not less than 1×10^7 viable spores per dose for cattle, buffaloes and horses, and not less than $1–5 \times 10^6$ viable spores per dose for sheep, goats and pigs.

d) Safety

Safety testing is performed on two healthy sheep or goats and consists of inoculating subcutaneously twice the recommended vaccination dose. The animals are observed for 10 days. The final bulk passes the test if no systemic reactions develop and if not more than a transient oedema is observed at the injection site. If the test is carried out in sheep only, a progressive oedema indicates that the vaccine may be unsuitable for goats.

e) Filling the containers

Distribution of aliquots of vaccine into single and multidose containers is performed as outlined in World Health Organization Technical Report No. 363 series entitled *General Requirements for Manufacturing Establishments and Control Laboratories* (Requirements for Biological Substances No. 1), 1965, 16–17. Basically, the final bulk is distributed to containers in an aseptic manner in an area not used for production, and any contamination or alteration of the product must be avoided. The vaccine may be lyophilised after distribution into appropriate dosage containers. Containers are sealed as soon as possible with a material that is not detrimental to the product and that is capable of maintaining a hermetic seal for the life of the vaccine.

3. In-process control

Purity tests consist of microscopic examination of stained smears with culture and motility tests as described in Section C.2.a.

4. Batch control and tests on the final product

a) Sterility

The vaccine is a live culture of *B. anthracis* spores; sterility does not apply, but the batches must be tested for freedom from contamination (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Efficacy

Efficacy or immunogenicity is tested on the final bulk as follows: at least ten healthy 300–500 g guinea-pigs are inoculated with a sheep dose of the vaccine. The guinea-pigs are observed for 21 days, and at least 80% of the animals must survive the observation period. Surviving immunised guinea-pigs and three non-vaccinated controls are challenged with an appropriate dose of virulent *B. anthracis.* A recommended challenge is 200 LD_{50} of the Pasteur II strain (17JB), which is available from the same source as the Sterne 34F2 vaccine strain. If, by 10 days after challenge, all vaccinated guinea-pigs survive and control animals die, the final bulk is deemed to be satisfactory. If any vaccinated animals die during the post-challenge observation period from a cause other than anthrax, and death is not associated with the vaccine, the test may be repeated.

c) Dose

The recommended dose for cattle and horses is a minimum of 1×10^7 culturable spores; for sheep, goats and pigs, it is $1–5 \times 10^6$ culturable spores. The vaccine should contain these spores in an appropriate volume, e.g. 1×10^7/ml.

d) Duration of immunity

Most experts agree that immunity is good for at least 1 year and it is recommended that an annual booster be given. Horses may be slow to develop immunity following initial vaccination; some manufacturers therefore recommend a two-dose initial vaccination, administered 1 month apart, followed by a single annual booster.

e) Stability

As there is no generally acceptable test for stability of anthrax vaccines, it is recommended that, in each filling lot, the number of culturable spores be determined before and after holding at an appropriate temperature for an appropriate period. There should be no evidence of a fall in the number of culturable spores.

f) Preservatives and storage

Bacillus anthracis spores are stable in unlyophilised or lyophilised vaccine and preservatives are not required. Storage under refrigeration is recommended (4°C).

g) Precautions (hazards)

The vaccine has been shown to cause disease in some goats and llamas; this may be related to the saponin adjuvant. The vaccine is not recommended for use in pregnant animals, nor in animals destined for slaughter within 2–3 weeks of vaccination. Local regulations may specify other time periods in some countries or regions, but there is no scientific reason for regarding meat from clinically healthy animals as unfit for human handling or consumption after a holding period of 2 weeks following vaccination. Concurrent administration of antibiotics to vaccinated animals is contraindicated as the antibiotic will interfere with the vaccine. Antibiotics should not be given for several days before and after vaccination. Leftover vaccine, empty vials, and equipment used for vaccinating are contaminated with the live spores and should be autoclaved, disinfected, or incinerated. Accidental human inoculation is treated by expressing as much of the inoculum as possible from the injection site and washing the wound thoroughly with soap and water. Medical attention should be sought if infection develops.

5. Tests on the final product

a) Safety

Every batch of vaccine will be tested for safety as described in Section C.2.d.

b) Potency

Every batch of vaccine will be tested for potency, as described in Section C.4.b.

6. Propagation of the diagnostic 'gamma' bacteriophage

Anthrax-specific phages were first isolated in the 1950s, and the specifically named gamma phage was first reported in 1955 and quickly became the standard diagnostic phage for anthrax. Gamma phage belongs to a family of closely related anthrax phages (11). On occasion a phage-negative *B. anthracis* or phage-positive *B. cereus* is encountered. The phage must be used in conjunction with other tests for confirmation, yet it is a useful and reliable test.

Phage suspensions may be obtained from central veterinary laboratories or central public health laboratories.

The phage can be propagated and concentrated by the following protocol. Store phage at 2–4°C and do not freeze phage as it will quickly become non-viable.

Stage one

i)   Spread a blood agar (BA) plate of the Sterne vaccine strain of *B. anthracis*. Incubate overnight at 37°C.

ii)   Inoculate approximately 10 ml of nutrient broth (NB) with growth from the BA plate and incubate at 37°C for approximately 4 hours or until just cloudy, then refrigerate.

iii)   Spread 100 µl of the culture from step ii on three pre-dried BA plates and incubate at 37°C for 30–60 minutes.

iv)   Spread 100 µl of the phage suspension to be amplified over the same plates. Incubate at 37°C overnight.

v)   Harvest the phage-lysed growth on the BA plate in 5 ml of NB followed by a second 'wash' of 5 ml NB. Incubate at 37°C overnight.

vi)   Filter (0.45 µm) and count by dropping 20 µl drops (three drops per dilution) of tenfold dilutions of the filtrate in saline onto lawns of the *B. anthracis* culture prepared as in step iii.

Stage two

This is essentially the same procedure as Stage one, only using the filtrate from step vi to harvest the phage from the plates.

vii)   Prepare three Sterne strain lawns on BA, as in step iii. Incubate at 37°C for 30–60 minutes.

viii) Spread 100 µl phage from step vi. Incubate at 37°C overnight.

ix)   To 9 ml of filtrate from step vi, add 1 ml of 10× concentrated NB.

x)   Harvest the phage from step viii with 5 ml of the solution from step ix, followed by a second 5 ml wash with the rest of the solution from step ix.

xi)   Add 10 ml of 1× NB.

xii)   Incubate at 37°C overnight, filter and count.

Stage three

xiii) Inoculate 100 ml of brain–heart infusion broth with approximately 2.5 ml of the culture from step ii. Incubate on a rotary shaker at 37°C until just turbid.

xiv)   Add the 20 ml of filtrate from step xii and continue incubation overnight.

xv)   The resultant filtrate is checked for sterility and titrated in ten-fold dilutions on lawns of the vaccine strain as in step vi to determine the concentration of the phage. This should be of the order of 10^8–10^9 plaque forming units per ml.

7. Polychrome methylene blue (M'Fadyean's stain)

Polychrome methylene blue is prepared as follows: 0.3 g of methylene blue is dissolved in 30 ml of 95% ethanol; 100 ml of 0.01% potassium hydroxide (KOH) is mixed with the methylene blue solution. Ideally, this should be allowed to stand exposed to the air, with occasional shaking, for at least 1 year to oxidise and mature. Addition of K_2CO_3 (to a final concentration of 1%) hastens the 'ripening' of the stain, but before it is regarded as diagnostically reliable, its efficacy should be established by testing it in parallel with an earlier, functional batch of stain on *bona fide* samples. It has now been found that stains that give positive reactions with cultures of *B. anthracis* cultured artificially in horse blood sometimes do not give positive results in the field.

In making smears for staining, only small drops of blood or tissue fluid are needed and a thin, small smear is best. After fixing and drying, a small (approximately 20 µl) drop of stain is placed on the smear and spread over it

with an inoculating loop. After 1 minute, the stain is washed with water, blotted, air-dried and observed initially using the ×10 objective lens under which the short chains appear like short hairs; once found, these can be observed under oil immersion (×1000) for the presence of the pink capsule surrounding the blue/black-staining bacilli. To avoid laboratory contamination, the slide and blotting paper should be autoclaved or left for some hours in a 10% sodium hypochlorite solution.

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NB: There are OIE Reference Laboratories for Anthrax (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.1.2.

AUJESZKY'S DISEASE

SUMMARY

Aujeszky's disease, also known as pseudorabies, is caused by an alphaherpesvirus that infects the central nervous system and other organs, such as the respiratory tract, in a variety of mammals except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks old, which die from encephalitis). The disease is controlled by containment of infected herds and by the use of vaccines and/or removal of latently infected animals.

A diagnosis of Aujeszky's disease is established by detecting the agent (virus isolation, polymerase chain reaction [PCR]), as well as by detecting a serological response in the live animal.

Identification of the agent: *Isolation of Aujeszky's disease virus can be made by inoculating a tissue homogenate, for example of brain and tonsil or material collected from the nose/throat, into a susceptible cell line such as porcine kidney (PK-15) or SK6, or primary or secondary kidney cells. The specificity of the cytopathic effect is verified by immunofluorescence, immunoperoxidase or neutralisation with specific antiserum. The viral DNA can also be identified using PCR; this can be accomplished using the real-time PCR techniques.*

Serological tests: *Aujeszky's disease antibodies are demonstrated by virus neutralisation, latex agglutination or enzyme-linked immunosorbent assay (ELISA). A number of ELISA kits are commercially available world-wide. An OIE international standard serum defines the lower limit of sensitivity for routine testing by laboratories that undertake the serological diagnosis of Aujeszky's disease.*

Since about 1990, it has become possible to distinguish between antibodies resulting from natural infection and those from vaccination by use of gene-deleted vaccines.

Requirements for vaccines and diagnostic biologicals: *Vaccines, either gene-deleted attenuated or inactivated virus vaccines should prevent or at least limit the excretion of virus from the infected pigs. More recently, these conventional vaccines have been supplemented by rDNA-derived gene-deleted or naturally deleted live Aujeszky's disease virus vaccines. The virus used in these new vaccines, sometimes referred to as marker vaccines, lacks a specific glycoprotein (gG, gE, or gC).*

A. INTRODUCTION

Aujeszky's disease, also known as pseudorabies, is caused by an alphaherpesvirus, a member of the family Herpesviridae. The virus infects the central nervous system and other organs, such as the respiratory tract, a variety of mammals except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks old, which die from encephalitis). The disease is controlled by containment of infected herds and by the use of vaccines and/or removal of latently infected animals.

Whereas isolation of the Aujeszky's disease virus will assist in a provisional diagnosis in the case of lethal forms of Aujeszky's disease or clinical disease in pigs, other techniques and serological tests are required for diagnosis of latent infections. Many affected animals, however, except pigs, do not live long enough to produce any marked serological response.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Virus isolation

The diagnosis of Aujeszky's disease can be confirmed by isolating PRV from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil swabs from living pigs, or from samples from dead pigs or following the presentation of clinical signs such as encephalitis in herbivores or carnivores. For post-mortem isolation of PRV, samples of brain, tonsil, lung and spleen are the preferred specimens. In cattle, infection is usually characterised by a pruritus, in which case a sample of the corresponding section of the spinal cord may be required in order to isolate the virus. In latently infected pigs, the trigeminal ganglion is the most consistent site for virus isolation, although latent virus is usually non-infective unless reactivated, making it difficult to recover in culture.

The samples are homogenised in normal saline or cell culture medium with antibiotics and the resulting suspension is clarified by low speed centrifugation at 900 **g** for 10 minutes. The supernatant fluid is used to inoculate any sensitive cell culture system. Numerous types of cell line or primary cell cultures are sensitive to PRV, but a porcine kidney cell line (PK-15) is generally employed. The overlay medium for the cultures should contain antibiotics (such as: 200 IU/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml polymyxin; and 3 µg/ml fungizone).

PRV induces a cytopathic effect (CPE) that usually appears within 24–72 hours, but cell cultures may be incubated for 5–6 days. The monolayer develops accumulations of birefringent cells, followed by complete detachment of the cell sheet. Syncytia also develop, the appearance and size of which are variable. In the absence of any obvious CPE, it is advisable to make one blind passage into further cultures. Additional evidence may be obtained by staining infected cover-slip cultures with haematoxylin and eosin to demonstrate the characteristic herpesviral acidophilic intranuclear inclusions with margination of the chromatin. The virus identity should be confirmed by immunofluorescence, immunoperoxidase, or neutralisation using specific antiserum.

The isolation of PRV makes it possible to confirm Aujeszky's disease, but failure to isolate does not guarantee freedom from infection.

b) Identification of virus by the polymerase chain reaction

The polymerase chain reaction (PCR) can be used to identify PRV genomes in secretions or organ samples. Many individual laboratories have established effective protocols, but there is as yet no internationally agreed standardised approach.

The PCR is based on the selective amplification of a specific part of the genome using two primers located at each end of the selected sequence. In a first step, the complete DNA may be isolated using standard procedures (e.g. proteinase K digestion and phenol–chloroform extraction) or commercially available DNA extraction kits. Using cycles of DNA denaturation to give single-stranded DNA templates, hybridisation of the primers, and synthesis of complementary sequences using a thermostable DNA polymerase, the target sequence can be amplified up to 10^6-fold. The primers must be designed to amplify a sequence conserved among PRV strains, for example parts of the gB or gD genes, which code for essential glycoproteins, have been used (10, 29).

The amplified product may be identified from its molecular weight as determined by migration in agarose gel, with further confirmation where possible by Southern hybridisation using a complementary probe. Recent techniques involve liquid hybridisation using enzyme-labelled probes, which give a colour reaction after incubation with the appropriate substrate. More recent techniques include the use of fluorescent probes linked to an exonuclease action and real-time monitoring of the evolution of product, enabling simultaneous amplification and confirmation of the template DNA thus increasing the rapidity and specificity of the PCR assays.

In all cases, the main advantage of PCR, when compared with conventional virus isolation techniques, is its rapidity; with the most modern equipment, the entire process of identification and confirmation can be completed within one day, However, because of the nature of the test, many precautions need to be taken to avoid contamination of samples with extraneous DNA from previous tests or from general environmental contamination in the laboratory (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials). This may limit the value of the test for many laboratories unless care is taken to avoid DNA carry-over contamination.

2.   Serological tests

Any serological technique used should be sufficiently sensitive to give a positive result with the OIE International Standard Reference Serum. This serum can be obtained from the OIE Reference Laboratory for Aujeszky's Disease in France (see Table given in Part 3 of this *Terrestrial Manual*). For international trade purposes, the test should be sensitive enough to detect the standard serum diluted 1/2.

Virus neutralisation (VN) has been recognised as the reference method for serology (4, 28), but for general diagnostic purposes it has been widely replaced by the enzyme-linked immunosorbent assay (ELISA) because of its suitability for large-scale testing (2, 12, 16, 18). The tests can be performed on a variety of matrices (e.g. serum, whole blood, milk) but the preferred matrix is serum.

A latex agglutination test has also been developed and can be used for screening for antibodies. Kits for the test are commercially available.

a)   Virus neutralisation (a prescribed test for international trade)

VN in cell culture can be performed in several ways, which vary according to the length of incubation of the virus/serum mixtures (e.g. 1 hour at 37°C or 24 hours at 4°C), and the presence or absence of complement. Most laboratories use a reaction period of 1 hour at 37°C in the absence of complement, because this is easy and rapid. However, the sensitivity can be improved by increasing the incubation period to 24 hours at 4°C, which facilitates the detection of antibody levels 10–15 times lower than in the 1-hour method. For international trade purposes, the test method should be validated as being sensitive enough to detect the OIE Standard Reference Serum diluted 1/2.

VN cannot be used to differentiate antibodies of vaccinal origin from those caused by natural infection. It is one of the two tests available that complies with the requirement in the OIE *Terrestrial Animal Health Code* chapter when it refers to 'a diagnostic test to the whole virus'.

Cells: Cells susceptible to infection with PRV are used; they may be cell lines (e.g. PK-15, SK6, MDBK), or primary or secondary cell cultures.

Cell culture medium: The medium depends on the type of cells. For example, the medium for PK-15 cells is Eagle's minimal essential medium (MEM) + 10% fetal bovine serum and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin, or alternatively, 50 µg/ml gentamycin).

Maintenance of the cells: The cells are cultured in cell culture vessels of, for example, 75 cm^2. They are trypsinised once or twice per week. For weekly trypsinisation, the cells are cultured in 50 ml of medium, with a multiplication rate of 5. For two trypsinisations a week, the cells are cultured in 30 ml of medium, with a multiplication rate of 3.

For trypsinisation, the growth medium is removed once the cell sheet is complete. The cell sheet is washed with about 5 ml of recently thawed trypsin/ethylene diamine tetra-acetic acid (EDTA) (0.25%) in an isotonic buffer. The washing fluid is discarded and the preparation is washed again, retaining only a few drops of trypsin. The container is placed in an incubator at 37°C for 5–10 minutes until the cells have become detached. Once the sheet is detached and the cells are well separated, they are suspended in 90 ml of growth medium, and this suspension is distributed into three 75 cm^2 cell culture bottles.

Virus: A suitable strain of PRV, such as the Kojnok strain, or NIA-3 strain, is stored at a temperature of –70°C or below, or in freeze-dried form at 4°C.

Preparation of stock virus suspension: The culture fluid is removed from a cell culture bottle containing a complete cell sheet. About 1 ml of stock virus suspension of known titre (about 10^7 TCID$_{50}$/ml [50% tissue culture infective dose]) is added, and the bottle is incubated at 37°C for 1 hour. Then, 30 ml of culture medium is added and the bottle is again incubated at 37°C. The bottle is examined frequently until there is about 75% cell destruction (after about 36–48 hours). It is then frozen at a temperature of –20°C or lower in order to disrupt the cells.

The bottle is then thawed and shaken vigorously. Medium is collected and centrifuged at 1500 *g* for 15 minutes. The supernatant fluid is divided into portions (of about 0.5 ml) in small tubes that are labelled (date and virus reference) before being stored at a temperature of –70°C or lower until required.

Titration of the stock virus suspension: Titration of the stock suspension is performed by the method of Reed & Muench or that of Kärber, and the titre is expressed per 50 µl and per ml.

The VN test requires an internal quality control serum with a known titre of neutralising antibody to PRV (it must be calibrated against an international standard serum or a secondary standard prepared from that serum), and a negative control serum (from a specific antibody free pig, e.g. from an official Aujeszky's disease free herd). The test sera themselves should be of good quality. Serum should be separated from the coagulum without delay, thereby preventing toxicity.

There are qualitative and quantitative procedures for VN, both of which are described below.

- **Qualitative technique**

i) Complement in the serum samples is destroyed by heating in a water bath at 56°C for 30 minutes.

ii) Each undiluted serum is placed in three wells, at 50 µl per well, of a 96-well cell-culture grade microtitre plate.

iii) 50 µl of virus suspension containing 100 $TCID_{50}$ (or 2×10^3 $TCID_{50}$/ml), obtained by diluting stock virus suspension of known titre with MEM, is added to each well.

iv) The plate is shaken and placed in an incubator for 1 hour at 37°C (CO_2 optional).

v) 150 µl of cell suspension containing about 150,000 cells/ml is added to each well.

vi) The plate is covered (for incubation in CO_2), or a plastic sheet is sealed carefully around the edges of the plate (for incubation in air). The plate is shaken lightly to obtain an even distribution of cells at the bottom of the wells, and placed in the incubator at 37°C (CO_2 optional).

vii) *Controls:* Each set of plates must include the following controls:

Virus control: This is to verify the amount of virus actually used for the test. The virus dose used for virus neutralisation (target titre 100 $TCID_{50}$/50 µl) is diluted with MEM at 1/10, 1/100 and 1/1000. Of each dilution, 50 µl is placed in at least eight wells, to which 50 µl of medium is added before the wells are incubated for 1 hour at 37°C. The cell suspension is added in the same way as for the sera under test.

Cell control: 150 µl cell suspension and 100 µl MEM are placed in each of at least two wells.

Positive serum control: A serum of known PRV neutralising antibody titre is used. Five dilutions are prepared in the same way as for the sera under test: a dilution corresponding to the serum titre, two-fold and four-fold dilutions, and 1/2 and 1/4 dilutions (equivalent to T, T/2, T/4, 2T and 4T, where T is the serum titre, i.e. undiluted serum for the qualitative test). To 50 µl of positive control sample dilutions, add 50 µl of virus suspension containing 100 $TCID_{50}$/50 µl. The cells are incubated and the cell suspension is added in the same way as for the sera under test.

Serum control: This is to verify the absence of a toxic effect of the sera on the cells. Wells containing 50 µl of each serum are incubated for 1 hour at 37°C in the presence of 50 µl of medium. Then, 150 µl of cell suspension is added in the same way as for the sera under test.

Negative serum control: This is done in the same way as for sera under test.

viii) *Reading the results:* An inverted-image microscope (×100) is used to examine the wells for toxic effects and CPEs after 48 and 72 hours. The controls must give the following results if the tests are to be considered valid:

Virus control: The titre of the viral suspension should be between 30 and 300 $TCID_{50}$/50 µl.

Cell control: The cell sheet must be intact.

Positive serum control: The titre obtained must be equal to the predicted titre, within one dilution.

Serum control: Examination for a CPE should take into account a possible toxic effect on cells.

Negative serum control: A CPE should be present.

ix) For the sera under test, the following results may be seen: presence of a CPE in three wells = negative result; absence of a CPE in three wells on day 3 = positive result; presence of a CPE in one well but not in the others = doubtful result, test must be repeated; small plaques indicating a CPE on day 3 = doubtful result, test must be repeated; toxicity in serum control and test wells = unreadable result, test must be repeated (NB replacement of medium with fresh medium after 16 hours' incubation will reduce the toxicity without affecting the titre of specific antibody).

x) *Interpretation of the results:* This test is capable of detecting the presence or absence of neutralising antibody to PRV. It is incapable of distinguishing vaccinated animals from infected animals.

The technique described (VN for 1 hour at 37°C) can give false-negative and false-positive results. The sensitivity can be increased (leading to fewer false negatives) by adopting a method based on neutralisation involving 24 hours of contact between virus and serum at 4°C, before the addition of cells.

A qualitative technique such as this one, which employs undiluted serum (1/2 final dilution), can give a false-positive result in certain cases due to nonspecific neutralisation of the virus. This problem can be addressed by carrying out a confirmatory test using the quantitative technique (see below).

- **Quantitative technique**

This is similar to the qualitative procedure, but each serum is used both undiluted and in a series of dilutions. Depending on the desired precision, the purpose of testing and the expected titre, two wells are used for each dilution of serum, and a greater or smaller range of dilutions. Ideally, the procedure may be described for a range of dilutions reaching an initial maximum of 1/256.

i)   Complement in the serum samples is destroyed by heating in a water bath at 56°C for 30 minutes.

ii)   50 µl of MEM is added to wells A3 to A6 of a 96-well cell-culture grade microtitre plate.

iii) 50 µl of undiluted serum is added to wells A1 to A3, and continued for wells in rows B, C, etc., with other serum samples.

iv)   Using a multichannel pipette, the contents of wells in row 3 are mixed, then 50 µl is transferred to row 4, and so on to row 6 or further to a predetermined row, using the same nozzles. The 50 µl portions remaining after the last row is discarded.

v)   Controls are set up as described for the qualitative technique.

vi)   50 µl of MEM is added to row 1 instead of virus: this is a control row of sera. Viral suspension is deposited in the wells of the other rows. Subsequent manipulations are the same as described for the qualitative technique.

vii) *Reading the results:* The neutralising titre of a serum is expressed by the denominator of the highest initial dilution that brings about complete neutralisation of the CPE of the virus in 50% of the wells. Neutralisation at any dilution (even undiluted, equivalent to a final dilution of 1/2) is considered to be positive. If the serum shows neutralisation only when undiluted (with growth of virus and CPE at the 1/2 and subsequent dilutions), it would be advisable to apply alternative tests (ELISA or latex agglutination) to provide confirmation of the result, or to request another sampling of the animal, at least 8 days after the first.

b) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

The sensitivity of the ELISA is generally superior to that of the VN test using 1-hour neutralisation without complement. Some weak positive sera are more readily detected by VN tests using 24-hour neutralisation, while others are more readily detectable by ELISA.

ELISA kits, which are available commercially, use indirect or competitive techniques for measuring antibody levels. They differ in their mode of preparation of antigen, conjugate, or substrate, in the period of incubation and in the interpretation of the results. Their general advantage is that they enable the rapid processing of large numbers of samples. This can also be automated and the results analysed by computer. Some of these kits make it possible to differentiate between vaccinated and naturally infected animals when used with a 'matching' vaccine (6, 21, 22). Alternatively, non-commercial ELISA protocols may be adopted (2, 18) provided they are shown to detect the OIE International Standard Reference Serum as positive at a dilution of 1/2 (the minimum sensitivity for international trade purposes). It is recommended to use a kit or in-house assay that has been validated to this standard by external quality control tests by an independent laboratory. A suitable test protocol for whole virus antibodies is presented below (18).

- **Preparation of antigen**

i)   A cell line sensitive to PRV is used, such as PK-15 or fetal pig testis. It must be free from extraneous viruses, such as bovine viral diarrhoea virus. The cells should be split and seeded into fresh 75 cm^2 flasks the day before inoculation. A suitable medium such as MEM, without serum, is used to overlay the cultures.

ii)   Virus inoculated, and control uninoculated flasks are processed in parallel throughout. A suitable well characterised strain of PRV is used, e.g. Kojnock strain. When a confluent cell monolayer has developed (approximately 24 hours after seeding), it is inoculated with 10^8 TCID$_{50}$ PRV in 5 ml medium; and 5 ml medium (without virus) is placed in control flasks. The cultures are left for adsorption for 30 minutes at 37°C, and then overlaid with 20 ml medium.

iii) When CPE is just beginning, the supernatant medium is discarded and 4 ml KCl (4 mM solution) and glass beads are added. The flasks are shaken gently to detach cells.

iv)   Cells are washed by centrifuging three times at 770 ***g*** in 4 mM KCl. The pellet is resuspended in 4 mM KCl with 0.2% Triton X-100 (1 ml per flask) by applying 60 strokes with a glass homogeniser

    v)   The cell homogenate is layered on to 0.25 mM sucrose in 4 mM KCl and centrifuged for 10 minutes at 770 ***g***.

    vi)   The pellet is resuspended in antigen-diluting buffer, pH 9.6 (0.1 M Tris, 2 mM EDTA, 0.15 mM NaCl) at 1/50 the volume of the original culture medium. It may then be stored in small aliquots at –70°C. Antigen is stable in this form for 2 years.

- **Coating microtitre plates**

    i)   Virus antigen and control (no virus) antigen are diluted in diluting buffer, pH 9.6 (see above) to a dilution predetermined in chequerboard titrations.

    ii)   200 µl of antigen is dispensed into each well of 96-well ELISA-grade plates, coating alternate rows with PRV positive and control antigen. Incubation is for 18 hours at 4°C.

    iii)   The plates are washed three times with washing solution (Tween 20, 0.5 ml/litre).

    iv)   Coated plates are stored at –20°C or –70°C. They are stable for several months.

- **Test procedure**

    i)   Test serum samples are diluted 1/30 in PBS/Tween buffer, pH 7.2 (137 mM NaCl, 9.5 mM phosphate buffer, 0.5 ml/litre Tween 20).

    ii)   Diluted samples are added to virus and control antigen coated wells, and incubated at 37°C for 30 minutes.

    iii)   The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

    iv)   Protein A/peroxidase conjugate is added to all wells at a predetermined dilution in PBS/Tween buffer, pH 7.2 (see above), with added bovine serum albumen fraction V (10 g/litre), and the plates are incubated at 37°C for 30 minutes.

    v)   The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

    vi)   A suitable chromogen/substrate mixture, such as tetra methyl benzidine (TMB)/hydrogen peroxide, is added to each plate.

    vii)   The reaction is stopped with 2 M sulphuric acid. The absorbance is read at 492 nm.

The test must be fully validated using known positive and negative sera, and calibrated against the OIE International Standard Reference Serum. All tests must include positive and negative internal controls, including a weak positive that, when diluted at the appropriate dilution for the test, has equivalent activity to a 1/2 dilution of the OIE International Standard Reference Serum. For further details see reference 18 and Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases. Commercial ELISA kits also have to be validated in the setting in which they are going to be used.

As well as testing sera, the ELISA can be adapted to test filter paper disks that have been moistened with a small quantity of blood obtained by puncturing a superficial vein. This technique makes it convenient to collect blood samples from large numbers of pigs (3, 19). The disks are air-dried before shipment to the laboratory.

Requirements for the detection of gE antibodies by ELISA in pigs destined for slaughter, that are to be introduced into zones free from Aujeszky's disease, have been defined by several control authorities. The OIE *Terrestrial Animal Health Code* specifies circumstances in which gE-specific tests may be used. The gE ELISAs can also be adapted to test blood on filter paper disks.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Aujeszky's disease may be controlled by the use of vaccines containing either modified live or inactivated virus antigens. In addition, these conventional vaccines have been supplemented by recombinant DNA-derived gene-deleted or naturally deleted live PRV vaccines. These vaccines, sometimes referred to as marker vaccines, are made with a virus that lacks a specific glycoprotein (most commonly gE-, although gG- or gC-deleted vaccines have also been described[1]). At least one commercially available vaccine has dual deletions. These gene-deleted marker vaccines have the advantage over conventional whole virus vaccines that it is possible to distinguish noninfected vaccinated animals from those with field infection. This is done by testing for the antibodies directed against the protein coded for by the deleted gene, which will be absent in noninfected marker-vaccinated pigs but

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1   The nomenclature for the genes changed several years ago, but the old designation is still in the literature. The old and the new nomenclature is: g11 – gB; g111 = gC; gp50 = gD; g1 = gE; gX = gG; gp63 = gl.

present in field-infected pigs. Therefore, in countries with infected pigs, where the eradication of Aujeszky's disease is planned, these marker vaccines are the vaccines of choice. Standards applicable to the manufacture of live and inactivated virus vaccines are described. For marker vaccines, the tests should include demonstrable absence of a serological response in vaccinated pigs to the protein coded for by the deleted gene, and in addition a demonstrable response to the same protein in vaccinated pigs that become infected by field virus.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Vaccines are made using a seed-lot system in which a master seed virus (MSV) is prepared from a suitable strain of Aujeszky's disease virus. A number of strains are used for vaccine manufacture. The antigen in an inactivated vaccine can be one of a number of wild-type strains, or the naturally deleted Bucharest virus, or rDNA-derived gene-deleted virus. Modified live conventional vaccines and rDNA-engineered vaccines use numerous strains, such as Bartha (8, 9, 11, 15, 17, 23, 27), or are derived from Aujeszky's original isolate or from other field isolates, such as the NIA-3 strain.

It is recommended that for differentiating between infected and vaccinated animals, deleted strains should be used.

A virus identity test (using either a fluorescent antibody test, neutralisation test, [constant serum/decreasing virus method], or any other suitable identity test) must be conducted on the MSV.

The MSV must be shown to be free from mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, porcine parvovirus, cytopathic and noncytopathic ovine and bovine pestiviruses and other extraneous agents, such as circovirus, as determined by culturing and by fluorescent antibody procedures or others, such as PCR.

b) Method of culture

Most of the cell lines used to propagate PRV are continuous lines, such as the PK-15 line. A master cell stock (MCS) is established at a specified passage level. The MCS and the highest passage level (MCS × n) intended for use in the preparation of a biological product is specified in an Outline of Production. Both MCS and MCS × n are monitored by a variety of procedures to characterise the cell line and to ensure freedom from adventitious agents. The extraneous agents to be detected are generally defined in monographs and/or guidelines (e.g. European Pharmacopoeia, US Code of Federal Regulations, EU guidelines, etc.). In general, the type of agents to be looked for is founded on a risk analysis depending on the history of the viral strain and cells on which the vaccinal strain was isolated and on which it is cultivated. The MCS must be monitored for species of origin. A minimum of 50 mitotic cells should be examined at both the MCS and MCS × n passage levels. The modal number in the MCS × n must not exceed 15% of the modal number of the MCS. Any marker chromosomes in the MCS must also be present in the highest cell passage.

If there is evidence that the cell line may induce malignancies in the species for which the product is intended, the cell line is tested for tumorgenicity and oncogenicity.

c) Validation as a vaccine

i) *Purity*

The MSV must be shown to be free from mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, porcine parvovirus, cytopathic and noncytopathic ovine and bovine pestiviruses and other extraneous agents, such as circovirus, as determined by culturing and by fluorescent antibody procedures or others, such as PCR.

ii) *Stability tests*

Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.

iii) *Safety tests*

Local and general reactions must be examined. When a live vaccine is used, it is necessary to differentiate the exact safety properties of the vaccinal strain from those of the finished product if this includes an adjuvant.

Objective and quantifiable criteria to detect and measure adverse reactions should be used; these would include temperature changes, weight gain, litter size, reproductive performance, etc., of vaccinated and control groups. The tests must be performed by administering the vaccine to the pigs in the recommended dose and by each recommended route of administration.

In general, safety is tested initially under experimental conditions. When the results of these preliminary tests are known, it is necessary to increase the number of animals vaccinated in order to evaluate the safety of the vaccine under practical conditions.

- **Laboratory testing**

    All tests must be carried out on pigs that do not have antibodies against Aujeszky's disease virus or against a subunit of the virus.

    ### a. General effects

    1. *Live vaccines*

    Intranasal tests and vaccination of 3–5-day-old piglets are very useful for ascertaining the degree of safety of a strain. At least five piglets should be used.

    It is also essential to assess the properties of a vaccine, especially live ones, in the target animals under normal conditions of use and at the youngest age intended for vaccination, e.g. fattening pigs, which are generally vaccinated when they are between 9 and 12 weeks old, and pregnant sows when this use of the vaccine is claimed by the manufacturer and is authorised. No clinical signs, including significant thermal reactions (data have to be recorded before vaccination and 6 hours, 24 hours and 48 hours later, then on a daily basis during the observation period) , should be observed after vaccination. These assays have to be performed on at least ten vaccinated pigs, with unvaccinated pigs as controls.

    Reversion to virulence following serial passage must be examined. Primary vaccination is done by the intranasal route. Series of at least five passages in piglets are made. No fewer than two fully susceptible animals must be used for each passage.

    The object of these assays is to test the genetic stability of live vaccine strains. The tests appear to be less necessary when a genetically modified live strain is concerned, especially if it is produced by gene deletion.

    It is recommended to test for possible excretion of the vaccine strain. For this purpose, no fewer than 14 piglets, 3–4 weeks old each receive one dose of vaccine by the recommended route and at the recommended site (except for vaccines administered by the intranasal route). Four unvaccinated piglets are kept as controls. Suitably sensitive tests for the virus are carried out individually on the nasal and oral secretions of vaccinated and in-contact pigs as follows: nasal and oral swabs are collected daily from 1 day before vaccination to 10 days after vaccination. Vaccine strains that are isolated from the nasal/oral secretion collected from pigs in which the vaccine was administered by the parenteral route are not recommended.

    The ability of the Aujeszky's disease vaccine strain to spread from a vaccinated pig to unvaccinated ones (lateral spread) must be tested by using the recommended route of administration that presents the greatest risk of spreading (except for vaccines administered by the intranasal route). A repetition of the assays (four times) is necessary as this phenomenon is difficult to detect. Four piglets should be used each time for vaccination and placed in contact, 1 day later, with two unvaccinated piglets. It may also be necessary to examine the spread of the strain to non target species that may be susceptible to the vaccine strain.

    Live attenuated vaccine strains are tested with regard to their general effects by administering to 5–10-day-old piglets ten times the field dose. This administration of an overdose makes it possible to detect reactions not produced under normal conditions of use. Such reactions may be produced inadvertently when large numbers of animals are vaccinated. If vaccines are administered by the intranasal route, the manufacturer has to indicate clearly that the vaccine will spread from vaccinated pigs to unvaccinated ones.

    2. *Inactivated vaccines*

    It is essential to test inactivated vaccines in the target animals under normal conditions of use for fattening pigs and for sows when this use is claimed by the manufacturer and authorised (26). As described previously, it is fundamental to use objective and quantifiable criteria to detect and to measure adverse reactions, such as temperature changes, weight performance, litter size, reproductive

performance, etc., on vaccinated and control groups. The tests must be performed by administering the vaccine in the recommended dose and by each recommended route of administration to the pigs for which it is intended.

Pigs or sows are usually observed until there is no further evidence of vaccine reaction. The period of observation must not be fewer than 14 days from the day of administration. This period has to be extended when, for example, the vaccine is used in pregnant sows and it is necessary to assess the possible effects of the vaccine on reproductive performance. In this case, the period of observation lasts the full duration of the pregnancy.

Control authorities generally request vaccination with a double dose so that adverse reactions, which may be at the limit of detection when a single dose is administered, are more likely to be detected.

b.   Local reactions

Local reactions are often associated with the use of inactivated vaccines, as these side-effects can be induced by adjuvants, particularly oil adjuvants (24). However, some Aujeszky's disease live vaccines are mixed with different adjuvants, which modify what has been observed in the past.

Local reactions are mainly inflammatory and can be more or less complicated (necrotic or suppurative), depending on the nature of the adjuvants used and the aseptic conditions of the vaccination. Oil adjuvants can induce a variety of effects including muscular degeneration, granuloma, fibrosis and abscessation. In addition to the nature of the oil used (the intensity of the reaction is reduced when metabolisable oils are used in the vaccine), the type of emulsion used (water/oil, oil/water, water/oil/water) induces these reactions to a greater or lesser extent. In consequence, it is necessary to observe the site of injection not only from the outside, but also by dissection after slaughter, especially for growing and finishing pigs.

- **Field testing**

    Field trials are necessary to assess the safety of an Aujeszky's disease vaccine in a large number of pigs or sows. In Europe (7), tests must be carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). At least three groups of no fewer than 20 animals each are used with corresponding groups of no fewer than 10 controls. The rectal temperature of each animal is measured at the time of vaccination, and 24 and 48 hours later. At slaughter, the injection site must be examined for local reactions. If the vaccine is intended to be used in sows, reproductive performances have to be recorded. Field trials are supplemented by laboratory studies of efficacy correlated to vaccine potency.

iv)   *Efficacy tests*

- **Laboratory trials**

    All tests must be carried out on pigs that do not have antibodies against Aujeszky's disease virus or against a subunit of the virus, except that some tests may be done using maternally immune animals.

a.   Assessment of passive immunity

To test the efficacy of vaccines, it is important to mimic the natural infection conditions (1). PRV infection gives rise to important losses of young piglets from nonimmune sows. Thus, when vaccinating sows, the main goal is to protect the young piglets through passive immunity conferred by the colostrum ingested immediately after birth, with the secondary objective of preventing abortion.

To measure this passive immunity and the protection induced by vaccinating the sows, experimental models have been established. The sows are vaccinated according to the vaccinal protocol during pregnancy. When the piglets are, for example, 6–10 days old they are given an intranasal challenge exposure with a virulent PRV strain. It is preferable to use a strain titrated in median lethal doses (LD_{50}). Pigs should be inoculated by the nasal route, 10^2 pig LD_{50} per pig in 1 ml. The efficacy of the vaccine is assessed by comparing clinical signs, but also and more importantly, mortality in piglets from unvaccinated dams with that observed in piglets from vaccinated sows.

Piglets from vaccinated sows can be found to have 80% protection against mortality compared with those from the control sows. In order for the results to be significant, it is recommended that eight vaccinated sows and four control sows be used (subject to satisfactory numbers of piglets from each sow).

b.   Assessment of active immunity

1.   *Clinical protection*

Several criteria can be considered when measuring active immunity induced by vaccinating pigs. Generally, pigs are vaccinated at the beginning of the growing period, i.e. when they are between 9 and

12 weeks old. Laboratory trials are performed by challenging pigs at the end of the finishing period, when they weigh between 80 and 90 kg.

In general, at least three criteria, such as rectal temperatures, weight losses and clinical signs, along with mortality, are used to measure the clinical protection of pigs after vaccination and challenge (5). The antibody titres have little predictive value for the efficacy of the vaccines. Weight loss compared between the vaccinated and control groups is the most reproducible and reliable parameter when the challenge conditions are well standardised. The measure of the difference in weight gain or loss between the two groups of pigs and, in the interval of time between challenge (day 0 and day 7), has a very good predictive value for the efficacy of the vaccines (14). Significant results can be obtained when weight performances are compared between one group of at least eight vaccinated pigs and another group of eight unvaccinated control pigs

For challenge, it is usually preferable to use a high titre of a virulent strain, as this makes it possible to obtain a more marked difference between vaccinated and control pigs. On the basis of previous work, a challenge dose with at least 10^6 $TCID_{50}$/ml virulent strain having undergone not more that three passages on primary cells can be sufficient, but a higher titre ($10^{7.5}$ $TCID_{50}$/ml) is recommended. The oro-nasal route should be used to challenge the pigs by introducing the virulent strain in an appropriately high volume (≥4 ml).

This method of evaluating the efficacy of PRV vaccines is now well tested and has made it possible to establish an objective index for determining the efficacy of a vaccine. This index, which compares the relative weight losses between vaccinated and control pigs, can also be used for potency testing batches before release and for batch efficacy testing. However, the value of the cut-off index will be different as the conditions of the assay will not be identical. The influence of passively acquired, maternally derived antibodies on the efficacy of a vaccine must be evaluated adequately.

2.   Virulent virus excretion

Additionally, it is desirable that vaccines should prevent or at least limit viral excretion from infected pigs (13, 20, 25). When a control programme against Aujeszky's disease is based on large-scale vaccination, it is essential to choose the vaccines or the vaccinal scheme that best limits the replication of virulent virus in infected pigs. Several assays have been performed to compare vaccines on that basis.

Generally, the pigs are vaccinated and challenged at different periods. It is better, but more time-consuming, to infect pigs at the end of the finishing period. To measure the virus excretion, nasal swabs (taken at 10 cm depth in the nostrils) are taken daily from each pig from the day before challenge to at least 12 days after challenge. The swabs can be weighed before the sampling and immediately after to calculate the exact weight of collected mucus. Medium is then added to each tube containing a swab. The virus is titrated from the frozen and thawed medium.

Different indexes can be used to express the quantity of virulent virus excreted by pigs, taking into consideration the duration and the level of viral excretion, and the number of pigs excreting virulent virus.

3.   Duration of immunity

It is recommended that any claims regarding the onset and duration of immunity should be supported by data from trials. Assessment of duration of immunity can be based on challenge trials or, as far as it is possible, on immunological and serological tests.

- **Field trials**

  In general terms, it is extremely difficult to assess vaccine efficacy in animal populations. In order to do this, it would be necessary to vaccinate the animals in the absence of the pathogen that the vaccine protects against, then to await the moment of infection and to compare the effects of infection in vaccinated animals (or the offspring of vaccinated dams) with the effects in the unvaccinated animals of the same age, in the same building and in the same batch as the vaccinated animals (or those protected passively). As all these conditions are difficult to achieve in the field, field trials are certainly more appropriate to safety testing than to efficacy testing.

2.   Method of manufacture

Only MSV that has been established as pure, safe and immunogenic may be used as seed for a vaccine product. Cells from the MCS are propagated in a variety of growth media. All batches of vaccine must be from the first to the twentieth passage of MCS.

3. In-process control

It is necessary to carry out tests at each critical step of the manufacturing process. The control tests are also carried out on intermediate products with a view to verifying the consistency of the production process and the final product.

4. Batch control

It is essential to differentiate the tests that are carried out on a routine basis to release batches of final product from those that are performed to define the biological properties of a vaccine. The trials carried out for batch release are not the same as the ones carried out once only to determine the safety and efficacy of a vaccine. The batch release controls are always short-term trials, as inexpensive as possible, and not always carried out in pigs. Their purpose is mainly to attest to the reproducibility of the quality of the finished product, which has to be in compliance with the quality initially defined in the application for marketing authorisation.

a) Sterility and purity tests

Tests must be carried out for sterility and freedom from contamination (see Chapter 1.1.9).

Each batch of PRV vaccines must be tested for freedom from extraneous viruses. Using a minimum amount of a monospecific antiserum, the live vaccinal strain is neutralised and inoculated into cell cultures known to be sensitive to viruses pathogenic for pigs. No CPE and no haemadsorbing agents should be detected. The vaccines have to be free from pestiviruses.

b) Inactivation

For inactivated vaccines, inactivation must be checked using two passages in the same type of cell culture as used in the production of the vaccine. Tests can be carried out by vaccinating susceptible animals such as rabbits.

c) Identity

Where necessary, a specific test for virus identification should be carried out.

d) Safety

Safety of live vaccines is tested by administering ten doses of the reconstituted vaccine by the route stated on the leaflet to each of at least two piglets of the minimum age recommended for vaccination that are free from PRV antibodies. Two piglets of the same origin and age are kept as controls. No abnormal local or systemic reaction should occur. The weight curve of the vaccinated piglets must not differ significantly from that of the controls.

For inactivated vaccines, safety is tested by injecting two doses into piglets under the same conditions as described previously.

e) Potency

The potency of the vaccine must be demonstrated using a suitable method, the results of which have to be correlated with the efficacy tests described previously.

In this kind of test, the most difficult point is to determine an acceptability threshold for using or rejecting the batch according to the results that are obtained.

Virus content tests should be carried out using each of at least three containers. The virus titre of the vaccine must be determined and must normally not be higher than 1/10 of the dose at which the vaccine has been shown to be safe, and not lower than the minimum release titre.

f) Preservatives

If no preservative is included in the final product, the manufacturer must demonstrate that the product remains acceptable for its recommended period of use after opening the vial.

The efficacy of preservatives in multidose containers must be demonstrated. The concentration of the preservative in the final filled vaccine and its persistence throughout shelf life must be checked.

g) Precautions (hazards)

All information about possible adverse reactions induced by the vaccine must be indicated. Any putative risk for human health if the user is accidentally given a small quantity of the product has to be indicated. The manufacturer should indicate all the conditions of use of the vaccine: mixing, reconstitution, storage, asepsis, length of needle, route of administration and health status of the vaccinated animals.

5. Tests on the final product

a) Safety

Every batch of vaccine must be tested for safety, as described in Section C.4.d.

b) Potency

Every batch of vaccine must be tested for potency, as described in Section C.4.e.

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NB: There are OIE Reference Laboratories for Aujeszky's disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

BLUETONGUE

SUMMARY

Bluetongue virus (BTV) infection involves domestic and wild ruminants such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and various other Artiodactyla as vertebrate hosts. This noncontagious, insect-borne viral Infection is inapparent in the vast majority of infected animals but causes fatal disease in a proportion of infected sheep, deer and wild ruminants. Although cattle rarely show clinical signs, they are important in the epidemiology of the disease due to the prolonged viraemia in the absence of clinical disease. Clinical signs range from mild to severe and vary not only between species but between breeds and within the flock or herd. BT disease is caused by fever and vascular permeability and includes hyperaemia and congestion, facial oedema and haemorrhages, and erosion of the mucous membranes. However in mild cases of the disease, a transitory hyperaemia and slight ocular and nasal discharge may be observed. In very severe cases the tongue may show hyperaemia, become oedematous and protrude from the mouth, or become cyanotic. Hyperaemia may extend to other parts of the body, particularly the coronary band of the hoof, the groin, axilla and perineum. There is often skeletal and cardiac muscle degeneration. Wool breaks may occur. Sheep may become lame as a result of laminitis and skeletal myopathy. A similar severe disease of wild ruminants is caused by epizootic haemorrhagic disease virus (EHDV), which, like BTV, is a member of the Orbivirus *genus, but is classified in a separate species.*

Identification of the agent: *BTV is a member of the* Orbivirus *genus of the family Reoviridae, one of 20 recognised species in the genus. The BTV species, or serogroup, contains 24 recognised serotypes. The orbiviral species are differentiated by immunological tests that detect viral proteins that are conserved within each, and hence are distinguishable by serogrouping tests. However there may be considerable cross-reaction between related species, and this is the case with the BT and EHD serogroups. The serotype of individual viruses in each species is identified on the basis of neutralisation tests and different strains within a serotype are identified by sequence analysis. Complete BTV particles are double-shelled icosahedral double-stranded RNA virus. The outer layer contains two proteins, one of which, VP2, is the major determinant of serotype specificity. The inner shell and core contains two major and three minor proteins and ten double-stranded RNA genetic segments. VP7 located in the inner shell is the major core protein possessing the species or serogroup-specific antigens. Virus identification traditionally requires isolation and amplification of the virus in embyonating hen eggs tissue culture or inoculations of susceptible ruminants and the subsequent application of serogroup- and serotype-specific tests. Reverse-transcription polymerase chain reaction (RT-PCR) technology has permitted rapid amplification of BTV RNA in clinical samples, and RT-PCR-based procedures are now available. These procedures can augment the classical virological techniques to provide information on virus serogroup, serotype and topotype.*

Serological tests: *Serological responses in ruminants appear some 7–14 days after BTV infection and are generally long-lasting. Historically, tests such as agar gel immunodiffusion and indirect enzyme-linked immunosorbent assay (ELISA) were used to detect BTV species-specific antibodies, but have the major drawback of being unable to consistently distinguish between antibodies to viruses in the BTV and EHDV species. A monoclonal antibody-based competitive ELISA has solved this problem and competitive ELISAs to specifically detect anti-BTV antibodies are recommended. Procedures to determine the serotype-specificity of antibodies in sera are more complex and time-consuming because they assess whether the sera inhibit the infectivity of panels of known virus serotypes in neutralisation tests.*

> ***Requirements for vaccines and diagnostic biologicals:*** *Vaccination is used in several countries to limit direct losses, minimise the circulation of BTV and allow safe movement of animals. For many years South Africa has used live, attenuated vaccines that are serotype-specific. Live attenuated vaccines are produced by adapting BTV field isolates to growth* in vitro *through serial passages in tissue culture or in embryonating chicken eggs. Stimulation of a strong antibody response by these vaccines is directly correlated with their ability to replicate in the vaccinated host. Live attenuated vaccines are cheap to produce in large quantities, they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease. Adverse consequences are depressed milk production in lactating sheep, and abortion/embryonic death and teratogenesis in offspring from pregnant females that are vaccinated during the first third of gestation. Another risk associated with the use of live attenuated vaccines is their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of vaccine virus genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but natural and local dissemination of vaccine strains has already been documented in Europe. The fact that attenuated viruses are teratogenic makes determination of transmissibility very important. Vaccine efficacy, teratogenic potential and transmissibility should be tested. Hence inactivated or recombinant vaccines would be preferred if effective. The inactivated vaccines are not teratogenic and have been used under government supervision since 2004 in the management of recent European outbreaks.*

A. INTRODUCTION

Midges of certain species in the genus *Culicoides* (the insect host) (39) transmit bluetongue virus (BTV) among susceptible ruminants, having become infected by feeding on viraemic animals (the vertebrate host). After a replication period of 6–8 days in the insect's salivary gland the virus can be transmitted to a new vertebrate host during feeding. Infected midges remain infective for life. The central role of the insect in BT epidemiology ensures that distribution and prevalence of the infection is governed by ecological factors, such as high rainfall, temperature, humidity and soil characteristics, which favour insect survival (6). In many parts of the world therefore, infection has a seasonal occurrence (43). It is accepted that BTV does not establish persistent infections in ruminants, and that survival of BTV in the environment is associated with insect factors (20, 23). Globally the distribution of BTV can be considered on the basis of epidemiological systems (episystems) based on the vector species present and their natural history (40).

The vertebrate hosts for BTV include both domestic and wild ruminants, such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and other Artiodactyla such as camels Although antibodies to BTV, and in some cases virus antigen, have been demonstrated in some carnivores, felids, black and white rhinoceroses and elephants, the roll of non-ruminant species in the disease in the wild is not known. The outcome of infection ranges from inapparent in the vast majority of infected animals, especially cattle, to fatal in a proportion of infected sheep, goats, deer and some wild ruminants (43). However clinical disease has been observed in cattle infected with BVT8 in Europe. Some breeds of sheep are more susceptible to disease than others, with the result that in some countries BTV infections of livestock can occur unobserved, and be detected only by active surveillance (9). Epizootic haemorrhagic disease virus (EHDV) can produce a disease in wild ruminants with clinical manifestations identical to those observed in response to BTV infection.

Clinical signs of disease in sheep vary markedly in severity, influenced by husbandry factors as well as by breed (43). In severe cases there is an acute febrile response characterised by hyperaemia and congestion, leading to oedema of the face, eyelids and ears, and haemorrhages and erosions of the mucous membranes. The tongue may show intense hyperaemia and become oedematous, protrude from the mouth and, in severe cases become cyanotic. Hyperaemia may extend to other parts of the body particularly the coronary band of the hoof, the groin, axilla and perineum. There is often severe muscle degeneration. Breaks in the wool may occur associated with pathology in the follicles. A reluctance to move is common, or even torticolis in severe cases. In fatal cases the lungs may show interalveolar hyperaemia, severe alveolar oedema and the bronchial tree may be filled with froth. The thoracic cavity and pericardial sac may contain several litres of plasma-like fluid. Most cases show a distinctive haemorrhage near the base of the pulmonary artery (43).

Taxonomically, BTV is classified as a species or serogroup in the Orbivirus genus in the family Reoviridae, one of 20 recognised species in the genus that also includes EHD and African horse sickness (AHS) (31). Within species, individual members are differentiated on the basis of neutralisation tests, and 24 serotypes of BTV have been described. There is significant immunological cross-reactivity between members of the BT and EHD viruses (31).

BTV particles are composed of three protein layers. The outer layer contains two proteins, VP2 and VP5. VP2 is the major neutralising antigen and determinant of serotype specificity. Removal of the outer VP2/VP5 layer

leaves a bi-layered icosahedral core particle that is composed of two major proteins, VP7 and VP3, three minor proteins and the ten species of double-stranded RNA. VP7 is a major determinant of serogroup specificity and the site of epitopes used in competitive enzyme-linked immunosorbent assay (C-ELISA) to detect anti-BTV antibody (30). VP7 can also mediate attachment of BTV to insect cells (46).

Genetic sequencing of BTVs allows differentiation and analysis of strains separately from serotype (16, 27, 36, 45). Even for strains within the one serotype it is possible to identify the likely geographical origin (16, 35). Such studies have led to the detection of international movements of BTV strains. Natural movements of vectors by climatic forces are believed to result in intercontinental movement of BTV. Identification of apparent associations between some genotypes of virus and some vector species has resulted in a concept of viral-vector ecosystems (9, 23, 40). A more complete understanding of such epidemiological aspects may further facilitate international trade in ruminants.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (a prescribed test for international trade)

a) Virus isolation

The same diagnostic procedures are used for domestic and wild ruminants. A number of virus isolation systems are in common use, but generally the most practical method is by inoculation of embryonated chicken eggs (ECE). Inoculation of sheep may also be a useful approach if the titre of virus in the sample blood is very low, as may be the case several weeks after virus infection. Attempts to isolate virus in cultured cells *in vitro* may be more convenient, but the success rate is frequently much lower than that achieved with *in vivo* systems (15). Cell culture has proven to be a more sensitive technique for isolation of EHDV.

- **Isolation in embryonated chicken eggs**

i)   Blood is collected from febrile animals into an anticoagulant such as EDTA (ethylamine diamine tetra-acetic acid), heparin or sodium citrate, and the blood cells are washed three times with sterile phosphate buffered saline (PBS). Washed cells are resuspended in PBS or isotonic sodium chloride and either stored at 4°C or used immediately for attempted virus isolation.

ii) For long-term storage where refrigeration is not possible blood samples are collected in oxalate–phenol–glycerin. If samples can be frozen, they should be collected in buffered lactose peptone or 10% dimethyl sulphoxide (41) and stored at –70°C or colder. The virus is not stable for long periods at –20°C.

iii) In fatal cases, spleen and lymph nodes are the preferred organs for virus isolation attempts. Organs and tissues should be kept and transported at 4°C to a laboratory where they are homogenised in PBS or isotonic saline, and used as described below, for blood cells.

iv) Washed blood cells are re-suspended in distilled water or sonicated in PBS and 0.1 ml amounts inoculated intravascularly into 5–12 ECE that are 9–12 days old. This procedure requires practice. Details are provided by Clavijo *et al.* (7).

v)   The eggs are incubated in a humid chamber at 32–33°C and candled daily. Any embryo deaths within the first 24 hours post-inoculation are regarded as nonspecific.

vi) Embryos that die between days 2 and 7 are retained at 4°C and embryos remaining alive at 7 days are killed. Infected embryos may have a haemorrhagic appearance. Dead embryos and those that live to 7 days are homogenised as two separate pools. Whole embryos, after removal of their heads, or pooled organs such as the liver, heart, spleen, lungs and kidney are homogenised and the debris is removed by centrifugation.

vii) Virus in the supernatant may be identified either directly by antigen-capture ELISA (17) or reverse transcription polymerase chain reaction (RT-PCR), or indirectly by antigen-detection methods such as immunofluorescence or immunoperoxidase after further amplification in cell culture, as described in the next section.

viii) If no embryos are killed following inoculation of sample material, an inoculum made from the first egg passage material may be repassaged in ECE or in cell culture.

- **Isolation in cell culture**

Virus isolation may be attempted in bluetongue virus susceptible cell cultures such as mouse L, baby hamster kidney (BHK-21), African green monkey kidney (Vero) or *Aedes albopictus* clone C6/36 (AA). The

efficiency of isolation is often significantly lower following inoculation of cultured cells with diagnostic samples compared with that achieved in ECE. Highest recovery rates are achieved by primary isolation of virus in ECE, followed by passage in AA cells for further replication of virus. Additional passages in mammalian cell lines such as BHK-21 or Vero are usually performed. A cytopathic effect (CPE) is not necessarily observed in AA cells but appears in mammalian cells. Cell monolayers are monitored for the appearance of a CPE for 5 days at 37°C in 5% CO_2 with humidity. If no CPE appears, a second passage is made in cell culture. The identity of BTV in the culture medium of cells manifesting a CPE may be confirmed by a number of immunological methods described below, including antigen-capture ELISA, immuno-fluorescence, immunoperoxidase, virus neutralisation (VN) tests, or by RT-PCR.

- **Isolation in sheep**

i)   Sheep are inoculated with washed cells from 10 ml to 500 ml of blood, or 10–50 ml tissue suspension. Inocula are administered subcutaneously in 10–20 ml aliquots. Large volumes may aid in the virus isolation attempts and should be administered intravenously.

ii) The sheep are held for 28 days and checked daily for pyrexia and weekly for antibody response using serological tests such as the C-ELISA as described below. Sheep blood collected at 7 to 14 days post-inoculation will usually contain the isolated virus, which can be stored viable at 4°C or –70°C.

b)   Immunological methods

- **Serogrouping of viruses**

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between members of the BT and EHD serogroups raises the possibility that an isolate of EHDV could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. For this reason, a BT serogroup-specific MAb can be used. A number of laboratories have generated such serogroup-specific reagents (2, 21). Commonly used methods for the identification of viruses to serogroup level are as follows.

i)   *Immunofluorescence*

Monolayers of BHK or Vero cells on chamber slides (glass cover-slips) are infected with either tissue culture-adapted virus or virus in ECE lysates. After 24–48 hours at 37°C, or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-BTV antiserum or BTV-specific MAbs and standard immunofluorescent procedures.

ii) *Antigen capture enzyme-linked immunosorbent assay*

Viral antigen in ECE and culture medium harvests (17), infected insects (28) and sheep blood may be detected directly. In this technique, virus derived proteins are captured by antibody adsorbed to an ELISA plate and bound materials detected using a second antibody. The capture antibody may be polyclonal or a serogroup-specific MAb. Serogroup-specific MAbs and polyconal antibody raised to baculovirus-expressed core particles have been used successfully to detect captured virus (17).

iii) *Immunospot test*

Small volumes (2 µl) of infected cell culture supernatant or lysed or sonicated infected cells are adsorbed to nitrocellulose and air-dried. Nonspecific binding sites are blocked by incubation in a solution containing skim milk protein. After incubation with a BTV serogroup-reactive MAb, bound antibody is detected using horseradish peroxidase-conjugated anti-mouse IgG (14).

- **Serotyping by virus neutralisation**

Neutralisation tests are type specific for the currently recognised 24 BTV serotypes and can be used to serotype a virus isolate or can be modified to determine the specificity of antibody in sera. In the case of an untyped isolate, the characteristic regional localisation of BTV serotypes can generally obviate the need to attempt neutralisation by all 24 antisera, particularly when endemic serotypes have been identified.

There is a variety of tissue culture-based methods available to detect the presence of neutralising anti-BTV antibody. Cell lines commonly used are BHK, Vero and L929. Four methods to serotype BTV are outlined briefly below. BTV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included.

i)   *Plaque reduction*

The virus to be serotyped is diluted to contain approximately 100 plaque-forming units (PFU), and incubated with either no antiserum or with dilutions of individual standard antisera to a panel of BTV

serotypes. Virus/antiserum mixtures are added to monolayers of cells. After adsorption and removal of inoculum, monolayers are overlaid with agarose. The neutralising antibody titres are determined as the reciprocal of the serum dilution that causes a fixed reduction (e.g. 90%) in the number of PFU. The unidentified virus is considered serologically identical to a standard serotype if the latter is run in parallel with the untyped virus in the test, and is similarly neutralised.

ii) *Plaque inhibition*

Tests are performed in 90 mm diameter Petri dishes containing confluent cell monolayers that are infected with approximately 5×10^4 PFU standard or untyped virus. After adsorption and removal of inoculum, monolayers are overlaid with agarose. Standard anti-BTV antisera are added to individual filter paper discs and placed on the agarose surface. Dishes are incubated for at least 4 days. A zone of virus neutralisation, with concomitant survival of the cell monolayer, will surround the disc containing the homologous antiserum.

iii) *Microtitre neutralisation*

Approximately 100 $TCID_{50}$ (50% tissue culture infective dose) of the standard or untyped virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of standard antiserum diluted in tissue culture medium. Approximately 10^4 cells are added per well in a volume of 100 µl, and after incubation for 4–6 days, the test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show convincing CPE. The unidentified virus is considered to be serologically identical to a standard BTV serotype if both are neutralised in the test to a similar extent.

iv) *Fluorescence inhibition test*

This rapid and simple neutralisation assay (5) requires varying concentrations of an unknown virus and standard concentrations of reference antisera. Virus isolates grown in cell culture are serially diluted starting and mixed with individual reference antisera in the wells of a Lab-Tek slide for 1 hour prior to addition of cells. After incubation for 16 hours, cells are fixed and probed by an immunofluorescent procedure using a BT serogroup-specific MAb. The serotype of the virus is indicated by the specificity of the antiserum causing the largest reduction in the number of fluorescent cells.

c) **Reverse-transcription polymerase chain reaction (a prescribed test for international trade)**

Primer-directed amplification of viral nucleic acid has revolutionised BT diagnosis (8, 27, 44). RT-PCR techniques have allowed the rapid identification of BT viral nucleic acid in blood and other tissues of infected animals. RT-PCR-based diagnostics should be interpreted with caution. The RT-PCR procedure will detect virus-specific nucleic acid, but this does not necessarily indicate the presence of infectious virus (24). RT-PCR can also be used to 'serogroup' Orbiviruses and may ultimately be possible to 'serotype' BTV within a few days of receipt of a clinical sample, such as infected sheep blood (29). Traditional approaches, which rely on virus isolation followed by virus identification serologically, may require up to 4 weeks to generate information on serogroup and serotype.

Oligonucleotide primers used so far have been derived from RNA 7 (VP7 gene) (44), RNA 6 (NS1 gene) (8), RNA 3 (VP3 gene) (36), RNA 10 (NS3 gene) (4) and RNA 2 (VP2 gene) (27). The size of the amplified transcripts is usually small – in the order of several hundred nucleotides – but can also be a full-length gene. In the procedure described in detail below, a 101-nucleotide stretch of RNA 6 is amplified. Primers derived from the more highly conserved genes, such as VP3, VP7 and NS1, may be used for serogrouping (i.e. will react with all members of the BT serogroup), while primers for which the sequence was determined from VP2 gene sequences provide information on virus serotype. A multiplex RT-PCR assay that depends on the size of the amplified products has been used to identify the five North American BTV serotypes, both alone and in mixtures, in a single reaction (18).

The nucleic acid sequence of cognate BTV genes may differ with the geographical area of virus isolation (16). This has provided a unique opportunity to complement studies of BTV epidemiology by providing information on the potential geographical origin of virus isolates, a process termed genotyping or topotyping. Thus, determination of the nucleic acid sequence of portions of RNA may provide information on where the virus came from. It appears likely that sequencing of BTV isolates from other parts of the world may permit finer discrimination of geographical origin. However, the relationship between sequence and geographical origin may not be straightforward. This sequencing information is important and all data regarding BTV segment sequences should be made widely available by submitting the data to officially recognised web sites.

http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ and
http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv_sequences.htm.
http://eubtnet.izs.it/btnet/index.htm

The web sites provide phylo-genetic tree analyses of BTV isolates based on the sequence of RNA segments. These compiled data will provide a resource for epidemiological studies, the identification of new isolates and the design of new primers for further RT-PCR and possibly serotype-specific assays for BTV.

It has been observed that BTV nucleic acid can be detected by RT-PCR from the blood of infected calves and sheep at least 30 days, and sometimes over 90 days, after the virus can be isolated The presence of virus-specific nucleic acid does not necessarily indicate the presence of infectious virus.

The capacity of RT-PCR assays to detect very small numbers of nucleic acid molecules means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids. The latter may include any primers in use in the laboratory or previously amplified polynucleotides. It is critical therefore to have a 'clean' area containing all equipment necessary for reagent and test preparation and a separate area with its own equipment for amplification. Impervious gloves should be worn and changed frequently at all stages of the procedure, particularly after working with sample RNA or amplified DNA. This will help protect reagents and samples from contamination by ubiquitous RNases and other agents and from cross-contamination by DNA. The possibility of false positives, due to sample contamination, highlights the importance of sequencing RT-PCR products to determine, for example, if the amplified sequence is identical to or different from that of the positive control. False negatives, due for example to poor sample quality or inappropriate primers, may be identified following the failure to amplify both BTV and a host gene, such as globin, from extracts of infected cells. This is covered in more detail in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

There are many RT-PCR assays currently in use that use different extraction methods, reverse transcriptases, amplification enzymes, primers and conditions. Technology is changing rapidly. Also the genetic diversity of the BTV genes makes the choice and validation of RT-PCR assays conditional on its application in a regional setting. This is also the case for real-time PCR, which will be developed for use in routine molecular diagnosis of BTV in the future. Therefore the procedures listed below are examples only. Increasingly it will be more important to maintain diagnostic testing under accreditation to an international standard such as ISO/ISEC 17025 and participate in proficiency testing. Systems for offering proficiency testing for RT-PCR tests are being developed in a number of countries.

The RT-PCR assay described here involves three separate procedures. In the first, BTV RNA is extracted from blood using a chaotropic agent such as guanidine thiocyanate (GuSCN) to denature protein and release viral RNA. A number of commercial kits are available for this purpose and the protocol below describes the use of one such kit, IsoQuick (Orca Research, Bothell, Washington, United States of America [USA]). The reagents provided with the kit are numbered and their use is indicated in the protocol below. Other kits are available and one, TRIZOL (Life Technologies, Grand Island, New York, USA), is particularly useful for the extraction of viral nucleic acid from spleen or blood clots. Operators should follow the procedures specified in each kit and use reagent solutions either provided or recommended for the kit of their choice. The second procedure is the denaturation of viral double-stranded RNA and reverse transcription to generate cDNA, which is amplified by RT-PCR. In the procedure described below, the Superscript[TM] Preamplification System (Life Technologies) is used to transcribe viral RNA, and reagents from Perkin-Elmer are used for the RT-PCR. Equivalent kits and reagents are available from other sources. The final step of the process is the analysis of the RT-PCR product by electrophoresis. Procedures used to determine the sequence of the amplified product are not described here.

- **Extraction of viral RNA**

i)   Whole blood is collected from test and uninfected control animals in EDTA tubes and centrifuged at 800–1000 ***g*** for 10 minutes. The plasma is aspirated and the red blood cells (RBCs) are gently resuspended in sterile PBS. RBCs are pelleted by centrifugation at 1000 ***g*** for 10 minutes and the supernatant is removed.

ii) Next, 400 µl of test RBCs is added to each of four 1.7 ml microcentrifuge tubes, and 400 µl of control RBCs is added to each of two microcentrifuge tubes. An equal volume of RNase-free water is added to each tube and the tubes are vortexed briefly to mix and lyse the cells. Two tubes containing test RBCs are frozen at –70°C for repository purposes and the extraction is continued in duplicate.

iii) Lysed test and control RBCs are centrifuged at 12,000–16,000 ***g*** for 10 minutes and the supernatant is discarded. Next, 800 µl RNase-free water is added and the tubes are vortexed and centrifuged again at the same speed for 10 minutes. The supernatant is removed and the RBC pellet is drained.

iv) A small volume of BTV (e.g. 5 µl containing from 10^3 to 10^7 PFU) is added to one of two control RBC pellets. This is the positive control. The other control RBC pellet remains as the negative control.

v)   Next, 75 µl of sample buffer (IsoQuick reagent A) is added to each pellet, and the pellets are then vortexed vigorously, followed by the addition of 125 µl of the GuSCN-containing lysis solution (IsoQuick reagent 1). The mixture is vortexed vigorously for 30 seconds.

vi) Before use the extraction matrix provided with the kit (IsoQuick reagent 2 plus dye 2A) is shaken vigorously and 500 µl is added to the sample lysates. Then, 400 µl extraction buffer (IsoQuick reagent 3) is added and the tubes are vortexed for 10 seconds.

vii) The tubes are incubated at 65°C for 10 minutes, vortexed briefly after 5 minutes and centrifuged at 12,000 *g* for 5 minutes.

viii) The aqueous phase (500 µl) is transferred to a new microcentrifuge tube and an equal volume of extraction matrix (IsoQuick reagent 2) is added. The tubes are vortexed for 10 seconds and centrifuged at 12,000 *g* for 5 minutes.

ix) The aqueous phase (330 µl) is transferred to a new microcentrifuge tube and a 10% volume (33 µl) of sodium acetate (IsoQuick reagent 4) and 365 µl isopropanol are added. After gentle mixing, the tubes are placed at –20°C for from 20 minutes to 1 hour.

x) The RNA is pelleted by centrifugation at 12,000 *g* for 10 minutes. The supernatant is decanted and 1.0 ml 70% ethanol is added and mixed gently. After centrifugation at 12,000 *g* for 5 minutes, the supernatant is decanted and 1.0 ml 100% ethanol is added. The tubes are stored at –70°C until ready for use in the RT-PCR.

- **Reverse-transcription polymerase chain reaction**

i) RNA in ethanol is centrifuged at 12,000 *g* for 5 minutes. The ethanol is decanted and the tubes are inverted and allowed to drain. The pellet, which may not be obvious, must not be allowed to dry out because this makes resuspension difficult. A dry pellet is also likely to fall out of the inverted tube.

ii) Next, 12 µl RNase-free water is added to each tube, mixed and heated at 65°C for 5–10 minutes. The samples are placed in ice.

iii) In a 'clean' biohazard hood, stock solutions containing 200 pmol/µl of primers A, B, C and D are prepared in RNase-free water and stored at –70°C.

First stage RT-PCR primers (to amplify RNA 6 from nucleotide 11 to 284)
Primer A: 5'-GTT-CTC-TAG-TTG-GCA-ACC-ACC-3'
Primer B: 5'-AAG-CCA-GAC-TGT-TTC-CCG-AT-3'

Nested RT-PCR primers (to amplify RNA 6 from nucleotide 170 to 270)
Primer C: 5'-GCA-GCA-TTT-TGA-GAG-AGC-GA-3'
Primer D: 5'-CCC-GAT-CAT-ACA-TTG-CTT-CCT-3'

iv) Primer stock solutions are diluted to a concentration of 15–20 pmol/µl. Primers for the first stage RT-PCR reaction are prepared by mixing equal volumes of A and B. Primers for the nested RT-PCR reaction are prepared by mixing equal volumes of C and D. Small aliquots of pooled primer mixes are frozen at –20°C.

v) RT-PCR reaction tubes are labelled and, for first stage synthesis, 4.0 µl of primer (A + B) mix is added to each tube. The tubes are held on ice.

vi) In a 'clean' fume hood methylmercuric hydroxide is diluted to 50 mM (1/20 dilution) and 2-mercaptoethanol is diluted to 350 mM (1/40 dilution) in RNase-free water. Methylmercuric hydroxide and 2-mercaptoethanol are considered to be extremely and highly toxic, respectively. Use both chemicals with extreme care and dispose of them and pipette tips as required by safety regulations. Alternative methods using heat denaturation have been described (22, 29).

vii) Next, 4 µl of test and positive and negative control RNA samples (step ii) are added to 4 µl of the primer mix in RT-PCR tubes (45).

viii) To each RT-PCR tube 2.0 µl of the 1/20 dilution of methylmercuric hydroxide is added with gentle mixing and allowed to sit at room temperature for 10 minutes prior to adding 2.0 µl of the 1/40 dilution of 2-mercaptoethanol. For safety reasons, some laboratories use formamide instead of methylmercuric hydroxide for double-stranded RNA denaturation. However, for optimum sensitivity, methylmercuric hydroxide is preferred.

ix) In a 'clean' hood a cDNA mix is prepared containing the following reagents in sufficient volume for the number of samples being tested. The amount given is per sample and the reagents are contained in the Superscript^{TM} Preamplification System (Life Technologies).

10 × Superscript^{TM} buffer (200 mM Tris/HCl, pH 8.4, and 500 mM KCl)	2.0 µl
MgCl$_2$ (25 mM)	2.0 µl
dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)	1.25 µl
Dithiothreitol (DTT) (0.1 M)	2.0 µl
Reverse transcriptase (200 units/µl)	0.75 µl

x) Then, 8.0 µl of the mix is added to each RT-PCR tube to a final volume of 20.0 µl.

xi) The RT-PCR tubes are placed in a thermal cycler, such as GeneAmp^{TM} PCR System 9600, which is programmed for reverse transcription as follows:

Hold 44°C   50 minutes
Hold 4°C   Forever

xii) The tubes are removed from the thermal cycler and 1.0 µl RNase H and a wax bead are added to each tube. The cycler is programmed as follows:

Hold 37°C   20 minutes
Hold 98°C   4 minutes
Hold 4°C   Forever

xiii) In a 'clean' hood a first stage amplification mix is prepared containing the following reagents and in a volume sufficient for the number of samples being tested. All these reagents except water are available from Perkin-Elmer. The amount given is per sample.

RNase-free water	62.0 µl
10 × PCR Perkin-Elmer buffer (100 mM Tris/HCl, pH 8.3, and 500 mM KCl)	7.0 µl
MgCl$_2$ (25 mM)	7.0 µl
dNTP mix (2.5 mM each dATP, dCTP, dGTP, dTTP)	4.0 µl
Taq DNA polmerase (5 units/µl)	0.85 µl

xiv) The first stage mix is removed from the 'clean' area to the thermal cycling area and 80 µl is overlaid in each sample tube. The wax layer must not be pierced. Each tube should now contain 101 µl.

xv) The tubes are placed in the thermal cycler, which is programmed as follows (correct for GeneAmp PCR System 9600 – programmes for other thermal cyclers would need to be determined) for first stage amplification:

One cycle:	Hold 95°C	3 minutes
	Hold 58°C	20 seconds
	Hold 72°C	30 seconds
40 cycles:	Hold 95°C	20 seconds
	Hold 58°C	20 seconds
	Hold 72°C	20 seconds
One cycle:	Hold 95°C	20 seconds
	Hold 58°C	20 seconds
	Hold 72°C	5 minutes
	Hold 4°C	Forever

xvi) RT-PCR reaction tubes are prepared for the nested reaction in a 'clean' hood 15 minutes before cycling is complete, and held on ice:

Rnase-free water	17 µl per tube
Nested primer mix (C+D)	4.0 µl per tube
Wax bead	

xvii) When first stage amplification is complete, the tubes are removed from the thermal cycler and placed in a biological safety cabinet (not the 'clean' hood). Then, 1.5 µl of the first stage product is transferred to the corresponding nested RT-PCR tube containing primer, water and a wax bead.

xviii) The tubes are placed in the thermal cycler, which is programmed as follows for wax layer formation:

Hold 98°C   4 minutes
Hold 4°C   Forever

xix) In a 'clean' hood the nested mix of the following reagents is prepared in sufficient volume for the number of samples being tested. The reagents used are the same as in the first stage (step xii). The amount given is per sample.

RNase-free water	17.0 µl
10 × PCR buffer	5.0 µl
MgCl$_2$	3.5 µl
dNTP mix	4.5 µl
Taq DNA polymerase	0.5 µl

xx) The nested mix is removed from the 'clean' hood to the thermal cycler and 30 µl is overlaid into each sample tube. Each tube should now contain 52 µl.

xxi) The tubes are placed in the thermal cycler, which is programmed as follows for nested amplification. After completion, the tubes are held at 4°C or at –20°C until electrophoresis:

One cycle:	Hold 95°C	3 minutes
	Hold 58°C	20 seconds
	Hold 72°C	30 seconds
40 cycles:	Hold 95°C	20 seconds
	Hold 58°C	20 seconds
	Hold 72°C	20 seconds
One cycle:	Hold 95°C	20 seconds
	Hold 58°C	20 seconds
	Hold 72°C	5 minutes
	Hold 4°C	Forever

- • **Electrophoretic analysis of RT-PCR product**

i) First, 1 × TBE buffer (0.045 mM Tris/borate, pH 8.6, and 1.5 mM EDTA) is prepared from a ×10 stock solution. For the Bio-Rad Wide Mini-Sub cell system, 700 ml buffer is prepared (100 ml for the gel and 600 ml for the tank buffer).

ii) A 3% solution of NuSieve 3/1 agarose (FMC Bioproducts, Rockland, Maine, USA) or an equivalent is prepared in TBE buffer. The solution is boiled until the agarose is completely dissolved, and then allowed to cool to 40°C. Ethidium bromide is added to a concentration of 0.5 µg/ml to both the agarose and the tank buffer. Ethidium bromide is a mutagen and is toxic. Gloves, protective clothing, and eye-wear must always be worn.

iii) The ends of the electrophoresis tray are taped and the agarose solution is poured. The comb is inserted and the agarose is allowed to solidify on a level surface for 30–60 minutes. The comb and the tape are gently removed from the electrophoresis tray.

iv) Pour the tank buffer into the electrophoresis apparatus and insert the tray with the agarose so that the buffer covers the agarose.

v) Test and positive and negative control samples are prepared for electrophoresis in 0.65 ml microcentrifuge tubes as follows:

Gel-loading solution (Cat. G-2526, Sigma, St Louis, Missouri, USA)	5.0 µl
Amplified DNA from each of the RT-PCR tubes and an extra tube is set up for a DNA ladder	15.0 µl
Gel-loading solution (Cat. G-2526, Sigma, St Louis, Missouri, USA)	5.0 µl
100 base-pair ladder (Cat. 15268-019, Life Technologies, Grand Island, New York, USA)	1.0 µl

vi) Samples are loaded into the appropriate wells in the gel and run at 65–75 volts for 1–1.5 hours or until the dye has travelled about half the length of the gel. The gel is transferred to a transilluminator and photographed for a permanent record. Use protective eye-wear to visualise the gel bands.

vii) BT-positive samples will have a band of 101 base pairs. For the test to be valid, the positive control must show a band of the correct size, and the negative and 'no RNA' controls show no band. Samples are considered to be positive if there is a band of the same size as the positive control. Duplicate samples should show the same reaction. If there is disparity, the test should be repeated.

viii) A destaining bag (Ameresco, Solon, Ohio, USA) is placed in the tank buffer overnight to remove the ethidium bromide. The buffer can then be poured down the drain and the destaining bag, after reuse 10–15 times, should be placed in a properly identified ethidium bromide waste container and ultimately incinerated.

2. Serological tests

Anti-BTV antibodies generated in infected animals can be detected in a variety of ways that vary in sensitivity and specificity. Both serogroup-specific and serotype-specific antibodies are elicited and if the animal was not previously exposed to BTV, the neutralising antibodies generated are specific for the serotype of the infecting virus. Multiple infections with different BTV serotypes lead to the production of antibodies capable of neutralising serotypes to which the animal has not been exposed.

a) Complement fixation

A complement fixation test to detect BTV antibodies was widely used until 1982, when it was largely replaced by the AGID test although the CF test is still used in some countries.

b) Agar gel immunodiffusion (a prescribed test for international trade)

The AGID test to detect anti-BTV antibodies is simple to perform and the antigen used in the assay relatively easy to generate. Since 1982, the test has been the standard testing procedure for international

movement of ruminants. However, one of the disadvantages of the AGID used for BT is its lack of specificity in that it can detect antibodies to other Orbiviruses, particularly those in the EHD serogroup. Thus AGID positive sera may have to be retested using a BT serogroup-specific assay. The lack of specificity and the subjectivity exercised in reading the results have encouraged the development of ELISA-based procedures for the specific detection of anti-BTV antibodies. The preferred format, a C-ELISA is described in the Section B.2.c.

- **Test procedure**

i) A 2.8 mm thick layer of 0.9% agarose in 0.85% NaCl is prepared and circular wells, 4.0 mm in diameter and 2.4 mm apart, are cut out with six wells arranged around a central well.

ii) Viral antigen is prepared by generating a crude soluble preparation from BHK or Vero cells infected with a single BTV serotype 24–48 hours previously. Antigen can be concentrated by precipitation or ultrafiltration.

iii) A reference positive serum and three test sera are placed in alternate wells in a six-well pattern surrounding antigen in a central well and the plates are incubated at 20–25°C in a humid environment for 24 hours.

iv) A series of precipitin lines form between the antigen and known positive sera and lines generated by strong positive test sera will join up with those of the positive controls. With weak positive samples the control lines bend toward the antigen and away from the test sample well, but may not form a continuous line between the control test wells. With negative samples, the precipitin lines will continue into the sample wells without bending toward the antigen.

v) All weak positive samples and other samples that produce questionable results should be repeated using wells that are 5.3 mm in diameter placed 2.4 mm apart or retested using the C-ELISA as described below.

c) **Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)**

The BT competitive or blocking ELISA was developed to measure BTV-specific antibody without detecting cross-reacting antibody to other Orbiviruses (1, 4, 21, 32, 37). The specificity is the result of using one of a number of BT serogroup-reactive MAbs, such as MAb 3-17-A3 (2) or MAb 20E9 (21) or MAb 6C5F4D7 (26). The antibodies were derived in a number of laboratories, and although different, all appear to bind to the amino-terminal region of the major core protein VP7. In the C-ELISA, antibodies in test sera compete with the MAbs for binding to antigen. The following procedure for the C-ELISA has been standardised after comparative studies in a number of international laboratories.

- **Test procedure**

There are several test procedures described; this is an example of one BT ELISA procedure.

i) First, 96-well microtitre plates are coated at 4°C overnight or at 37°C for 1 hour with 50–100 µl of either tissue culture-derived sonicated cell culture antigen (2) or the major core antigen VP7 expressed in either baculovirus (33) or yeast (26) and diluted in 0.05 M carbonate buffer, pH 9.6.

ii) The plates are washed five times with PBST (0.01 M PBS containing 0.05% or 0.1% Tween 20, pH 7.2).

iii) Next, 50 µl of test sera is added in duplicate at a single dilution, either 1/5 (1) or 1/10 (21) in PBST containing 3% bovine serum albumin (BSA).

iv) Immediately, 50 µl of a predetermined dilution of MAb diluted in PBST containing 3% BSA is added to each well. MAb control wells contain diluent buffer in place of test serum.

v) Plates are incubated for 1 hour at 37°C or 3 hours at 25°C, with continuous shaking.

vi) After washing as described above, wells are filled with 100 µl of an appropriate dilution of horseradish peroxidase-labelled rabbit anti-mouse IgG (H+L) in PBST containing 2% normal bovine serum.

vii) Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates are washed five times using PBS or PBST. Wells are filled with 100 µl substrate solution containing 1.0 mM ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]), 4 mM H_2O_2 in 50 mM sodium citrate, pH 4.0, and the plates are shaken at 25°C for 30 minutes. (Other substrates may be used and the reaction continued with shaking for an appropriate length of time to permit colour development.)

viii) The reaction is stopped by addition of a stopping reagent, such as sodium azide.

ix) After blanking the ELISA reader on wells containing substrate and stopping reagent, the absorbance values are measured at 414 nm. Results are expressed as per cent inhibition and are derived from the mean absorbance values for each sample by the following formula.

% inhibition = 100 – [(Mean absorbance test sample)/(Mean absorbance MAb control) × 100].

NB: Some laboratories prefer to use a negative control serum that has previously been shown to have a percentage inhibition of zero as an alternative to the MAb control.

x) Percentage inhibition values >50% are considered to be positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the test sera duplicates can vary as long as the results do not lie either side of the positive cut -off.

xi) Strong and weak positive sera and a negative serum should be included on each plate. The weak positive should give 60–80% inhibition and the negative should give less than 40% inhibition.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Both live attenuated and killed BTV vaccines are currently available for use. Recombinant vaccines based on various approaches are under development but none has been licensed and these vaccines will not be addressed here. In South Africa live attenuated vaccines have been used for over 40 years and are known to induce an effective and lasting immunity (11). Live attenuated vaccines are produced by adapting BTV field isolates to growth *in vitro* through serial passages in tissue culture or in embryonated chicken eggs. Stimulation of a strong antibody response by these vaccines is directly correlated to their ability to replicate in the vaccinated host. Live attenuated vaccines are cheap to produce in large quantities; they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used (10). However, live attenuated BTV vaccines suffer from a variety of documented or potential drawbacks, including under-attenuation, the impact of which may vary with different breeds of sheep. Potential adverse consequences are depressed milk production in lactating sheep, and abortion/embryonic death and teratogenesis in offspring when used on pregnant females in the first period of pregnancy. Another risk associated with the use of live attenuated vaccines is that of their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of modified live virus (MLV) genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but natural and local dissemination of vaccine strains has already been documented in the USA and Europe (25, 42). Therefore inactivated vaccines, if effective, are preferred. Virus inactivation eliminates risks of vector transmission, reversion to virulence, fetal abnormalities and the possibility of viral reassortment.

1. Seed management

a) Characteristics of seed

For live, attenuated vaccines the master or primary virus seed is prepared from a single plaque of serially passaged, attenuated BTV. Vaccine viruses have been attenuated by either passage in ECE, tissue culture cells or a combination of both. Each primary seed virus lot should also be tested for transmissibility and reversion to virulence prior to vaccine manufacture. Samples of vaccine prepared from secondary seed virus at the maximum permitted passage level should be tested in sheep for avirulence, safety and immunogenicity.

For killed vaccines the issues of attenuation do not apply, and the approach adopted has been to use field strains of low passage level with the intent of achieving high antigenicity.

Primary seed virus must be free of contaminating bacteria, viruses, prions, fungi and mycoplasmas, particularly pestivirus contamination. For the latter, particular attention should be paid to the fetal bovine serum used in cell cultures, as it may be contaminated. Seed viruses must be shown to have the desired serotype specificity.

BTV seed lot viruses should be sequenced and the data made available to relevant databases (34).

Secondary seed lots, which are used as inocula for vaccine production, are usually not more than three passages beyond the primary seed lot.

b) Method of culture

Although the first attenuated BT vaccines were propagated in ECE subsequently cell cultures have been used for tissue culture adaptation and serial passage. These include primary bovine embryo, lamb and fetal lamb kidney cells, and the continuous BHK cells. Cell cultures must be thoroughly checked for the presence of contaminating viruses.

BTV for inactivated vaccines is produced in large-scale suspension cell systems that have been shown to be susceptible to bluetongue virus.

c) Validation as a vaccine

Attenuated BT vaccines must be safe and efficacious, and a brief description of appropriate tests for these parameters is given below. In addition, attenuated viruses should not revert to virulence during replication in vaccinated animals or be transmitted from such animals by insect vectors. The latter criterion is very important because insect-mediated transmission of attenuated virus from vaccinated to nonimmune animals, with the subsequent replicative steps in each host species, increases the possibility of reversion to virulence. Although tests for reversion to virulence and transmissibility are rarely, if ever performed, a brief description of what may be necessary is outlined.

There is a variation in bluetongue susceptibility between breeds of sheep; it is important that sheep that that have been proven to be susceptible to infection with BTV be used for vaccine validation.

i) *Safety*

All vaccines must be safety tested. Safety tests for attenuated vaccines do not address the issue of their teratogenicity. Attenuated virus vaccines are teratogenic and should not be administered to pregnant sheep during the first half of pregnancy as this may cause fetal abnormalities and embryonal death (12, 20).

Demonstration of avirulence is necessary for live, attenuated vaccines. A number of sheep, seronegative by an appropriate, sensitive serological test (that will reliably detect antibodies even in vaccinated animals), are inoculated with the primary seed stock. Temperatures are noted twice daily. The animals are monitored at regular intervals over a period of 28 days for clinical signs and any local or systemic reactions to ensure avirulence and innocuity. Blood samples removed at regular intervals can be used to measure level of viraemia and antibody responses. The test shall be valid if all of the vaccinated sheep show evidence of virus replication and do not display signs of disease other than mild transient illness. In South Africa, a clinical reaction index is calculated for each animal between days 4 and 14 and must be below a specific standard value.

ii) *Efficacy*

Vaccinated and unvaccinated sheep should be challenged with virulent homologous serotype. It is recommended that the challenge model use wild type virus preferably passaged only in ruminant animals and with no ECE or cell culture passages. Passage in such an isolation system results in viral cultures that might induce clinical bluetongue disease that is milder than the natural disease (12). Animals are monitored for clinical signs of BT, rectal temperatures are taken twice daily and blood samples removed at regular intervals to measure viraemia and antibody responses. Unvaccinated control sheep should show clinical signs of BT and viraemia. Unvaccinated control sheep should show clinical signs of BT. However, it is difficult to be certain of the appearance of clinical disease following inoculation of sheep with certain BTV serotypes and isolates, and consequently, evidence of infection of unvaccinated control sheep may rest on the appearance of a temperature rise of at least 1.7°C over the pre-challenge mean and a viraemia. As a further evidence of infection pre- and post-vaccination sera are checked for the presence of neutralising antibody.

iii) *Transmissibility*

Transmissibility is an issue with live attenuated vaccines but not with killed vaccines. Procedures to determine if attenuated virus can be transmitted by insects that feed on vaccinated, viraemic sheep are difficult to perform and analyse statistically, and consequently, this criterion of vaccine validation is rarely sought. Laboratory data indicate that laboratory-adapted viruses can be transmitted by insect vectors (13, 31, 39). A suitable procedure to determine attenuated virus transmissibility requires that sheep be vaccinated and, during viraemia, that they be exposed to competent, uninfected *Culicoides*, which are then permitted to feed on uninfected animals that are monitored for the presence of BTV and anti-BTV antibody. Due to the fact that the titre of attenuated virus in the blood of vaccinated sheep is low, very large numbers of *Culicoides* would be needed and only a small proportion of these would become infected and live long enough to feed on and potentially transmit the virus to other uninfected sheep. It is difficult to design a laboratory experiment that takes account of the large numbers of vaccinated sheep and insects that would be present in field situations. Current data indicate that during viraemia and in contrast to wild-type virus, laboratory-adapted strains of BTV may be found in the semen of bulls and rams (19, 38). A recent study of semen from rams vaccinated with BTV2 live attenuated vaccine showed that even if BTV was not detected in the semen, the vaccine caused a decrease in the quality of the semen (3).

A suitable procedure to determine attenuated virus transmissibility requires that sheep be vaccinated and, during viraemia, that they be exposed to competent, uninfected *Culicoides*, which are then permitted to feed on uninfected animals that are monitored for the presence of BTV and anti-BTV antibody. Due to the fact that the titre of attenuated virus in the blood of vaccinated sheep is low, very large numbers of *Culicoides* would be needed and only a small proportion of these would become infected and live long enough to feed on and potentially transmit the virus to other uninfected sheep. It is difficult to design a laboratory experiment that takes account of the large numbers of vaccinated

sheep and insects that would be present in field situations. Although virus titres in blood less than $10^3 TCID_{50}$/ml have traditionally been considered a "safe" threshold, authentic instances of insects acquiring BTV from animals with viremic titres less than $10^3 TCID_{50}$/ml have been reported. Given the complex interaction of BTV, *Culicoides* vectors and animal hosts in the life cycle of infection, virus titres induced by live attenuated vaccine should be kept to an absolute minimum specially if field transmission of vaccine strains is a concern.

Current data indicate that during viraemia and in contrast to wild-type virus, laboratory-adapted strains of BTV may be found in the semen of bulls and rams (19, 38). The implications of these observations for virus transmissibility are unclear.

iv) *Reversion to virulence*

Validation studies confirm that attenuated viruses do not revert to virulence in vaccinated sheep. However, if attenuated viruses can be transmitted from vaccinated animals, reversion to virulence during a number of sheep-insect replication cycles becomes a distinct prospect. The only appropriate way to monitor for reversion to virulence under these circumstances is to compare the virulence of the vaccine virus with that which had been subject to several sheep–insect replication cycles as described above. As indicated, this is difficult to achieve. Consequently, the effect of a number of sheep–insect passages on the virulence of attenuated viruses has not been determined. In South Africa, it is accepted that if blood from vaccinated animals during the viraemic stages is serially passaged three times in sheep without reversion to virulence, the chances of reversion in the field will be infinitely small.

2. Method of manufacture

Attenuation of field isolates of BTV was first achieved by serial passage in ECE. Because of the concern about transmission of the egg propagated attenuated virus, it has been recommended that animals receiving vaccines produced in ECE should not be moved internationally (34). More recently, it is clear that passage in cultured cells will also result in attenuation of virulence. No studies have been done to precisely relate passage number and extent of attenuation for individual virus isolates or serotypes. To prepare attenuated virus, field isolates are adapted to cell culture and passaged *in vitro* up to 40 times or more. Ideally, a number of plaque-purified viruses are picked at this stage and each is examined to determine the level of viraemia they generate and their ability to elicit a protective immune response in vaccinated sheep. The most suitable virus is one that replicates to low titre but generates a protective immune response, and this may represent the source of vaccine primary seed stock virus.

BTV for killed vaccines is produced in large-scale suspension cell systems under aseptic and controlled conditions. Cell lines adapted for large scale industrial cultures are used, and these are proven to be free from contaminating microorganisms. When the viral suspension virus reaches its maximum titre, cell disruption is performed and the culture is clarified and filtered. Subsequently inactivation is performed according to processes adopted by the manufacturer, such as by addition of binary ethyleneimine (BEI) or other inactivants. The process must comply with legislation relevant for the intended market, be validated to ensure complete inactivation and be supported by the appropriate documentation. The inactivation process should not significantly alter the immunogenic properties of the viral antigens. Purification is carried out by chromatography. The inactivated virus is then concentrated by ultrafiltration and stored. The inactivated, chromatography-purified and concentrated BTV antigens are made into vaccine by dilution in a buffer solution and addition of adjuvants.

3. In-process control

All ingredients of animal origin, including serum and cells must be checked for the presence of viable bacteria, viruses, fungi or mycoplasmas.

Virus concentration of attenuated vaccines is assessed by infectivity and ELISAs.

For inactivated vaccines, during inactivation of the virus, timed samples are taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of BHK-21 or other appropriate cell cultures. At the end of the inactivation process, the vaccine is checked to ensure that there is no live virus.

4. Batch control

a) Sterility

Every batch of vaccine should be tested for the presence contaminant viruses of viable bacterial, fungal or mycoplasmal contamination. For example, in South Africa a pool of ten randomly selected ampoules are

inoculated into soya broth and thioglycollate broth, and incubated at room temperature and 37°C, respectively, for 14 days. If contaminated, the batch is disqualified.

b)   Safety

Every batch of attenuated vaccine is safety tested in newborn and adult mice, guinea-pigs and sheep. If any adverse reactions or significant signs are noted, the test is repeated. Any increase in the body temperature of the target animal that is above the level expected for the particular strain of attenuated virus under test should be regarded as symptomatic. If the results are unsatisfactory after a second attempt, the batch is disqualified.

Safety testing of inactivated vaccines is conducted in sheep to ensure side effects are not observed.

c)   Potency

Each batch is tested by inoculation of susceptible sheep. Prevaccination, and 21- and 28-day post-vaccination sera are tested by VN assay to determine neutralising antibody levels. To be passed, the antibody titre must be equal to or higher than a set standard based on international vaccine standards.

d)   Duration of immunity

Studies with live attenuated BTV vaccine have shown that antibodies in sheep may appear before day 10 post-vaccination, reach a maximum approximately 4 weeks later and persist for well over a year. There is a temporal relationship between the increase in neutralising antibody titre and clearance of virus from the peripheral circulation. Live attenuated BTV vaccines have been in use for over 40 years and are known to induce an effective and lasting immunity (43). Many serotypes of BTV may be present in endemic areas of South Africa, and polyvalent vaccines are used. The inclusion of 15 serotypes in the vaccine means that an effective immune response is not generated to all serotypes, presumably because of the antigenic mass of individual serotype-specific antigens is small. In an attempt to broaden the response, vaccination is repeated annually (11).

Initial studies with inactivated vaccines show that antibody against BTV can be detected by day 7 post-vaccination and increase in titre to days 14–21. A second dose of vaccine boosts the titre. Data to demonstrate the expected duration of immunity is under development.

e)   Stability

Procedures have been developed for attenuated vaccines. Stability should be tested over a period of 2 years. Vaccines in liquid and lyophilised forms are deemed to have shelf lives of 1 and 2 years, respectively. Each batch of vaccine is subjected to an accelerated shelf-life test by storing it at 37°C for 7 days. It is then titrated and evaluated according to a set standard, as determined in the initial testing of the vaccine.

Inactivated vaccines have been used to the present time in emergency situations where shelf life has not been an issue. Requirements and procedures for routine commercial use have not been developed.

f)   Precautions (hazards)

Attenuated vaccines should be used in the cooler months when the *Culicoides* population and its typical activity are at the lowest level. They should not be used in ewes during the first half of pregnancy and in rams 2 months before the breeding season.

5.   Tests on the final product

a)   Safety

See C.4.b.

b)   Potency

See C.4.c.

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NB: There are OIE Reference Laboratories for Bluetongue (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

ECHINOCOCCOSIS/HYDATIDOSIS

SUMMARY

Diagnosis of echinococcosis in dogs or other susceptible carnivores relies on the demonstration of adult cestodes of the Echinococcus *genus or their eggs in their faeces or small intestine. Recently, coproantigen and copro-DNA assays have proven useful for safe, fast and accurate diagnosis. In intermediate hosts, diagnosis depends on detection of the larval cyst form that can infect almost any organ, particularly the liver and lungs.*

Identification of the agent: *At present, five species of the genus* Echinococcus *are regarded as taxonomically valid. These are* E. granulosus, E. multilocularis, E. oligarthrus, E. vogeli *and* E. shiquicus. Echinococcus oligarthrus *and* E. vogeli *occur less frequently than the first two species. Until recently* E. shiquicus *had been discovered only in a specific region of the People's Republic of China. These five species are morphologically distinct in both adult and larval stages. A number of intraspecific variants have been described for* E. granulosus*, which exhibit morphological and biological characteristics, and these can reliably be differentiated by DNA analysis. Some of the* E. granulosa *genotypes have been recommended for elevation to species status.*

Larval forms of Echinococcus *can usually be visually detected in organs. Special care has to be taken for a specific diagnosis of* E. granulosus *in instances where* Taenia hydatigena *in sheep is also a problem. Histological examination may confirm the diagnosis after formalin-fixed material is processed by conventional staining methods. The presence of a periodic-acid-Schiff positive, acellular laminated layer with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of metacestodes of* Echinococcus*. The identification of larval* E. multilocularis *in rodents and other hosts is possible by macroscopic or microscopic examination and by DNA detection using the polymerase chain reaction* (*PCR*).

The small intestine is required at necropsy for the detection of adult Echinococcus *spp. in wild or in domestic carnivores. The technique of carrying out surveys with the use of arecoline has been generally adopted for determining the prevalence of* E. granulosus *in dogs. Handling infected material presents a risk to the operator of contracting a potentially fatal disease. Significant progress is being made in the development of immunological tests for the diagnosis of intestinal* Echinococcus *infections by use of coproantigen detection. The technique has been used successfully in surveys of* E. granulosus *in dogs and is currently used in surveys for* E. multilocularis *in populations of dogs, foxes, and cats. Coproantigen detection is possible in faecal samples collected from dead or living animals or from the environment.*

PCR/DNA methods for the detection of E. multilocularis *and more recently* E. granulosus *in definitive hosts have now been established in specialised laboratories as diagnostic techniques.*

Serological tests: *Antibodies directed against oncosphere, cyst fluid and protoscolex antigens can be detected in the serum of infected dogs and sheep, but this approach is presently of limited practical use as it does not distinguish between current and previous infections. Cross-reactivity between* Echinococcus *and* Taenia *species also may occur.*

Requirements for vaccines and diagnostic biologicals: *Progress has been made in the development of an effective vaccine against infection with the larval stage of* E. granulosus *in sheep and cattle.*

A. INTRODUCTION

Species under genus *Echinococcus* are small tapeworms of carnivores with larval (metacestode) stages known as hydatids proliferating asexually in various mammals including humans. There were four morphologically distinct species in this genus until recently when *Echinococcus shiquicus* was added to the previously known species: *E. granulosus*, *E. multilocularis*, *E. oligarthus*, and *E. vogeli*. Discovered in the Shiqu County, the Qinghai-tibet plateau region of western Sichuan, the People's Republic of China (57, 58), *E. shiquicus* is morphologically distinct both in adult and larval stages from other species.

A number of interspecific and intraspecific variants have been described for *E. granulosus*. Some genotypes of *E. granulosus* exhibit characteristic features that would justify the recognition as separate species according to some authors. Recently other species and genotypes of *Echinoccocus* have been proposed (51). Further studies are needed to define the full range of genetic diversity (32, 37, 43, 50). *Echinococcus granulosus* has a global distribution; *E. multilocularis* occurs in wide areas of the Northern Hemisphere, *E. shiquicus* is found in the People's Republic of China and *E. oligarthrus* and *E. vogeli* are confined to Central and South America. All five species are infective to humans causing various forms of echinococcosis. Human cystic echinococcosis, caused by *E. granulosus* and alveolar echinococcosis, caused by *E. multilocularis*, are important public health threats in many parts of the world (56).

Table 1. Useful characteristics for identification of *Echinococcus* species. Source: Xiao *et al.* (58)

	E. granulosus	*E. multilocularis*	*E. oligarthus*	*E. vogeli*	*E. shiquicus*
Distribution	Cosmopolitan	Holoarctic region	Neotropical region	Neotropical region	Tibet plateau
Definitive Host	Dogs	Foxes	Wild felids	Bush dog	Tibetan fox
Intermediate Host	Ungulates	Microtine rodents	Neotropical rodents	Neotropical rodents	Plateau pika
Adult:					
Body length (mm)	2.0–11.0	1.2–4.5	2.2–2.9	3.9–5.5	1.3–1.7
No. segments	2–7	2–6	3	3	2–3
Length of large hooks (μm)	25.0–49.0	24.9–34.0	43.0–60.0	49.0–57.0	20.0–23.0
Length of small hooks (μm)	17.0–31.0	20.4–31.0	28.0–45.0	30.0–47.0	16.0–17.0
No. testes	25–80	16–35	15–46	50–67	12–20
Position of genital pore					
a. Mature segment	Near to middle	Anterior to middle	Anterior to middle	Posterior to middle	Near to upper edge
b. Gravid segment	Posterior to middle	Anterior to middle	Near to middle	Posterior to middle	Anterior to middle
Gravid uterus	Branching laterally	Sac-like	Sac-like	Tubular	Sac-like
Metacestode	Unilocular cysts in viscera	Multilocular cysts in viscera	Polycystic cysts in muscles	Polycystic cysts in viscera	Unilocular cysts in viscera

- ### *Echinococcus granulosus*

The parasite is transmitted between the domestic dog and a number of domestic ungulate species. The dog/sheep cycle is most important. Sylvatic definitive and intermediate hosts also occur, e.g. wolf/cervid. The adult varies between 2 and 11 mm in length and usually possesses from two to seven segments, averaging from

three to four segments. The penultimate segment is mature, and the genital pore normally opens posterior to the middle in both mature and gravid segments. The last (gravid) segment is usually more than half the length of the entire worm. There are rostellar hooks of various sizes on the protoscolex in two rows. The size of the hooks varies between 25 to 49 µm in the first row, and between 17 and 31 µm in the second row. The gravid uterus has well-developed sacculations.

The larval stage is a fluid-filled bladder or hydatid cyst that is unilocular, although communicating chambers also occur. Growth is expansive, and endogenous daughter cysts may be produced. Individual bladders may reach up to 30 cm in diameter and occur most frequently in liver and lungs, but may develop in other internal organs. The infection with this stage is referred to as cystic echinococcosis.

The strain specificities of *E. granulosus* in domestic cycles include, dog/sheep in the Mediterranean region, South America (Argentina, Brazil, Chile, Peru and Uruguay), Africa (Ethiopia, Kenya and Sudan), the Middle East and Levant regions, Russia, Central Asia (Kazakhstan, Kyrgyzstan and Uzbekistan), Mongolia, the People's Republic of China, Oceania and the United Kingdom; dog/horse in Belgium, Ireland and the United Kingdom; dog/cattle in Belgium, Germany, South Africa and Switzerland; dog/swine in Poland; and dog or wolf/reindeer in sub-Arctic regions of Norway, Finland and Alaska. The status of dog/camel strains requires further elucidation. This strain has recently been identified in human cases in Argentina, Nepal, the People's Republic of China and Iran (3, 19, 44, 59). To date, all genotypes of *E. granulosus* except the dog/horse (G4) and the Finnish cervid (G10) strains have been found to infect humans.

- *Echinococcus multilocularis*

The parasite is transmitted primarily between wild definitive hosts (e.g. *Vulpes vulpes, V. ferrilata, V. corsac, Alopex lagopus, Canis latrans*) and small arvicolid rodents (voles and lemmings). The adult varies between 1.2 and 4.5 mm in length and usually possesses from two to six segments, with an average of four to five. The penultimate segment is characteristically mature, and the genital pore is anterior to the midline in both mature and gravid segments. The gravid uterus is sac-like. On the rostellum, the larger hooks of the first row vary in size between 24.9 and 34.0 µm and the smaller hooks of the inner row between 20.4 and 31.0 µm.

The metacestode is a multivesicular structure consisting of conglomerates of small vesicles, usually not exceeding a few millimetres in diameter. Unlike *E. granulosus*, the larval mass often contains a semisolid rather than a fluid matrix. It proliferates by exogenous budding, which results in infiltration of tissues. Infection with this stage is commonly referred to as alveolar echinococcosis. There is no clear evidence for distinct strains or genotypes of *E. multilocularis,* though regional variations at the continental scale have been described (56).

This zoonotic parasite is found in the Northern Hemisphere, and its life cycle is mainly maintained in wildlife (25). The sylvatic cycle involves foxes and many species of wild rodents. Coyotes, raccoon dogs, wolves, wild cats, domestic dogs and cats (20, 27), however, may serve as definitive hosts while pigs, horses, primates and humans can be infected as intermediate hosts (25).

- *Echinococcus oligarthrus*

The parasite typically uses neptropical wild felids as definitive hosts (e.g. *Felis concolor, F. jaguarundi*) and large rodents (e.g. *Dasyprocta* sp., *Cuniculus paca*) as intermediate hosts. The adult varies between 2.2 and 2.9 mm in length, and normally possesses three segments, the penultimate of which is mature. The genital pore is anterior to the middle in mature segments and approximately at the middle in gravid segments. The gravid uterus is sac-like.

The metacestode is polycystic and fluid-filled with a tendency to become septate and multichambered. The rostellar hooks of the protoscolex vary in length between 25.9 and 37.9 µm. The hooks are described in more detail in the next section and compared with those of *E. vogeli*. The single cyst may reach a diameter of approximately 5 cm. Predilection sites are internal organs and muscles. To date, there have only been a few reports of human disease. The parasite appears not to mature in dogs.

- *Echinococcus vogeli*

The parasite typically uses the South American bush dog (*Speothus venaticus*) as a wild definitive host, but the domestic dog is susceptible, as are large rodents (e.g. *Cuniculus paca*) as intermediate hosts. The adult varies between 3.9 and 5.5 mm in length, and usually has three segments, the penultimate of which is mature. The genital pore is situated posterior to the middle in both the mature and gravid segments. The gravid uterus has no lateral sacculations and is characterised by being relatively long and tubular in form, compared with the other segments, which are sac-like.

The metacestode is similar to that of *E. oligarthrus*. It has been reported that the two species can be distinguished by comparing differences in the dimensions and proportions of the rostellar hooks on the protoscolex. The hooks of *E. oligarthrus* vary in length between 25.9 and 37.9 µm (average 33.4 µm) and

between 22.6 and 29.5 µm (average 25.45 µm) for large and small hooks, respectively. Those of *E. vogeli* vary between 19.1 and 43.9 µm (average 41.64 µm) and between 30.4 and 36.5 µm (average 33.6 µm) for the large and small hooks, respectively. Also the hook-guard for *E. oligarthrus* divides the hook 50:50, compared with 30:70 for *E. vogeli*.

Echinococcus vogeli is a zoonotic agent with approximately 100 human cases reported in South America. The infection caused by the larval stage of this species is commonly referred to as polycystic echinococcosis.

- **Echinococcus shiquicus**

The parasite was found in the Tibetan fox (*Vulpes ferrilata*) its definitive host and the plateau pika (*Ochotona curzoniae*), the intermediate host. In most species of *Echinococcus*, the gravid segment is connected to a mature segment; however, a strobila consisting of only two segments (a gravid segment directly attaching to a premature segment) is unique to this species (56). The adult stage is morphologically similar to *E. multilocularis* but differs by its smaller hooks, fewer segments, upper position of genital pore in the premature segment and fewer eggs in the gravid segment. It is easily distinguishable from *E. granulosus* by its shorter length, branchless gravid uterus and anterior position of genital pore in the gravid segment. The adult measures 1.3 to 1.7 mm.

The metacestode is found in the liver and is essentially a unilocular minicyst containing fully developed brood capsules; however, oligovesicular forms have also been observed. It is differentiated from *E. granulosus* having no daughter cysts appearing within the fertile cyst (56).

A detailed description of echinococcosis in humans and animals can be found in the WHO/OIE Manual on echinococcosis (56).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

In the intermediate host, diagnosis depends on the detection of the larval cyst form, which can occur in almost any organ, but particularly in the liver and lungs. The diagnosis of echinococcosis in dogs or other carnivores requires the demonstration of the adult cestodes of *Echinococcus* spp. in their faeces or the small intestine or the detection of specific coproantigens or coproDNA.

Investigators carrying out these procedures are exposed to the risk of infection and severe disease, which must be minimised by appropriate procedures. Infective material can be decontaminated by freezing at –80°C (core temperature) for 48 hours, or –70°C for 4 days or by heating to 70°C for 12 hours (41, 45). Face masks, disposable gloves and an apron must be worn. Chemical disinfection is not reliable, although sodium hypochlorite may destroy a proportion of eggs (8). Contaminated material must be destroyed by heat; hot water, at temperature of 85°C or above, is very effective. The decontamination of laboratories can be achieved at reduced humidity (40%) combined with increased room temperature (30°C) for at least 48 hours.

a) Diagnosis of *Echinococcus* eggs in environmental samples

- **Faecal samples (22, 49)**

This is a concentration method in which a saturated solution is used to separate *Echinococcus* eggs from faeces. A faecal sample of 0.5–2 g is mixed with water or 0.3% Tween 20 in 1% formalin (42) in a 10–15-ml test tube and centrifuged (1000 *g* for 10 minutes) once or twice until the supernatant is clear. Sediment is mixed with either zinc sulphate 33% (1.18 sp. Gr.) or sucrose solution (1.27 sp. Gr.) and centrifuged at 1000 *g* for 5–10 minutes. The test tube is filled to the top and a cover-glass is placed on the tube. The cover-glass is examined microscopically 2–16 hours later.

- **Soil samples (35)**

A 20-g soil sample is placed in a 50-ml conical tube to which is added 40 ml of 0.05% Tween 80. The mixture is stirred vigorously and sieved through a 100-µm mesh. The suspension is centrifuged at 1000 *g* for 5 minutes and the supernatant is discarded. The remaining procedure follows the concentration method used for faecal sample examination.

b) Diagnosis of larval echinococcosis

- **Necropsy**

Whereas surveillance for *E. granulosus* in domestic animals may take place in licensed slaughter houses, that for *Echinococcus* sp. in wildlife must be done by field surveys. Specimens should be preserved by

removal of tissue and fixation in 4% formal saline or kept cool at +4°C and deep-frozen at –20°C for subsequent examination. When undertaking surveillance work with *E. granulosus* in intermediate hosts, it is vitally important that data are stratified and reported according to the age of animals slaughtered. Prevalence rates are strongly age dependent (53) and reports from abattoirs that may slaughter only young animals will substantially under-represent the true situation. This is because older animals may be heavily infected even when animals have very few larvae.

Larvae can be observed in many organs, but in large animals, such as sheep and cattle, palpation or incision should be done. Pigs, cattle, sheep and goats may be infected with larval *Taenia hydatigena,* and it is sometimes difficult to differentiate between these two parasites when they occur in the liver. In wild animals, such as ruminants and rodents, several other larval cestodes should be considered for differential diagnosis.

Formalin-fixed material can be stained by conventional histological techniques. The presence of a periodic-acid-Schiff (PAS) positive acellular laminated layer, underlying a connective tissue layer, and with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of the metacestodes of *Echinococcus spp.* The presence of protoscoleces within brood-capsules or in hydatid sand is also diagnostic for the genus. Genotyping of *E. granulosus* or *E. multilocularis* is usually done on DNA derived from protoscoleces or larval tissue material that is frozen, refrigerated or preserved in 90% ethanol.

c)   Diagnosis of adult parasites in carnivores

-   **Necropsy**

Necropsy is invariably employed in studies of echinococcosis in wildlife and is useful if domestic dogs are humanely culled. It should be emphasised that it is necessary to isolate and identify the adult *Echinococcus*, because under normal conditions of faecal examination, the eggs of *Echinococcus* cannot be differentiated from those of *Taenia* spp. The eggs of *E. granulosus* and *E. multilocularis* can now be identified and differentiated from other taeniid eggs by polymerase chain reaction (PCR).

The small intestine is removed as soon as possible after death, and tied at both ends. If the material is not frozen or formalin fixed (4–10%), it should be examined quickly, as the parasite can be digested within 24 hours. Formalin does not kill eggs. The fresh intestine is divided into several sections and immersed in 0.9% saline at 37°C for examination. Worms adhering to the intestinal wall may be observed and counted by means of a hand lens (for *E. granulosus* and *E. vogeli*). For accurate counts, the unfixed intestine is best divided into four or six sections, opened up and immersed in 0.9% saline at 37°C for 30 minutes to release the parasites. The contents are washed into another container for detailed examination, and the intestinal wall is scraped with a spatula. All material is boiled and washed by sieving to eliminate most of the particulate material and to make it noninfectious. The washed intestinal contents and scrapings are placed on a black tray, and the worms are counted with the aid of a hand lens or stereoscopic microscope. *Echinococcus granulosus* is usually found in the first third of the small intestine of dogs and *E. multilocularis* in the mid/posterior sections.

Necropsy is considered to be the most reliable form of diagnosis for *E. multilocularis* in definitive hosts. It is an inexpensive method for determining the prevalence in a population and the best way to determine worm burden (15). Carcasses or intestines of definitive hosts for examination should be deep frozen at between –70°C and –80°C for 3–7 days before necropsy to kill any eggs. Eggs of *E. multilocularis* are resistant to freezing to –50°C.

Methods for quantitative determination of *E. multilocularis* in definitive host's intestine.

-   **Sedimentation and counting technique (SCT; 16, 21)**

This technique can be regarded as the 'gold standard' for assessing the sensitivity and specificity of other techniques.

i)   The small intestine is incised longitudinally and cut into 20 cm long segments or into 5 pieces of approximately the same length. These pieces are transferred to a glass bottle containing 1 litre physiological saline (0.9% NaCl) solution.

ii)   The glass bottle is shaken vigorously for a few seconds and the pieces of intestine are removed. The superficial mucosal layer is stripped by exerting pressure between thumb and forefinger to dislodge attached helminths.

iii)   The glass bottle is left for 15 minutes for sedimentation to occur; the supernatant is then decanted. The glass bottle is refilled with physiological saline solution. This procedure is repeated 2–6 times until the supernatant is cleared of coloured particles.

iv) The sediment fraction is examined in small portions of about 5–10 ml in rectangular plastic or Petri dishes with a counting grid (9 × 9 cm) in transmission light under a stereomicroscope at a magnification of ×120.

v) If up to 100 worms are found, the entire sediment fraction is checked; if higher numbers are present, the total worm burden is calculated from the count of one subsample.

- **Intestinal scraping technique (IST; 12, 17)**

i) Deep mucosal scrapings are taken at nearly equal distances from the small intestine using microscope slides (75 × 25 × 1 mm). Five mucosal scrapings from proximal, middle and posterior thirds of the small intestine (total 15) are recommended. Adherent materials are transferred to a square plastic Petri dish.

ii) Scrapings are squashed between slides and examined under a stereoscopic light microscope (×120). Three slides are placed in one plastic dish and examined. *Echinococcus multilocularis* is usually found in the second half of the small intestine.

- **'Shaking in a vessel' technique (SVT; 15)**

i) A plastic vessel (1 litre), which has a plastic screw-on lid with a central hole 6–7 cm in diameter is used. The hole is covered with a high-grade steel mesh (mesh size 500 µm) fixed into the remaining plastic ring with a hot soldering iron. Silicone is applied to seal the edges of the steel mesh.

ii) The longitudinally opened small intestine is transferred to the vessel with all its contents; the vessel is closed with the lid and filed with water.

iii) The vessel is inverted and shaken; the water is decanted. Vessel is refilled with water, and the process is repeated until the decanted water is clear.

iv) The half-filled vessel is opened and the intestines are removed. The intestines are stripped between the thumb and forefinger to dislodge parasites stuck to the mucosa into the vessel.

v) The vessel is closed again, refilled and shaken one last time draining as much water from it as possible.

vi) The remaining sediment is filled into a 1 litre plastic jug and stored at 4°C. For prolonged storage, a 0.9% NaCl solution is added to the sediment to prevent the parasites from shrinking.

vii) For analysis, the materials are placed into small glass Petri dishes and scanned along engraved lines using the stereomicroscope as above.

- **Preserving specimens**

Intact worms are fragile and for morphological studies are best handled in normal saline with a Pasteur pipette. They are washed free of other material and left for approximately 30 minutes for all movement to cease. After removal of the fluid, cold 5–10% formalin (5°C) or FAA fixative (95% ethanol [80 ml], 37–40% formaldehyde [10 ml], and glacial acetic acid [5 ml]) is added and the worms are left for a further 12 hours. For staining, the worms are washed in water for 15 minutes and transferred to Mayer's paracarmine (carminic acid [1.0 g], aluminium chloride [0.5 g], calcium chloride [4.0 g], and 70% ethanol [100 ml]) for 12–24 hours. Excess stain is removed by immersion in 0.5–1.0% hydrochloric acid solution for a few seconds. Dehydration is accomplished by serial passage in ascending concentrations of alcohol (41, 50, 70, 85, 95, and 100%) for at least 15 minutes in each, with two changes in 100%. The alcohol is removed by xylol (10 minutes) and cleared with methyl salicylate or creosote. Prior to mounting in any suitable medium such as balsam, picolyte, etc., the specimens should be returned to the xylol for a few minutes. Persons involved in such examinations should receive serological screening for anti-*Echinococcus* serum-antibodies at least once a year (56).

Recently, some methods have been developed with the aim of simplifying and improving epidemiological investigations in final host populations and of allowing diagnosis in living animals. These methods include the detection of coproantigens and PCR DNA detection (see below).

d)   Arecoline surveys and surveillance

Arecoline has been used to perform surveys of tapeworm infections in dog populations. Its use as a control agent has now been superseded by praziquantel. Arecoline is a parasympathomimetic agent. Its action results in sweating, and stimulation of salivary, lachrymal, gastric, pancreatic, and intestinal glands. It increases intestinal tonus and the mobility of smooth muscle, and this effect is responsible for purgation. The liver is the principal site of detoxification. Arecoline also has a direct action on the worm itself, by causing paralysis, but not death, and thereby making it relax its hold on the intestinal wall. Thus, it must be administered by the oral route. The accompanying purgation carries the worms out with the faeces. It is particularly suitable for baseline surveys of *E. granulosus,* however, 15–25% of dogs may not purge.

Arecoline may also be used to purge dogs infected with *E. multilocularis.* In animals, arecoline purgation has been useful; again, the recovered tapeworms are identified morphologically. Products containing arecoline are no longer available as an anthelmintic, but can be obtained from chemical supply companies. As it has side-effects, old, infirm and pregnant animals should be excluded from treatment. A dose of 4 mg/kg should result in purgation in under 30 minutes. Walking and abdominal massage of recalcitrant cases or enema for constipated dogs may avoid the use of a second dose (2 mg/kg), which should be given only sparingly.

Dogs that are purged successfully may produce at least two motions; the first will be formed faeces and can be ignored (or collected for laboratory tests as described later), but the mucus that follows may be productive. This can be divided into several samples and each examined separately, but this method is not recommended as the worms will be difficult to detect. Preferably, the mucus sample (about 4 ml) is diluted with 100 ml of tap water, covered with a thin layer of 1 ml of kerosene (paraffin) and boiled for 5 minutes. The kerosene prevents foaming and reduces the smell.

Investigators carrying out these procedures are exposed to risk of infection and severe disease. Personnel should wear whole body coveralls, boots, disposable gloves and a face mask. Coveralls should be boil washed after use, and boots disinfected in 10% sodium hypochlorite solution. The purge should be boiled as soon as possible after collection. Dogs may continue to pass eggs, proglottides and worms after the first purge, therefore, they should remain tethered for 2 hours after purgation and given access to drinking water. After arecoline testing, the area of ground used to tether dogs should be sprayed with kerosene and flamed.

e) **Coproantigen tests**

An alternative to arecoline testing, based on a faecal antigen-detection antibody sandwich enzyme-linked immunosorbent assay (ELISA), has been developed and has shown particular promise as coproantigens can be detected shortly after infection (10–14 days) and the level declines rapidly following expulsion of the worms. The sensitivity and specificity of the test have been estimated at 70% and 98%, respectively (2, 5, 8, 12, 13).

Both qualitative and quantitative results can be obtained from arecoline testing, which is most useful for base-line epidemiological studies on the comparative rates of infection with Taeniidae in dogs. Further studies may show that the coproantigen test may be more cost-effective than arecoline testing during routine surveillance of *E. granulosus* in the dog population (6).

ELISAs for specific coproantigen have now been developed that have sufficient specificity and sensitivity to replace arecoline testing for detecting *Echinococcus* in dogs and other definitive hosts (8). When testing for genus-specific *Echinococcus* coproantigens (against necropsy as a gold standard), specificity is around 98% and overall sensitivity approximately 70%; however, when mean worm burdens are >50–100, sensitivity approaches 100% (2, 8, 9, 13). Dogs, dingoes, foxes and wolves have been screened successfully for coproantigen ELISAs and, importantly, *E. multilocularis* worm infestations are also detectable in red foxes and domestic dogs (13, 46). When the capture ELISA uses either anti-ES or anti-somatic proglottid antibodies to *E. granulosus*, the sensitivity for *E. multilocularis* infection may be reduced, though genus specificity remains intact. Polyclonal- or monoclonal-antibody-based ELISAs for coproantigens exhibit high sensitivity and specificity to *E. granulosus* (~80%), even though they were developed for *E. multilocularis* (11, 44). However, for low worm burdens (<50), the sensitivity of the *E. multilocularis* coproantigen ELISA is below that of the mucosal smear method at necropsy (11).

The exact nature of *Echinococcus* antigens released in faeces for coproantigen detection has not been fully characterised. However, their stability in 5% formal saline after boiling and susceptibility to periodate treatment suggest involvement of large (>150 KDa) of carbohydrate antigen(s) with α-D-mannose, α-D-glucose, β-galactose and N-acetyl-β-glucosamine residues (8, 18).

Coproantigens can be detected prior to release of eggs by *Echinococcus* worms, and therefore are not related to egg antigen(s) (13, 44). This has the advantage of detection of prepatent infections. Furthermore, coproantigen levels return to the preinfection baseline within 5 days of anthelminthic treatment of infected dogs (13). More importantly, it reduces the biohazardous risk of exposure of personnel to potentially infective eggs during purgation or necropsy (26).

For detection of *E. multilocularis* infection of foxes, necropsy is time-consuming. Coproantigen testing by ELISA offers a specific practical alternative. Fox faecal samples should be taken at post-mortem from the rectum rather than from the small intestine. *Echinococcus* coproantigens are also stable in fox or dog

faeces left at 20°C for 1 week and in frozen dog faeces. Coproantigen testing has also been successfully used to evaluate he efficacy of deworming wild foxes infected with *E. multilocularis* using praziquantel-laced bait, which proved to be a successful combination of eliminating the source of infection (24).

- **Coproantigen test procedure (*Echinococcus granulosus*) (2, 9)**

i) The faecal sample (collected per rectum or from the ground) is mixed with an equal volume of phosphate buffered saline (PBS), pH 7.2, containing 0.3% Tween 20 (PBST), in a capped 5 ml disposable tube. This is shaken vigorously and centrifuged at 2000 *g* for 20 minutes at room temperature. Faecal supernatants can be tested immediately or stored at –20°C or lower. Supernatants that appear very dark or viscous are still acceptable for use.

ii) A 96-well ELISA microtitre plate (Immulon #4, Thermo Electron Corporation) is coated with optimal concentration (typically 5 µg per ml) of a protein A purified IgG fraction of rabbit anti-*E. granulosus* proglottid extract (2) in 0.05 M bicarbonate/carbonate buffer, pH 9.6 (100 µl per well). The plate is covered and incubated overnight at 4°C.

iii) The wells are rinsed three times in PBST with 1 minute between washes; 100 µl of the same buffer is added to each well, and the plate is incubated for 1 hour at room temperature.

iv) The PBST is discarded and 50 µl of neat fetal calf serum is added to all wells. This is followed by the addition of 50 µl per well of faecal sample supernatants is added (in duplicate wells). The plate is incubated at room temperature for 1 hour with clingfilm seal covering the plate.

v) The wells are rinsed as in step iii, but the contents are discarded into a 10% bleach (hypochlorite) solution.

vi) An optimal dilution concentration of around 1 µg/ml of an IgG rabbit anti-*E.-granulosus* proglottid extract peroxidase conjugate (2) in PBST is prepared and 100 µl per well is added to all wells. The plate is incubated for 1 hour at room temperature (22–24°C).

vii) The wells are rinsed as in step iii.

viii) Next, 100 µl per well of tetramethyl benzidene substrate (TMB, KPL Labs) is added and the plate is left in the dark for 20 minutes at room temperature (22–24°C).

ix) Absorbance of wells is read at 630 nm. The enzyme-substrate reaction is stopped by adding 100 µl of 1 M phosphoric acid (H_3PO_4) to each well. The colour turns from blue to yellow if positive.

x) Laboratories should establish their own end-point criteria using standard positive and negative samples. Standards can also be obtained from the OIE Reference Laboratories (see Table given in Part 3 of this *Terrestrial Manual*). Usually, the positive to negative threshold is taken as 3 standard deviations above the mean absorbance value of control negatives, or against a reference standard control positive using absorbance units equivalence.

- **Coproantigen test procedure (*Echinococcus multilocularis*) (39, 42)**

Sandwich ELISA using a monoclonal antibody EmA9 raised against adult *E. multilocularis* somatic antigen (28).

i) 0.5 g of each faecal sample is placed in a centrifuge tube and a 1% formalin solution containing 0.3% Tween 20 is added to a total volume of 15 ml.

ii) After adequate mixing, the faecal solution is centrifuged at 1200 *g* for 10 minutes at room temperature. A supernatant fraction is used for the coproantigen detection assay.

iii) Flat-bottomed microtitre plates (Immulon 600, Greiner, Germany) are coated with 50 µl/well of 1 µg/ml rabbit IgG directed against adult *E. multilocularis* excretory/secretory (ES) products in 0.05 M $NaHCO_3$/Na_2CO_3 buffer (pH 9.6) and are left overnight at 4°C.

iv) The plates are washed three times with 250 µl/well PBS (pH 7.4) containing 0.05% Tween 20 (PBST), and blocked using 100 µl/well 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (22–24 C).

v) The plates are washed three times (with the wash disinfected with 10% bleach) and 50 µl of faecal supernatant is added to each well and the plates are incubated for 2 hours.

vi) The plates are again washed four times and 0.5 µg/ml of the biotinylated monoclonal antibody in 0.5% BSA/0.5% casein in PBST is added to each well and the plates are incubated for 1 hour.

vii) The plates are washed four times and streptavidine-biotinylated horseradish peroxidase complex (Amersham Life Science), diluted 1/1000 in 0.5% BSA/0.5% cCasein in PBST is added to each well and the plates are incubated for 1 hour.

viii) The plates are washed five times and 100 μl/well of substrate solution (20 mg of phenylenediamine (Wako) in 50 ml of 0.1 M citric phosphate buffer with 10 μl of H_2O_2) is added.

ix) The plates are shaken immediately and placed in a 37°C incubator for 30 minutes. The reaction is stopped by adding 50 μl/well of 4 N H_2SO_4. The optical densities (OD) of the plates are read at 490 nm.

x) The cut-off value is calculated as the mean OD value plus 3 standard deviations of samples from uninfected animals.

This procedure was also used in a sandwich ELISA for *E. granulosus* coproantigen detection (45). In 2008, a latex agglutination test and immunochromatography in-house kit using EmA9 became available for coproantigen detection (23, 41).

f) DNA recognition methods

Definitive hosts: Copro-DNA has proven to be of value for the diagnosis of Echinococcosis in animal definitive hosts. DNA isolation from the faeces, however, is laborious.

PCR is a technically demanding and expensive technique. It is currently used mainly for confirmatory testing of coproantigen-positive samples or for identification of taenid eggs recovered from faeces. Table 2 presents the different PCR primers used for identification of copro-DNA from faeces in definitive hosts of genus *Echinococcus*.

Differential diagnosis of *E. granulosus* and *E. multilocularis* infections in definitive hosts may be achieved by specific detection of PCR-amplified DNA from *E. multilocularis* eggs present in faeces (4, 32). In practice, it is recommended to screen definitive hosts (e.g. foxes) using the coproantigen test and confirm with the PCR DNA test. In Europe, transmission of *E. multilocularis* generally occurs in regions where *E. granulosus* is not endemic or appears very infrequently. In other regions, including parts of the Near East (Turkey and Iran), Central Asia, Russia and the People's Republic of China, these two species may occur together (10). Further evaluation of *E. multilocularis* infection is required to investigate intermittent shedding and duration of shedding of parasite DNA. Recently PCR has been developed for the detection of copro-DNA for *E. granulosus* and for genotypic differentiation. (36, 55)

As PCR is generally used as a confirmatory test, it is suggested to concentrate the taeniid eggs by sequential sieving and an in-between concentration method step. DNA isolation from these eggs can be achieved using a simplified protocol of the alkaline lysis method combined with a commercial kit with no need for organosolvent extractions (30).

Table 2. *PCR primers used for copro-DNA detection (modified from Mathis & Deplazes [34])*

Primer designation: primer sequences (5'–3')	Ref.	Target, comments
E. multilocularis GTG-AGG-CGA-TGT-GTG-GTG-ATG-GAG-AGA-AGG CAA-GTG-GTC-AGG-GGC-AGT-AG	4	U1 sRNA gene: may yield non-specific products when used with metacestode material containing host DNA (unpublished observation) Mitochondrial 12S RNA gene; used in two-tube nested PCR
Outer primers: (P60 forward) TTA-AGA-TAT-ATG-TGG-TAC-AGG-ATT-AGA-TAC-CC (P375 reverse) AAC-CGA-GGG-TGA-CGG-GCG-GTG-TGT-ACC Inner primers: (Pnest forward) ACA-ATA-CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC (Pnest reverse) ATA-TTT-TGT-AAG-GTT-GTT-CTA	14	Mitochondrial 12S RNA gene; modified from ref. 14 for use in one-tube nested PCR

Table 2 cont. *PCR primers used for copro-DNA detection* (*modified from Mathis & Deplazes* [*34*])

Primer designation: primer sequences (5'–3')	Ref.	Target, comments
Outer primers:	54	Mitochondrial 12S RNA gene; modified from ref. 14 for use in single PCR
(Em-1)		
TAA-GAT-ATA-TGT-GGT-ACA-GGA-TTA-GAT-ACC-C		
(Em-2)		
GGT-GAC-GGG-CGG-TGT-TGT-A		
Inner primers:		
(Em-3)		
ATA-TTA-CAA-CAA-TAT-TCC-TAT-C		
(Em-4)		
ATA-TTT-TGT-AAG-GTT-GTT-CTA		
(EM-H15)	48	NADH dehydrogenase subunit 1 (ND1) of mtDNA; cleavage with enzyme *Cfo1* distinguish *E. multilocularis* from *E. granulosus*
CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC		
(EM-H17)		
GTG-AGT-GAT-TCT-TGT-TAG-GGG-AAG		
E. multilocularis and *E. granulosus*	38	Repeated sequences from *E. granulosus*. 'sheep strain'; yields banding pattern upon electrophoresis
ND1		
(NDfor2-)		Mitochondrial 12SRNA gene; specific for *E. granulosus* 'sheep strain'
AGT-TTC-GTA-AGG-GTC-CTA-ATA		
(NDrev2-)		Amplify a fragment of the *coxl* genespecific fo *E. granulosus*
CCC-ACT-AAC-TAA-CTC-CCT-TTC		
E. granulosus	1	
(Eg1121a)		
GAA-TGC-AAG-CAG-CAG-ATG		
(Eg1122a)		
GAG-ATG-AGT-GAG-AAG-GAG-TG		
(Eg1f)	47	
CATTAATGTATTTTGTAAAGTTG		
(Eg1r)		
CAC-ATC-ATC-TTA-CAA-TAA-CAC-C		
(EgO/DNA-IM1)	40	
forward		
TCA-TAT-TTG-TTT-GAG-KAT-YAG-TKC		
reverse		
GTA-AAT-AAM-ACT-ATA-AAA-GAA-AYM-AC		

Table 2 cont. *PCR primers used for copro-DNA detection* (*modified from Mathis & Deplazes* [*34*])

Primer designation: primer sequences (5'–3')	Ref.	Target, comments
E. multilocularis, *E. granulosus* and *Taeniid spp.*	52	
(JB11)		Cestodes
AGA-TTC-GTA-AGG-GGC-CTA-ATA		
(JB12)		
AC-CAC-TAA-CTA-ATT-CAC-TTT-C		
(60.for.-mod)		Cestodes
ATG-TGG-TAC-AGG-ATT-AGA-TAC-CC		
(375.rev.-mod)		
GGT-GAC-GGG-CGG-TGT-GTA-CC		
(Eg1f)	52	*Echinococcus granulosus* (sheep strain)
CAT-TAA-TGT-ATT-TTG-TAA-AGT-TG		
(Eg1r)		
CAC-ATC-ATC-TTA-CAA-TAA-CAC-C		
(EM-H15)	52	*E. multilocularis*
CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC		
(EM-H17)		
GTG-AGT-GAT-TCT-TGT-TAG-GGG-AAG		
(Cest1)	52	*E. multilocularis*
TGC-TGA-TTT-GTT-AAA-GTT-AGT-GAT-C		
(Cest2)		
CAT-AAA-TCA-ATG-GAA-ACA-ACA-ACA-AG		
(Cest4)	52	*E. granulosus*
GTT-TTT-GTG-TGT-TAC-ATT-AAT-AAG-GGT-G		
(Cest5)	52	*Taenia* spp.
GCG-GTG-TGT-ACM-TGA-GCT-AAA-C		
(Cest3)	52	*Taenia* spp. (Sequencing primer for the 267 bp amplicon of the multiplex PCR)
YGA-YTC-TTT-TTA-GGG-GAA-GGT-GTG		
(Cest5)		
GCG-GTG-TGT-ACM-TGA-GCT-AAA-C		
(Cest5seq)		
GAT-TCT-TTT-TAG-GGG-AAG-G		

Intermediate hosts: DNA hybridisation methods are not currently used for the detection of *E. granulosus* in livestock intermediate hosts. Molecular methods are, however, important in identification of isolates or strains of *E. granulosus* for epidemiological purposes (37).

For the identification of small, degenerated or calcified lesions of *E. multilocularis* in intermediate or aberrant hosts, PCR is of great value (33).

2. Serological tests

a) Intermediate hosts

Immunological tests, useful in humans, are less sensitive and specific in livestock and at present cannot replace necropsy (8, 30).

b) **Definitive hosts**

An extensive programme has been initiated to develop immunodiagnostic tests to control canine echinococcosis. Following ingestion of a cyst, dogs will be exposed at the intestinal level to various antigens during the establishment of the parasite and its development and oogenesis. Specific antibodies against oncosphere and protoscolex antigens can be readily detected in the serum of infected dogs. This methodology has not reached a practical stage as it does not differentiate between current and previous infections and false positives may occur with infections of *Taenia* species.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Intermediate hosts

Application of an effective vaccine to reduce hydatid infection in livestock would be likely to have a substantial impact on the rate of transmission of the disease to humans (29). As *E. granulosus* belongs to the Taeniid family, many aspects of its immunological relationship with its intermediate host are similar to that occurring in *Taenia* species. Moreover, it was considered that the vaccine development approach used in *Taenia* species such as the native host-protective antigens of *T. ovis* would also be successful for *E. granulosus*. Using recombinant DNA technology, an oncosphere antigen vaccine EG95 was shown to be capable of inducing a high level of protection against experimental challenge infection with *E. granulosus* eggs in sheep (31).

The EG95 vaccine has been licensed by the University of Melbourne and AgResearch New Zealand to a commercial group in the People's Republic of China (29).

2. Definitive hosts

While considerable research has been undertaken with crude antigens to protect dogs from echinococcosis, only limited evidence has been demonstrated so far. Recent studies using recombinant protoscolex antigens, however, look encouraging (7). Basic research on canine mucosal immunology and *Echinococcus* infection is required for progress.

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NB: There are OIE Reference Laboratories for Echinococcosis/Hydatidosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.1.5.

FOOT AND MOUTH DISEASE

SUMMARY

Foot and mouth disease (FMD) is the most contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus, namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Infection with one serotype does not confer immunity against another. FMD cannot be differentiated clinically from other vesicular diseases, including swine vesicular disease, vesicular stomatitis and vesicular exanthema. Laboratory diagnosis of any suspected FMD case is therefore a matter of urgency.

Typical cases of FMD are characterised by a vesicular condition of the feet, buccal mucosa and, in females, the mammary glands. Clinical signs can vary from mild to severe and fatalities may occur, especially in young animals. In some species the infection may be subclinical, e.g. African buffalo (Syncerus caffer). The preferred tissue for diagnosis is epithelium from unruptured or freshly ruptured vesicles or vesicular fluid. Where collecting this is not possible, blood and/or oesophageal–pharyngeal fluid samples taken by probang cup in ruminants or throat swabs from pigs provide an alternative source of virus. Myocardial tissue or blood can be submitted from fatal cases, but vesicles are again preferable if present.

It is vital that samples from suspected cases be transported under secure conditions and according to international regulations. They should only be dispatched to authorised laboratories.

Diagnosis of FMD is by virus isolation or by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis and antibodies to viral nonstructural proteins (NSPs) can be used as indicators of infection, irrespective of vaccination status.

Identification of the agent: *The demonstration of FMD viral antigen or nucleic acid is sufficient for a positive diagnosis. Due to the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a laboratory that meets the OIE requirements for Containment Group 4 pathogens.*

Complement fixation (CF) has been replaced in most laboratories by the enzyme-linked immunosorbent assay (ELISA), as it is more specific and sensitive and is not affected by pro- or anti-complement factors. If the sample is inadequate or the diagnosis remains uncertain, sample materials should be inoculated on to susceptible cell cultures or into 2–7-day old unweaned mice to amplify any live virus that may be present. The cultures should preferably be of primary bovine (calf) thyroid, but pig, lamb or calf kidney cells, or cell lines of comparable sensitivity may be used. Once a cytopathic effect (CPE) is complete in the cultures, the fluids can be used in CF tests ELISAs or by reverse transcription polymerase chain reaction (RT-PCR). Similar tests can be performed on homogenised suspensions of the dissected musculo-skeletal tissues of any mice that die.

Nucleic acid recognition tests, such as the RT-PCR are being used increasingly as rapid and sensitive diagnostic methods. Electron microscopic examination of lesion material is sometimes used to differentiate FMD from disease caused by other viruses.

Serological tests: *The demonstration of specific antibodies to structural proteins in nonvaccinated animals, where a vesicular condition is present, is sufficient for a positive diagnosis. This is particularly useful in mild cases or where epithelial tissue cannot be collected. Tests for antibodies to some NSPs of FMD virus are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins, are highly*

conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted.

*Virus neutralisation (*VN*) tests and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VN tests depend on tissue cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for antibodies have the advantage of being faster, and are not dependent on cell cultures. The ELISA can be performed with inactivated antigens, thus requiring less restrictive biocontainment facilities.*

Requirements for vaccines and diagnostic biologicals: *Inactivated virus vaccines of varying composition are available commercially. Typically, virus is used to infect a suspension or monolayer cell culture and the resulting preparation is clarified, inactivated with ethyleneimine and blended with adjuvant. Many FMD vaccines are multivalent to provide cover against the different serotypes likely to be encountered in a given field situation.*

The finished vaccine must be shown to be free from residual live virus. This is most effectively done using in-vitro *tests on concentrated inactivated virus preparations prior to formulation of the vaccine and freedom from live virus is subsequently confirmed during* in-vivo *and/or* in-vitro *tests on the finished product. Challenge tests are also conducted in vaccinated cattle to establish a PD_{50} (50% protective dose) value or protection against generalised foot infection (PGP), although a serological test is considered to be satisfactory where a valid correlation between the amount of antigen present in the vaccine, the observed protection, and the specific antibody response has been established.*

FMD vaccine production facilities should also meet the OIE requirements for Containment Group 4 pathogens.

*Diagnostic and reference reagents are available from the OIE Reference Laboratories for FMD or the FAO (*Food and Agriculture Organization of the United Nations*) World Reference Laboratory for FMD. The Institute for Animal Health Pirbright Laboratory has dual designations as both the FAO World Reference Laboratory and as an OIE Reference Laboratory for FMD.*

A. INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of FMD virus, namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven-hoofed animals. Infection with any one serotype does not confer immunity against another. Within serotypes, many strains can be identified by biochemical and immunological tests.

In Africa, FMD viruses are maintained by cattle and African buffalo (*Syncerus caffer*) and they are usually the most common host. Available evidence indicates that although other domestic and wild species become infected, they are unable to maintain the infection for more than a few months in the absence of cattle or African buffalo. Elsewhere in the world cattle are usually the main reservoir, although in some instances the viruses involved appear to be specifically adapted to domestic pigs or sheep and goats. It is probable that these adapted viruses are able to modify their adaptation and affect other species if given the opportunity. However, the pig-adapted Cathay strain of FMD virus apparently does not infect large ruminants in the field or experimentally and requires cells of porcine origin for primary isolation. Wildlife outside Africa has not, so far, been shown to be able to maintain FMD viruses. The evidence indicates that infection of deer in the past was derived from contact, direct or indirect, with infected domestic animals.

Of the domesticated species, cattle, pigs, sheep, goats and buffalo are susceptible to FMD (30). In addition, many species of cloven-hoofed wildlife, such as deer, antelope and wild pigs may become infected, although, apart from the African buffalo they have not been shown to play a significant role in the epidemiology of FMD. Strains of FMD virus that infect cattle have been isolated from wild pigs and deer. For the diagnosis of FMD in wild species, procedures similar to those described for farm animals can be applied.

Infection of susceptible animals with FMD virus leads to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. Coronary band lesions may give rise to growth arrest lines whose progress down the side of the hoof can be used to indicate the time since infection occurred. In severe infections of the feet, hooves may be shed. Mastitis is a common sequel of FMD in dairy cattle. Vesicles can also occur at other sites, such as inside the nostrils and at pressure points on the limbs – especially in pigs. The severity of clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and its degree of immunity (44). The signs can range from a mild or inapparent infection to one that

is severe. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals: myositis may also occur in other sites.

On premises with a history of sudden death in young cloven-hoofed livestock, close examination of adult animals may often reveal the presence of vesicular lesions if FMD is involved. The presence of vesicles in fatal cases is variable.

In animals with a history of vesicular disease, the detection of FMD virus in samples of vesicular fluid, epithelial tissue, oesophageal–pharyngeal (OP) sample, milk, or blood is sufficient to establish a diagnosis. Diagnosis may also be established by the isolation of FMD virus from the blood, heart or other organs of fatal cases. A myocarditis may be seen macroscopically (the so-called "tiger heart") in a proportion of fatal cases.

FMD virus can replicate and be excreted from the respiratory tract of animals. Airborne excretion of virus occurs during the acute phase of infection. FMD viruses may occur in all the secretions and excretions of acutely infected animals including expired air. Transmission is generally effected by direct contact between infected and susceptible animals or, more rarely, exposure of susceptible animals to the excretions and secretions of acutely infected animals. Following recovery from the acute stage of infection, infectious virus disappears from all secretions and excretions with the exception of OP fluids from some ruminants, where live virus may continue to be recovered. Animals in which the virus persists in the OP for more than 28 days after infection are referred to as carriers. Pigs do not become carriers. Circumstantial evidence indicates, particularly in the African buffalo, that carriers are able, on rare occasions, to transmit the infection to susceptible animals with which they come in close contact: the mechanism involved is unknown. The carrier state in cattle usually does not persist for more than 6 months, although in a small proportion it may last up to 3 years. In African buffalo individual animals have been shown to harbour the virus for at least 5 years, but it is probably not a lifelong phenomenon. Within a herd of buffalo, the virus may be maintained for 24 years or longer. There is no information on the duration of the carrier state in another domestic buffalo, the swamp buffalo of East Asia. Domestic buffalo, sheep and goats do not usually carry FMD viruses for more than a few months.

Due to the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a facility that meets the requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE FMD Reference Laboratory. Vaccine production facilities should also meet the requirements for Containment Group 4 pathogens.

Diagnostic and standard reagents are available in kit form or as individual items from OIE Reference Laboratories for FMD. The use of inactivated antigens in the enzyme-linked immunosorbent assay (ELISA), as controls in the antigen-detection test or to react with test sera in the liquid-phase blocking or solid-phase competitive ELISA, reduces the disease security risk involved compared to the use of live virus. Reagents are supplied freeze-dried or in glycerol or non-glycerinated but frozen and can remain stable at temperatures between +1°C and +8°C, –30°C and –5°C and –90°C and –50°C, respectively, for many years. The International Atomic Energy Agency has produced a manual that includes a recommended test and quality control protocols.

B. DIAGNOSTIC TECHNIQUES

For laboratory diagnosis, the tissue of choice is epithelium or vesicular fluid. Ideally, at least 1 g of epithelial tissue should be collected from an unruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet. To avoid injury to personnel collecting the samples, as well as for animal welfare reasons, it is recommended that animals be sedated before any samples are obtained.

Epithelial samples should be placed in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, preferably with added antibiotics (penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], mycostatin [100 IU]). If 0.04 M phosphate buffer is not available, tissue culture medium or phosphate buffered saline (PBS) can be used instead, but it is important that the final pH of the glycerol/buffer mixture be in the range pH 7.2–7.6. FMD virus is extremely labile in low pH and buffering of the transport media is critical for successful sample collection. Samples should be kept refrigerated or on ice until received by the laboratory.

Where epithelial tissue is not available from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of OP fluid can be collected by means of a probang (sputum) cup (or in pigs by swabbing the throat) for submission to a laboratory for virus isolation or reverse-transcription polymerase chain reaction (RT-PCR). Viraemia may also be detected by examining serum samples by means of RT-PCR or virus isolation. For the collection of throat swabs from pigs, the animal should be held on its back in a wooden cradle with the neck extended. Holding a swab in a suitable instrument, such as an artery forceps, the swab is pushed to the back of the mouth and into the pharynx.

Before the collection of OP samples from cattle or large ruminants (e.g. buffaloes), 2 ml transport fluid (composed of 0.08 M phosphate buffer containing 0.01% bovine serum albumin, 0.002% phenol red, antibiotics [1000 units/ml penicillin, 100 units/ml mycostatin, 100 units/ml neomycin, and 50 units/ml polymyxin], and adjusted to pH 7.2) should be added to a container of around 5 ml capacity capable of withstanding freezing above solid carbon dioxide (dry ice) or liquid nitrogen (39).

An OP sample is collected by inserting a probang over the tongue into the oro-pharyngeal area and then passing it vigorously backwards and forwards 5–10 times between the first portion of the oesophagus and the back of the pharynx. The purpose is to collect oro-pharyngeal fluid and especially superficial epithelial cells from these areas, including the proximal part of the oesophagus, the walls of the pharynx, the tonsillar crypts and the surfaces of the soft palate. If the sample does not contain adequate cellular debris the actions may be repeated.

After collection of OP fluid by probang, the contents of the cup should be poured into a wide-necked transparent bottle of around 20 ml capacity. The fluid is examined, and should contain some visible cellular material. Of this, 2 ml is then added to the 2 ml of transport fluid, ensuring that cellular material is transferred; the mixture is shaken gently and should have a final pH of around pH 7.6. Samples contaminated with ruminal contents may be unsuitable for culture. Samples seen to contain blood are not entirely satisfactory. Repeat sampling can be done after the mouth and throat of the animal have been rinsed with water or PBS. Where several animals are to be sampled the probang must be cleaned and disinfected between each animal. This is done by washing the probang in tap water, then immersing it in a suitable disinfectant (e.g. 0.5% [w/v] citric acid in tap water) and then rinsing off the disinfectant well with water before sampling the next animal.

OP samples from small ruminants are collected by putting 2 ml of transport fluid into a wide-necked bottle of about 20 ml capacity and, after collection, rinsing the probang cup in this transport fluid to discharge the OP sample. This is then transferred to a container of about 5 ml capacity for transport. The small container should be capable of withstanding freezing above solid carbon dioxide or liquid nitrogen (39).

Samples of OP fluid should be refrigerated or frozen immediately after collection. If they are to remain in transit for more than a few hours, they should preferably be frozen by being placed either above solid carbon dioxide or liquid nitrogen. Before freezing, the containers should be carefully sealed using airtight screw caps or silicone. This is particularly important when using solid carbon dioxide, as introduction of CO_2 into the OP sample will lower its pH, inactivating any FMD virus that may be in the samples. Glass containers should not be used because there is a risk that they will explode on defrosting in the event of liquid nitrogen leaking into them. Samples should reach the laboratory preferably in a frozen state or, if this is not feasible, under refrigeration.

Special precautions are required when sending perishable suspect FMD material both within and between countries. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) has explicit requirements for packaging and shipment of diagnostic specimens by all commercial means of transport. These are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

1.   Identification of the agent

A range of sample types including epithelium, OP samples and serum may be examined by virus isolation or RT-PCR. By contrast, ELISA is suited to the examination of epithelial suspensions, vesicular fluids or cell culture supernatants, but is insufficiently sensitive for the direct examination of OP samples or serum.

a)   Virus isolation

The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000 *g* for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMD virus are inoculated onto cell cultures or into unweaned mice. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IB-RS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity (19). The sensitivity of any cells used should be tested with standard preparations of FMD virus. The use of IB-RS-2 cells aids the differentiation of swine vesicular disease (SVD) from FMD (as SVD virus will only grow in this cell type) and is often essential for the isolation of porcinophilic strains, such as O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. Unweaned mice are an alternative to cell cultures and should be 2–7 days old and of selected inbred strains. Some field viruses may require several passages before they become adapted to mice (58). In the case of OP fluids, pre-treatment with an equal volume of chloro- fluoro- carbons may improve the rate of virus detection by releasing virus from immune complexes.

b)   Immunological methods

- **Enzyme-linked immunosorbent assay**

The preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (28, 53). This is an indirect sandwich test in which different rows in multiwell plates are coated with rabbit antisera to each of the seven serotypes of FMD virus. These are the 'capture' sera. Test sample suspensions are added to each of the rows, and appropriate controls are also included. Guinea-pig antisera to each of the serotypes of FMD virus are added next, followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents. A colour reaction on the addition of enzyme substrate and chromogen indicates a positive reaction. With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at an appropriate wavelength. In this case, an absorbance reading greater than 0.1 above background indicates a positive reaction; the serotype of FMD virus can also be identified. Values close to 0.1 should be confirmed by retesting or by amplification of the antigen by tissue culture passage and testing the supernatant once a CPE has developed. A suitable protocol is given below. Other protocols are available with slightly different formats and interpretation criteria (3, 6).

Depending on the species affected and the geographical origin of samples, it may be appropriate to simultaneously test for SVD virus or vesicular stomatitis (VS) virus. Ideally a complete differential diagnosis should be undertaken in all vesicular conditions.

Rabbit antiserum to the 146S antigen of each of the seven serotypes of FMD virus (plus SVD virus or VS virus if required) is used as a trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Control antigens are prepared from selected strains of each of the seven types of FMD virus (plus SVD virus or VS virus if appropriate) grown on monolayer cultures of BHK-21 cells (IB-RS-2 cells for SVD or VS virus). The unpurified supernatants are used and pretitrated on ELISA plates. The final dilution chosen is that which gives an absorbance at the top of the linear region of the titration curve (optical density approximately 2.0), so that the five-fold dilutions of the control antigens used in the test give two additional lower optical density readings from which the titration curve can be derived. PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST).

Guinea-pig antisera prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes of FMD virus (plus SVD virus if required) and preblocked with normal bovine serum (NBS) is used as the detecting antibody. Predetermined optimal concentrations are prepared in PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used at a predetermined optimum concentration in PBSTM. As an alternative to guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody.

- **Test procedure**

i)   ELISA plates are coated with 50 µl/well rabbit antiviral sera in 0.05 M carbonate/bicarbonate buffer, pH 9.6. Rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVD virus or VS virus (optional).

ii)   Leave overnight at 4°C in a stationary position or place on an orbital shaker set at 100–120 revolutions per minute in a 37°C incubator for 1 hour.

iii) Prepare test sample suspension (with 10% original sample suspension or undiluted clarified cell culture supernatant fluid).

iv)   The ELISA plates are washed five times in PBS.

v)   On each plate, load wells of columns 4, 8 and 12 with 50 µl PBST. Additionally, add 50 µl of PBST to wells 1, 2 and 3 of rows A to H on plate 1. To well 1 of row A of plate 1 add 12.5 µl of control antigen type O, to well 1 of row B add 12.5 µl of control antigen type A; continue in this manner for control antigen of types C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VS (if appropriate) in order to well 1, rows C to H. Mix diluent in well 1 of rows A to H and transfer 12.5 µl from well 1 to 2 (rows A to H), mix and transfer 12.5 µl from well 2 to 3, mix and discard 12.5 µl from well 3 (rows A to H) (this gives a five-fold dilution series of each control antigen). It is only necessary to change pipette tips on the micropipette between antigens. The remainder of the plate can be loaded with the test sample(s). Add 50 µl of sample one to wells 5, 6 and 7 of rows A to H, the second sample is placed similarly in columns 9, 10 and 11, rows A to H.

If more than two samples are to be tested at the same time, the other ELISA plates should be used as follows:

Dispense 50 µl of the PBST to the wells (rows A to H) of columns 4, 8 and 12 (buffer control columns). Note that the control antigens are not required on these plates. These test samples may be added in 50 µl volumes in rows A to H to columns 1, 2, 3; 5, 6, 7; 9, 10, 11, respectively.

vi) Cover with lids and place on an orbital shaker at 37°C for 1 hour.

vii) Wash the plates by flooding with PBS – wash three times as before and empty residual wash fluid. Blot the plates dry.

viii) Transfer 50 µl volumes of each guinea-pig serum dilution to each plate well in the appropriate order, e.g. rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVD virus or VS virus (optional).

ix) Cover the plates with lids and replace on the orbital shaker. Incubate at 37°C for 1 hour.

x) The plates are washed again three times, and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

xi) The plates are washed again three times, and 50 µl of substrate solution, containing 0.05% % H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

xii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1.25 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

- **Complement fixation test**

In general, the ELISA is preferable to the complement fixation (CF) test because it is more sensitive and it is not affected by pro- or anti-complementary factors. If ELISA reagents are not available, the CF test may be performed as follows:

Antisera to each of the seven types of FMD virus are diluted in veronal buffer diluent (VBD) in 1.5-fold dilution steps from an initial 1/16 dilution to leave 25 µl of successive antiserum dilutions in U-shaped wells across a microtitre plate. To these are added 50 µl of 3 units of complement, followed by 25 µl of test sample suspension(s). The test system is incubated at 37°C for 1 hour prior to the addition of 25 µl of 1.4% standardised sheep red blood cells (SRBC) in VBD sensitised with 5 units of rabbit anti-SRBC. The reagents are incubated at 37°C for a further 30 minutes and the plates are subsequently centrifuged and read. Appropriate controls for the test suspension(s), antisera, cells and complement are included. CF titres are expressed as the reciprocal of the serum dilution producing 50% haemolysis. A CF titre ≥36 is considered to be a positive reaction. Titre values of 24 should be confirmed by retesting an antigen that has been amplified through tissue culture passage.

c) Nucleic acid recognition methods

RT-PCR can be used to amplify genome fragments of FMD virus in diagnostic materials including epithelium, milk, serum and OP samples (7, 13). RT combined with real-time PCR has a sensitivity comparable to that of virus isolation (2, 51) and automated procedures enhance sample throughput (52). Specific primers have been designed to distinguish between each of the seven serotypes. *In situ* hybridisation techniques have been developed for investigating the presence of FMD virus RNA in tissue samples (63). These techniques are only in use in specialised laboratories, although simplified systems for potential field-use are under development (18).

- **Agarose gel-based RT-PCR assay**

The procedure used at the OIE Reference Laboratory at Pirbright is described (50). The RT-PCR assay consists of the three successive procedures of (i) extraction of template RNA from the test or control sample followed by (ii) RT of the extracted RNA, (iii) PCR amplification of the RT product and (iv) detection of the PCR products by agarose gel electrophoresis.

- **Test procedure**

i) Add 200 µl of test sample to 1 ml of TRIzol® Reagent in a sterile tube. Store at –70°C until required for RNA extraction.

ii) Transfer 1 ml of the solution from i) into a fresh, sterile tube containing 200 µl of chloroform. Vortex mix for about 10–15 seconds and leave at room temperature for 3 minutes.

iii) Centrifuge for 15 minutes at 20,000 **g**.

iv) Transfer 500 µl of the aqueous phase into a fresh, sterile tube containing 1 µl of glycogen (20 mg/ml) and add 500 µl of iso-propyl-alcohol (propan-2-ol). Vortex mix for a few seconds.

v) Leave at room temperature for 10 minutes then centrifuge for 10 minutes at 20,000 **g**.

vi) Discard the supernatant fluid from each tube and add 1 ml of 70% ethanol. Vortex mix for a few seconds.

vii) Centrifuge for 10 minutes at 20,000 **g**.

viii) Carefully remove the supernatant fluid from each tube taking care not to dislodge or lose any pellet at the bottom of the tube.

ix) Air dry each tube at room temperature for 2–3 minutes.

x) Re-suspend each pellet by adding 20 µl of nuclease-free water to the tube.

xi) Keep the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at –70°C.

xii) For each sample to be assayed, add 2 µl of random hexamers (20 µg/ml) and 5 µl of nuclease-free water into a sterile 0.5 ml microcentrifuge tube. It is recommended to prepare the dilution in bulk for the total number of samples to be assayed but allowing for one extra sample.

xiii) Add 5 µl of RNA from the extraction procedure described above to give a volume of 12 µl in each tube. Mix by gently pipetting up and down.

xiv) Incubate at 70°C for 5 minutes.

xv) Cool at room temperature for 10 minutes.

xvi) During the 10-minute incubation period, prepare the RT reaction mixture described below for each sample. Prepare the reaction mixture in bulk in a sterile 1.5 ml microcentrifuge tube for the number of samples to be assayed plus one extra sample.

First strand buffer, 5× conc. (4 µl); bovine serum albumin (acetylated), 1 mg/ml (2 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); DTT, 1 M (0.2 µl); Moloney Murine Reverse Transcriptase, 200 U/ µl (1 µl).

xvii) Add 8 µl reaction mix to the 12 µl of random primer/RNA mix. Mix by gently pipetting.

xviii) Incubate at 37°C for 45 minutes.

xix) Keep the RT products on ice if the PCR amplification step is about to be performed, otherwise store at –20°C.

xx) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (35 µl); PCR reaction buffer, 10× conc (5 µl); MgCl$_2$, 50 mM (1.5 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer 1, 10 pmol/µl (1 µl); primer 2, 10 pmol/µl (1 µl); *Taq* Polymerase, 5 units/µl (0.5 µl).

xxi) Add 45 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample to be assayed followed by 5 µl of the RT product to give a final reaction volume of 50 µl.

xxii) Spin the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

xxiii) Place the plate in a thermal cycler for PCR amplification and run the following programme:

94°C for 5 minutes: 1 cycle;

94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes: 30 cycles;

72°C for 7 minutes: 1 cycle.

xxiv) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.5% agarose gel. After electrophoresis a positive result is indicated by the presence of a 328 bp band corresponding to FMDV sequence in the 5' untranslated region of the genome.

- **Stock solutions**

i) Nuclease-free water, TRIzol® Reagent, chloroform, glycogen, iso-propyl-alcohol (propan-2-ol), ethanol, random hexanucleotide primers, First strand buffer, BSA (acetylated), dNTPs, DTT, Moloney Murine Reverse Transcriptase, PCR reaction buffer (10×), MgCl$_2$ and *Taq* Polymerase are commercially available.

ii) Primers at a concentration of 10 pmol/µl: Primer 1 sequence 5'-GCCTG-GTCTT-TCCAG-GTCT-3' (positive strand); Primer 2 sequence 5'-CCAGT-CCCCT-TCTCA-GATC-3' (negative strand).

- **Real-time RT-PCR assay**

The procedure used at the OIE Reference Laboratory at Pirbright is described. The real-time RT-PCR assay uses the same procedures of extraction of total RNA from the test or control sample followed by RT of the

extracted RNA as for the conventional agarose gel-based procedure. Automated extraction of total nucleic acid from samples followed by automated pipetting programmes for the RT and PCR steps (52) can be used as an alternative to the manual procedures described above. PCR amplification of the RT product is performed by a different procedure. Detection of the PCR products in agarose gels is not required following real-time amplification.

i)   Take the RT products from step xix (see above).

ii)   Prepare the PCR mix described below for each sample. Again it is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample: nuclease-free water (6 µl); PCR reaction master mix, 2× conc. (12.5 µl); real-time PCR forward primer, 10 pmol/µl (2.25 µl); real-time PCR reverse primer, 10 pmol/µl (2.25 µl); TaqMan® probe, 5 pmol/µl (1 µl).

iii)   Add 24 µl PCR reaction mix to a well of a real-time PCR plate for each sample to be assayed followed by 1 µl of the RT product to give a final reaction volume of 25 µl.

iv)   Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

v)   Place the plate in a real-time PCR machine for PCR amplification and run the following programme:

        50°C for 2 minutes: 1 cycle;

        95°C for 10 minutes: 1 cycle;

        95°C for 15 seconds, 60°C for 1 minute: 50 cycles.

vi)   *Reading the results:* Assign a threshold cycle (CT) value to each PCR reaction from the amplification plots (a plot of the fluorescence signal versus cycle number; different cut-off values may be appropriate for different sample types; 51). The CT values used to assign samples as either FMDV positive or negative should be defined by individual laboratories using appropriate reference material. For example at the OIE Reference Laboratory at Pirbright, negative test samples and negative controls should have a CT value at >50.0. Positive test samples and positive control samples should have a CT value <40. Samples with CT values falling within the range 40–50 are designated "borderline" and can be re-tested. Strong positive FMD samples have a CT value below 20.0 (51).

- **Stock solutions for real-time PCR assay**

i)   Nuclease-free water and real-time PCR reaction master mixes are available from commercial suppliers.

ii)   Either of the two following primers and probe sets can be used for real-time PCR of FMDV:

        5'UTR (51) Forward primer: CACYT YAAGR TGACA YTGRT ACTGG TAC; Reverse primer: CAGAT YCCRA GTGWC ICITG TTA and TaqMan® probe: CCTCG GGGTA CCTGA AGGGC ATCC.

        3D (18) Forward primer: ACTGG GTTTT ACAAA CCTGT GA; Reverse primer: GCGAG TCCTG CCACG GA and TaqMan® probe: TCCTT TGCAC GCCGT GGGAC.

- **Molecular epidemiology**

The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene (encoding the VP1 viral protein) have been published. Reverse-transcription PCR (RT-PCR) amplification of FMD virus RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. Many laboratories have developed techniques for performing these studies, and reference laboratories hold databases containing over 3000 partial sequences.

The recommended method is to:

i)   Extract FMD virus RNA directly from epithelial suspensions or from a low cell culture passage.

ii)   Perform an RT-PCR of the complete 1D gene (or if only part of the 1D gene, then the 3' end of the gene is more useful).

iii)   Determine the nucleotide sequence of the PCR product (or at least 170 nucleotides [preferably 420 for the SAT types] at the 3' end of the gene).

A protocol, complete with primer sequences, is available from the OIE Reference Laboratories on request or can be downloaded from the following World Wide Web URLs:

http://www.iah.bbsrc.ac.uk/virus/picornaviridae/aphthovirus/fmd.htm

http://bvs.panaftosa.org.br/textoc/SerManDid17.pdf

2. Serological tests

Serological tests for FMD are performed in support of four main purposes namely: 1) to certify individual animals prior to import or export (i.e. for trade); 2) to confirm suspected cases of FMD; 3) to substantiate absence of infection; 4) to demonstrate the efficacy of vaccination. For substantiating freedom from infection, different approaches are required according to whether the population has been vaccinated or not and if vaccination has been used, whether this has been applied as an emergency application or as part of an ongoing programme of vaccination. Different tests and different interpretations of test results will be appropriate according to the above-mentioned purposes and the validation of the selected procedure must take account of the purpose. For example, test cut-offs may be set at a different threshold for herd-based serosurveillance than is appropriate for certifying freedom from infection for individual animals for the purposes of international trade.

Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs).

The SP tests are serotype-specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralisation (VN) test (32), the solid-phase competition ELISA (SPCE; 41, 46) and the liquid-phase blocking ELISA (LPBE; 35, 36). These tests are serotype-specific and are highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field. They are the prescribed tests for trade and are appropriate for confirming previous or ongoing infection in non-vaccinated animals as well as for monitoring the immunity conferred by vaccination in the field. The VN test requires cell culture facilities, the use of live virus and takes 2–3 days to provide results. The ELISA tests are blocking- or competition-based assays that use serotype-specific polyclonal or monoclonal antibodies, are quicker to perform and are not dependent on tissue culture systems and the use of live viruses. Low titre false-positive reactions can be expected in a small proportion of the sera in either ELISA test. An approach combining screening by ELISA and confirming the positives by the VN test minimises the occurrence of false-positive results. Reference sera to standardise FMD SP serological tests for some serotypes and subtypes are available from the Reference Laboratory at Pirbright.

The detection of antibody to the NSPs of FMD virus can be used to identify past or present infection with any of the seven serotypes of the virus, whether or not the animal has also been vaccinated. Therefore the tests can be used to confirm suspected cases of FMD and to detect viral activity or to substantiate freedom from infection on a population basis. For certifying animals for trade, the tests have the advantage over SP methods that the serotype of virus does not have to be known. However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results (17). These assays measure antibody to NSPs using antigens produced by recombinant techniques in a variety of *in-vitro* expression systems. Antibody to the polyproteins 3AB or 3ABC are generally considered to be the most reliable indicators of infection (42). In animals seropositive for antibody to 3AB or 3ABC, antibody to one or more of the other NSPs can aid in the final interpretation of the test (14, 42). However, lack of vaccine purity may affect diagnostic specificity as the presence of NSPs in some vaccine preparations may result in misclassification in animals that have been repeatedly vaccinated. Procedures for evaluating vaccine purity are covered in Section D of this chapter.

International standard sera for NSP testing of cattle have been developed and are available from the OIE Reference Laboratory, Panaftosa, PAHO/WHO. In the future, standard sera will also be made available for sheep and pigs. Bovine serum panels have been established to compare the sensitivity of NSP tests at OIE Reference Laboratories.

a) Virus neutralisation test (a prescribed test for international trade)

The quantitative VN microtest for FMD antibody is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue-culture grade microtitre plates.

Stock virus is grown in cell monolayers and stored at –20°C after the addition of 50% glycerol. (Virus has been found to be stable under these conditions for at least 1 year.) The sera are inactivated at 56°C for 30 minutes before testing. The control standard serum is 21-day convalescent or post-vaccination serum. A suitable medium is Eagle's complete medium/LYH (Hank's balanced salt solution with yeast lactalbumin hydrolysate) with hepes buffer and antibiotics.

The test is an equal volume test in 50 µl amounts.

- **Test procedure**

i) Starting from a 1/4 dilution, sera are diluted in a twofold, dilution series across the plate, using at least two rows of wells per serum , preferably four rows, and a volume of 50 µl.

ii)   Previously titrated virus is added; each 50 µl unit volume of virus suspension should contain about 100 $TCID_{50}$ (50% tissue culture infective dose) within an accepted range (e.g. 32–320 $TCID_{50}$).

iii) Controls include a standard antiserum of known titre, a negative serum, a cell control, a medium control, and a virus titration used to calculate the actual virus titre used in the test.

iv)   Incubate at 37°C for 1 hour with the plates covered.

v)   A cell suspension at 10^6 cells/ml is made up in medium containing 10% bovine serum (specific antibody negative) for cell growth. A volume of 50 µl of cell suspension is added to each well.

vi)   Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 3–5% carbon dioxide at 37°C for 2–3 days.

vii) Microscope readings may be feasible after 48 hours. The plates are finally fixed and stained routinely on the third day. Fixation is effected with 10% formol/saline for 30 minutes. For staining, the plates are immersed in 0.05% methylene blue in 10% formalin for 30 minutes. An alternative fixative/stain solution is naphthalene blue black solution (0.4% [w/v] naphthalene blue black, 8% [w/v] citric acid in saline). The plates are rinsed in tap water.

viii) Positive wells (where the virus has been neutralised and the cells remain intact) are seen to contain blue-stained cells sheets; the negative wells (where virus has not been neutralised) are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected (38). The test is considered to be valid when the amount of virus used per well is in the range log_{10} 1.5–2.5 $TCID_{50}$, and the positive standard serum is within twofold of its expected titre.

ix)   Interpretation of tests can vary between laboratories in regard to the negative/positive cut-off threshold. Laboratories should establish their own criteria by reference to standard reagents that can be obtained from the OIE Reference Laboratory at Pirbright. In general, a titre of 1/45 or more of the final serum dilution in the serum/virus mixture is regarded as positive. A titre of less than 1/16 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of 1/16 to 1/32 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/16 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/45 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

b) Solid-phase competition enzyme-linked immunosorbent assay (a prescribed test for international trade)

Rabbit antiserum to the 146S antigen of one of the seven types of FMD virus is used as the trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Antigens are prepared by inactivating viruses propagated in cell culture with ethyleneimine using the procedures described for vaccine manufacture. The final dilution chosen is that which, after addition of an equal volume of diluent, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20, 10% normal bovine serum and 5% normal rabbit serum and phenol red indicator is used as a diluent (blocking buffer).

Guinea-pig antisera, prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes and preblocking with normal bovine serum, is used as the detecting antibody. Predetermined optimal concentrations are prepared in blocking buffer PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used as conjugate at a predetermined optimum concentration in PBSTM blocking buffer.

Test sera are diluted in PBST blocking buffer.

The solid-phase competitive ELISA is more specific but as sensitive as the liquid-phase blocking ELISA (41, 46). Methods have been described for the development of secondary and working standard sera (33) and for charting assay performance (34).

- **Test procedure**

i)   ELISA plates are coated with 50 µl/well rabbit anti-FMD virus antigen diluted in carbonate/bicarbonate buffer, pH 9.6, and left overnight in a humid chamber at 4°C.

ii)   The ELISA plates are washed three times with PBS.

iii) Then 50 µl of the FMD virus antigen diluted in blocking buffer is added to each well of the ELISA plates. (Blocking buffer: 0.05% [w/v] Tween 20, 10% [v/v] normal bovine serum, 5% [v/v] normal rabbit serum.) The plates are covered and placed on an orbital shaker at 37°C for 1 hour, with continuous shaking.

iv) After washing three times with PBS, 40 µl of blocking buffer is added to each well, followed by 10 µl of test sera (or control sera), giving an initial serum dilution of 1/5.

v) Immediately 50 µl of guinea-pig anti-FMD virus antiserum diluted in blocking buffer is added, giving a final serum dilution of 1/10.

vi) The plates are covered and incubated on an orbital shaker at 37°C for 1 hour.

vii) After washing three times with PBS, 50 µl of anti-guinea-pig Ig conjugate (pre-blocked by incubation for 1 hour at room temperature with an equal volume of NBS) diluted in blocking buffer is added. The plates are covered and incubated for 1 hour at 37°C on an orbital shaker.

viii) After washing three times with PBS, 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

ix) The reaction is stopped after 10 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

x) *Controls:* On each plate two wells are used for conjugate control (no guinea-pig serum), four wells each for strong and weak positive sera, two wells for negative sera, and four wells for 0% competition (no test sera).

xi) *Interpretation of the results:* A percentage of inhibition is calculated for each well, either manually or using a suitable computer programme (100 – [optical density of each test or control value/mean optical density of the 0% competition] × 100%), representing the competition between the test sera and the guinea-pig anti-FMD virus antisera for the FMD virus antigen on the ELISA plate. Laboratories should validate the assay in terms of the cut-off value above which sera should be considered positive in relation to (i) the particular serotypes and strains of virus under investigation (ii) the purpose of testing (iii) the population under test, using the methods described in Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases. At the OIE Reference Laboratory at Pirbright, for serotype O, for all species, for the purposes of demonstrating freedom from infection in a naïve population, greater than 60% inhibition is considered positive (46). For maximum sensitivity, for example when certifying individual animals for international trade, an inconclusive range may be set between 40 and 60%.

c) **Liquid-phase blocking enzyme-linked immunosorbent assay (a prescribed test for international trade)**

Antigens are prepared from selected strains of FMD virus grown on monolayers of BHK-21 cells. The unpurified supernatants are used and pretitrated in a twofold dilution series but without serum. The final dilution chosen is that which, after addition of an equal volume of diluent (see below), gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST). The other reagents used in the test are the same as those in the solid-phase blocking ELISA. An example of the test procedure is described below. Temperature and incubation times can vary depending on the protocol.

• **Test procedure**

i) ELISA plates are coated with 50 µl/well rabbit antisera to the 14S antigen being tested for and left overnight in a humid chamber at room temperature.

ii) The ELISA plates are washed three times with PBS.

iii) In U-bottomed multiwell plates (carrier plates) 50 µl of a duplicate, twofold series of each test serum is prepared, starting at 1/8. To each well, 50 µl of a constant dose of viral antigen that is homologous to the rabbit antisera used to coat the plates is added and the mixtures are left overnight at 4°C, or incubated at 37°C for 1 hour. The addition of the antigen increases the final serum dilution to 1/16.

iv) Then 50 µl of serum/antigen mixtures is transferred from the carrier plates to the rabbit-serum coated ELISA plates and the plates are incubated at 37°C for 1 hour on a rotary shaker.

v) After washing, 50 µl of guinea-pig antiserum homologous to the viral antigen used in the previous step (iv) (pre-blocked with normal bovine serum and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are then incubated at 37°C for 1 hour on a rotary shaker.

vi) The plates are washed and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase (pre-blocked with normal bovine serum and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

vii) The plates are washed again three times and 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

viii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

ix) *Controls:* A minimum of four wells each of strong positive, weak positive and negative bovine reference sera at a final dilution of 1/32 should be included on each plate together with an equivalent number of reaction (antigen) control wells containing antigen in diluent alone without serum. For end-point titration tests, duplicate twofold dilution series of positive and negative homologous bovine reference sera should be included on at least one plate of every run.

x) *Interpretation of the results:* Antibody titres are expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera results in an optical density equal to 50% inhibition of the median optical density of the reaction (antigen) control wells (38). The median is calculated as the mean of two mid-values of the reaction control wells, eliminating from the calculation the highest and lowest values (alternatively, the mean value can be used after setting suitable tolerance limits to control for inter-well variation). In general sera with titres greater than or equal to 1/90 are considered to be positive. A titre of less than 1/40 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of greater than 1/40, but less than 1/90 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/40 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/90 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

d) Nonstructural protein antibody tests

Antibody to expressed recombinant FMD virus NSPs (e.g. 3A, 3B, 2B, 2C, 3ABC) can be measured by different ELISA formats or immunoblotting. These ELISAs either use purified antigens absorbed directly to microplates or use polyclonal or monoclonal antibodies to trap specific antigens from semi-purified preparations (14, 20, 42, 59). The index screening method used in Panaftosa is described in detail below. Other indirect and competitive ELISAs detecting bovine antibodies to 3ABC have been shown to have equivalent diagnostic performance characteristics (17). This same study corroborates preliminary data from Panaftosa that suggests that the diagnostic performance characteristics of these tests are similar in cattle, sheep and pigs.

• **Indirect enzyme-linked immunosorbent assay**

  • **Preparation of recombinant antigens (see Section B.2.d Enzyme-linked immunoelectrotransfer blot assay below)**

  • **Test procedure**

i) Microplates are coated overnight at 4°C with 1 µg/ml of the fusion antigen 3ABC in carbonate/ bicarbonate buffer, pH 9.6 (100 µl per well). Antigen 3ABC was expressed and purified as indicated for the EITB (enzyme-linked immunoelectrotransfer blot) tests (45).

ii) The plates are washed six times with PBS, pH 7.2, supplemented with 0.05% Tween 20 (PBST).

iii) Test sera (100 µl per well) are added in a 1/20 dilution in blocking buffer consisting of PBS, 0.05% Tween 20, 5% nonfat dry milk, 10% equine sera and 0.1% *Escherichia coli* lysate. Each plate includes a set of strong and weak positive and negative controls calibrated against the International Standard Sera described below.

iv) The plates are incubated for 30 minutes at 37°C and washed six times in PBST.

v) Horseradish-peroxidase-conjugated rabbit anti-species IgG is diluted optimally in the blocking buffer, added at 100 µl per well and the plates are incubated for 30 minutes at 37°C.

vi) After six washings, each well is filled with 100 µl of 3'3', 5'5'-tetramethylbenzidine plus 0.004% (w/v) H_2O_2 in phosphate/citrate buffer, pH 5.5.

vii) The reaction is stopped after 15 minutes of incubation at room temperature by adding 100 µl of 0.5 M H_2SO_4. Absorbance is read at 450 nm and at 620 nm for background correction.

viii) *Interpreting the results:* Test results are expressed as per cent positivity relative to the strong positive control [(optical density of test or control wells/optical density of strong positive control) × 100] or alternatively as a test to control (T/C) index relative to a cut-off (i.e. threshold positive) control. Profiling the NSP antibody reactivity levels in herds along with age/vaccination stratification aids interpretation of herd infection status in vaccinated populations (15). Test cut-off values, with or without suspicious zones, need to be determined with consideration to the purpose of testing and the intended target population. Inconclusive results may be followed up using confirmatory tests. In case of multi-vaccinated animals, EITB is the recommended approach, whereas, in animals that have received only one or two vaccinations, inconclusive results are resolved and positive results confirmed by retesting with a second NSP ELISA (taking account of the conditional dependence of the two tests). This must

take into account the overall test system sensitivity and specificity when designing the serosurveillance programme. Although not a prescribed test for trade, NSP ELISAs may be a valuable adjunct in circumstances where the serotype or subtype of virus in the originating country is not known.

• **Enzyme-linked immunoelectrotransfer blot assay (EITB)**

The EITB assay has been widely applied in South America as a confirmatory test for the above-described index screening method. Further information is available from the OIE Reference Laboratory, Panaftosa, PAHO/WHO.

• **Preparation of test strips containing the recombinant antigens**

i) The five bioengineered FMD virus NSPs 3A, 3B, 2C, 3D and 3ABC are expressed in *E. coli* C600 by thermo-induction. The 3D polypeptide is expressed in its complete form (45), whereas the rest of the proteins are obtained as fusions to the N-terminal part of the MS-2 polymerase gene (60).

ii) The expressed polymerase is purified over phosphocellulose, followed by poly(U) Sepharose columns. The fused proteins 3A, 3B, 2C and 3ABC are purified by sequential extraction of the bacterial extracts with increasing concentrations of urea. The 7M fraction containing the fusion proteins is further purified on a preparative 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The fusion protein band is excised from the gel and electroeluted (45).

iii) A mixture containing 20 ng/ml of each one of the purified recombinant polypeptides is separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose (45).

• **Test procedure**

i) The required amount of test strips should be assessed, taking into account that for each nitrocellulose sheet, which defines one transferred gel, a positive, a weakly positive, a cut-off and a negative control serum should be assayed. In general, 24 nitrocellulose strips, each 3 mm wide, should result from a gel.

ii) A volume of 0.8 ml of saturation buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 0.2% Tween 20; 5% nonfat dry milk; and 0.05% bacterial *E. coli* lysate) is added to each well. The antigen-coated strips are blocked by placing the trays on a rocker and agitating for 30 minutes at room temperature (20–22°C).

iii) A dilution of 1/200 of test sera and of each of the controls is added to the appropriate trough. The strips must be completely submerged and facing upwards, and maintained in that position during the whole process.

iv) Strips are incubated for 60 minutes on a rocker at room temperature.

v) Liquid is removed from the trays, and each test strip is washed three times with washing solution (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; and 0.2% Tween 20) by agitation for 5 minutes.

vi) The alkaline-phosphatase-conjugated rabbit anti-bovine solution is added to each test well, and the strips are incubated with shaking for 60 minutes at room temperature.

vii) The liquid is removed from the trays and each test strip is washed three times with washing solution as above.

viii) Substrate solution (0.015% bromochloroindolylphosphate/0.03% nitroblue tetrazolium) is prepared in substrate buffer (100 mM NaCl; 5 mM $MgCl_2$; and 100 mM Tris/HCl, pH 9.3), and is added to each test well.

ix) Strips are incubated by placing the test tray on the orbital mixer and agitating until the cut-off control shows five distinct, discernible bands. Strips are washed with running deionised water and air-dried.

x) *Interpreting the results:* The EITB may be scanned with a densitometer but visual reading, although more subjective, is considered suitable as well. Individual control sera are run that exhibit minimal but consistent staining for each of the five antigens. A test sample is considered positive if antigens 3ABC, 3A, 3B and 3D (±2C) demonstrate staining densities equal to or higher than that of their appropriate controls. A sample is considered negative if two or more antigens demonstrate densities below their control sera. Test samples not fitting either profile are considered indeterminate.

C. VACCINE MATCHING TESTS

1. Introduction

The selection of vaccine strains has recently been reviewed (48). Vaccination against one serotype of FMDV does not cross-protect against other serotypes and may also fail to protect fully or at all against other strains of

the same serotype. The most direct and reliable method to measure cross-protection is to vaccinate relevant target species and then to challenge them by exposure to the virus isolate against which protection is required. This will take account of both potency and cross-reactivity. However, such an approach is slow and expensive, and the use of animals for such studies should be avoided where possible by the use of *in vitro* alternatives.

A variety of *in vitro* serological methods can be used to quantify antigenic differences between FMDV strains and thereby estimate the likely cross-protection between a vaccine strain and a field isolate. Genetic characterisation and antigenic profiling can also reveal the emergence of new strains for which vaccine matching may be required and, conversely, may indicate that an isolate is similar to one for which vaccine matching information is already available.

Appropriate vaccine strain selection is an important element in the control of FMD and is necessary for the application of vaccination programmes in FMD-affected regions as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions.

Vaccine potency also contributes to the range of antigenic cover provided by a vaccine. A highly potent vaccine that stimulates a strong immune response may give greater protection against a heterologous virus than an equally cross-reactive vaccine that stimulates a weaker immune response. Furthermore, booster doses of vaccine can increase efficacy and the subsequent breadth of antigenic cover provided by a given vaccine, although the onset of full protection may be delayed.

2.   Selection of field viruses for vaccine matching

Serological matching of field isolates to vaccine strains requires that isolates have been serotyped and adapted to growth in cell cultures. The serotype is usually determined by ELISA or CFT using type-specific serological reagents, although methods based on monoclonal antibodies or genetic typing may also be used. BHK or IB-RS-2 cell cultures are usually used for *in vitro* virus replication. For vaccine matching, preferably, at least two isolates should be evaluated from any outbreak and inconsistent results should be followed up to determine whether this is due to genuine antigenic differences or is an artefact of testing.

Viruses can be selected based on epidemiological information, for instance isolation at different stages of an epidemic, from different geographic locations or from different hosts (4). Field evidence for a suspected lack of vaccine efficacy, as shown by reduced apparent protection, is an important criterion for vaccine matching.

Antigenic profiling by CFT or ELISA, or sequence analysis of the VP1 gene, are suitable approaches for selecting representative virus isolates for vaccine matching. Antigenic profiling is performed by CFT using panels of hyperimmune guinea-pig sera raised against epidemiologically relevant field isolates (16) or by ELISA using panels of well-characterised monoclonal antibodies (5).

3.   Selection of vaccine strains to be matched

The serotype of the virus, the region of origin and any information on the characteristics of the field isolate may give indications as to the vaccine strains most likely to provide an antigenic match. The availability of reagents for matching to particular vaccine strains may limit the extent of testing that is possible. Antigenic characterisation has two purposes; first, to chose the most effective vaccine strain for use in a particular circumstance and, second, to monitor, on an ongoing basis, the suitability of vaccine strains maintained in strategic antigen reserves.

4.   Choice of vaccine matching test

The serological relationship between a field isolate and a vaccine virus ('r' value) can be determined by CFT, ELISA or VNT (40, 49, 55). One way testing is recommended (r_1) with a vaccine antiserum, rather than two way testing (r_2) which also requires an antiserum against the field isolate to be matched. Due to the inherently low repeatability of the assays used, tests need to be repeated to be confident of the results (56). *In vitro* neutralisation may be more relevant to *in vivo* protection than other measures of virus-antibody interaction, although non-neutralising antibodies may also be protective (43). Advantages of ELISA are that the test is rapid and uses smaller volumes of post-vaccination sera, which are often available in only limited quantities. ELISA and CFT are recommended to be used as screening methods, whereas VNT or the expected percentage of protection (EPP) method provides more definitive results. For either VNT or ELISA, post-vaccination sera should be derived from at least five cattle 21–30 days after immunisation. The titre of antibody to the vaccine strain is established for each serum. Sera are used individually or pooled, after excluding low responders. The CFT method uses guinea-pig sera raised against vaccine strains.

A more thorough evaluation is provided by the EPP method (4), which measures the reactivity of a panel of post-vaccination antisera using either VNT or ELISA and relates the serological titres to the probability of protection, established through correlation tables associating antibody titres with protection against the relevant vaccine

strain. These correlation tables derive from previously performed vaccine-specific challenge tests. However, the requirement for a panel of antisera and accompanying challenge test data for the vaccine in question currently cannot be met for a wide range of vaccine strains.

a)   **Vaccine matching by ELISA**

This test uses an antiserum raised against a vaccine strain. The blocking ELISA titres of this reference serum against antigens prepared from the homologous vaccine strain and are compared with the corresponding titres of the serum against a field isolate to determine how antigenically 'similar' the field virus is to the vaccine virus.

The test procedure is similar to that of the liquid phase blocking ELISA (see Section B.2.c). Additional biological reagents are: 21–30 day post-vaccination bovine vaccine sera (inactivated at 56°C for 45–60 minutes); the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain.

• **Test procedure**

i)   Grow the field isolate and the vaccine strain in BHK or IB-RS-2 cells. The number of cell culture passages should be kept to a minimum (normally less than four) to avoid selection of antigenic variants unrepresentative of those in the original material. A sufficient quantity of virus should be present if cell cultures show CPE within 24 hours of inoculation.

ii)   Harvest and titrate the vaccine and field viruses using a panel of trapping rabbit antisera and detector guinea pig antisera raised against the same or closely related vaccine strains. If necessary, the virus antigens may be inactivated prior to use using binary ethyleneimine.

iii)   Select the optimum trapper/detector combination and the working dilution of the field virus. This should not be less than 1/6. If there is no suitable trapper/detector combination then a back-titration of the antigen stock must be performed to confirm that sufficient virus is present. If it is confirmed that the field virus is present at high tire, this indicates that none of the available vaccine strains are suitable.

iv)   Titrate 21–30 day post-vaccination serum of a chosen vaccine strain against the field isolate and the homologous vaccine strain. The titre against the vaccine strain should not fluctuate more than twofold either side of the running mean value for the virus stock.

v)   To determine the serum titre, calculate the average optical density (OD) of 24 antigen control wells without blocking serum. This represents the maximum OD value for the test, i.e. the 100% control value. Divide this by 2 to determine the 50% inhibition value. Score wells with blocking serum positive if the OD is less than or equal to 50% and negative if the OD value is greater than this. The end-point is defined as the dilution at which half of the wells show 50% inhibition or more (i.e. identify the dilution at which one out of the two duplicate wells has an OD less than 50% of the antigen control). If the end-point falls between two dilutions, it is taken as the mid-point between these dilutions as estimated by the Spearman–Kärber method.

Derive the 'r' value, the relationship between the field and the vaccine strain, as:

$$r_1 = \frac{\text{reciprocal titre of reference serum against field virus}}{\text{reciprocal titre of reference serum against vaccine virus}}$$

At least two consistent results are needed for acceptance.

vi)   *Interpretation of the results:* for r_1 values derived by ELISA the following guidelines are used for interpretation (29):

0.4–1.0: Close relationship between field isolate and vaccine strain. A potent vaccine containing the vaccine strain is likely to confer protection.

0.2–0.39: The field isolate is antigenically related to the vaccine strain. The vaccine strain might be suitable for use if no closer match can be found provided that a potent vaccine is used and animals are preferably immunised more than once.

<0.2: The field isolate is only distantly related to the vaccine strain and the vaccine strain is unlikely to protect against challenge with the field isolate.

b)   **Vaccine matching by two-dimensional neutralisation test**

This test also uses an antiserum raised against a vaccine strain. The titres of this serum against 100 $TCID_{50}$ of the homologous vaccine strain and the same dose of a field isolate are compared to determine how antigenically 'similar' the field virus is to the vaccine strain.

The procedure is similar to that of the microtitre plate virus neutralisation test (see Section B.2.a). Additional biological reagents are: 21–30 day post-vaccination bovine vaccine sera (inactivated at 56°C for 45–60 minutes); the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain.

i) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre ($\log_{10}$ $TCID_{50}$/ml) by end-point titration.

ii) For each test and vaccine virus a chequerboard titration is performed of virus against vaccine serum along with a back-titration of virus alone. Cells are added and incubated at 37°C for 48–72 hours after which time CPE is assessed.

iii) Antibody titres of the vaccine serum against the vaccine strain and field isolate for each virus dose used are calculated using the Spearman-Kärber method. The titre of the vaccine serum against 100 $TCID_{50}$ of each virus can then be estimated by regression. The relationship between the field isolate and the vaccine strain is then expressed as an 'r' value as described for vaccine matching by ELISA.

iv) *Interpretation of the results:* in the case of neutralisation, r_1 values greater than 0.3 indicate that the field isolate is sufficiently similar to the vaccine strain that use of the vaccine is likely to confer protection against challenge with the field isolate (54). Conversely, values less than 0.3 suggest that the field isolate is so different from the vaccine strain that the vaccine is unlikely to protect. In these cases, either the field isolate should be examined against alternative vaccine strains or, rarely, it will be necessary to adapt a suitable field isolate to become a new vaccine strain.

v) Tests should always be repeated more than once. The confidence with which 'r' values can be taken to indicate differences between strains is related to the number of times that the examination is repeated. In practice, a minimum of at least three repetitions is advised.

c) Vaccine matching by CFT

The relationship between a field isolate and a vaccine strain can also be determined by complement fixation using a guinea-pig antiserum raised against the relevant vaccine strain.

CFT 50% titres of this reference serum against antigens prepared from the homologous vaccine strain and a field isolate are compared to determine how antigenically 'similar' the field virus is to the homologous vaccine virus.

i) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, the virus titre that fixes 2.5 CFU_{50} (50% complement fixing units) is determined.

ii) A relationship is established by titration of the guinea-pig antisera through a twofold dilution series against 2.5 CFU_{50} of the homologous and heterologous antigens in veronal buffer diluent (VBD) or borate saline solution (BBS) placed in separate tubes. Four haemolysis units of complement are then added to each reaction.

iii) The test system is incubated at 37°C for 30 minutes prior to the addition of 2% of standardised sheep red blood cells (SRBC) in VBD or BSS sensitised with rabbit anti SRBC. Reagents are incubated at 37°C for a further 30 minutes and the tubes are subsequently centrifuged and read.

iv) The CFT 50% titres are calculated by the Spearman-Kärber method and an 'r' value is derived from the relationship between the reactivity of the field isolate and the vaccine strain, as:

$$r_1 = \frac{\text{Reciprocal titre of hyperimmune serum against field virus}}{\text{Reciprocal titre of hyperimmune serum against vaccine virus}}$$

v) *Interpretation of the results:* in the case of CFT, r_1 values greater than 0.25 indicate that the field isolate is sufficiently similar to the vaccine strain and that use of the vaccine is likely to confer protection against challenge with the field strain (3).

d) Expected percentage of protection

The expected percentage of protection (EPP) estimates the likelihood that cattle would be protected against a challenge of 10,000 infective doses after a single or boosted vaccination.

i) Individual sera are required from 16 or 30 18–24 month-old cattle at 30 days post-vaccination and 30 days post-re-vaccination, using a full dose of the vaccine strain to be matched.

ii) This panel of sera is tested for antibody titres to the homologous FMD vaccine strain and the field isolate to be matched using VNT or LPB-ELISA (see Sections B.2.a and B.2.c).

iii) If necessary, the antigens used in the ELISA may be inactivated prior to use using binary ethyleneimine.

iv) The EPP is determined from the serological titre obtained, for each individual serum, by reference to predetermined tables of correlation between serological titres and clinical protection. The mean EPP is then calculated from the EPP for each individual serum.

v) The clinical protection data are derived from previously performed experiments carried out on hundreds of cattle that have been immunised using the vaccine strain in question and challenged with a homologous virus (similar to the PGP potency tests described in Section D.4.b). Each animal is scored as protected or not and tables of correlation based on logistic regression models are established between antibody titre and clinical protection.

vi) An EPP <75% (when sera from a group of 16 re-vaccinated animals are used) and <70% (when sera from a group of 30 re-vaccinated animals are used) is an indication that the vaccines will give a low protection against the field strain (47).

D. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The control of FMD is usually a national responsibility and, in many countries, the vaccine may be used only under the control of the Competent Authority.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions in order for manufacturers to obtain an authorisation or licence for a veterinary vaccine. Where possible, manufacturers should seek to obtain such a license or authorisation for their FMD vaccines as independent verification of the quality of their product.

Virulent FMD virus must be used to produce FMD vaccine; consequently, the FMD vaccine production facility should operate under the appropriate biosecurity procedures and practices. The facility should meet the requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.2 of this *Terrestrial Manual.*

Routine vaccination against FMD is used in many countries or zones recognised as free from foot and mouth disease with vaccination and in countries where the disease is endemic. In contrast, a number of disease-free countries have never vaccinated their livestock but have preferred the use of strict movement controls and culling of infected and contact animals when outbreaks have occurred. Nevertheless, many disease-free countries maintain the option to vaccinate and have their own strategic reserves of highly concentrated inactivated virus preparations. Such antigen reserves offer the potential of supplying formulated vaccine in an 'emergency' at short notice (26).

FMD vaccines may be defined as a fixed formulation containing defined amounts (limits) of one or more chemically inactivated cell-culture-derived preparations of a seed virus strain blended with a suitable adjuvant/s and excipients. The vaccines are formulated for their specific purpose and in the case of vaccines destined for use in swine, oil adjuvants are preferred. Oil adjuvanted vaccines can also be used in ruminants and may have advantages in terms of less interference from maternal antibody and a longer duration of immunity. FMD vaccines may be classified as either 'standard' or 'higher' potency vaccines. Standard potency vaccines are formulated to contain sufficient antigen to ensure that they meet the minimum potency level required (recommended at Section D.4.b as 3 PD_{50} [50% protective dose]). Higher potency vaccines are formulated with an increased amount of antigen such that the potency is in excess of the minimum requirement to provide particular features such as a more rapid onset of immunity and a wider spectrum of immunity against relevant field viruses. Higher potency vaccines are thus well suited for emergency use. Live FMD vaccines are not acceptable due to the danger of reversion to virulence and as their use would prevent the differentiation of infected from vaccinated animals.

Because of the presence of multiple serotypes of the virus, many FMD vaccines are multivalent and it is common practice to prepare vaccines from two or more different virus strains. In certain areas, it may be advisable to include more than one virus per serotype to ensure broad antigenic coverage against prevailing viruses.

1. Seed virus management

a) Characteristics of the seed virus

Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, stability and broad antigenic spectrum (57). MSVs should be characterised and distributed by

the official control laboratories in regions where such laboratories exist; they should be selected in accordance with the epidemiological importance of each variant.

b) Method of culture

Where no suitable established vaccine strain exists, new vaccine strains are derived through the establishment of MSVs from local field isolates by adapting them to growth in suspension or monolayer cells by serial passage. In order to remove the risk of contaminating lipid-enveloped viruses, it is recommended that putative MSVs undergo a validated organic solvent treatment prior to, or during, adaptation. It is preferable to keep the number of passages in cell culture to a minimum as there is evidence of antigenic 'drift' of FMD virus during this procedure.

c) Validation as a vaccine strain

MSVs must be antigenically and, if possible genetically, characterised and proven to be pure and free from all extraneous agents listed by the appropriate licensing authorities. Homology should be established with the original candidate isolates and effectiveness against the circulating strains from which they were developed should be proven. This often encompasses a number of methods, the most reliable being *in vivo* cross protection assays. Alternatively, *in vitro* tests (preferably virus neutralisation) can also be used, which require the availability of post-vaccination sera against these master seeds. Seed viruses may be stored at –20°C if glycerinated or at a lower temperature (e.g. –70°C) if not glycerinated. Working seed viruses may be expanded in one or a few more passages from the master seed stock and used to infect the final cell culture at an approximate rate of 1 PFU (plaque-forming unit) per 100 cells. Whenever possible, the exact source of the isolate should be recorded and should include details such as the location, species and the type of material from which the virus was derived. The *in-vitro* passage history of the virus should be recorded. Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (TSEs) by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

2. Method of manufacture

The recommended method of virus propagation for antigen production is the growth of FMD virus in large-scale suspension cultures or monolayers using cell lines under sterile conditions. Primary cell culture may be acceptable for vaccine production in some countries but only if the method of production is entirely compliant with Good Manufacturing Practice, a validated procedure is applied to ensure inactivation of all possible extraneous agents and adequate in-process and final products tests are in place to ensure consistency and safety of the final product. It is essential that all pipework and vessels be thoroughly sterilised ensuring that no areas in the system harbour microorganisms. In addition to general considerations of sterility, it is important to note that the virus is vulnerable to attack by proteolytic enzymes, such as those produced by microorganisms (22). Control of pH and temperature are also critical because of the acid and temperature lability of the virus (21). Optimum temperature for cell, virus growth and inactivation, normally around 37°C and 26°C, respectively, should be precisely controlled. During other stages of manufacture, the temperature should be reduced to 4–6°C. Virus should be maintained at approximately pH 7.6 and should never be below pH 7.0.

A suitable strain of the virus is used to infect a suspension or monolayers of an established cell line, such as BHK. Such cell cultures should be proven to be free from contaminating microorganisms. It is common practice to keep stocks of BHK cells over liquid nitrogen and revive as necessary. On revival, they are expanded in nutrient medium to a volume and cell density appropriate to seeding the main culture. As an approximation, the main culture is seeded to give an initial density of $0.2–0.5 \times 10^6$ cells/ml, which is allowed to multiply to $2–5 \times 10^6$ cells/ml before being infected with virus.

When the virus has reached its maximum titre, which is variously determined by infectivity, CF or other tests, the culture is clarified, often by chloroform treatment followed by centrifugation and filtration. The virus is subsequently inactivated by addition of ethyleneimine, usually in the form of binary ethyleneimine (BEI). This is usually prepared by dissolving, to a concentration of 0.1 M, 2-bromoethylamine hydrobromide in 0.2 N sodium hydroxide solution, and incubating at 37°C for 1 hour (9, 10). The BEI formed is then added to a virus suspension held at 26°C, to give a final concentration of 3 mM. Inactivation is usually continued for 24 hours, followed by a second dose of BEI for a further 24 hours. The time period for BEI treatment and temperature used for inactivation must be validated for the actual conditions and equipment used. After inactivation any residual BEI in the harvest can be neutralised by adding sodium thiosulphate solution to a final concentration of 2%. To decrease the likelihood of live virus failing to contact the EI at the second application, it is essential to transfer the vessel contents immediately to a second sterile vessel where inactivation is allowed to go to completion at 48 hours.

The inactivated virus may be concentrated by ultrafiltration, polyethylene glycol precipitin or polyethylene oxide adsorption (1, 62) concentrated inactivated virus may be purified further by procedures such as chromatography. These concentrated antigens can be kept at –70°C or lower temperatures for many years, if necessary, and made into vaccine when required by dilution in a suitable buffer and addition of adjuvants (24).

Conventional FMD vaccines are usually formulated as aqueous or oil adjuvanted. The aqueous vaccine, which is most commonly used for cattle is prepared by adsorbing the virus on to aluminium hydroxide gel, one of the adjuvant constituents of the final vaccine blend. Other components of the final blend include antifoam, phenol red dye (if permitted by the country requiring vaccine), lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins and buffer salts. A second adjuvant, saponin, derived from the South American tree *Quillaja saponaria mollina*, is also incorporated, as well as a preservative such as merthiolate or chloroform.

Oil-adjuvanted vaccines are usually formulated using mineral oils, such as Marcol and Drakeol, as adjuvants. These preparations offer a number of advantages over the standard aluminium hydroxide/saponin vaccine, not least of which is their efficacy in pigs. They are widely used for vaccinating cattle in South America because of the longer duration of immunity. The mineral oil is usually premixed with an emulsifying agent, such as mannide monooleate, before the addition of a proportion, or all, of the aqueous phase of the vaccine, and emulsified by use of a colloid mill or continuous mechanical or flow ultrasonic emulsifier. More complex double emulsions (water/oil/water) may be produced by emulsifying once more in an aqueous phase containing a small amount of detergent such as Tween 80 (37).

A further alternative are the 'ready-to-use' oil adjuvants. Oils containing esters of octadecenoic acid and 2,5 anhydro-d-mannitol, for example, readily form double or mixed emulsions (water/oil/water) that are both stable and of low viscosity, without the requirement of sophisticated emulsification equipment (11, 26). When using novel components, including adjuvants, in any vaccine it is important to take into account that its status with regard to residues in products derived from food producing species must be assessed to ensure that adequate assurance can be giving to licensing authorities in relation to safety for consumers. This requirement limits considerably the choice of adjuvants for use in food producing species.

3. In-process control

In general, virus titres reach optimum levels within about 24 hours of the cell culture being infected. The time chosen to harvest the culture may be based on a number of assays; for instance cell death. Virus concentration may be assessed by infectivity test, sucrose density gradient (23) or serological techniques. It is preferable to use a method for measuring antigenic mass, such as sucrose density gradient analysis, as well as one that measures infectivity, as the two properties do not necessarily coincide and the different methods may complement one another.

During inactivation of the virus, timed samples should be taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of cell cultures proven to be highly susceptible to FMD virus, e.g. BHK or bovine thyroid cells. Such cultures permit the testing of statistically meaningful samples under reproducible conditions. The $\log_{10}$ infectivities of the timed samples are plotted against time, and the inactivation procedure is not considered to be satisfactory unless at least the latter part of the slope of the line is linear and extrapolation indicates that there would be less than one infectious particle per 10^4 litres of liquid preparation at the end of the inactivation period.

4. Tests on the final product

a) Safety

Tests for innocuity (non-infectivity) are most effectively carried out on the bulk, concentrated, inactivated viral harvest (see Sections D.3 and D.5.b, below). Although it may be possible to confirm innocuity by testing virus eluted from the vaccine, this is not universally applicable to all formulations and is not as reliable as testing concentrated antigens. For example, saponin influences greatly the elution of FMD virus from aluminium hydroxide/saponin vaccines (25). If the elution procedure is appropriate to a particular formulation, then it may be validated by seeding parallel samples of vaccine with trace amounts of live virus (12).

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in an *in-vivo* test in an appropriate number of cattle (27). Double dose and repeat dose tests using vaccines formulated to contain the maximum permitted amount and number of antigens should be conducted using a similar protocol described below for batch safety tests.

b) Potency

Cattle of at least 6 months of age, obtained from areas free from FMD that have not previously been vaccinated against FMD and are free from antibodies to the different types of FMD virus should be used. Three groups of no fewer than five cattle per group should be vaccinated by the route recommended by the manufacturer. The vaccine should be administered at different doses per group by injecting different volumes of the vaccine. For example, if the label states that the injection of 2 ml corresponds to the administration of 1 dose of vaccine, a 1/4 dose of vaccine would be obtained by injecting 0.5 ml, and a

1/10 dose would be obtained by injecting 0.2 ml. These animals and a control group of two nonvaccinated animals are challenged either 3 weeks (aqueous) or up to 4 weeks (oil) after vaccination with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating the equivalent of a total of 10,000 ID_{50} (50% bovine infectious dose) intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for at least 8 days. Unprotected animals show lesions at sites other than the tongue. Control animals must develop lesions on at least three feet. From the number of animals protected in each group, the PD_{50} (50% bovine protective dose) content of the vaccine is calculated. There are a variety of methods for calculating PD_{50} (31), but procedures based on the Kärber (38) method are generally preferred. The vaccine should contain at least 3 PD_{50} per dose for cattle, when employed for routine prophylactic use, although 6 PD_{50} per dose is more commonly preferred. In some cases, vaccine of high potency will prevent the development of local tongue lesions at the site of challenge.

For the PGP test (percentage of protection against generalised foot infection) a group of 16 FMD-seronegative cattle of at least 6 months of age, with the same characteristics described for the PD_{50} test, are vaccinated with a full vaccine dose by the route recommended by the manufacturer. These animals and a control group of two nonvaccinated animals are challenged 4 weeks or more after vaccination with the challenge strain, which is a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating a total of 10,000 BID_{50} (50% bovine infectious dose), intradermally into at least two sites on the upper surface of the tongue. Unprotected animals show lesions at sites other than the tongue within 7 days after inoculation. Control animals must develop lesions on at least three feet; for routine prophylactic use, the vaccine should protect at least 12 animals out of 16 vaccinated. Animals are observed at 7–8 days after challenge (61).

Potency tests in other target species, such as sheep, goats or buffalo are either different or not yet standardised. In general, a successful test in cattle is considered to be sufficient evidence of the quality of a vaccine to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in a species other than cattle, it may be more appropriate to potency test the vaccine in that same species. With respect to African or Asiatic buffalo (*Bubalus bubalis*) and sheep, due to the often inapparent nature of the disease in these species, potency results from a cattle test may be a more reliable indicator of vaccine quality than attempting a potency test reliant on the detection of clinical signs in these other species.

A similar protocol to the cattle test can be adopted for potency testing FMD vaccines in pigs using three groups of five pigs, not less than 2 months old and free from antibodies neutralising the different serotypes of FMD virus. One group is vaccinated with the full pig dose recommended by the manufacturer, one group receives a reduced dose e.g. 1/4 dose, and a third group receives a further reduced dose e.g. 1/16 dose of the vaccine. Traditionally, the response to oil vaccine is allowed longer to develop, and not until day 28 after vaccination are the three groups, plus two unvaccinated control pigs challenged. However, depending on the formulation, this interval could be reduced to that used in the cattle test. It is important that the different dose groups are individually separated from each other during the trial and that animals are removed as soon as they appear to be developing generalised FMD to avoid excessive challenge to those remaining. Challenge is by intradermal injection into the heel bulbs of one foot with 10,000 $TCID_{50}$ (0.2 ml), as calculated by growth in a suitable pig cell culture, of a virulent challenge virus homologous to a strain used in the vaccine and that normally results in generalised disease in the pig. Alternatively, the challenge virus may be administered into one site in the muscular part of the neck behind the ear, using a dose of virus known to cause generalised disease by this route. The animals are observed daily for 10 days after challenge for clinical signs of FMD. Both control animals should develop clinical signs on more than one foot. From the number of animals protected in each group, the PD_{50} content of the vaccine is calculated. There are a variety of methods for calculating PD_{50} (31), including procedures based on Kärber. The vaccine should contain at least 3 PD_{50} per dose for pigs. Likewise, a similar protocol to the PGP test in cattle can be adopted for pigs using a group of 16 animals vaccinated with a full vaccine dose and two non-vaccinated controls. Challenge is by intradermal injection into the heel bulb of one foot with 10,000 $TCID_{50}$ (0.2 ml) of a virulent challenge virus homologous to the strain used in the vaccine and that is known to induce clinical signs in pigs.

Indirect tests, including measurement following vaccination of virus neutralising antibodies in cell culture, or ELISA antibodies, or serum-protecting antibodies in suckling mice, may be used to assess the potency of a vaccine provided that a statistical evaluation has established a satisfactory correlation between the results obtained by the test on the relevant vaccine serotype and the potency test in cattle (60). For example, the expected percentage of protection is used to analyse the sera of a group of at least 16 vaccinated cattle and to express the probability of an animal being protected by measuring neutralising, ELISA or protecting antibodies. In a single group of animals given a full dose of vaccine, the mean individual expected percentage protection should be equal to or greater than 75% when 16 animals are used or 70% when 30 animals are used in the experimental group.

The presence of more than one serotype in a vaccine does not diminish the induction of antibodies against another serotype or the correlation of antibody titre with protection.

c)   **Purity: testing for antibody against nonstructural proteins**

The OIE *Terrestrial Animal Health Code* stipulates that a criterion for regaining FMD free status following an outbreak, if vaccine is used, is to test the vaccinated animals for antibody against NSP. Likewise, countries wishing to be recognised as FMD free with vaccination must demonstrate the absence of virus circulation by showing that vaccinated animals are free from antibody to NSPs arising as a result of infection. Consequently, FMD antigens used to formulate vaccines that may be used in these circumstances should be purified to reduce the NSP content. With current manufacturing techniques it is possible to exclude the majority of NSPs so that they induce little, if any, NSP specific antibody. Under these circumstances, detection of NSP antibodies can provide evidence that vaccinated animals have been exposed to FMD virus. Vaccine manufacturers may wish to exploit this potential by including a claim that their vaccines do not induce antibody to one or more NS proteins and can be used in conjunction with an appropriate diagnostic test. In addition to providing supporting documentation on the processes involved in such purification, manufacturers should demonstrate lack of immunogenicity against NS proteins as part of the licensing procedure in order to make such a claim on their product literature. A test method that can be used is to vaccinate an appropriate number of calves, preferably with at least a double dose of a trial blend of the vaccine containing the maximum number and amounts of antigen permitted on the authorisation (these calves may be the same as those used for the safety test described in Section D.4.a of this chapter). Calves should be vaccinated at least three times over a period of 3–6 months and then tested 30–60 days after the last vaccination for the presence of antibody to NSPs using the tests described in Section B.2.d of this chapter. Negative results in these NSP assays support claims that the vaccine does not induce antibody to NSPs.

At the batch level, confirmation of vaccine purity can be shown by demonstrating a lack of increase in reactivity against NS proteins of the sera from the animals used in the potency test obtained 30 days after primovaccination and before challenge, when compared with the sera of the same animals prior to vaccination.

d)   **Duration of immunity**

In order to establish a satisfactory level of immunity it is usual to give a primary course consisting of two inoculations, 2–4 weeks apart, followed by revaccination every 4–12 months. The frequency of revaccination will depend on the epidemiological situation and the type and quality of vaccine used. Where access to the animals is difficult, it is preferable to use oil adjuvanted vaccine at 4 months and 1 year of age, followed by annual revaccination. Wherever possible, vaccine manufacturers should demonstrate the duration of immunity for their specific formulation in each species for which it is indicated.

For calves born of vaccinated dams, the first vaccination should be delayed as long as possible to allow decline of maternal antibody. This period should not be extended beyond 4 months, as by this age a high proportion can be expected to respond effectively to vaccination. For calves born to non-vaccinated dams, the first vaccination may be administered from as early as 1 week of age for some vaccines (8).

e)   **Stability**

The shelf life of conventional FMD vaccines is usually 1–2 years at 4°C (maximum range 2–8°C), but they are temperature labile and should never be frozen nor stored above a target temperature of 4°C. The stability of all vaccines, but particularly oil emulsion vaccines, should be demonstrated as part of the shelf life determination studies for authorisation.

f)   **Preservatives**

The most commonly used preservatives are chloroform and merthiolate. The latter is used at a final concentration of 1/30,000 (w/v).

g)   **Precautions (hazards)**

Current FMD vaccines are innocuous and present no toxic hazard to the user. Care must be taken to avoid self-injection with oil-emulsion vaccines.

5.   Batch control

a)   **Sterility**

The bulk inactivated antigen, the adjuvants, the dilution buffers and the final formulated product should all undergo sterility testing. This may be carried out directly with components of the vaccine and the final product, but the preferred method is to collect any contaminating microorganisms by membrane filtration of the material to be examined and to detect any organisms present by incubation of the membranes with culture media. The latter procedure allows the removal of preservatives, etc., which may inhibit the detection of microorganisms. Guidelines on techniques and culture media, which allow the detection of a

wide range of organisms, are described in the European Pharmacopoeia 2008 (27; also refer to Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Innocuity

The test for innocuity is an in-process test that should be carried out for every batch of antigen. Following inactivation, a sample of each batch of inactivated antigen representing at least 200 doses should be tested for freedom from infectious virus by inoculation of sensitive monolayer cell cultures, preferably of the same origin as those used for the production of antigen. It may be preferable to concentrate the antigen to do this, in which case it must be shown that the concentrated material does not interfere with the sensitivity or reading of the assay. The cell sheets are examined daily over a period of 3 days, after which the spent medium is transferred to fresh monolayers and the original monolayers are replenished with fresh medium. Using this method, traces of live virus can be amplified by the passage procedure and detected on the basis of CPE observed. Two to three passages of the original virus preparation are commonly used. A variant on this method is to freeze–thaw the old monolayers to release intracellular virus, which can be detected by further passage.

c) Safety

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions. The test may also confirm innocuity but is not as sensitive as the *in- vitro* tests described above. For the purposes of batch release, each of at least two healthy seronegative cattle is inoculated by the recommended route of administration with double the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Should any of the animals develop clinical signs of FMD, the vaccine fails the safety test. Equally, any undue toxicity attributable to the vaccine should be assessed and may prevent acceptance of the batch. Ideally, vaccines prepared for species other than cattle should also be safety tested in the species for which they are intended, administering a double dose of vaccine according to the manufacturer's recommended route and dose volume. The animals should be examined daily for a minimum of 14 days for evidence of toxicity or signs of FMD.

d) Potency

Potency is only examined on the final formulated product (see Section D.5.b). Antigen load can be used as an indirect indicator of potency, provided a correlation has previously been established between antigen load, serological response and protection against challenge, and provided that a suitable alternative test measuring the serological response to immunisation has been carried out with satisfactory results.

6. Storage and monitoring of antigen concentrates

The process of storing concentrated antigens at ultra-low temperature for later formulation into FMD vaccine is becoming an increasingly popular option for vaccine manufacture. It not only forms the basis for the storage of antigens in a strategic reserve for emergency purposes (see Chapter 1.1.10 Guidelines for International Standards for Vaccine Banks), but allows the manufacturer immediate access to many different antigen strains which can be rapidly formulated and dispatched to the customer. Such stockpiling minimises delays subsequent to an order, particularly where a multivalent vaccine is requested. Another advantage of this procedure is that much of the quality testing can be completed well in advance of shipment.

a) Pre-storage criteria

It is necessary to state that antigens have to be controlled using standards indicated in Sections D.1–4.

Special attention should be paid to the following:

- freedom from extraneous agents;

   antigens should be proven free from all extraneous agents listed by the appropriate licensing authorities.

- sensitivity of the cell line used to detect residual virus;

   Cells used to test for absence of residual live virus are not suitable if use of an amount of virus corresponding to 1 µg of 146S antigen gives a titre of less than 10^6 CC ID$_{50}$ (27).

- emergency procedures for provisional acceptance of new Master Seed Virus (MSV), and subsequent release of formulated vaccines.

In the case of incursion in a region of a new strain that is antigenically distinct from existing vaccine strains, it may be necessary to develop a new vaccine strain from a representative field isolate. Before the new MSV can be accepted, full compliance should be demonstrated with the relevant guidelines to demonstrate freedom from all extraneous agents listed by the appropriate licensing authorities using both general and specific tests, and to establish homology to the original candidate isolates. The time taken to raise the specific antisera necessary to neutralise the new strain for use in the general tests for detection of extraneous agents and to conduct other specific tests that require specialised techniques may be lengthy. Therefore, in emergency situations where there is insufficient time to complete full testing of the MSV, provisional acceptance of the new strain should be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account that a validated procedure to inactivate enveloped viruses must be used when establishing the MSV and that the virus is inactivated using a chemical inactivant with first order kinetics. Further assurance is provided by the requirement for the kinetics of inactivation to be monitored and recorded for each production batch.

In order to accelerate the release of batches of vaccine formulated to contain new vaccine strains, it may be acceptable for batch potency testing to be carried out using a vaccine formulated using an intermediate antigen lot pending production of all of the batches of antigen that are intended to constitute the final antigen lot. This will allow the potency of antigen derived from a new MSV to be determined whilst the manufacturer continues to build up stocks of this new antigen.

b) Storage criteria

- **Facilities**

It is important that all aspects of the storage of antigen concentrate conform fully to internationally accepted standards of Good Manufacturing Practice.

- **Containment of stored antigens**

The dose numbers or volumes stored are an important consideration, particularly where a reserve is shared between Member Countries and there is variation in number of doses perceived to be needed by each member in an emergency. It may be advisable to store antigen concentrates in user-friendly units to allow better usage of storage space and capability in producing smaller vaccine batches. One to two litre sized containers can accommodate in excess of 30,000 bovine doses. Where the requirement is for a large stockpile of a particular vaccine strain that can only be produced from several separate production runs, vaccine bank managers must consider the need to either formulate each lot into a representative final blend for testing purposes or mixing the individual batches, at some convenient point, for ease of formulating and/or testing.

The type of container used to hold antigen concentrate is important. Under ultra-low temperature conditions it is important to use containers made from materials that do not become brittle and fragile, a good example being fluoropolymer based moulded bottles. Polyfluoro-alkoxy (PFA) based bottles, for example, have a temperature resistance range of between –270°C and +250°C.

- **Labelling of stored antigens**

Although there are national and international guidelines on the required labelling of veterinary medical products, there are no such guidelines for emergency stored materials such as the antigen component of a vaccine, as these are essentially regarded in regulatory terms as 'in process' materials. Under ultra-low temperature conditions, the method of labelling must be of a durable nature. From experience, wire tagging bottles is the most preferred option using a metal tag sizeable enough to allow the necessary detail. Such detail should include the antigen/vaccine strain, batch number, date received and should also include an individual container or stock number. This information should be clear to read and marked on the tag using an indelible marker pen. Aluminium metal tags have been used for such purpose and these can be obtained with different colour coatings to allow better identification and accessibility, particularly when different antigen strains are housed in the same container. Metal tags also allow information to be permanently engraved.

- **Monitoring**

It is vitally important that antigen concentrates are optimally maintained and routinely monitored in order to have some assurance that they will be efficacious when needed. Therefore arrangements should be made to monitor these antigen concentrates on a routine basis and to include where necessary, and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product. For example, storage temperature monitoring is normally undertaken and recorded in FMD vaccine banks, as well as periodic inspection of the bottles containing the antigen for cracks or leakage. Depending on type, volume and how they are stored, there may also be value in

weighing antigen deposits annually to ensure they have not lyophilised. Some FMD vaccine banks have incorporated physico-chemical tests like sucrose density gradient analyses to monitor virus integrity and hence stability and some have also carried out in-vivo tests. However, since it has been shown that the shelf-life of FMD antigen concentrates are likely to be well in excess of 15 years when stored at ultra-low temperature, a physico-chemical approach would appear sufficient.

The following timetable of tests is proposed as suitable for validation and re-validation of stored antigens.

Time	Test
On receipt (year 0) and every 5 years thereafter	146S quantification* Potency test in cattle that may rely on serological techniques where potency has been adequately correlated with immunogenicity for the antigen concerned or, at the discretion of the bank holder, may be a 'truncated' test** to demonstrate that the minimum potency of the vaccine remains greater than the minimum requirement; however, truncation may underestimate vaccine potency
Years 2 and 4, and immediately before formulation if the need arises	146S quantification
Every 5 years	Evaluation of all data for the preceding 5 years to assess need to replace antigen

* Other physiochemical tests such as SDS-PAGE have been used to evaluate integrity of VP1 but are not sufficiently validated for routine use.

** In a truncated test all animals in the next lower volume group are assumed to have not been protected. The test therefore gives an artificially low PD_{50} value but reduces the number of animals required.

To support these testing requirements for depositories of antigen, concentrates should include a number of small samples that are representative of the larger stock. Small aliquots/stocks of FMD antigen have usually consisted of a volume representing approximately one milligram of antigen.

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NB: There are OIE Reference Laboratories for Foot and mouth disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

HEARTWATER

SUMMARY

Heartwater (also known as cowdriosis) is an acute, fatal, non-contagious, infectious, tick-borne rickettsial disease of ruminants caused by Ehrlichia ruminantium (*formerly* Cowdria ruminantium) *and transmitted by* Amblyomma *ticks. It occurs in nearly all sub-Saharan countries of Africa, in its neighbouring islands, and also in the Caribbean, threatening the American mainland. The disease can cause high mortality (up to 90%) in susceptible domestic ruminants. Goats and sheep are more susceptible than cattle, and European breeds are generally more susceptible than indigenous African breeds.*

Clinically, the disease most commonly presents as an acute form characterised by a sudden high fever, depressed demeanour, nervous signs and a high mortality. Hydropericardium and hydrothorax and lung oedema are commonly associated post-mortem signs. Acute and peracute clinical forms of the disease occur: In the former, there are high death rates without many clinical manifestations and in the latter, there is a higher recovery rate.

Recovered animals become carriers of infection. Certain wild animals can play a role as reservoir; Rusa deer, white tailed deer, and springbok are susceptible to this infection and can experience high mortality.

Identification of the agent: *The specific diagnosis of heartwater is based on the observation of colonies of* E. ruminantium *in capillary endothelial cells of the brain. In the absence of adequate tools, a piece of cerebellum can easily be removed with a curette through the foramen magnum after cutting off the head, while a sample of cerebral cortex can be obtained through a hole made in the skull with a hammer and a large nail. Brain smears are prepared by crushing to a paste and spreading thinly a small piece of cerebral or cerebellar cortex between two microscope slides. The capillaries are spread out in a single cell layer by drawing one slide across the other. The smears are air-dried, fixed with methanol and stained with Giemsa. With fast stains, the smears can be fixed and stained in less than 1 minute. The colonies (clusters) are reddish-purple to blue, and very often close to the nucleus of the infected endothelial cell. They can be scanty and difficult to find, particularly in peracute cases, but they are always present in the brain of a ruminant that died from heartwater, if not treated with drugs. Colonies are not likely to be detected in animals that were treated with antibiotics. The colonies are still visible 2 days after death in a brain that has been stored at room temperature (20–25°C) and up to 34 days in a brain that has been stored in a refrigerator at 4°C.*

Fresh whole blood collected from suspect animals can be inoculated intravenously into a susceptible sheep or goat. The development of clinical signs and the demonstration of E. ruminantium *in the brain of the inoculated animal during the febrile reaction are diagnostic for heartwater. Due to animal welfare concerns, this method should be avoided.*

Ehrlichia ruminantium *can be isolated from the blood of an infected host using cultivation on ruminant endothelial cells. When a cytopathic effect consisting of plaques of cell lysis appears, the presence of characteristic morulae is confirmed by staining the cell monolayer with eosin– methylene blue or by immunofluorescence or immunoperoxidase techniques using a specific antiserum.*

DNA probes and especially the more sensitive polymerase chain reaction (PCR) techniques are available to reveal the presence of E. ruminantium *in the blood of animals with clinical signs, and in the tick vectors, to a lesser extent in the blood or bone marrow of carrier animals. Apart from*

diagnosis, PCR is widely used for research on the E. ruminantium *genome and for epidemiological studies.*

Serological tests: *Serological tests available include indirect fluorescent antibody tests, enzyme-linked immunosorbent assays (*ELISAs*) and Western blot. However, when the whole* E. ruminantium *is used as antigen, cross-reactions with* Ehrlichia *spp. occur in all of these tests. Serology has limited diagnostic applications.*

*One recently developed ELISA uses a recombinant antigen expressed as a partial fragment of the recombinant major antigenic protein 1 (*MAP1*) antigens –the MAP1-B ELISA. This test has shown a dramatic improvement in specificity compared with previous tests. Although this test is more specific, it still detects cross-reacting antibodies to other* Ehrlichial *organisms. Hence, definitive proof of heartwater must rely on epidemiological evidence and additional molecular testing indicating the presence of the organism. This ELISA has made the interpretation of serological results more reliable in regions where* Ehrlichia *infections occur in ruminants. It can help to monitor experimental infections and to measure the immune response of immunised animals, whose pre-immunisation serological history is known. Serology has very limited diagnostic use as clinically infected animals remain sero-negative during the febrile reaction and sero-convert after they recover from the infection.*

Serology is also not an effective import test. Prior to importation of animals from a heartwater endemic region, it is important to study the epidemiological data to try to establish that the herd and the resident ticks are not infected; in addition repeated PCR testing should be carried out to demonstrate that the pathogenic agent is not present in the herd.

Requirements for vaccines and diagnostic biologicals: *The immunisation against heartwater by the 'infection and treatment' method using infected blood is still in use in some countries. A first-generation vaccine consisting of inactivated purified elementary bodies of* E. ruminantium *emulsified in Montanide ISA 50 adjuvant has given promising results in experimentally controlled conditions and has demonstrated significant protection in the field. An additional isolate, Welgevonden, has been attenuated and shown to confer good protection, and significant protection has also been obtained using DNA vaccination. However, none of these new experimental vaccines has been fully validated under field conditions. Field trials have revealed that antigenic diversity is important in formulating effective vaccines, and further investigations are critical for the delivery of any vaccine in the field.*

A. INTRODUCTION

Heartwater (cowdriosis) is a rickettsial disease of domestic and wild ruminants caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) and transmitted by *Amblyomma* ticks (3, 8, 36). It is also known by the synonyms malkopsiekte (Afrikaans), péricardite exsudative infectieuse (French), hidrocarditis infecciosa (Portuguese), idropericardite dei ruminanti (Italian), and a variety of names in different African languages (5). *Ehrlichia ruminantium* is classified in the order Rickettsiales and in the family Anaplasmataceae, together with the genera *Anaplasma*. Although ruminants remain the primary target of the pathogen, in South Africa a possible canine *E. ruminantium* infection has been reported (1), and, more recently, *E. ruminantium* has been strongly suspected in several cases of rapidly fatal encephalitis in humans (14). However, in all cases, evidence of *E. ruminantium* infection was based on molecular detection. Isolation and characterisation of the infectious agent is necessary before *E. ruminantium* can be considered an emerging pathogen in species other than ruminants and especially in humans.

Heartwater occurs in nearly all the sub-Saharan countries of Africa where *Amblyomma* ticks are present and in the surrounding islands: Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands and Sao Tomé. The disease is also reported in the Caribbean (Guadeloupe, Marie-Galante and Antigua) (34), from where it threatens the American mainland. All domestic and wild ruminants can be infected, but the former appear to be the most susceptible. Indigenous domestic ruminants are usually more resistant to the disease. Wild animals could play a role as reservoir, but Rusa deer, white tailed deer, springbok, chital, timor deer, which are used in wildlife farming, seem to be the main wild ruminant species in which heartwater can have a significant economic impact (36).

The average natural incubation period is 2–3 weeks, but can vary from 10 days to 1 month. In most cases, heartwater is an acute febrile disease, with a sudden rise in body temperature, which may exceed 41°C within 1–

2 days after the onset of fever. It remains high for 4–5 weeks with small fluctuations and drops shortly before death

Fever is followed by inappetence, sometimes listlessness, diarrhoea, particularly in cattle (4), and dyspnoea indicative of lung oedema. Nervous signs develop gradually. The animal is restless, walks in circles, makes sucking movements and stands rigidly with tremors of the superficial muscles. Cattle may push their heads against a wall or present aggressive or anxious behaviour. Finally, the animal falls to the ground, pedalling and exhibiting opisthotonos, nystagmus and chewing movements. The animal usually dies during or following such an attack.

Subacute heartwater with less pronounced signs, and peracute heartwater with sudden death, can also occur, according to the breed of ruminant and the strain of *E. ruminantium* involved.

The most common macroscopic lesions are hydropericardium, hydrothorax, pulmonary oedema, intestinal congestion, oedema of the mediastinal and bronchial lymph nodes (4), petechiae on the epicardium and endocardium, congestion of the brain, and moderate splenomegaly.

A tentative diagnosis of heartwater is based on the presence of *Amblyomma* vectors, nervous signs, and presence of transudates in the pericardium and thorax on post-mortem examination. When making a diagnosis based on clinical signs, the following other diseases should be considered: bovine cerebral babesiosis and theileriosis, anaplasmosis, botulism, haemonchosis in small ruminants, rabies and poisoning.

B. DIAGNOSTIC TECHNIQUES

1.   Identification of the agent

During the febrile reaction, *E. ruminantium* can be readily isolated in culture from blood or plasma; however, it is difficult to detect these organisms in a blood smear. Typical colonies of *E. ruminantium* can be observed in brain smears made after death and this represents a definitive diagnosis for heartwater.

Opening the cranium is not necessary. An alternative method (42) is to cut off the head in front of the first cervical vertebra. Then, introduce a curette through the foramen magnum, between the medulla and the meninges. The curette is turned over towards the brain and removed with a piece of cerebellum. Another method consists of making a hole in the skull with a hammer and a large nail and aspirating a sample of brain cortex with a needle attached to a syringe. These methods also lessen the danger to the operator in cases where the nervous signs have been caused by rabies.

In the live animal, a brain biopsy may be obtained aseptically and harmlessly after local anaesthesia, although with difficulty; appropriate restraint must be used especially with large and horned animals. Colonies of *Ehrlichia* are observed during the febrile period. This method is useful for experimental studies, but not suitable for routine diagnosis.

Colonies of *E. ruminantium* are still present 48 hours after death in a brain that has been stored at room temperature (20–25°C) and for up to 34 days in a brain that has been stored in a refrigerator at 4°C (5).

A small fragment of grey matter (approximately the size of a match head) is placed on a microscope slide, crushed to a paste consistency by another slide and, while maintaining pressure, the slides are drawn over each other lengthwise to produce a single layer of cells. The slides are air-dried, fixed in methanol, stained with Giemsa diluted with Sörensen buffer (2.54 g KH_2PO_4; 8.55 g $Na_2HPO_4.H_2O$; *q.s.* to 5 litres with distilled water), pH 7.2, and washed with tap water. Fast Giemsa stains (DiffQuick, RAL555, Field's stain, CAM's Quick stain) give quicker results, but the colour contrast is usually poorer. Some 'fast' stains do provide excellent contrast, e.g. Hema 3 stain.

The slides are examined under a microscope at a low magnification (×10 objective) to locate the cerebral capillaries. An oil-immersion lens with a magnification of at least ×50 is useful for identifying the colonies of rickettsiae. Experience is required *E. ruminantium* colonies must be differentiated from other haemoparasites (*Babesia bovis*), certain blood cells (thrombocytes, granulocytes), normal subcellular structures (mitochondria, mast cell granules), or stain artefacts (stain precipitates), etc. The specificity of the reading can be improved by staining formalin-fixed brain sections using immunoperoxidase techniques.

Ehrlichia ruminantium colonies are formed from clusters of granules (0.2–0.5 µm), sometimes arranged in the shape of a ring or a horseshoe (1–3 µm), that are placed close to the nucleus inside the endothelial cell. The granules can be scanty, particularly in peracute cases, but they are always present in the brain of an animal that died from heartwater. However, if the animal has been treated with doxycyclin or oxytetracyclin 48 hours before, the granules of *Ehrlichia* tend to fuse making the diagnosis very difficult, and sometimes impossible.

Fresh whole blood collected from suspect animals can be inoculated intravenously into a susceptible sheep or goat. The development of clinical signs and the demonstration of *E. ruminantium* in the brain of the inoculated ruminant are diagnostic for heartwater.

Transmission electron microscopy has been used to demonstrate that the *E. ruminantium* organisms develop inside a vacuole-like structure, which is surrounded by a membrane in the endothelial cell's cytoplasm (39). Each organism is enclosed by a double membrane. Within the vacuole-like structure, *E. ruminantium* electron-dense forms (elementary bodies), as well as intermediate reticulate forms, are identified.

a)   Isolation of *Ehrlichia ruminantium* using *in-vitro* culture

Although numerous cell lines have been shown to support growth of *E. ruminantium,* isolation is not the first choice of test for a rapid diagnosis of cowdriosis as isolation is a labour intensive and time-consuming laboratory procedure. For a rapid diagnosis, polymerase chain reaction (PCR)/molecular diagnosis is preferable. However, *E. ruminantium* isolation should be encouraged for typing the strains present in a region for the purpose of vaccination programmes. *Ehrlichia ruminantium* can be isolated from the blood of reacting animals by cultivation on ruminant endothelial cells (45). Endothelial cells from umbilical cord, aorta, or the pulmonary artery of different ruminant species (cattle, goat, sheep) are used most often for isolation, although other endothelial cell types (brain capillaries, circulating endothelial cells, etc.) have been described for the routine culture of the microorganism. Endothelial cell lines from sable, eland, buffalo, kudo and bush pig can also be used to grow *E. ruminantium*. No standard cell line has yet been designated for isolation.

- **Isolation procedure**

i)   The blood of the reacting animal (optimal time for detection of the organism is the second or third day of febrile reaction) is collected in anticoagulant (heparin or sodium citrate, not ethylene diamine tetra-acetic acid) and diluted 1/2 in the culture medium consisting of Glasgow minimal essential medium (MEM) supplemented with 10% inactivated fetal bovine serum, 2.95 mg/ml tryptose phosphate broth, 200 mM L-glutamine, and antibiotics if necessary (penicillin 100 international units/ml, streptomycin 100 µg/ml).

ii)   The culture medium is poured off the endothelial cell monolayer, and infective blood (approximately 2 ml for a 25 cm^2 flask) is added. The flask is incubated at 37°C on a rocking platform for 2 hours.

iii) After incubation, the blood is poured off and the monolayer is gently washed three times with culture medium prewarmed at 37°C.Fresh culture medium is added and the flask is incubated at 37°C. The medium is changed twice weekly.

(The *use of plasma instead of blood is more efficient* when taken from an animal with a febrile reaction >41°C. In this case, steps ii and iii above may be replaced with the following:

- Seed 4 ml of plasma (smaller inoculum can be used if there is a limited amount of plasma available) on to a susceptible endothelial cell culture and incubate for 1 hour at 37°C on a rocking platform.

- Wash off plasma with growth medium and then add 5 ml of growth medium (per 25 cm^2 flask) and observe for development of cytopathic effect.)

iv)   The monolayer is inspected regularly for the appearance of small plaques of cell lysis. The first plaques generally appear after about 2 weeks. Passaging on uninfected cell monolayers is performed when the lysis reaches 80% of the cell layer. The remaining cells are stained with eosin/methylene blue or Giemsa or DiffQuick and examined microscopically for the presence of *E. ruminantium* morulae. Alternatively, cells can be stained by an indirect fluorescent antibody (IFA) test or an immunoperoxidase test using an *E. ruminantium*-specific antiserum; the immunoperoxidase test is not commonly used.

b)   Isolation of *Ehrlichia ruminantium* using *in-vivo* culture

It is feasible to assess the presence of heartwater in a herd, a region or a country, or to isolate a strain of *E. ruminantium* by inoculating blood or tick homogenate into a susceptible animal. However, due to the animal welfare concerns, this method is not recommended. Blood from individual animals, or pooled blood, is injected slowly at a dose of 10–100 ml intravenously into a susceptible sheep or goat. Blood as an inoculum, to determine infection status of herd, will be infective if there are clinically infected donor animals present; however, the method will rarely detect infection in carrier/recovered animals. Another method consists of collecting and homogenising adult *Amblyomma* ticks, and after centrifuging the homogenate and then inoculating the resulting supernatant into susceptible hosts. This method can be more sensitive than blood from suspect animals (especially if blood is from recovered animals) because the concentration of *E. ruminantium* is higher in the tick than in the blood. However, the tick infection rate in the field is variable and sometimes as low as 1% (6). In this case, to detect an infection as many ticks as possible should be

used; at least 100 ticks are needed. In both cases, the inoculum, to which 10% dimethyl suphoxide (final concentration) has been added, can be stored in liquid nitrogen for several years. Note that inoculation of tick homogenates into susceptible animals may cause anaphylaxis, which can be prevented by the simultaneous administration of adrenaline. The development of clinical signs and the detection of circulating rickettsiae by molecular methods and/or the demonstration of *E. ruminantium* in the brain of the inoculated ruminant, on the second or third day of fever, are diagnostic for heartwater. In addition, confirmation could be accomplished by *in-vitro* isolation on endothelial cells using plasma from the inoculated animals.

2. Molecular methods

a) Detection of *Ehrlichia ruminantium* using DNA probes

A genomic DNA fragment pCS20 specific for *E. ruminantium* has been cloned and used as a nucleic acid probe (24, 50). It recognises all strains of *E. ruminantium* tested to date. This probe, designated pCS20, readily detects infection in clinically ill animals and experimentally infected *Amblyomma* ticks (18, 20, 24, 51). However, it is not sufficiently sensitive to detect most carrier animals or low level infections in ticks (35, 36). The pCS20 probe proved nevertheless to be more sensitive than 16S and MAP1 (major antigenic protein 1) probes for the detection of *E. ruminantium* in ticks when hybridised on a PCR-amplified product of the homologous DNA fragment (2).

b) Detection of *Ehrlichia ruminantium* using PCR and nested PCR

Two primers –AB128 (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-TAT-3') and AB129 (5'-TGA-TAA-CTT-GGT-GCG-GGA-AAT-CCT-T-3') – have been designed from the DNA sequence of the pCS20 probe (24) for use in a PCR. These primers amplify a 279 base pair DNA fragment which is specific only for *E. ruminantium.* Hybridisation of the amplified PCR products to a labelled pCS20 probe, as an additional step, resulted in a 350-fold more sensitive assay than using the nucleic acid probe to detect *E. ruminantium* directly in DNA extracted from ticks. Low levels of infection in animals and in ticks fed on carrier animals are detected by PCR, while a hybridisation reaction with the pCS20 probe alone (without PCR first) usually remains negative (37). Experimentally, the detection limit of the conventional PCR assay was found to be between 10 and 10^2 organisms, whereas it was between 1 and 10 organisms after PCR/hybridisation. The PCR/ hybridisation has been shown to detect 37 strains from all endemic areas with a specificity of 98%. However, the sensitivity of the PCR assay is variable, ranging from 88 to 97% with tick samples containing 10^7 to 10^4 organisms, and dropping to 61% and 28% with samples containing 10^3 and 10^2 organisms, respectively (35). Consequently, the rate of 86% of ticks testing positive when fed on a clinically reacting animal dropped to 21% when fed on carrier animals due to a lower rickettsemia in such animals. The PCR/hybridisation assay has been used widely to define the epidemiology of heartwater in southern Africa.

Two nested PCR assays have been developed to enhance detection of low levels of rickettsemia and to do away with the hybridisation step (30, 43). Both use the pCS20 region as the target sequence. The Semu *et al.* assay uses two external primers U24 (5'-TTT-CCC-TAT-GAT-ACA-GAA-GGT-AAC-3') and L24 (5'-AAA-GCA-AGG-ATT-GTG-ATC-TGG-ACC-3') and then the AB 128 and AB 129 for the nested reaction. The sensitivity of detection of this assay is one gene copy of the pCS20 fragment or 1 organism. The other nested PCR assay (30) uses a pair of external primers comprises the AB128 sense primer together with an anti-sense primer called AB130. These amplify a 413 bp fragment used as a template in a second round PCR using AB128 and AB129 as internal primers. The use of AB128 and AB129 primers avoids the need to repeat a full evaluation of the test specificity. The nested PCR shows a hundred-fold improvement in sensitivity compared with a simple PCR, and an average detection limit of 6 organisms. The direct implication of this was an increase in the detection rate in wild ticks of from 1.7% to 36% in an epidemiological study in the Caribbean. The detection limit is comparable to that of the PCR/hybridisation method, which is nevertheless much more complex and time-consuming to perform. The pCS20 nested PCR allowed regular detection of *E. ruminantium* organisms from ticks, blood, brain and lungs from infected animals, whether the samples were processed fresh, or after freezing or preservation in 70% ethanol. One drawback of the nested PCR is that extreme care needs to be exercised to prevent introduction of contamination due to repeated opening of the tubes containing the first PCR reaction when conducting the nested reaction.

A nested PCR targeting the entire *map*1 polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism or sequencing of the amplification fragment directly from the pathological samples testing positive in the pCS20 nested PCR (30). An additional nested PCR targets the polymorphic map1 gene and can be used to type circulating heartwater strains for vaccine selection and disease management. PCR amplicons are analysed by restriction fragment length polymorphisms or sequencing. A high genetic diversity of *E. ruminantium* is observed in the field may influence the formulation of vaccines and needs to be further investigated. The *map*1 nested PCR performs well although with a slightly lower sensitivity than the pCS20 nested PCR. Its detection limit was evaluated at around 60 organisms and only 91% of samples testing positive in the pCS20 nested PCR also tested positive in the *map*1 nested PCR; some positives of low intensity found using the pCS20 nested PCR were negative in the *map*1 PCR.

Primers 32F1 and 32R1 designed from the sequence of the MAP1 gene of *E. ruminantium* as well as additional primer sets designed to target the MAP1, MAP2, gltA, and 16SrDNA genes of *E. ruminantium* have been used in PCR to detect the pathogen in tick, blood and bone marrow of carrier sheep and wild African ungulates, but these methods has not been widely evaluated and used.

Although the PCR methods have proved highly effective in detecting infection in ticks or in animal samples during the clinical phase of the disease or after death, only limited studies have been done to evaluate their value in healthy carrier ruminants. *Ehrlichia ruminantium* can easily be demonstrated in the blood of infected animals just before the onset of the febrile period and for a few days after recovery (24, 43), but after that period, its detection is sporadic and appears to be dependant on the rickettsemia levels. In one study in Zimbabwe only between 3.3 and 26.7% of cattle, and 23.3% of goats were found to be positive, while data from ticks collected in the same area would suggest that given the age of the cattle or goats, they should have all been exposed or infected with *E. ruminantium* (19). A comparison of the indirect MAP1-B ELISA and the pCS20 PCR/hybridisation assay, to evaluate their respective detection sensitivity levels over a period of 8 weeks (tests performed every 2 weeks), was done on 15 cattle located in Zimbabwe on a heartwater-endemic farm where tick control was minimal and the infection pressure was high (44). The *E. ruminantium* tick infection rate on this farm was between 10 and 12%. The data demonstrated that the pCS20-PCR assay was more reliable in detecting infection in blood of these cattle than detection of antibodies by the indirect MAP1-B ELISA. These cattle were not always PCR positive or positive for antibodies at every testing time and some cattle were negative by PCR throughout the study. These data suggest that the rickettsemia levels fluctuate from high to low, and that the PCR detects infection when the levels are high. Hence detecting carrier/recovered animals is less reliable than detecting clinically infected animals. This highlighted the fact, that for determination of infection status of sub-clinical animals, it is advisable to repeatedly test the blood of such animals for *E. ruminantium* by the pCS20-PCR assay. Whether the absence of detection in most carrier animals is due to an insufficient sensitivity of the PCR methods for detecting very low rickettsemia, or is due to an intermittent release of organisms in the circulation, is not fully understood. A useful technique for confirming the status of a suspected carrier animal, whose blood is PCR negative, is to feed batches of naive ticks on the animal and then test the ticks by a pCS20 semi-nested PCR. It is not known whether ticks act simply by concentrating circulating organisms, or also by amplifying their number or even by inducing release of micro-organisms in the circulation during feeding.

c) **Detection of *Ehrlichia ruminantium* using the reverse line blot technique**

The reverse line blot technique (RLB) has been used for the simultaneous detection and identification of *Anaplasma* and *Ehrlichia* species known to occur in ruminants on the basis of differences in the small subunit rRNA gene (3). Primers 16S8FE and B-GA1B-new were designed from conserved domains and used to amplify a 492–498 bp fragment of the 16S rRNA gene spanning the variable V1 region. Species-specific oligonucleotide probes were designed in this V1 loop to allow species-specific detection of *E. ruminantium*, *E. ovina*, *E.* sp. strain Omatjenne, *Anaplasma marginale*, *A. centrale*, *A. bovis*, *A. ovis* and *A. phagocytophilum*. One oligonucleotide probe cross-reactive with all species (catch-all probe) was also designed to serve as control in case a PCR product does not hybridise to any of the species-specific probes. In the method, the species-specific probes are covalently linked to the hybridisation membrane, which is hybridised with the PCR product obtained using primers 16S8FE and B-GA1B-new. PCR products obtained from all above-mentioned microorganisms were shown to bind with specific oligonucleotide probes only. No PCR product was detected and no hybridisation occurred when the PCR-RLB was applied to *Theileria annulata*, *Babesia bigemina* or mammalian DNA. Similarly, negative control ticks were always negative in the RLB assay whereas it was possible to detect *Ehrlichia ruminantium* infection in 15–70% of ticks fed on experimentally infected or long-term carrier sheep. In Mozambique, *E. ruminantium* could also be detected in the blood of 12 sentinel small ruminants placed in the field with the infected animals; mixed infection was detected in five of the infected sentinel animals, thus demonstrating the usefulness of the method for detecting multiple infections. However, the sensitivity of the assay has not yet been determined and there is a need to further validate the technique in large epidemiological studies.

d) **Detection of *Ehrlichia ruminantium* using real-time PCR**

Two real-time PCR (QPCR) tests have been described for the detection and quantitative determination of *E. ruminantium* organisms. In a first test, a 182 bp fragment from the non-polymorphic *map1-1* gene was amplified and detection carried out using the SYBR Green method (41). DNA from six different isolates was successfully amplified. The detection limit mentioned was higher than 0.1 organism/µl, but this finding was not subjected to an in-depth investigation. Counting *E. ruminantium* under the microscope after Giemsa staining does not give very precise results. The method does not significantly improve the detection sensitivity of a nested PCR, although it does allow *E. ruminantium* organisms to be quantified. In addition to limited laboratory validation, the QPCR method was used in only one study aimed at following the *E. ruminantium* kinetics in the blood of experimentally infected sheep. *Ehrlichia ruminantium* was detected only during the hyperthermia reaction period. QPCR thus does not improve detection of asymptomatic carriers compared with nonquantitative PCR.

A second SYBR Green-based real-time PCR has been described and fully validated for use in the characterisation of *E. ruminantium* replication and release kinetics in endothelial cell cultures and its subsequent use to control the mass production process in bio-reactors (40). The product is an 873 bp fragment from the *map1* gene. The external standard for quantifying *E. ruminantium* is a pCI-neo plasmid containing one copy of the *map1* target sequence, and is a more precise method of quantifying the organisms than the method described previously where the standard is based on the counting of *E. ruminantium* bodies under the microscope. The dynamic quantitative range allows accurate measurements to be taken in samples containing 10^2 to 10^8 gene copies. The method was successfully applied to four different isolates but has not been validated for use on diagnostic samples.

• **Reading the results**

As *E. ruminantium* is an obligate intra-cellular bacteria that cannot be cultivated in acellular media and its isolation is complex and takes several weeks, molecular detection techniques are the best methods for the diagnosis of cowdriosis. PCR proves to be easier to perform and more sensitive than DNA probes. With all PCRs, however, care must be taken to ensure that no cross-contamination occurs between samples. Negative and positive controls must be included in each test. As heartwater serology has several limitations (see Section B.3), the PCR could be used to help confirm if seronegative animals, originating from an endemic area, are not infected, prior to translocating them to a heartwater-free area that has the risk of becoming infected, because of the presence of potential vectors. However, despite interesting experimental results in detecting subclinical carriers, there is not enough available information on the reliability of carrier detection by PCR; more extensive field studies need to be conducted to recommend the best protocol of detecting carrier animals. It is nevertheless clear from the Zimbabwe study in cattle that detection of infection in carrier hosts is going to be difficult and will require repeated testing to confirm status of infection (44). The current results obtained with the PCR, the nested PCR, the RLB assay and more recently the QPCR, show that the direct detection of *E. ruminantium* in the blood is only reliable during and around the febrile phase of the disease. PCR-based methods appear to be more reliable in detecting infection in ticks, and this could have epidemiological value in determining the geographical distribution of *E. ruminantium*. In addition, when necessary in endemic areas, the inclusion of testing (originally naive) ticks fed on a suspect animal would greatly improve the sensitivity of carrier detection when serology and PCR on blood have failed. The procedure is nevertheless not convenient for routine diagnostic laboratories as it requires the maintenance of tick colonies and the capacity to experimentally infect animals.

3. Serological tests

Various serological tests for diagnosing heartwater have been described: an IFA test with *E.-ruminantium*-infected endothelial cell culture as antigen (CIFA test), indirect ELISA, a competitive ELISA (C-ELISA), and a Western blot. The IFA test using *E.-ruminantium*-infected mouse peritoneal macrophages (MIFA) (10) is no longer used.

The major drawback of all of these tests is the detection of false-positive reactions due to common antigenic determinants between the *E. ruminantium* MAP1 (11) and similar proteins in several *Ehrlichia* species (23, 43). Almost all of these tests are no longer used to study the epidemiology or for diagnosis. The CIFA test is still used in some places, but care must be taken when interpreting the results because of the problem of false-positive reactions.

To minimise the problem of cross-reactions with *Ehrlichia*, two ELISAs based on a recombinant MAP1 antigen have been developed. The first is an indirect ELISA that uses an immunogenic region of the MAP1 protein (called MAP1-B) and gives far fewer cross-reactions with *Ehrlichia spp.* (MAP1-B ELISA) (49). The second is a competitive ELISA that uses the MAP1 gene cloned in a baculovirus and monoclonal antibodies (MAbs) raised against the MAP1 protein (MAP1 C-ELISA) (12). Both tests have dramatically improved specificity, but they still show some reactivity with high titre sera against *E. canis*, *E. chaffeensis* and an unclassified white-tailed deer agent. The MAP 1-B ELISA has been the most extensively used and will be described in detail. The MAP1-B ELISA does detect antibodies to *E. muris* (Mahan S.M., pers. comm.) an Ehrlichial agent that is very closely related to *E. ruminantium; this agent* is found in white tailed deer in Georgia USA and is transmitted by *Amblyomma americanum* ticks (15). Hence serology as a diagnostic tool for detecting of individual animals exposed specifically to *E. ruminantium* is unreliable. Serology should be considered at a herd level taking into consideration the epidemiological environment and, if necessary, be complemented by molecular techniques.

a) **Indirect fluorescent antibody test with infected endothelial cell tissue culture as antigen (CIFA test) (29)**

To prepare the antigen, an *E. ruminantium* strain is cultivated in ruminant endothelial cell cultures. When most cells are lysed, the remaining adherent cells are scraped and mixed with the supernate. The cells are centrifuged three times with phosphate buffered saline (PBS) at 200 ***g*** for 10 minutes. Of the washed cell suspension, 10 µl are placed in every well of an immunofluorescence slide. The antigen slides are dried, fixed in acetone and stored at –20°C.

- **Test procedure**

i) The sera to be tested are diluted 1/20 or a higher dilution in PBS, added to the antigen wells and incubated for 30 minutes in a humid chamber at 37°C.

ii) The slides are then washed in PBS buffer for 15 minutes.

iii) The appropriate anti-species conjugate, usually diluted 1/60, is added to cover the wells. The slides are incubated again for 30 minutes at 37°C.

iv) After a second washing, the slides are mounted in glycerine buffer under a cover-slip and examined under a fluorescence microscope.

v) Control positive and negative sera are included on each slide.

b) MAP1-B enzyme-linked immunosorbent assay (43, 49)

Using the vector pQE9, the PCR fragment MAP1-F2R2, which encodes the amino acids 47–152 of the MAP1 protein including the immunogenic region MAP1-B, is expressed in *Escherichia coli* M15[pREP4] as a fusion protein containing six additional histidine residues. The recombinant MAP1-B is purified using Ni^{2+}-NTA agarose (nitrilotriacetic acid agarose) under denaturing conditions as described by the manufacturer. The antigen is preserved at 4°C and each batch is titrated.

The antigen is diluted at 0.5 µg/ml in 0.05 M sodium carbonate buffer, pH 9.6, and immobilised on to polystyrene plates by incubation for 1 hour at 37°C, and stored at 4°C until use. However, in initial trials, an antigen concentration of 2 µg/ml reduced background noise and improved specificity (data not shown, 43).

- **Test procedure**

i) Plates are blocked for 30 minutes by adding 100 µl per well of 0.1 M PBS, pH 7.2, supplemented with 0.1% Tween 20 and 3% non fat dry milk (PBSTM).

ii) The plates are washed three times with PBS supplemented with 0.1% Tween 20 (PBST) and twice with distilled water.

iii) 100 µl of test serum diluted 1/100 in PBSTM is added in duplicate to wells, which are then incubated for 1 hour at 37°C.

iv) Plates are washed three times in PBST and twice in distilled water.

v) Horseradish-peroxidase-conjugated anti-species IgG optimally diluted in PBSTM is added at 100 µl per well and the plate is incubated for 1 hour at 37°C.

vi) After washing as in step iv, each well is filled with 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml orthophenylene-diamine and 3 µl/ml of 9% H_2O_2.

vii) The reaction is stopped after 30 minutes of incubation at room temperature (20–25°C) by adding 50 µl of 2 N H_2SO_4. Absorbance is read at 495 nm. Positive and negative controls are included in each plate.

c) MAP1 competitive enzyme-linked immunosorbent assay (32)

Recombinant MAP1 antigen is prepared as follows: 8-day-old *Trichoplusia ni* insect larvae are infected by a baculovirus expressing the *map1* gene and moribund larvae are homogenised (10% [w/v]) in PBS supplemented with 0.001% (v/v) Triton X-100.

Anti-MAP1 MAb is prepared as follows: spleen cells of BALB/C mice previously inoculated with larval homogenate are fused to SP2/0 cells. Supernatant fluids from hybridoma cell cultures are screened for reactivity with MAP1 by immunoblotting and immunoperoxidase methods. A reactive cell culture is subcloned, isotyped and subsequently used for ascites production.

After a further 1/800 (v/v) dilution in PBS, the antigen is immobilised on to polystyrene plates (Nunc-Immuno Plates PolySorp) by incubation overnight at 4°C, and stored at –70°C.

- **Test procedure**

i) Prior to use, the plates are blocked for 30 minutes by adding 100 µl per well of PBS, pH 7.2, supplemented with 0.05% Tween 20 and 5% nonfat dry milk.

ii) Plates are washed three times with PBS/Tween, 50 µl/well of test serum diluted 1/50 in PBS supplemented with 0.05% Tween 20 and 1% nonfat dry milk (PBSTM) is added in duplicate and the plates are incubated for 30 minutes at 37°C.

iii) Without an intervening washing step, 75 µl/well of the MAb diluted 1/4000 (v/v) in PBSTM is added and the plates are incubated for another 30 minutes at 37°C.

iv) Plates are washed three times in PBS/Tween and horseradish-peroxidase-conjugated anti-mouse IgG optimally diluted in PBSTM is added at 50 µl per well. The plate is incubated for 1 hour at 37°C.

v) After three washings as before, 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml O-phenylene diamine and 3 µl/ml of 9% H_2O_2 are added to each well. After 30 minutes of incubation at room temperature in the dark, the reaction is stopped by adding 50 µl of 2 N H_2SO_4 and the absorbance is read at 495 nm. Positive and negative controls are included in each plate.

- **Reading the results**

All serological tests based on non-recombinant *E. ruminantium* antigens, such as CIFA, ELISAs, and Western blot, are still used for experimental studies but are no longer used for sero-epidemiological studies. The tests have been compared and applied to known positive and negative sera to *E. ruminantium* (9). No false-positive reactions were observed with any of the tests against known negative sera. There is good correlation among tests, but the specificity of all five tests is low because cross-reactions occur with certain *Ehrlichia* spp.

The interpretation of results of the various tests applied to field surveys is thus difficult in areas where *E. ruminantium* infections occur in ruminants, which is probably the case in most of the heartwater-endemic regions of Africa. This situation has also been demonstrated in farms without *Amblyomma* but infected with tick species not known to be vectors of *E. ruminantium* (13, 49).

Both the MAP1-B ELISA and the MAP1 C-ELISA have shown a high specificity after evaluation in 3000 ruminant sera (goat, sheep and cattle) collected from 14 *A.-variegatum*-infested islands of the Lesser Antilles, among which only three are known to be infected by *E. ruminantium* (32). Overall specificity calculated from the 11 heartwater-free islands was 98.5% and 99.4% for the MAP1 C-ELISA and the MAP1-B ELISA, respectively. Although a few false-positive sera are still found, these tests are likely to solve much of the specificity problems of the earlier serological tests. However, high seroprevalence in vector-free areas of Zimbabwe or South Africa has also been reported although not explained (it may be caused by a cross-reacting agent not transmitted by *Amblyomma*) and should be kept in mind when interpreting the results.

Evaluating the sensitivity of the tests is more problematic as it would require knowledge of the exact status of a high number of animals sampled in the field. As mentioned before there is currently no simple technique available to confirm if an animal is infected. Experimentally, the sensitivity of the C-ELISA in goats was reported to be 91.6–95.4% for the MAP1-B ELISA, and 96.3–96.9% for the MAP1 C-ELISA (32). However, in another study the sensitivity averaged 95% for cut-off values set at 31% and 26.6% of the positive control serum for sheep and goat sera, respectively (31). Indeed, calculations are based on a limited number of experimentally inoculated animals in a period of time soon after inoculation, when almost all the animals are still positive. Sensitivity in cattle is even lower and several reports show that after infection most of the animals become seronegative again in less than 6 months and some animals never seroconvert (21, 43). This observation is in line with the difference in antibody prevalence observed between small ruminants and cattle in epidemiological surveys that cannot be explained by a lower risk of infection of the latter. For example, in Zimbabwean farms situated in endemic areas, more than 90% of goats presented antibodies in their serum compared with only 33% of cattle maintained in the same conditions (21). Similar observations were made in the Caribbean. In addition, some areas of Zimbabwe, which was labelled heartwater-free, had a large number of goats positive for MAP1-B antibodies; this further complicates the sero-diagnosis of heartwater (13).

Serological tests are useful for the assessment of heartwater antibody response in vaccinated animals. The tests should not be used to screen animals for importation into heartwater-free areas. Antibodies are maintained at detectable levels in naturally infected domestic ruminants for a few months only and circulating antibodies disappear more rapidly in cattle than in small ruminants. It is thus possible that serologically negative animals may be carriers of infection. Serology should therefore only be regarded as a diagnostic method to be applied at the herd level and not at the individual animal level (38). When interpreting diagnostic serology results, other epidemiological parameters must be considered.

Molecular methods, such as PCR assay, could potentially help in detecting carrier animals, but this approach has still significant drawbacks (see Section B.2 Molecular methods).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No commercial vaccines are available at present. The only method of immunisation against heartwater remains the 'infection and treatment' method using infected blood followed by treatment of reacting animals with

tetracycline (4). This method is still in use in several areas, but it is likely to be replaced soon by preparations using attenuated or inactivated organisms, which have given promising research results.

1. Inactivated vaccine preparations

Vaccination with preparations of inactivated *E. ruminantium* elementary bodies emulsified in oil adjuvants was shown to be possible following the demonstration that susceptible goats can be protected by inactivated *Ehrlichia* in Freund's adjuvant (27). This vaccine also protected against challenge in sheep (16) using different strains of *E. ruminantium* (17), and in cattle (47) using the same strain as in goats. A first generation vaccine preparation of inactivated *Ehrlichia* in Montanide ISA 50 oil adjuvant was shown to be similarly effective to the Freund's adjuvant preparation on laboratory challenge of immunised goats and sheep (28).

In initial vaccine trials, animals were immunised with two subcutaneous injections of 250–1000 µg of antigen (depending on trial) emulsified (50/50) in Montanide ISA 50 adjuvant in a volume of 2 ml. It has recently been shown in goats in experimental conditions that the vaccine dose can be lowered to 35 µg of antigen without decreasing the effect on protection (48). From the initial description, this represents a 28-fold reduction in the dose of vaccine from 1 mg to 35 µg of *E. ruminantium* without any modification to the protective effect. The process for the mass production of *E. ruminantium* has been developed in parallel (25). Critical parameters have been determined and optimised for the production of *E. ruminantium* in endothelial cells in stirred-tank bioreactors. Using serum-free medium in such bioreactors, *E. ruminantium* production yields reached a 6.5-fold increase compared with conventional methods. Using 2-litre bioreactors and the estimated efficient 30 µg vaccine dose, the cost estimation for one vaccine dose was around 0.11 euro, which makes it affordable in countries with limited resources. Efficacy trials conducted with vaccine preparations entirely produced using the mass production and purification process followed by preservation of the product in various solutions (NaCl versus PBS) and at different temperatures (–20°C, 4°C) have demonstrated that the efficacy of the vaccine is maintained after the entire mass production and preservation process (26).

In Zimbabwe, field trials of the inactivated vaccine emulsified in ISA 50 adjuvant have also demonstrated protection of sheep against natural tick challenge (17). In larger field trials conducted in East and South Africa, a significant reduction in mortality has been achieved in cattle, goats and sheep using either a prototype strain from Zimbabwe (Mbizi strain) or a local strain from the experimental sites (22). However, in three out of four sites, the vaccine prepared from the local isolate was less effective than the prototype Mbizi vaccine, strongly suggesting an inadequate coverage of the antigenic repertoire of isolates present in each site. Lack of cross-protection between *E. ruminantium* isolates due to disparities of antigenic composition is well established, but the complexity of the *E. ruminantium* population structure in the field has been underestimated. It has recently been demonstrated in large field evaluation trials carried out in several farming systems in West Africa that, in limited geographical areas, more than 10 genotypes with differing cross-protection capacities can be present and have a significant influence on protection with inactive vaccine preparations (unpublished data).

The Mbizi strain inactivated vaccine is being developed commercially by Onderstepoort Biological Products in South Africa (Mahan S.M., pers. comm.). These inactivated vaccines do not prevent infection but do prevent or reduce death of vaccinated animals when exposed to live virulent challenge. The advantage however is that several field strains can be incorporated to make the vaccine more widely cross-protective.

A major challenge remains the characterization of the extent of strain diversity in a region to be covered by an appropriate formulation of the vaccine. This knowledge will also be essential for new generation vaccines that will be developed in the future.

2. Attenuated vaccine preparations

Infection of ruminants with live *E. ruminantium* strains induces a strong long-lasting protection against an homologous isolate. This is the basis for infection and treatment using virulent isolates. Isolates of attenuated virulence that do not necessitate treatment of animals would be ideal but a limited number of such attenuated isolates are available. An attenuated Senegal isolate has been obtained and shown to confer 100% protection against an homologous lethal challenge, but very poor protection against a heterologous challenge. The Gardel isolate, which gives a significant level of cross-protection with several isolates (although far from complete), has also been attenuated. Recently, a third isolate named Welgevonden from South Africa has been attenuated and shown to confer complete protection against four heterologous isolates under experimental conditions (46). The main drawback of attenuated vaccines is their extreme lability, which necessitates their storage in liquid nitrogen and their distribution in frozen conditions. In addition, they have to be administered intravenously.

3. Recombinant vaccine preparations

Several reports show partial protection of mice using *map1* DNA vaccination and an improvement of protection by vaccination following a prime (plasmid) – boost (recombinant MAP1) protocol (33). However, protection of

ruminants has never been demonstrated using this strategy. In opposition, significant protection of sheep was reported against homologous and heterologous experimental challenge following plasmid vaccination using a cocktail of four ORFs (open reading frames) from the 1H12 locus in the *E. ruminantium* genome (7). No further results have been described since then. Recombinant vaccines will probably not be available in the near future.

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NB: There is an OIE Reference Laboratory for Heartwater (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

JAPANESE ENCEPHALITIS

SUMMARY

Japanese encephalitis virus is a mosquito-borne flavivirus that causes encephalitis, principally in horses. It also infects humans, and causes abortions in pigs. Pigs act as amplifiers of the virus, and birds can also be involved in its spread. The disease has been observed in large parts of Asia and recently in the western Pacific region.

A definitive diagnosis of Japanese encephalitis in horses depends on the isolation of virus from dead or sick animals. As the virus is difficult to isolate, clinical, serological and pathological findings are useful in diagnosis.

Identification of the agent: *For virus isolation, brain material is collected from sick or dead horses that have demonstrated the clinical signs of encephalitis. Isolation procedures include the inoculation of mice and cell cultures. A suspension of brain tissue is inoculated intracerebrally into 2–4-day-old mice. If the mice show neurological signs followed by death within 14 days, then virus identification can be carried out by cell culture. The virus can also isolated in cell cultures made from chicken embryos, porcine or hamster kidney cells, the African green monkey kidney (*Vero*) cell line, the MDBK (*Madin-Darby bovine kidney*) cell line, and mosquito cell lines. Identification of the virus isolated in mice or in tissue cultures is confirmed by serological or nucleic acid detection methods such as reverse-transcription polymerase chain reaction assay.*

Serological tests: *Antibody assay is a useful technique for determining the prevalence of infection in a horse population, and also for diagnosing Japanese encephalitis in diseased individuals. The assay methods include virus neutralisation (*VN*), haemagglutination inhibition, and complement fixation tests. There is serological cross reactivity with other flaviviruses, such as West Nile, which can confuse the diagnosis. The plaque reduction VN test is the most specific and can be used to differentiate Japanese encephalitis virus infection from other flavivirus infections.*

Requirements for vaccines and diagnostic biologicals: *There is an inactivated vaccine prepared from a virus suspension derived from infected mouse brains or cell cultures.*

A. INTRODUCTION

Japanese encephalitis (JE) is a disease of horses caused by a mosquito-borne flavivirus that elicits clinical signs of encephalitis in infected animals and can be fatal (9, 12). It also infects humans, and causes stillbirths and abortions in pigs. Pigs act as amplifiers of the virus, and birds can also be involved in its spread. JE virus (JEV) is widespread in eastern, south-eastern and southern Asian countries and has recently spread to western India and to the western Pacific region including the eastern Indonesian archipelago, New Guinea and Northern Australia (17). Only a single serotype of JEV has been identified, although antigenic and genetic differences among JEV strains have been demonstrated by several techniques including complement fixation, haemagglutination inhibition, neutralisation tests using polyclonal or monoclonal antibodies (1, 2, 10, 11, 15) and oligonucleotide fingerprints of viral RNA (3, 13). Based on the 240-nucleotide sequence analysis of the viral premembrane (prM) region, JEV strains are classified into four distinct genotypes (5, 6). Recently the envelope (E) gene analysis was shown to be good representative for the phylogenetic analysis of JEV. To date, five genotypes of JEV have been described based on the phylogenetic analysis of the viral E gene (18, 20, 21).

B. DIAGNOSTIC TECHNIQUES

The definitive diagnosis of Japanese encephalitis in horses depends on the isolation of the causal virus. The isolation rate of virus from diseased or dead horses is usually very low, which may be due to the instability of the

virus under certain environmental conditions, and also to the presence of antibody in infected animals. Clinical, serological and pathological findings are of assistance in diagnosis. Diagnosis is also possible by the detection of specific IgM and IgG antibodies in cerebrospinal fluid by enzyme linked immunosorbant assay (ELISA) methods (4). Viral nucleic acid has been detected in the brain of infected horses by reverse transcription polymerase chain reaction (RT-PCR) (16).

The specimens collected for virus isolation are portions of the corpus striatum, cortex or thalamus of the brain of affected horses. The virus can also be isolated from blood and spinal cord samples. All materials should be refrigerated immediately after collection and frozen to –80°C if specimens are to stored for more than 48 hours. Any potentially infected materials must be handled following containment level 3 procedures (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities), to prevent the risk of human infection. Humans may be infected by direct contact of infectious material with broken skin or mucous membranes, accidental parenteral inoculation or aerosol. Diagnosticians collecting samples should also take the appropriate precautions. A human vaccine is available and at risk field veterinarians and laboratory workers should be vaccinated.

1. Identification of the agent

Samples of brain and spinal cord are homogenised in a 10% suspension in buffered saline, pH 7.4, containing calf serum (2%) or bovine serum albumin (0.75%), streptomycin (100 µg/ml) and penicillin (100 units/ml). The calf serum should be free from Japanese encephalitis antibodies. The suspension is centrifuged at 1500 **g** for 15 minutes, and the supernatant fluid is removed for inoculation: 0.02 ml is inoculated intracerebrally into 2 to 4-day-old mice. The inoculated mice are kept under clinical observation for 14 days. No clear clinical signs may develop, but anorexia becomes evident by the disappearance of the white milk spot on the abdomen. The skin then changes colour from pinkish to dark red, and convulsions develop immediately before the mice die. Brains of dead or moribund mice are collected and stored at –80°C for a further passage.

To identify the virus, sucrose/acetone-extracted antigen is prepared from the infected mouse brains of a second passage in mice as described in Section B.2.b.1. This antigen is checked for its ability to agglutinate the red blood cells (RBCs) of 1-day-old chickens or of geese at different pH levels between pH 6.0 and 7.0, at intervals of pH 0.2, according to the method described (8). Briefly, RBC suspensions of 1/24 dilution are prepared in the diluent with different pH values. In a 96-well plate with a U-shaped bottom, 25 µl volumes of the extracted antigen is diluted serially. Then, 25 µl of the diluted RBCs is added to each well. The plate is incubated at 37°C for 1 hour, and the haemagglutination result is read. If the antigen is able to haemagglutinate red blood cells, it is used in a haemagglutination inhibition (HI) test using a Japanese encephalitis antiserum.

Primary cultures of chicken embryo, African green monkey kidney (Vero), baby hamster kidney (BHK) cells, or the C6/36 mosquito cell line (a cloned cell line from *Aedes albopictus*) may be used for virus isolation. The specimens, such as brain and blood taken from animals suspected of being infected, and the brain suspension from mice after inoculation, are inoculated onto the cell cultures. Monoclonal antibodies specific to flavivirus and Japanese encephalitis virus are used to identify the virus in the indirect fluorescent antibody test (16). RT-PCR assay can also be used for identification of JEV in clinical specimens or cell culture fluid using appropriate primers specific for JEV (7, 14, 16, 19).

2. Serological tests

Serological tests are useful to determine the prevalence of infection in an animal population, the geographical distribution of the virus, and the degree of antibody production in vaccinated horses. If serology is to be used for the diagnosis of the disease in individual horses, it should be remembered that horses in an endemic area may have been inapparently infected with the virus or may have been immunised with a vaccine. Diagnosis requires a significant rise in antibody titre in paired sera collected during the acute and convalescent phases. The specificity of each serological test should also be considered. A latex agglutination test to detect swine antibodies to Japanese encephalitis has recently been described (22).

In some regions of the world, there is a need to perform additional tests for related viruses before an unequivocal diagnosis of Japanese encephalitis can be made. For example, in Australia antigenically closely related viruses of Murray Valley encephalitis and Kunjin virus occur. Recent expansion of the distribution of West Nile virus in North America, where St Louis encephalitis virus was known to be endemic, further illustrates the flexibility of flaviviruses to adapt to new environments. The presence of antibody to these other flaviviruses can make serological diagnosis of Japanese encephalitis difficult. There is some cross reactivity with other flaviviruses on all the tests; the plaque reduction virus neutralisation test is the most specific.

a) Virus neutralisation

The plaque reduction test using chicken embryo primary cultures, African green monkey kidney (Vero) cells or baby hamster kidney (BHK) cells is sensitive and the most specific serological procedure available. The cross reaction with other flaviviruses is minimal; however, if an animal has a high titre to another flavivirus, such as West Nile, there may be a low neutralising antibody titre to Japanese encephalitis.

Japanese encephalitis virus (Nakayama strain or JaGAr-01 strain) is propagated by intracerebral inoculation in 1-day-old mice. Brains are collected from moribund or dead mice and a 10% suspension is prepared in phosphate buffered saline (PBS), pH 7.2, containing 10% fetal calf serum. The suspension is centrifuged at 5000 *g* for 20 minutes at 4°C. The supernatant is stored in aliquots at –80°C. The supernatant fluid of virus infected cell cultures could also be used.

- **Test procedure**

i) Inactivate sera for 30 minutes in a water bath at 56°C.

ii) Make twofold serial dilutions of the sera in cell culture medium, starting with a 1/10 dilution, in a 24-well (17 mm in diameter) flat-bottomed microplate or test tubes.

iii) Dilute stock virus in cell culture medium to make 100 plaque-forming units (PFU)/0.2 ml.

iv) Mix one volume of each diluted serum with an equal volume of diluted virus. Include culture medium, negative serum control and positive serum controls in each plate.

v) Incubate for 90 minutes at 37°C.

vi) Add 200 µl of the virus/serum mixture to each well of BHK-21 cell monolayer in 24-well culture plates.

vii) Incubate the plates in a CO_2 atmosphere for 90 minutes at 37°C.

viii) Remove the inoculum and add 1 ml of overlay medium (1.5% carboxymethyl cellulose, 1% fetal calf serum in Eagle's medium).

ix) Incubate the plates in a CO_2 atmosphere for 4 days at 37°C.

x) After removing the culture fluid, fix the plates in a solution containing 2.5% potassium dichromate, 5% glacial acetic acid and 5% formalin for 30 minutes at room temperature. Wear rubber gloves when handling the fixing solution.

xi) Stain the plate in 0.1% crystal violet solution for 30 minutes at room temperature.

xii) Discard the stain and rinse the cells with tap water.

xiii) Air dry the cells and count the plaques.

xiv) Estimate the serum dilution that reduces the number of plaques by 50% or more of the control without serum.

b) **Haemagglutination inhibition**

The HI test is widely used for the diagnosis of Japanese encephalitis, but has cross-reactivity with other flaviviruses. For this test, the sera must first be treated with acetone or kaolin, and then adsorbed with homotypic RBCs to remove any nonspecific haemagglutinins. The RBCs of geese or of 1-day-old chickens are used at the optimum pH (6.6–7.0). The test should be conducted with the treated sera and 8 units of standard antigen; this is commercially available in some countries.

- **Haemagglutination (HA)**

- **Preparation of virus antigen**

1. *Sucrose–acetone extraction of antigen from infected suckling mouse brains (SMB)*

   i) Homogenise infected SMB with 4 volumes of 8.5% sucrose.

   ii) Add the homogenate drop-wise to 20 times its volume of cold acetone.

   iii) Centrifuge (500 *g* for 5 minutes), then remove the supernatant.

   iv) Resuspend the sediment with the same volume as above of cold acetone, and keep in an ice bath for 1 hour.

   v) Centrifuge (500 *g* for 5 minutes), then remove the supernatant.

   vi) Pool the sediment with cold acetone in a single tube.

   vii) Centrifuge (500 *g* for 5 minutes), then remove the supernatant.

   viii) Spread the sediment inside the tube and vacuum dry for 1–2 hours.

   ix) Dissolve the dry sediment with saline: 0.4 volume of original homogenate.

   x) Centrifuge (8000 *g* for 1 hour, 4°C). The supernatant is ready for use.

2. *Infected fluid of* Aedes albopictus*, clone C6/36, cell line*

   i) Harvest the infected fluid after incubation of the infected cultures at 28°C for 1 week.

   ii) Centrifuge (1000 *g* for 15 minutes). The supernatant is ready for use.

- **Preparation of goose red blood cells**

1. *Solutions*

   Acid-citrate-dextrose (ACD): 11.26 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$); 4.0 g citric acid ($H_3C_6H_5O_7.H_2O$); 11.0 g dextrose ($C_6H_{12}O_6$); distilled water to a final volume of 500 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes.

   Dextrose-gelatine-veronal (DGV): 0.58 g veronal (Barbital); 0.60 g gelatine; 0.38 g sodium veronal (sodium barbital); 0.02 g (0.026 g) $CaCl_2$ (for $CaCl_2.2H_2O$); 0.12 g $MgSO_4.7H_2O$; 8.50 g NaCl; 10.0 g dextrose; distilled water to a final volume of 1000 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes (five times stock volume is easier to prepare).

2. *Blood collection*

   1.5 ml of ACD + 8.5 ml of blood (0.5 ml of ACD + 2.8 ml of blood).

3. *Washing (sterile)*

   i)   Total blood + 2.5 volume of DGV. Centrifuge (500 **g** for 15 minutes), then remove the supernatant.

   ii) Resuspend the sedimented RBCs in three volumes (total blood) of DGV.

   iii) Centrifuge (500 **g** for 15 minutes), then remove the supernatant. Repeat steps 2 and 3 twice more (total four spin cycles).

   iv) Transfer the final RBC suspension to a flask with aluminium foil cover.

4. *Adjusting the RBC concentration*

   i)   0.2 ml of the RBC suspension + 7.8 ml of 0.9% NaCl (1/40 dilution).

   ii) Read the optical density (OD)$_{490}$ in a spectrophotometer with 10 mm tube.

   iii) Adjust the RBC stock so that 1/40 dilution gives 0.450 of OD$_{490}$. (Final volume = Initial volume × absorbance OD$_{490}$/0.450.)

   iv) Store the RBC stock in a refrigerator for up to 3 weeks.

   v)   Before use, resuspend the RBCs gently and dilute 1/24 in virus-adjusting diluent (VAD).

- **Antigen dilution**

1. Stock solutions (should be kept at 4°C): *1.5 M NaCl:* 87.7 g NaCl and distilled water to a final volume of 1000 ml; *0.5 M boric acid:* 30.92 g H_3BO_3 and hot distilled water to a final volume of 700 ml (dissolve boric acid and cool down); *1 N NaOH:* 40.0 g NaOH and distilled water to a final volume of 1000 ml; *borate saline (BS), pH 9.0:* 80 ml 1.5 M NaCl, 100 ml 0.5 M H_3BO_3, 24 ml 1.0 N NaOH, and distilled water to a final volume of 1000 ml; *4% bovine albumin:* 4 g bovine albumin fraction V (Armour), 90 ml BS, pH 9.0, adjust pH to 9.0 with 1 N NaOH, and BS, pH 9.0, to make a final volume of 1000 ml.

2. *Antigen diluent:* 0.4% bovine albumin/borate saline (BABS): 10 ml 4% bovine albumin, pH 9.0, and 90 ml BS, pH 9.0.

3. Twofold serial dilution of antigen with BABS on U-bottom microtitre plate.

- **Addition of goose red blood cells**

1. *Stock solutions*

   1.5 M NaCl

   0.5 M Na_2HPO_4: 70.99 g Na_2HPO_4 (for Na_2HPO_4, 12 H_2O: 179.08 g), and distilled water to a final volume of 1000 ml.

   1.0 M NaH_2PO_4: 138.01 g $NaH_2PO_4.H_2O$ (for Na_2PO_4, 2H_2O: 156.01 g), and distilled water to a final volume of 1000 ml.

2. *Working solution: virus adjusting diluent (VAD)*

VAD	1.5 M NaCl	0.5 M Na$_2$HPO$_4$	1.0 M NaH$_2$PO$_4$	
6.0	100	32	184	
6.2	100	62	160	Add distilled
6.4	100	112	144	water to a
6.6	100	160	120	final volume of
6.8	100	192	104	1000 ml
7.0	100	240	80	

Values of VADs are not the pH of each VAD, but the pH after each VAD is mixed with
an equal volume of BABS, pH 9.0.

3. *Procedures*

i) 1 volume of stock goose RBCs + 23 volumes of VAD (1/24 dilution).

ii) Add 25 µl of diluted RBCs to each well on microtitre plate containing diluted antigen (25 µl/well).

iii) Incubate at 37°C for 1 hour, then read the result.

++ Complete agglutination (uniformly thin pellicle of RBCs following the curvature of the well bottom)

+ Partial agglutination (a ring associated with a rough or thinner pellicle)

± Minimal agglutination (a button on a thin or scattered pellicle)

– Negative agglutination (clearly defined button with no RBC film)

End point is the last dilution (highest dilution) in which ++ or + is observed.

Titre: the reciprocal of the end point dilution.

• **Haemagglutination inhibition**

• **Preparation of test sera**

1. *Blood collection and separation of the sera*

i) Incubate blood specimen at 37°C for 1 hour and then at 4°C overnight. If the test must be performed immediately, incubating the sample for 2–3 hours at 37°C can replace the overnight incubation.

ii) Centrifuge (2000 ***g*** for 15 minutes) to separate the serum from the clot.

iii) Heat inactivate at 56°C for 30 minutes.

iv) Store at –20°C if not processed immediately.

2. *2-mercaptoethanol treatment* (*perform this step when IgM antibody titres should be determined*)

i) Place 50 µl of the sera into two small test tubes.

ii) Add 150 µl of 0.13 M 2-mercaptoethanol in PBS into one test tube, and 15 µl PBS into another tube.

iii) Incubate at 37°C for 1 hour, then cool in an ice bath.

3. *Acetone extraction*

i) Place 2.5 ml of cold acetone into each tube. Apply rubber stoppers and extract for 5 minutes in an ice bath.

ii) Centrifuge cold (1500 ***g*** for 5 minutes), then remove the supernatant.

iii) Repeat steps i and ii once more.

iv) Spread the sediment inside tubes and vacuum dry at room temperature for 1 hour.

v) Add 0.5 ml of BS, pH 9.0, to each tube. Apply rubber stoppers. Dissolve the sediment overnight at 4°C to make 1/10 dilution of the sera.

4.   *Kaolin extraction as an alternative to acetone extraction*

    i)   25% acid-washed kaolin (Fischer) in BS, pH 9.0.

    ii)   1 volume of sera + 4 volumes of BS + 5 volumes of 25 % kaolin.

    iii) Extract at room temperature for 20 minutes with occasional shaking.

    iv)   Centrifuge (1000 *g* for 30 minutes). The supernatant is 1/10 dilution of the sera.

5.   *Adsorption with goose RBCs*

    i)   To each treated serum add 1/50 volume of packed goose RBCs.

    ii)   Adsorb for 20 minutes in an ice bath.

    iii) Centrifuge (800 *g* for 10 minutes). The supernatant is ready for the HI test (1/10 dilution).

- **Haemagglutination inhibition test**

1.   *Primary haemagglutination titration of antigen*

    Dilute the antigen to make 8 units/50 µl.

2.   *Serial twofold dilution of test sera on microtitre plate*

    Serum–antigen reaction

    Add 25 µl of diluted antigen into each well containing diluted test sera. Place the remainder of the antigen in empty wells and incubate at 4°C overnight.

3.   *Secondary haemagglutination titration of the antigen*

    i)   Serially dilute the prepared antigen (8 units/50 µl) twofold in a 25 µl system.

    ii)   Add 25 µl of BABS to each well to make 50 µl/well.

4.   *Addition of goose RBCs*

    i)   Dilute RBC stock (1/24) in VAD.

    ii)   Distribute 50 µl into each well containing 50 µl of serum antigen mixture or secondary titration of antigen.

    iii) Incubate at 37°C for 1 hour then read the result.

          Serum HI titre: the reciprocal of the highest dilution of the test sera showing complete inhibition of HA.

5.   *Interpretation of the results*

    Four-fold difference between the titre in the acute and convalescent sera is considered to be a significant rise or fall and is diagnostic of infection with a virus antigenically related to that used in the test.

c)   **Complement fixation**

Complement fixation (CF) is sometimes used for serological diagnosis. The antigen for this test is extracted with acetone/ether from the brains of inoculated mice.

- **Antigen preparation**

    i)   Extract and weigh the brains of the inoculated dead mice.

    ii)   Add to the brains 20 volumes of cold acetone, kept at –20°C, and homogenise.

    iii) Centrifuge the suspension at 5000 *g* for 5 minutes at 4°C, and remove the supernatant.

    iv)   Add to the pellet the same volume of cold acetone as used in step ii above, and mix well.

    v)   Extract with acetone by keeping the pellet at –20°C for 20 minutes, and repeat the centrifugation described in step iii above.

    vi)   Repeat steps iv and v.

    vii) Repeat steps iv and v, but this time use cold acetone/ether (equal volume mixture).

    viii) Repeat steps iv and v twice using cold ether.

    ix)   Remove the supernatant by aspirator and spread the pellet over the centrifuge tube.

x) Vacuum dry for 1–2 hours.

xi) Dissolve the pellet in cold saline (2 ml/g of brain) and keep at 4°C overnight.

xii) Centrifuge at 5000 *g* for 1 hour. The supernatant is the antigen.

- **Test procedure**

i) Heat-inactivate the test sera at 1/4 dilution in gelatin–veronal buffer.

ii) Serially dilute the sera twofold in a 96-well microtitre plate (25 µl).

iii) Add 25 µl of 4 units of antigen and mix by vibration.

iv) Add 50 µl of 2 units of complement (pooled fresh guinea-pig serum).

v) Mix by vibration and incubate at 4°C for 18 hours.

vi) Leave the microtray at room temperature for 15 minutes.

vii) Add 25 µl of sensitised sheep RBCs to each well.

viii) Mix by vibration and incubate at 37°C for 30 minutes, then read the result.

ix) The highest dilution of test sera showing no haemolysis is the titre of the sera by CF test. A rise or drop of four-fold or more in the titre is considered to be significant.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The vaccine for Japanese encephalitis in horses is prepared by the inactivation of a virus suspension derived from infected mouse brains or cell cultures.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

The Beijing-1 strain of Japanese encephalitis virus is used for vaccine production in Japan. The strain must be lethal for mice when inoculated intracerebrally, and must be able to grow in a primary culture of porcine kidney. This strain has the capacity to haemagglutinate the RBCs of geese, 1-day-old chickens or pigeons. The virus must be able to be neutralised by a standard antiserum to Japanese encephalitis virus.

b) Method of culture

The original and seed viruses should be grown in mouse brains or cell cultures. The passage levels should not exceed three more than the original virus and two more than the seed virus.

Sara, I'm not sure about the above change. Are you marking these changes for the author to review after you get MC comments included? If not, let me know and I will try to remember to add with MC comments

c) Validation as a vaccine

The vaccine product from this strain provides immunity to encephalitis in equines and prevents stillbirths in pregnant sows.

It is recommended that the original and seed viruses be maintained below –70°C, or below 5°C after lyophilisation.

2. Method of manufacture

The virus is grown in the brains of 3–4 week old mice or in a monolayer culture. The cultures should be tested to confirm that they do not contain adventitious agents (Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials). Seed virus is inoculated intracerebrally into mice. The brains of those mice

that show severe clinical signs of encephalitis are collected. These brains are homogenised in PBS, centrifuged at 1500 *g* for 30 minutes, and the supernatant fluid is processed as the virus suspension.

Seed virus is inoculated into cell cultures and the fluids are later harvested separately from each batch when virus replication is at its maximum. This fluid is filtered, or centrifuged at 1500 *g* for 30 minutes, and the supernatant fluid is processed as the virus suspension.

Formalin (0.5%) is added to the suspension to inactivate any live virus; this is considered to be the 'undiluted virus suspension'. Adjuvant may be added to enhance its immunogenicity.

3. In-process control

The virus suspension should be examined for bacterial contamination by culture techniques and for virus infectivity by intracerebral mouse inoculation or inoculation into cell cultures. The inactivated undiluted virus suspension should be re-examined for contamination by culture and by microscopy after staining, and should be checked by intracerebral mouse inoculation to ensure complete inactivation by the formalin.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Ten 3-day-old mice are inoculated intracerebrally with 0.02 ml of the final product, and observed for 14 days to ensure (by the absence of any deaths) the complete inactivation of live virus.

5. Tests on the final product

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

See Section C.4.b.

c) Formalin determination

The formalin concentration should be less than 0.2% (v/v) by general quantification procedures.

d) Potency

The final product must be checked for immunogenicity by mouse protection tests. The product is diluted one part to ten parts of PBS; 30 mice aged 2–3 weeks are inoculated intraperitoneally with 0.1 ml of the diluted product twice at 3-day intervals. There should be an equivalent uninoculated control group. All mice are challenged intraperitonally with graded doses of live virus 8 days following the first inoculation, and observed for 14 days. The survival rate should be more than 40% in the immunised group and the mortality rate in the control group should be more than 90%. The titre of challenge virus should be not less than 10^3 LD_{50} (50% lethal dose) per 0.2 ml.

e) Stability

The final product must be shown to be fully effective for 12 months when stored at 4°C.

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LEISHMANIOSIS

SUMMARY

Leishmaniosis is not a single entity but comprises a variety of syndromes due primarily to at least 16 species and subspecies of Leishmania. *Dogs are commonly affected by* L. infantum *and* L. chagasi (*now regarded as synonyms*), *but canine infections with* L. tropica, L. major *and* L. braziliensis *have also been reported. In humans, the clinical spectrum ranges from asymptomatic infections to those with high mortality, with three distinct forms being classically described: visceral (*VL*), cutaneous (*CL*) and mucocutaneous (*MCL*). The vectors of these diseases are phlebotomine sandflies belonging to the genera* Phlebotomus *and* Lutzomyia.

Identification of the agent: *When clinical signs and characteristic lesions are present in affected humans and animals, the demonstration of the parasites in stained smears of splenic, bone marrow and lymph node aspirates, of skin scrapings, and in tissue biopsies, gives a positive diagnosis. If the infection is low grade, detection of parasites is possible only by attempting* in-vitro *or* in-vivo *isolation or by polymerase chain reaction (*PCR*). As there are very few morphological differences among various species, any isolated* Leishmania *organism must be identified by molecular, biochemical and/or immunological methods. Several centres throughout the world are presently using isoenzyme, DNA and antigen characterisation to identify the agent.*

Serological tests: *Serology is the preferred method for diagnosis of canine leishmaniosis and VL, even during the early stages of the disease. In subclinical forms, seropositive cases are confirmed by parasitological diagnosis or PCR. Serology is of less value for CL and MCL. Of the several serological techniques available, the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay are the most suitable. Serodiagnostic antigens need to be prepared in the laboratory, though some commercial products are under evaluation.*

Delayed hypersensitivity test: *The leishmanin skin test is useful for determining the distribution of human infections, distinguishing immune from nonimmune cases. The test is positive in CL, MCL and cured VL, but negative in active VL.*

Requirements for vaccines and diagnostic biologicals: *There is no effective vaccine available at present for use in dogs or humans. Leishmanin is no longer available commercially and needs to be standardised.*

A. INTRODUCTION

Leishmaniosis is caused by the vector-borne protozoan parasite, *Leishmania*. Various forms of clinical manifestations of human leishmaniosis have been described and divided into three entities: visceral leishmaniosis (VL, kala azar), cutaneous leishmaniosis (CL, oriental sore, uta, pian bois, chiclero's ulcer) and mucocutaneous leishmaniosis (MCL, espundia) (50). In the New World[1], leishmanioses are caused by *L. braziliensis* complex (MCL and CL), *L. mexicana* complex (CL), *L. peruviana* (CL) and *L. infantum* (VL and CL); in the Old World, the aetiological agents are *L. donovani* (VL), *L. infantum* (VL and CL), *L. tropica* (CL), *L. major* (CL) and *L. aethiopica* (CL). *Leishmania infantum* and *L. chagasi* have been found to be identical by biochemical genotyping and should be regarded as synonyms (29). The diseases are mainly zoonoses with two exceptions, that of CL due to *L. tropica* in urban areas of Near and Middle East, and that of VL due to *L. donovani* the Indian sub-continent (northern India, Nepal and Bangladesh). Canine leishmaniasis (CanL) is a chronic viscero-cutaneous disease caused by *L. infantum* (= *L. chagasi*), for which the dog acts as the source reservoir. In some instances, parasites belonging to *L. braziliensis* complex, *L. major* and *L. tropica* have been isolated from this

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1   In this chapter, the term 'New World' refers to the Americas, and the term 'Old World' refers to Europe, Africa and Asia.

host (31, 40). The vectors of leishmanioses are phlebotomine sandflies belonging to the genera *Lutzomyia* (New World) and *Phlebotomus* (Old World).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Clinical examination of suspected cases, parasitological diagnosis and immunodiagnosis are the routine methods available for the diagnosis of leishmaniosis. However, the demonstration of the parasite is the only way to confirm the disease conclusively. In VL and CanL, isolation and identification of the parasite from biopsies (lymph node, bone marrow, and spleen aspirate) coupled with molecular and immunodiagnostic tests are recommended. Parasitological diagnosis is necessary for confirmation of CL (through lesion scraping or needle aspiration from the edge of the lesions) as neither clinical examination nor serology is adequate. Smears of biopsy material are stained with Giemsa stain and examined microscopically at ×600–1000 magnification. Material should also be cultured in appropriate media at 22–26°C.

Morphological characteristics of amastigotes (in humans and mammalian hosts) and promastigotes (in phebtomine sand flies and in cultures) are the following:

- *Amastigote:* small intracellular rounded or oval body, 1.5–3 × 2.5–6.5 µm in size, found in vacuoles within the cytoplasm of the macrophages. There is no free flagellum. The organism has a relatively large nucleus and a kinetoplast consisting of a rod-like body and a dot-like basal body;

- *Promastigote:* elongated extracellular organism, body size 15–20 × 1.5–3.5 µm with a single flagellum 15–28 µm long, arising close to the kinetoplast at the anterior. The nucleus is situated centrally.

The choice of the isolation and culture methods will depend on the immediate circumstances and on the technical capability and experience of the laboratory staff (45). *In-vitro* isolation offers certain advantages over the *in-vivo* methods: cultures become positive more rapidly (5–30 days compared with months for lesions to appear on an animal) and the materials are less expensive. However, for *in-vitro* isolation, the techniques used should be carried out under strictly sterile conditions, which is rarely feasible in the field. Unfortunately, there is still no 'universal' culture medium in which all the different leishmanias will grow easily, and it is almost impossible to predict which medium will be best suited to the growth of a particular isolate of *Leishmania*. Individual laboratories have to find the most suitable medium among biphasic blood agar media and tissue culture media supplemented with fetal calf serum (14). When attempting primary isolation of unknown organisms, a blood agar-based medium should be used – preferably NNN medium (Novy, McNeil and Nicolle), otherwise brain–heart infusion (BHI) agar medium or EMTM (Evan's modified Tobie's medium) should be used. For bulk cultivation of established isolates, suitable media are reported in Section B.1.a (see ref. 14 for media composition). The organisms from patients with VL and MCL can be very difficult to cultivate. The parasites sometimes die when subcultured, even when the initial isolation is successful. This seems especially common when the initial isolation has been into a rich medium. Often this can be overcome if subcultures are made into less nutritionally rich media, such as NNN, or one of the semisolid media such as 'sloppy Evans' or semisolid Locke blood agar.

Hamster (*Mesocricetus auratus*) is the most commonly used animal for *in-vivo* isolation. Tissue suspensions or aspirates are inoculated intradermally into the nose and/or feet in the case of detection of dermotropic parasites. When the material is suspected to be infected with parasites causing VL, the inoculation should preferably be made by the intraperitoneal route. The resulting infection becomes apparent, weeks or months later, by the development of a nodule or ulcer at the site of inoculation, and in case of viscerotropic parasites, the infection becomes apparent, some months later, by massive infection of internal organs. The examination of Giemsa-stained smears of hamster tissue suspension/aspirate will show amastigotes. BALB/c mice are commonly used for the diagnosis of *L. major*.

Several techniques are now being used in many centres to identify the different *Leishmania* species, subspecies or strains.

a) Isoenzyme characterisation, also known as MLEE (multi-locus enzyme electrophoresis), is the reference method for species identification (23, 39, 45, 50), although this technique requires cultivation of a large number of parasites (5×10^9–1×10^{10}). The principles of enzyme electrophoresis are as follows: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM/FCS/EBLB [minimal essential medium/fetal calf serum/Evans' blood lysate broth] medium, Schneider's *Drosophila* medium). A small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is usually chosen so that the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins will have moved in the matrix

towards the anode, depending on the amount of negative charge. If stained at this stage with a general protein stain, many bands will be seen. However, the high substrate and cofactor specificity of enzymes make it possible to stain only these proteins. Hence, the electrophoretic mobility of one particular enzyme can be compared among several organisms. The stained matrix with its collection of stained isoenzyme bands is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. Most enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined; organisms showing identical zymograms are classified into zymodemes of a given species.

b)   The monoclonal antibody (MAb) technique is applied to the analysis and classification of *Leishmania* species and subspecies (20). For the production of the antibodies, BALB/c mice are immunised with membrane preparations from either promastigotes or amastigotes. Antibody-secreting hybridoma cultures are then selected and cloned by limiting dilutions. Specificity to *Leishmania* strains is assessed through immunofluorescence or immunoradiometric assays. This analysis should be quantitative, as the amount of the same surface antigen may vary among *Leishmania* species. Monoclonal antibodies have also been used in immunohistochemical techniques applied to tissue biopsies.

c)   DNA hybridisation probes are a very specific tool the principle of which is to allow labelled, single-stranded nuclear or kinetoplast DNA sequences from well characterised standard strains to find and hybridise with homologous DNA sequences from or within unknown *Leishmania* isolates (19, 44). Only complementary DNA sequences will form double-stranded DNA, which can be detected by autoradiography if the probe is radiolabelled, or by immunoenzymatic reaction. These techniques are sensitive enough to identify 10^2–10^3 organisms spotted on to nylon filters. Much fewer parasites (<10) are required for identification through the *in situ* hybridisation technique.

d)   Polymerase chain reaction (PCR)-based methods are available for diagnosis and/or identification of *Leishmania* from different types of human and canine samples. Essentially, techniques developed either to detect organisms from fresh or frozen, formalin-fixed and paraffin-embedded biopsies, or to identify established isolates of *Leishmania* include: (a) digestion of material with proteinase K and DNA extraction; (b) standard PCR amplification using oligonucleotide sequences (primers) selected from the small-subunit rRNA gene (28), kinetoplast DNA minicircles (25) or other highly repetitive genomic DNA sequences (9, 36); (c) analysis of amplification products by 1–2% agarose gel. To increase sensitivity, a nested or semi-nested PCR using internal primers from the above sequences can be performed. In human VL, PCR has a sensitivity comparable with that of culture-based methods, but gives results much faster. In CanL, the diagnostic efficacy of PCR as compared with serology depends on the natural course of the disease, the sensitivity being highest shortly after infection (37). In American CL and MCL, PCR appears to be consistently more sensitive than any previously recommended method of diagnosis (13). Different techniques have been described that improve both sensitivity and specificity of the method, such as the PCR-RFLP (restriction fragment length polymorphism) analysis in which the PCR products are digested by appropriate restrictions enzymes and the resulting restriction fragment pattern is analysed for species or strain identification (30, 46). Real-time PCR methods, which allow the continuous monitoring of the accumulation of PCR products during amplification, have been described and are available commercially. They can be more sensitive than conventional PCR, and are mainly addressed to study the kinetics of infection and monitoring therapeutic response (3, 7). In addition, real-time PCR has been reported to be useful for evaluating infections in less invasive samples such as blood (15).

2.   Serological tests

Several serological tests are used for detecting anti-leishmanial antibodies. Sensitivity values reported below for each test, however, apply only to individuals who are not immunocompromised. A high percentage of patients with VL co-infected with human immunodeficiency virus (HIV) have been reported to be seronegative for anti-leishmanial antibodies (18).

a)   Indirect fluorescent antibody test

The indirect fluorescent antibody (IFA) test is widely used because it is easy to perform. The test is genus specific, although significant cross-reactions have been reported in individuals infected with *Trypanosoma cruzi*. For these subjects, serological tests based on specific recombinant *Leishmania* antigens would be more appropriate (see Section B.2.b and d below). In Chagas' disease-free areas, the IFA test for the diagnosis of clinical VL or CanL has a sensitivity of 96% and specificity of 98%, which is similar to the ELISA. Although amastigotes from frozen sections or smears of infected organs can be used as antigen, cultured promastigotes represent the commonest antigen source.

- **Antigen preparation**

i)   Harvest 3–4 ml of the liquid media of a 3-day-old culture showing flourishing promastigote growth (see Section B.1 for culture media).

ii) Wash the organisms three times with phosphate buffered saline (PBS), pH 7.2–7.4, by centrifugation at 350 **g** for 15 minutes at room temperature.

iii) Resuspend the final cell pellet in PBS and adjust the promastigote concentration to approximately 4×10^6/ml with the aid of a haemocytometer.

iv) Distribute 30 µl of the promastigote suspension on to each circle of a multispot slide and allow to dry at room temperature.

v) Fix the promastigotes in cold acetone for 10 minutes, then put the slides into a plastic box and keep in a deep freezer (–35°C) for no longer than 2–3 months.

- **Test procedure**

i) Wash the frozen antigen-coated slides in PBS and allow to dry at room temperature.

ii) Inactivate the sera for 30 minutes in a water bath at 56°C.

iii) Make doubling dilutions of test sera from 1/80 to 1/10,240 for human VL, and from 1/40 to 1/5120 for CanL. Positive and negative control sera, at dilutions of 1/80 and 1/160 for human VL, and of 1/40 and 1/80 for CanL, are also included in the test. No standard sera are available, but internal standards should be prepared and titrated.

iv) Distribute 30 µl of diluted serum samples on to each slide circle and incubate for 30 minutes at 37°C.

v) Remove the serum samples by vigorous washing in PBS, followed by immersion of the slides in PBS for 10 minutes. Allow the slides to dry.

vi) Distribute 30 µl of diluted fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin on to each slide circle and incubate for 30 minutes at 37°C. FITC-conjugated anti-human and anti-dog immunoglobulins are commercially available. Follow the instructions for the appropriate dilution.

vii) Repeat step v and mount with a cover-slip in a few drops of PBS/glycerol (50% [v/v] of each).

viii) Read the slides under a fluorescent microscope. The highest dilution showing fluorescent promastigotes is taken to be the antibody titre. In human VL, the threshold titre usually ranges from 1/80 to 1/160, while in CanL it ranges from 1/40 to 1/160. As IFA test performance may vary in different laboratories, it is better for each laboratory to define its own threshold titre using defined positive and negative reference sera.

b) Enzyme-linked immunosorbent assay

The ELISA can be carried out on serum or on a measured volume of blood. The blood is collected by needle-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted and tested at a single dilution previously determined to give an acceptable sensitivity and specificity. This test can be used for seroepidemiological surveys under field conditions.

In the classical method, the antigen is prepared as follows: promastigotes harvested from cultures are washed four times with PBS, pH 7.2, at 1000 **g** for 15 minutes. The packed promastigotes are resuspended in twice their volume of distilled water, and then sonicated at medium amplitude in an ice bath. The suspension is left at 4°C overnight to allow the proteins to come into solution. After a final centrifugation at 4000 **g** for 10 minutes to eliminate the cellular debris, the overlay, representing the concentrated soluble antigen, is dispensed into vials and stored at –20°C until required. For use in the test, it is reconstituted with PBS to the predetermined optimal protein concentration (around 20 µg/ml) as measured by Lowry's method. The ELISA is useful for the diagnosis of Old and New World leishmanioses. There is little or no cross-reaction with other diseases and, according to the *Leishmania* strain used, sensitivity can range from 86% to 99%.

A version of the ELISA called the Falcon assay screening test and enzyme-linked immunosorbent assay (FAST-ELISA) and which uses antigen-coated beads, is considered to be a sensitive, specific and field-adaptable test for visceral CanL with comparable sensitivity and specificity to the IFA test and ELISA. Whole blood or plasma can be evaluated quickly without the use of a microscope or spectrophotometer (1).

A detergent-soluble promastigote antigen has been used in ELISA instead of the crude lysate, for the diagnosis of CanL. The detergent was Triton X-100 and the proteic extract was protected with protease inhibitors. Using this method, ELISA sensitivity increased to 99.5%, while its specificity was comparable with that of the IFA test (97%) (26).

The ELISA methods described above are all based on crude antigenic preparations. More recently, a recombinant antigen from a cloned protein of *L. infantum*, called rK39, has been reported to be highly reactive to sera from human and canine visceral leishmaniosis cases when run in an ELISA format. Using 25–50 ng of the antigen, 99% specificity and sensitivity was consistently found for immunocompetent

human patients with clinical VL and for dogs with parasitologically proven disease (2, 41). In HIV-positive patients, K39-ELISA showed higher sensitivity (82%) than the IFA test (54%) (24). The K39 antigen, which shows remarkable stability and reproducibility, is now produced commercially.

c) Direct agglutination test

The direct agglutination test (DAT) has been described for the diagnosis of VL and CanL. After test improvement, DAT has been validated as a specific and sensitive assay for field investigations (4, 10, 35). The antigen consists of promastigotes harvested from cultures, washed in PBS, pH 7.2, treated with 0.4% trypsin (for 45 minutes at 37°C and then washed again), and stained with 0.02% Coomassie brilliant blue. Twofold serial dilutions of serum in PBS are made in V-bottomed microtitre-plate wells; 50 µl of antigen preparation is added to each well, and the plate is then carefully shaken by hand and left for 18 hours at room temperature. The test is read visually against a white background. Positive reactions are indicated by a clear sharp-edged blue spot.

A modified DAT for detection of specific anti-leishmanial antibodies in canine reservoir hosts is considered to be highly suitable for wide-scale epidemiological and ecological field work and diagnosis of CanL, having 100% sensitivity and 98.9% specificity (21, 22). The reliability of the test was improved by treating the test sera with 0.2 M 2-mercaptoethanol and incubating them at 37°C.

d) Rapid immunochromatographic assay (dipstick or strip-test)

A rapid immunochromatographic assay using rK39 as antigen (K39 dipstick or strip-test, commercially available) has been evaluated in different endemic settings of VL. The nitrocellulose membrane of the test kit holds an absorbent pad at one end, a band of immobilised anti-protein A antibody (used to detect IgG) at the other (control region), and a band of rK39 antigen in the middle (test region). A protein-A-colloidal gold conjugate is used as the immunochromatographic detection reagent. One small drop (20 µl) of the serum to be examined is placed on the absorbent pad before two large drops (100 µl) of test buffer are added to the pad, and the mixture is allowed to migrate up the strip by capillary action. After 2–10 minutes, the result is positive if two distinct red lines appear (one in the test region and another in the control region), it is negative when no red line appears in the test region, and it is invalid if the control line fails to appear.

In clinical cases of human VL, the K39 dipstick showed 100% sensitivity and 93% specificity in India (43), 90% sensitivity and 100% specificity in Brazil (11), and 100% sensitivity and specificity in the Mediterranean basin (8). In parasitologically proven CanL, in both asymptomatic and symptomatic cases, the sensitivity of the K39 dipstick was 97% and the specificity 100% (34).

3. Delayed hypersensitivity test

Delayed hypersensitivity is an important feature of all forms of human leishmaniosis and can be measured by the leishmanin test, also known as the Montenegro reaction (27). The leishmanin skin test has no value for the diagnosis of CanL. Leishmanin is a killed suspension of whole (0.5–1 × 10^7/ml) or disrupted (250 µg protein/ml) promastigotes in pyrogen-free saline containing phenol. A delayed reaction develops and is read at 48–72 hours.

The false-positive reaction rate in otherwise healthy people is approximately 1%, but this can be higher in areas where there is a background of leishmaniosis, as many of the healthy population may show quite high rates of leishmanin sensitivity. Although there is complete cross-reactivity among all strains of *Leishmania,* although heterologous antigens often give smaller reactions, which may be caused by difficulty in standardisation. The leishmanin test is used in the clinical diagnosis of CL and MCL. In VL it will only measure past infections because during active disease, a complete anergy is found. Leishmanins are not available commercially.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccine

There is no effective vaccine available for prophylactic immunisation against leishmaniosis. Until now, the only dependable vaccination against *Leishmania* has been limited to the protection of humans from both *L. tropica* and *L. major* by prior syringe-induced infection with *L. major* organisms. The promastigotes are injected into the arm or other parts of the body. The living promastigotes used must either be freshly extracted from cultures or may be preserved in liquid nitrogen. The infection is allowed to run a natural course and after recovery, the individual is firmly immune to subsequent infection with both Leishmania species. This type of immunisation has been practised on a limited scale in hyperendemic areas of CL (due to *L. major*) in Israel, Iran and the former Union of Soviet Socialist Republics (42). *Leishmania major* causes cross-protection against *L. tropica*, but the reverse is probably not true. However, this species cannot be considered to be totally safe and this type of immunisation should be used only for humans moving into high-risk areas. Moreover, it is not beneficial in highly endemic areas as individuals contract infection long before this type of preparation confers protection (i.e.

approximately 3 months after vaccination). Standardisation and quality control of such vaccines, presently not available, are needed.

At present, a number of promising anti-leishmanial vaccines are under development (12, 16). Among the first-generation vaccines, the glycoprotein-enriched fraction of *L. donovani* known as 'fucose-mannose ligand' (FML), developed in Brazil, represents the first licensed veterinary vaccine against CanL. Field studies showed about 80% clinical protection conferred by the antigen administered with QuilA saponin as adjuvant (5), and also good immunotherapeutic efficacy when used in sick dogs (6). Killed *Leishmania* organisms mixed with a low concentration of BCG as adjuvant have undergone phase I–II and phase III trials for immunisation against CL *Leishmania* agents in humans and against VL in humans and in dogs, with limited success (32, 33).

Second-generation vaccines, most of which are at predevelopment stage, consist of genetically reconstructed *Leishmania* parasites incapable of producing disease, recombinant molecules or their corresponding DNAs, or recombinant organisms carrying leishmanial genes and expressing parasite antigens. A chimeric antigen generated from three recombinant *Leishmania* antigens screened for their ability to elicit cellular immune responses (known as Leish-111f), entered Phase I clinical testing in healthy volunteers in January 2003 (38). The same polyproteinic antigen, administered with monophosphoryl lipid A – stable emulsion (MPL-SE) or Adjuprime as adjuvants, failed to protect dogs from *L. infantum* infection in a phase III trial (17).

2. Immunodiagnostic antigens

Neither the leishmanin used for skin tests nor the antigens commonly employed in serodiagnosis in leishmaniosis are internationally standardised (the recombinant K39 antigen, which is virtually standardised, is patent-protected and is not widely available). The leishmanin test is group-specific, not species-specific, and the leishmanin prepared from one clinical type of leishmaniosis will cause the development of delayed hypersensitivity to the same or other clinical types. Similarly, serological cross-reactions are common among leishmanial species.

a) Leishmanin

The leishmanin test is described in Section B.3. Sterility, safety and potency tests are required for leishmanin preparations.

b) Antigens for serological tests

Commercial antigens for the IFA tests and ELISAs have been produced are still under evaluation. The main reason for unsatisfactory results with these antigens is the poor stability of leishmanial antigens. They can be obtained in the laboratory by growing a *Leishmania* strain in a suitable culture medium. For the IFA test and the DAT, crude particulate antigens, i.e. intact promastigotes, are required, whereas for ELISAs a soluble form of the antigen is needed.

3. Seed management

a) Characteristics of the seed

Strains of *Leishmania* species used to prepare biological products should be identified at species and subspecies level by appropriate identification tests given in Section B.1. Once the organisms have been isolated and established in the laboratory, they must be assigned an International Code (45, 50). This Code should consist of four elements separated by oblique strokes: (a) the type of host from which the strain was isolated (M for Mammalia and I for Insecta followed by three letters indicating the generic name of the host); (b) the country where isolation was made, indicated by a two-letter code; (c) the year of isolation indicated by the last two digits, and (d) the original laboratory code given to the isolate (for example, MHOM/IN/80/DD8). The parasites must be free from contaminating organisms and should be capable of yielding a product that conforms to the norms. Standard strains are available on request from the World Health Organization (WHO) Collaborating Centres in Madrid (Spain), Montpellier (France) and Jerusalem (Israel). A list of Identification Centres has been published by WHO (50).

b) Method of culture

The strain of the parasite used for preparing leishmanin should be capable of producing a product that conforms to national/international norms. It should be free from ingredients causing toxic or allergic reactions. There is no single specific antigen standardised for use in serodiagnostic tests, but when these antigens are prepared in the laboratory, they must be standardised for their sensitivity depending on the requirement. For the preparation of leishmanin as well as serodiagnostic antigens, the organisms should be grown in a suitable culture medium (such as those recommended in Section B.1 for *Leishmania* isolation and bulk cultivation). Normally, good growth of parasites is obtained 7 days after inoculation, and care must

be taken that leishmanial stocks are not lost by overgrowth of the flagellates, which may occur after approximately 10 days.

c) Cryopreservation

Promastigote cultures and tissue infected with amastigotes may easily be conserved in the living state at low temperatures. Both forms can be cryopreserved for years at low temperatures in mechanical freezers (–70°C), in solid carbon dioxide containers (–76°C), or in liquid nitrogen containers (–196°C) (45). A sterile cryoprotectant is required – glycerol, to give a final concentration of 7.5–10%, or dimethyl sulphoxide (DMSO), to a final concentration of 5–7.5%. The cryoprotected samples are transferred to the sterile containers in which they are to be frozen. These may be 2 ml plastic freezing tubes with airtight screw-caps, hard glass, heat-sealed ampoules, or glass/plastic capillaries. A slow cooling rate (approximately 1°C/minute) is essential for the cryopreservation of *Leishmania*. This can be obtained by cooling samples to 4°C and keeping them at this temperature for a minimum of 1 hour; they are then transferred to a –20°C freezer and left for 24 hours, then removed to a –70°C freezer for at least 24 hours. They can be permanently stored at this temperature, or else transferred into liquid nitrogen or solid carbon dioxide. If possible, a programmable freezing unit should be used. When the cryopreserved material is required, the sample is taken out and thawed rapidly in a water bath at 37°C.

d) Validation

Cultures for leishmanin or serodiagnostic antigens should be checked for sterility before use. Leishmanin is stored at 4°C and serodiagnostic antigens at –20°C or –70°C until required. The latter should be reconstituted with PBS, pH 7.2, before use. Viable *Leishmania* cultures can be kept at –70°C for 3–4 years or at –196°C indefinitely. Because of nonavailability of suitable vaccine, it has not been possible to validate the currently developed immunising agents. Live or attenuated promastigotes of *L. major* used in some areas are far from being satisfactory. Leishmanin should be tested for allergenicity in guinea-pigs before use. Serodiagnostic antigens should be tested for their efficacy and sensitivity by proper standardisation for a particular test. If a batch of antigen has not been used for a long time, it should be rechecked before being used in the test.

4. Method of manufacture

As standardised immunodiagnostic antigens are not available commercially, they need to be prepared in the laboratory. Workers in the laboratory can be at risk of laboratory acquired infection, especially by injection. Appropriate biosafety precautions are therefore essential to minimise the risks (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

a) Leishmanin

Leishmania species are grown, preferably in blood-free liquid media such as Schneider's *Drosophila* medium and RPMI (Rosewell Park Memorial Institute) medium, in order to avoid blood–antigen contamination. The promastigotes are harvested during the log phase, washed four times in pyrogen-free saline at 1000 ***g*** for 15 minutes, and resuspended in pyrogen-free saline containing 0.5% phenol (w/v) to obtain a final concentration of $0.5–1 \times 10^7$/ml. Leishmanin can also be made with disrupted promastigotes obtained as above and sonicated. The filtrate is adjusted to a final protein concentration of 250 µg/ml with pyrogen-free saline containing Tween 80 (0.0005% [v/v]) and phenol (0.28% [w/v]).

b) Antigens for serological tests

Methods of antigen preparation for various tests are given in Section B.2.

5. In-process control

One or more batches of leishmanin should be tested in guinea-pigs by allergic test. Sensitivity and specificity of the leishmanin should preferably be determined by performing the test in appropriate animal models (different inbred mice according to the *Leishmania* species), or in patients who have recovered from leishmanial infections, and in an unexposed control population.

6. Batch control

The WHO has suggested guidelines for the production of leishmanin (48, 49). It is recommended that the source material be controlled by using isoenzyme analysis to type the *Leishmania* strains used in preparing leishmanin.

a) Sterility

Each filling lot should be tested for bacterial and mycotic sterility according to WHO (47). Absence of live leishmaniae is checked by inoculating one sample of each lot in an appropriate blood–agar medium, which

is then incubated at 23°C for at least 15 days. One sample is injected intradermally (for dermotropic leishmaniae) or intraperitoneally (for viscerotropic leishmaniae) in mice or hamsters. These animals are observed during a period of 30–90 days.

b) Safety

Samples from each filling lot should be tested for abnormal toxicity by appropriate tests in guinea-pigs and mice. For each lot, five mice weighing 17–22 g and two guinea-pigs weighing 250–350 g are injected subcutaneously and intraperitoneally with one human dose of the product. The animals are then observed for at least 7 days for death or signs of disease.

c) Potency

The leishmanin is tested on animal models (according to the *Leishmania* species involved) that have been previously infected by the same strain used for leishmanin production. Lots of at least five infected animals and control animals are injected intradermally into one of the posterior footpads with 50 µl of leishmanin. After 2–3 days, all the infected animals should show a significant enlargement of the footpad compared with control animals.

7. Tests on the final product

a) Safety

See Section C.6.b.

b) Potency

See Section C.6.c.

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LEPTOSPIROSIS

SUMMARY

Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus Leptospira. *Laboratory diagnosis of leptospirosis can be complex and involves tests which fall into two groups. One group of tests is designed to detect anti-leptospiral antibodies and the other group of tests is designed to detect leptospires, leptospiral antigens, or leptospiral nucleic acid in animal tissues or body fluids. The particular testing regimen selected depends on the purpose of testing* (*e.g. herd surveys or individual animal testing*) *and on the tests or expertise available in the area.*

Identification of the agent: *The isolation or demonstration of leptospires in:*

a)   *the internal organs* (*such as liver, lung, brain, and kidney*) *and body fluids* (*blood, milk, cerebrospinal, thoracic and peritoneal fluids*) *of clinically infected animals gives a definitive diagnosis of acute clinical disease or, in the case of a fetus, chronic infection of its mother.*

b)   *the kidney, urine, or genital tract of animals without clinical signs is diagnostic only of a chronic carrier state.*

Isolation of leptospires from clinical material and identification of isolates is time-consuming and is a task for specialised reference laboratories. Isolation followed by typing from renal carriers is important and very useful in epidemiological studies to determine which serovars are present within a particular group of animals, an animal species, or a geographical region.

The demonstration of leptospires by immunochemical tests (*immunofluorescence and immunohistochemistry*) *is more suited to most laboratory situations. However, the efficacy of these tests is dependent on the number of organisms present within the tissue, and these tests lack the sensitivity of culture. Unless specially prepared reagents are used, immunochemical tests do not identify the infecting serovar and results must be interpreted in conjunction with serological results. Reagents for immunofluorescence are best prepared with high IgG titre anti-leptospire sera, which are not available commercially. Rabbit leptospiral-typing serum or monoclonal antibodies can be used for immunohistochemistry and are available from leptospiral reference laboratories.*

Genetic material of leptospires can be demonstrated in tissues or body fluids using a variety of assays based on the polymerase chain reaction (*PCR*), *either in real-time or traditional formats. PCR assays are sensitive, but quality control procedures and sample processing for PCR are critical and must be adjusted to the tissue, fluid and species being tested. Like immunochemical tests, PCR assays do not identify the infecting serovar.*

Serological tests: *Serological testing is the most widely used means for diagnosing leptospirosis, and the microscopic agglutination test* (*MAT*) *is the standard serological test. Antigens selected for use in the MAT should include representative strains of the serogroups known to exist in the particular region plus those known to be maintained elsewhere by the host species under test.*

The MAT is used to test individual animals and herds. As an individual animal test, the MAT is very useful for diagnosing acute infection: a four-fold rise in antibody titres in paired acute and convalescent serum samples is diagnostic. To obtain useful information from a herd of animals, at least ten animals, or 10% of the herd, whichever is greater, should be tested and the vaccination history of the animals documented.

The MAT has limitations in the diagnosis of chronic infection in individual animals and in the diagnosis of endemic infections in herds. Infected animals may abort or be renal/genital carriers with MAT titres below the widely accepted minimum significant titre of 1/100 (final dilution).

Enzyme-linked immunosorbent assays (ELISAs) can also be useful for detection of antibodies against leptospires. Numerous assays have been developed and are primarily used for the detection of recent infections and the screening of experimental animals for use in challenge studies. Animals that have been vaccinated against the serovar of interest may be positive in some ELISAs, thus complicating interpretation of the results.

Requirements for vaccines and diagnostic biologicals: *Vaccines for veterinary use are most often suspensions of one or more serovars of* Leptospira *spp. inactivated in such a manner that immunogenic activity is retained. While a range of experimental vaccines based on cellular extracts have been tested, commercial vaccines are, with few exceptions, whole cell products. The leptospires are grown in suitable culture media, which often contain serum or serum proteins. If used, serum or serum proteins should be removed from the final products. Vaccines may contain suitable adjuvants.*

A. INTRODUCTION

Leptospirosis is a transmissible disease of animals and humans caused by infection with the spirochete *Leptospira*. All the pathogenic leptospires were formerly classified as members of the species *Leptospira interrogans*, however the genus has recently been reorganised and pathogenic leptospires are now identified in 17 named species and four genomospecies of *Leptospira* (14, 61, 71, 105). There are more than 200 distinct leptospiral serovars recognised and these are arranged in 23 serogroups (52, 98).

The use, interpretation, and value of laboratory diagnostic procedures for leptospirosis vary with the clinical history of the animal or herd, the duration of infection, and the infecting serovar. Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and acute renal failure or jaundice in dogs. Chronic leptospirosis should be considered in the following cases: abortion, stillbirth, birth of weak offspring (may be premature); infertility; chronic renal failure or chronic active hepatitis in dogs; and cases of periodic ophthalmia in horses. Two major chronic microbiological sequelae of leptospiral infection present particular diagnostic problems: the localisation and persistence of leptospires in the kidney and in the male and female genital tract. Chronically infected animals may remain carriers for years to life and serve as reservoirs of the infection for other animals and humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The demonstration of leptospires in blood and milk of animals showing clinical signs suggestive of acute leptospirosis is considered to be diagnostic. However, isolation from blood is not often successful because bacteraemia is transient and not always accompanied by clinical signs. Dogs are often treated with antibiotics before samples are collected for testing for *Leptospira,* which further decreases the likelihood of identifying the agent in blood. The demonstration of generalised leptospiral infection in a range of organs taken at necropsy is also considered to be diagnostic. However, if the animal lives long enough or has been treated with antibiotics, it may be difficult to detect intact organisms systemically; immunohistochemistry can be particularly helpful in identifying residual leptospiral antigen in these cases. Demonstration of leptospires in the genital tract, kidneys, or urine only must be interpreted with full consideration of the clinical signs and serological results as these findings may merely indicate that the animal was a carrier.

Failure to demonstrate leptospires in the urine of an animal does not eliminate the possibility that the animal is a chronic renal carrier, it merely indicates that the animal was not excreting detectable numbers of leptospires at the time of testing. Collection of urine following treatment of the animals with a diuretic enhances the chances of detecting the organism (63). In important cases involving individual animals (e.g. clearing an infected stallion to return to breeding), negative tests on three consecutive weekly urine samples has been considered to be good evidence that an animal is not shedding leptospires in the urine.

The demonstration of leptospires in body fluids or internal organs (usually kidney, liver, lung, brain, or adrenal gland) of aborted or stillborn fetuses is considered to be diagnostic of chronic leptospirosis of the mother, and is evidence of active infection of the fetus.

In experienced hands, the isolation of leptospires is the most sensitive method of demonstrating their presence, provided that antibiotic residues are absent, that tissue autolysis is not advanced, that tissues are processed for culture rapidly after collection, and – in the case of urine – at a suitable pH. If tissues or fluids cannot be transported promptly to the laboratory for leptospiral culture, the sample should be kept at 2–5°C to prevent overgrowth with other bacteria and autolysis of tissue samples. Liquid culture medium or 1% bovine serum albumin (BSA) solution containing 5-fluorouracil at 100–200 µg/ml should be used as transport medium for the submission of samples.

Culture should be carried out in a semisolid (0.1–0.2% agar) medium containing BSA and either Tween 80 (e.g. Tween 80/BSA medium or EMJH) (44) or containing BSA and a combination of Tween 80 and Tween 40 (30). Contamination may be controlled by the addition of a variety of selective agents, e.g. 5-fluorouracil (48), nalidixic acid (49), fosfomycin (64), and a mixture of rifamycin, polymyxin, neomycin, 5-fluorouracil, bacitracin, and actidione (1). However, use of selective agents may reduce the chances of isolation when there are only small numbers of viable leptospires, and some strains of leptospires will not grow in selective media containing multiple antibiotics. Addition of 0.4–1% rabbit serum to semisolid culture medium enhances the chances of isolating fastidious leptospiral serovars.

Cultures should be incubated at 29 ± 1°C for at least 16 weeks, and preferably for 26 weeks (30). The time required for detection of a positive culture varies with the leptospiral serovar and the numbers of organisms present in the sample. Less fastidious serovars (e.g. Pomona and Grippotyphosa) may result in positive cultures as soon as 7–10 days after inoculation; other serovars (e.g. Hardjo and Bratislava) may take much longer. Cultures should be examined by dark-field microscopy every 1–2 weeks. It is important to use a 100 watt light source and a good quality dark-field microscope.

Leptospires may also be demonstrated by a variety of immunochemical staining techniques, e.g. immunofluorescence (10, 31), and various immunohistochemical techniques (5, 33, 79, 81, 99, 106). These are useful in diagnosing infection in pathological material that is unsuitable for culture or where a rapid diagnosis is required. As the success of these techniques is dependant on the number of organisms present, they are less suitable for diagnosing the chronic carrier state, where the numbers of organisms may be very low or localised. Leptospires do not stain satisfactorily with aniline dyes, and silver-staining techniques lack sensitivity and specificity, although they are a useful adjunct for histopathological diagnosis (7).

Polymerase chain reaction (PCR)-based assays are now used in some diagnostic and many reference laboratories for the detection of leptospires in tissues and body fluids of animals. A variety of primer sets for the conduct of PCR assays have been described (3, 6, 13, 37, 39, 44, 50, 51, 54, 59, 60, 66, 84, 93, 97, 103) with some primers only specific for the genus *Leptospira* and others designed to identify only pathogenic species. These assays do not identify the infecting serovar, although some primer sets may permit further identification to the species or serovar level if the PCR amplicons are sequenced. This further analysis is not a routine diagnostic method. Many of the PCR primer sets have been designed and evaluated for use in human rather than animal specimens and general agreement about the PCR primers to be used for testing of animal samples is lacking. Therefore, the individual laboratory is generally responsible for the validation of the particular assay they use for the tissue, fluid, and species being tested. PCR assays can be quite sensitive, but lack of specificity (i.e. false-positive results) can be a problem. Presence of amplification inhibitors in clinical samples can cause false-negative results, particularly in animal specimens that may be compromised by contamination with faeces or autolysis. Quality control of PCR assays used for diagnosis of leptospirosis requires careful attention to laboratory design and workflow to prevent contamination of reagents, and use of appropriate control samples (29, 57). In addition, sample processing for PCR is critical and must be suited to the tissue, fluid, and species being tested. A procedure for the preparation of urine samples for PCR using magnetic beads coated with anti-leptospiral antibody shows promise in enhancing the detection of pathogenic leptospires in urine (88).

The identification of leptospiral isolates is a task for specialised reference laboratories. For complete identification, a combination of procedures is used to determine: 1) if the isolate is a pathogen or a saprophyte; 2) the species of *Leptospira* to which the isolate belongs; and 3) the serogroup and serovar of the isolate. A pure leptospiral culture may be identified as belonging to a pathogenic or saprophytic species by a variety of tests: the ability to infect animals; the relative resistance to 8-azaguanine; lipase activity; salt and temperature tolerance (46, 47); PCR-assay-based amplification of 23S rDNA (102); and G+C content of DNA (46).

New leptospiral species have been identified based on DNA–DNA hybridisation analysis (14, 71, 105). In most cases, the type strain for each serovar was used in these analyses; for a few serovars, clinical isolates have also been tested to determine the new species designations. Different isolates belonging to a single serovar usually belong to the same species, but this is not always the case. Species identification of field isolates is still cumbersome but can be done by sequence analysis of the 16S rDNA, by genetic analysis of the 16S or 23S ribosomal RNA genes (17, 53, 61, 68, 70, 100, 101), by multilocus sequence typing (4, 77), by sequencing the DNA gyrase subunit B encoding gene (82), or by PCR using species-specific *ompL1* primer sets (73).

Strains belonging to *Leptospira* can be differentiated to the serogroup level by cross-agglutination reactions (28). Further differentiation to the serovar level was traditionally by cross-agglutination absorption, although for most isolates this is now being done using less time-consuming methods: factor analysis (28), monoclonal antibodies (MAbs) (89, 90), restriction endonuclease analysis (43, 56, 91, 92), and various other molecular strategies (17, 21, 26, 38, 61, 69, 70, 72, 75, 77, 78, 82, 83, 107, 108). However, genetic-based tests may not always give the same results as the cross-agglutination absorption test.

2. Serological tests

Serological testing is the laboratory procedure most frequently used to confirm the clinical diagnosis, to determine herd prevalence, and to conduct epidemiological studies. Leptospiral antibodies appear within a few days of onset of illness and persist for weeks or months and, in some cases, years. Unfortunately, antibody titres may fall to undetectable levels while animals remain chronically infected. To overcome this problem, sensitive methods are needed to detect the organism in urine or the genital tract of chronic carriers.

A wide variety of serological tests, which show varying degrees of serogroup and serovar specificity, have been described. Two tests have a role in veterinary diagnosis: the microscopic agglutination test (MAT) and the enzyme-linked immunosorbent assay (ELISA).

a) Microscopic agglutination test

The MAT using live antigens is the most widely used serological test. It is the reference test against which all other serological tests are evaluated and is used for import/export testing. For optimum sensitivity, it should use antigens representative of all the serogroups known to exist in the region in which the animals are found and, preferably, strains representing all the known serogroups. The presence of a serogroup is usually indicated by frequent reaction in serological screening but can only be definitively identified by isolation of a serovar from clinically affected animals. The sensitivity of the test can be improved by using local isolates rather than reference strains, but reference strains assist in the interpretation of results between laboratories.

The specificity of the MAT is good; antibodies against other bacteria usually do not cross-react with *Leptospira* to a significant extent. However, there is significant serological cross-reactivity between serovars and serogorups of *Leptospira* and an animal infected with one serovar is likely to have antibodies against the infecting serovar that cross-react with other serovars (usually at a lower level) in the MAT. Therefore, serology cannot be used to definitively identify the infecting serovar in an individual infection or outbreak – this requires isolation of the agent. However, in areas where the serovars of *Leptospira* present have been well described by isolation studies, serological examination of the infected animal(s) may suggest, but not definitively identify, the infecting serovar. In addition, animals that have been vaccinated against leptospirosis may have antibodies against the serovars present in the vaccine used. Therefore, it is particularly important to consider the vaccination history of the animals under test. The two methods for carrying out the test have been described in detail (36, 62).

The strains selected should be grown in liquid leptospiral culture medium (e.g., EMJH, Tween 80 BSA, or other suitable medium) at 29 ± 1°C and the culture should be at least 4 days old, but no more than 8 days. Live cultures with densities of approximately 2×10^8 leptospires per ml are to be used as the antigens. The culture density can be determined by counting the cells directly using a bacterial counting chamber and dark-field microscopy. Alternatively, cell counts can be estimated by measuring transmittance in a spectrophotometer with a 400 nm filter or by nephelometry. If indirect methods are used, direct bacterial cell counts should be correlated with the readings on the specific instrument being used. The number of antigens to be used is determined and a screening test may be performed with a 1/50 serum dilution (or a different starting dilution based on the purpose of the test). A volume of each antigen, equal to the diluted serum volume, is added to each well, making the final serum dilution 1/100 in the screening test. The microtitration plates are incubated at 29 ± 1°C for 2–4 hours. The plates are examined by dark-field microscopy.

The endpoint is defined as that dilution of serum that shows 50% agglutination, leaving 50% free cells compared with a control culture diluted 1/2 in phosphate buffered saline. The result of the test may be reported as the endpoint dilution of serum (e.g. 1/100 or 1/400) or as a titre that is the reciprocal of the endpoint serum dilution (e.g. 100 or 400). Many laboratories perform a screening test at a final serum dilution of 1/100 and then retest sera with titres of ≥100 to determine an endpoint using doubling dilutions of sera beginning at 1/100 through to 1/12,800 or higher.

Identity of antigens is a crucial factor in conducting the MAT. Antigens should be evaluated for identity, using hyperimmune rabbit sera, MAbs, or a molecular method that confirms passages over time, preferably each time the test is run, but at least twice a year. Hyperimmune rabbit serum for this purpose can be obtained from a reference laboratory or prepared using a protocol such as that given by the Subcommittee on the Taxonomy of *Leptospira* (45). Briefly, healthy rabbits weighing 3–4 kg that lack detectable anti-

leptospiral antibodies are selected. Each rabbit is given an intravenous injection in a marginal vein of the ear with a well-growing live or formalin-treated cloned culture with a density of approximately 2 × 10^8 leptospires/ml. The culture should be grown in Tween 80 BSA medium or another appropriate medium. Five injections of 1 ml, 2 ml, 4ml, 6 ml, and 6 ml each are given at 7-day intervals. One week following the final injection, the homologous antibody titre is determined by MAT. If the titre is ≥1/12,800, the rabbit is anaesthetised and bled by cardiac puncture 7 days later (i.e. 14 days after the final injection). If the titre is <1/12,800, a further injection of 6 ml of culture can be given; 7 days after this injection the homologous titre is again determined. Unless the titre is ≥1/12,800 the procedure should be repeated with another rabbit. Two rabbits are used to prepare each antiserum. If the titres are satisfactory in both rabbits, the sera may be pooled. To preserve potency, it is preferable to freeze-dry the antiserum in 2 ml volumes and store it at 2–5°C. Alternatively, the serum can be stored in 2 ml volumes at –15 to –20°C. All animal inoculations should be approved and conducted according to the relevant standards for animal care and use. Other immunisation protocols may be considered based on the intended use of the antiserum.

Purity of antigens used in the MAT should be checked regularly by culture on blood agar and in thioglycolate broth. Stock cultures of antigens may be stored at –70 to –80°C or in liquid nitrogen. There may be a low survival rate of leptospires after lyophilisation. Repeated passage of antigens in liquid medium results in a loss of antigenicity. In this case, a new liquid culture should be derived from the stock culture.

As an individual animal test, the MAT is very useful in diagnosing acute infection; the demonstration of a four-fold change in antibody titres in paired acute and convalescent serum samples is diagnostic. In addition, a diagnosis of leptospirosis is likely based on the finding of very high titres in an animal with a consistent clinical picture. The test has limitations in diagnosis of chronic infection in individual animals, both in the diagnosis of abortion (32) and in the identification of renal or genital carriers (30). This is particularly true with the host-adapted leptospiral infections, e.g. serovar Hardjo infection in cattle: when a titre of 1/100 or greater is taken as significant, the sensitivity of the test is only 41%, and even when the minimum significant titre is reduced to 1/10, the sensitivity of the test is only 67% (30). The demonstration of antibodies in fetal blood is diagnostic, but the titres are often very low, i.e. 1/10, requiring a modified testing procedure for most laboratories.

As leptospirosis is a herd problem, the MAT has much greater use as a herd test. To obtain useful information, Cole *et al.* (20) suggested that samples be taken from at least ten animals, or 10% of the herd, whichever is the greater. In a study of Hardjo infection in cattle, Hathaway *et al.* (42) found that a 10-cow sample usually indicated the presence or absence of infection in a herd. Increasing the sample size markedly improved epidemiological information, investigations of clinical disease, and public health tracebacks.

In making a serological diagnosis of leptospirosis, the infecting serovar and the clinical condition involved must be fully considered. In the case of serovar Pomona-induced abortion in cattle, a high titre is commonly found at the time of abortion because the clinical incident occurs relatively soon after infection. Abortion in cattle due to serovar Hardjo is a chronic event; in this case, the serological response at the time of abortion is more variable, with some animals seronegative and others showing high titres. Cattle may experience a drop in milk production during the acute phase of Hardjo infection and this clinical sign is associated with high titres. Vaccination history must also be considered in the interpretation of MAT results as widespread vaccination contributes significantly to the number of seropositive animals and may mask the presence of chronic infections in the herd – particularly with serovar Hardjo.

b)   Enzyme-linked immunosorbent assays

ELISAs for detection of anti-leptospiral antibodies have been developed using a number of different antigen preparations, assay protocols and assay platforms, including plate tests and dipstick tests. Information regarding the surface antigens of *Leptospira* has been reviewed (25). In general, ELISAs are quite sensitive, but lack the serovar specificity of the MAT. An ELISA that measures canine IgG and IgM against various leptospiral serovars has been developed and evaluated in Europe (40, 41). Anti-leptospiral IgM is detectable in this assay as early as 1 week after infection, before agglutinating antibodies are present. IgG antibodies are detectable in infected dogs beginning 2 weeks after infection and persist for long periods of time. Therefore, dogs with acute leptospirosis have high IgM titres and relatively low IgG titres; dogs that are vaccinated or have had previous leptospiral infections have high IgG titres but low IgM titres. Similar assays to detect anti-leptospiral bovine, porcine, and ovine antibodies have also been developed (2, 19, 24, 58, 74, 85–87, 94, 104). The major identified role of ELISA in livestock species is the use of an IgM ELISA for identification of recent infections (24) and for screening herds in regions where vaccination for leptospirosis is not practiced. A total-Ig ELISA is useful in identification of fully susceptible animals suitable for experimental challenge work (34). ELISAs have also been developed for use in milk from individual cows or in bulk tank milk for the detection of serovar Hardjo antibodies. These tests have been helpful in identifying Hardjo-infected herds. However, herds that are vaccinated against serovar Hardjo will also be positive in these various ELISAs decreasing their usefulness in regions where vaccination is a routine

practice. New ELISAs have been developed based on detection of antibodies against surface proteins or lipoproteins of *Leptospira* (12, 27, 55, 65, 67) but these tests are not yet widely available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Leptospiral vaccines for veterinary use are suspensions of one or more strains of pathogenic *Leptospira* inactivated in such a manner that immunogenic activity is retained. While experimental vaccines based on cellular extracts have been tested (9), commercial vaccines are, with few exceptions, whole-cell products. The leptospires are grown in suitable culture media that may contain serum or serum proteins. If used, serum or serum proteins should be removed from the final product. Vaccines may contain suitable adjuvants.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Proper selection of vaccine production strains is of utmost importance. Immunity induced by vaccination is largely serovar specific (18). A vaccine should be formulated for use in a particular animal species in a particular geographical region. It should contain only those serovars – and preferably those genotypes – that cause problems in the animal species, or that are transmitted by the animal species to other species in the region. Strains selected for use as master seed culture should be cloned on solid medium to ensure the absence of saprophytic *Leptospira* contaminants and uniformity of the culture.

Suitable strains should be further selected by their ability to grow to high yields under batch culture conditions.

b) Methods of culture

Each component strain to be included in the final vaccine should be grown separately in liquid medium; preferably in a protein-free (8, 80) or low-protein medium (8).

The volume of each master seed culture should be amplified by growth for 2–10 days at 29°C ±1°C in a series of subcultures until a volume sufficient for use as a production seed culture is achieved. Cultures should be aerated and agitated as required.

Each subculture of the master seed culture should be checked for purity and for satisfactory growth. Purity can be checked by inoculating a loopful of culture into blood agar plates or into thioglycolate broth for incubation at 35–37°C for 2–5 days, and by examining a Gram-stained smear of culture sediment. Growth can be checked by dark-field microscopy. Each production seed culture should also be checked against its' homologous rabbit antiserum (28) to ensure purity and homology. MAbs may also be used for this purpose.

c) Validation as a vaccine

There is a large volume of literature describing the efficacy of leptospiral vaccines. In most cases, vaccines provide significant protection against disease produced by homologous challenge under field conditions.

Vaccines are less efficacious at preventing infection in animals and a percentage of vaccinated animals will become infected with the relevant serovar and may shed the organism in their urine despite a lack of clinical signs of disease.

Efficacy trials and vaccine validation must be conducted in the target species for the vaccine. The vaccine should be administered as recommended on the label, and immunity should be tested by challenge with virulent field strains of each serovar by natural routes of infection, i.e. by conjunctival and/or vaginal challenge. Validation studies have often been conducted with challenge of immunity by intravenous or intramuscular injections of leptospires. Vaccines validated in this way have not always been shown to be protective against field challenge, which occurs by exposure of mucous membranes of the eye, mouth, and genital tract to leptospires. Most notably, commercial leptospiral vaccines containing serovar Hardjo have not always protected cattle from conjunctival or field challenge with serovar Hardjo (11). A draft monograph for the efficacy testing of serovar Hardjo vaccines has been prepared and specifies the use of more natural routes of challenge (35).

2. Method of manufacture

Manufacture is carried out by batch culture in appropriately sized fermentor vessels. These should be equipped with ports for the sterile addition of seed culture, air, and additional medium. They should also have sampling ports so that the purity and growth of the production culture can be monitored.

Ideally, low-protein or protein-free media are used for production. However, some strains require the presence of animal protein to achieve suitable yields; this is usually supplied as BSA. All media components that are not degraded by heat should be heat sterilised. This reduces the risk of contamination by water-borne saprophytic leptospires that are not removed by filter sterilisation.

After addition of the seed culture, the growth of the production culture is monitored at frequent intervals for the start of log-phase growth. Once this is observed, the vessel is then agitated and aerated. The final yield can often be improved by the addition of more Tween 80 to the culture when log-growth is first observed to be slowing down. Adequate growth may require up to 10 days of incubation at 29 ± 1°C.

Inactivation is usually by the addition of formalin, but phenol, merthiolate, and heat inactivation have also been used.

After the appropriate inactivation period, the culture may be concentrated and extraneous protein material may be removed by ultrafiltration. Suitable volumes of the various strains to be included in the final vaccine can then be blended, and adjuvant and preservative added, if appropriate.

3. In-process control

During production, daily or twice daily subsamples should be taken and monitored for growth of leptospires and absence of contaminants. Growth is monitored either by counting leptospires in a counting chamber under dark-field microscopy or by a nephelometer. The absence of contamination can be monitored by the microscopic examination of Gram-stained preparations of centrifuged culture.

Immediately prior to inactivation, a sample should be taken for checking against its homologous antibody in a MAT. The inactivated culture must be checked for freedom from viable leptospires. This is done by inoculating aliquots of inactivated culture into an appropriate growth medium, such as the medium of Johnson & Harris (47), incubating at 29 ± 1°C for at least 4 weeks, and examining weekly by dark-field microscopy for the presence of viable leptospires.

After blending, the levels of free inactivating agents, minerals present in adjuvants (such as aluminum), and preservative (such as thiomersal) must be within prescribed limits.

4. Batch control

a) Sterility

Selected samples of the completed vaccine should be tested for the absence of viable bacteria and fungi (16, 22, 23, 95). Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Samples of completed product should be tested for safety. Methods for this have been described elsewhere (15, 22, 96). The test should be carried out for each route of inoculation indicated on the label and in two healthy animals of each category (e.g. pregnant animals, young stock) for which the vaccine is intended. The animals must be susceptible to the serovars used in the vaccine and their sera must be free from agglutinating antibodies to those serovars. Each animal is given an injection of the vaccine by the recommended route with twice the recommended dose, as stated on the label. The animals are observed for 14 days and should show no adverse local or systemic effects attributable to the vaccine.

c) Potency

Samples of completed vaccine should be tested for potency in hamsters or guinea-pigs. Potency is usually measured by the vaccine's ability to prevent the death of the animal when challenged with between 10 and 10,000 LD_{50} (50% lethal dose). With some serovars that are not hamster or guinea-pig lethal, such as serovar Hardjo, potency is measured against prevention of renal infection when the animals are challenged with between 10 and 10,000 ID_{50} (50% infectious dose) or by induction of a suitable antibody titre in rabbits.

An example protocol is to inject 1/40 dog dose of the vaccine into each of ten healthy hamsters no more than 3 months old. After 15–30 days, each vaccinated hamster, and each of ten unvaccinated hamsters of the same age, is injected intraperitoneally with a suitable quantity of a virulent culture of leptospires of the serovar used to make the vaccine (or a suspension of liver or kidney tissue collected from an experimentally infected animal). In the case of bivalent vaccines, each serovar is tested separately. For the vaccine to pass the test, at least 8/10 of the vaccinated animals should remain in good health for 14 days after the death of the controls. Other protocols may apply to cattle and pig vaccines, which contain as many as five or six components.

In-vitro potency tests for leptospiral vaccines are being developed based on quantifying the protective antigen in the vaccine using MAbs in a capture ELISA (76). These assays are being standardised using reference vaccines and correlation with existing hamster or antibody-based potency assays and target–host efficacy data.

d) Duration of immunity

Duration of immunity should be determined in the animal species for which the vaccine is intended using natural routes of challenge (11). Duration of immunity should not be estimated based on the duration of MAT titres in vaccinated animals as protection against clinical disease may be present with very low titres. Vaccinal immunity should persist for at least 6 months or longer depending on the label claim.

e) Stability

When stored under the prescribed conditions, the vaccines may be expected to retain their potency for 1–2 years. Stability should be assessed by determining potency after storage at 2–5°C, room temperature, and 35–37°C.

5. Tests on the final project

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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NB: There are OIE Reference Laboratories for Leptospirosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

NEW WORLD SCREWWORM
(*Cochliomyia hominivorax*) AND
OLD WORLD SCREWWORM (*Chrysomya bezziana*)

SUMMARY

The New World screwworm[1] (*NWS*), Cochliomyia hominivorax (*Coquerel*)*, and the Old World screwworm*[1] (*OWS*)*, Chrysomya bezziana Villeneuve, are both obligate parasites of mammals during their larval stages. Both species are in the subfamily Chrysomyinae of the family Calliphoridae of the order Diptera (true flies). Larvae feeding on the skin and underlying tissues of the host cause a condition known as wound or traumatic myiasis, which can be fatal. Infestations are generally acquired at sites of previous wounding, due to natural causes or to animal husbandry practices, but they may also occur in the mucous membranes of body orifices.*

*Female flies are attracted to wounds at the edges of which each female lays an average of 175 (*OWS*) to 340 (*NWS*) eggs. The larvae emerge within 12–24 hours and immediately begin to feed, burrowing head-downwards into the wound. After developing through three larval stages (instars) involving two moults, the larvae leave the wound and drop to the ground into which they burrow to pupate. The duration of the life-cycle off the host is temperature dependent, being shorter at higher temperatures, and the whole cycle may be completed in less than 3 weeks in the tropics.*

Treatment is generally effected by application of organophosphorus insecticides into infested wounds, both to kill larvae and to provide a residual protection against reinfestation. Preventive measures include the spraying or dipping of susceptible livestock with organophosphorus compounds and, more recently, use of avermectins (especially doramectin) as subcutaneous injections to animals 'at risk'. Strict control of the movement of animals out of affected areas also acts as a preventive measure.

Identification of the agent: *The larvae of NWS and OWS can be easily confused with each other and with the larvae of other agents of myiasis. Accurate diagnosis involves the identification of larvae extracted from the deepest part of an infested wound. The mature, third instar larvae are most reliable for this purpose and those of NWS can be identified by their darkly pigmented dorsal tracheal trunks extending from the twelfth segment forward to the tenth or ninth. This pigmentation is unique to the larvae of NWS among the species encountered in wound myiasis. Confirmation of OWS relies on the recognition of a characteristic combination of spinulation, the number of lobes on the anterior spiracles (*4–6*), and pigmentation of secondary tracheal trunks.*

In the adult stage, species in the genus Cochliomyia *can be separated from other genera involved in wound myiasis by confirmation of a body colour that is usually a metallic blue/green with three dark longitudinal stripes always present on the thorax. The separation of NWS from the very similar* C. macellaria *and the identification of adult OWS are discussed in this chapter.*

Serological tests: *At present there are no applicable serological tests, nor are they indicated in the identification of this disease. However, serology may have a future role in studies of the prevalence of myiasis.*

Requirements for vaccines and diagnostic biologicals: *There are no vaccines or biological products available except for the use of sterilised male flies in the sterile insect technique (*SIT*). In this technique, vast numbers of sterilised male flies are sequentially released into the environment,*

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[1] In this chapter, the term 'New World' refers to the Americas and the term 'Old World' refers to Europe, Africa and Asia.

> *where their matings with wild females produce infertile eggs, leading to an initial population reduction and, progressively, eradication.*

A. INTRODUCTION

The New World screwworm fly (NWS), *Cochliomyia hominivorax* (Coquerel), and the Old World screwworm fly (OWS), *Chrysomya bezziana* Villeneuve, are species of two genera of the subfamily Chrysomyinae of the Dipteran family Calliphoridae (blowflies). Both species are obligate parasites of mammals including humans and, rarely, birds. Despite being in different genera and geographically separated, the two species have evolved in remarkable parallel. They have almost identical life histories because they fill identical parasitic niches in their respective geographical zones. The following discussion will relate to both species, except where indicated.

Unlike most other species of blowflies, adult female screwworms do not lay their eggs on carrion. Instead, they lay them at the edges of wounds on living, injured mammals or at their body orifices. Virtually any wound is attractive, whether natural (from fighting, predators, thorns, disease, and/or tick and insect bites) or man made (from shearing, branding, castrating, de-horning, docking, and/or ear-tagging). Commonly infested natural wounds are the navels of newborn animals and the vulval and perineal regions of their mothers, especially if traumatised. If eggs are deposited on mucous membranes, the larvae can invade undamaged natural body openings such as the nostrils and associated sinuses, the eye orbits, mouth, ears, and genitalia.

Within 12–24 hours of the eggs being laid, larvae emerge and immediately begin to feed on the wound fluids and underlying tissues, burrowing gregariously head-downwards into the wound in a characteristic screwworm fashion. As they feed, tearing the tissue with their hook-like mouthparts, the wound is enlarged and deepened, resulting in extensive tissue destruction. Infested wounds often emit a characteristic odour, which can be the first indication that at least one animal in a group is infested. Although the odour is not always apparent to humans, it is obviously highly attractive to gravid females (19), which lay further batches of eggs so increasing the extent of the infestation. A severe infestation that is left untreated may result in the death of the host.

Screwworm larvae pass through three stages (or instars), separated by cuticular moults that facilitate rapid growth, and they reach maturity about 5–7 days after egg hatch. They then stop feeding and leave the wound, falling to the ground into which they burrow and pupariate. The pupa develops within the puparium, a barrel-shaped protective structure formed by hardening and darkening of the cuticle of the mature larva. On completion of development, adult flies usually emerge from the puparium in the morning and work their way up to the soil surface, where they extend their wings for hardening prior to flight. Males become sexually mature and able to mate within 24 hours, but the ovaries of females need to mature over 6–7 days, and females only become responsive towards males and mate when about 3 days old. About 4 days after mating, female flies are ready to oviposit. They seek a suitable host and lay their eggs, all oriented in the same direction, like a tiled roof, firmly attached to each other and to the oviposition substrate. The numbers of eggs laid per batch vary depending on many factors (e.g. fly strain, disturbance during oviposition), but the average first batch has in the order of 175 eggs for OWS and 340 for NWS (43). Following the first egg batch, further batches are laid at intervals of 3–4 days (51). Adult flies live on average for 2–3 weeks in the field during which time they feed at flowers, and the females also take in protein, e.g. from serous fluids at animal wounds.

The rate of development of the immature stages is influenced by environmental and wound temperatures, being slower at low temperatures, although true diapause does not occur. This effect is most pronounced in the off-host pupal stage, which can vary from 1 week to 2 months' duration depending on the season (24). Thus, the complete life cycle of NWS may take 2–3 months in cold weather (36), whereas in temperate conditions with an average air temperature of 22°C, it is completed in about 24 days (22), and in tropical conditions averaging 29°C it is completed in about 18 days (51).

The degree to which NWS and OWS can tolerate cold has had a major influence on their distributions, best documented for NWS. Historically, the range of NWS extended from the southern states of the United States of America (USA), through Mexico, Central America, the Caribbean islands and northern countries of South America to Uruguay, northern Chile and northern Argentina (22). This distribution contracted during the winter months but expanded during the summer months, producing a seasonality at its edges and year round populations in the central areas – the New World tropics. Use of the sterile insect technique (SIT) in major programmes has resulted in eradication of NWS from the USA (6), Mexico (17), Curacao, Puerto Rico, and the Virgin Islands and, in Central America, from Guatemala, Belize, El Salvador, Honduras, Nicaragua and, in 2000, Costa Rica (55). The Central American eradication programme is continuing in Panama, where sterile flies were first released in July 1998. The ultimate objective is to establish a barrier zone in Panama that will become the future northern limit of NWS in the Americas. A NWS eradication programme was also officially launched in Jamaica in July 1998, as part of a plan to eradicate the species from the entire Caribbean. This programme has encountered severe setbacks due to a complex combination of management and technical difficulties, but is ongoing although with an uncertain future (12, 53). Although NWS is a New World species, in 1988, it was detected in Libya in North Africa where it threatened to become firmly established. However, it was eradicated in

1991 by an intensive SIT campaign (14, 26). The threat of spread of screwworms aided by modern rapid transport systems is ever present, necessitating constant vigilance from quarantine and other front-line animal health and medical officers in unaffected areas. Imported cases of NWS have been reported recently in Mexico, USA, and even in the United Kingdom (30).

The distribution of OWS is confined to the Old World, as the name suggests, throughout much of Africa (from Ethiopia and sub-Saharan countries to northern South Africa), the Gulf countries, the Indian subcontinent, and South-East Asia (from southern China [People's Rep. of] through the Malay Peninsula and the Indonesian and Philippine islands to Papua New Guinea) (22, 43, 47, 56). OWS was reported from Hong Kong for the first time in 2000, infesting dogs, and a first human case was reported in 2003 (35). OWS myiasis has also been reported from Algeria (1), in a local shepherd, but in the absence of other reported cases, particularly animal cases, a continuing presence there seems unlikely and the original case could have been a misidentification. The situation in the Gulf area and surrounding regions is dynamic with recent reports confirmed from Iran (34) and Iraq (2). Epidemics of traumatic myiasis can follow introductions into such areas, especially where the livestock owners and veterinarians are unfamiliar with OWS (38). The climatic requirements of the two screwworm species are very similar and their potential distributions, if unrestrained, would overlap considerably (47).

Organophosphorus insecticides such as dichlofenthion, fenchlorphos, and in particular, coumaphos are recommended for the treatment of wounds infested with OWS and NWS (16, 37, 45). They have the effect of expelling the larvae, which die on the ground. To provide residual protection against reinfestation, they must be applied at 2–3-day intervals until the wound has healed. The contents of individual wound treatment sachets, e.g. 5 g of 5% coumaphos wettable powder, should be either sprinkled directly on to a wound or, more effectively, brushed into the wound as a paste after mixing with ordinary cooking oil (33 ml). Organophosphorus compounds may also be applied as aerosol sprays, in which marker dyes and bacteriostats are included, or as dusts that are puffed into the wound from plastic squeeze bottles. Dichlorfenthion is used in South America as a 1% aerosol to treat NWS cases and is also effective against OWS (37). Any larvae that die in the wound should be removed to prevent sepsis. Close attention should always be paid to the manufacturers' safety instructions.

Direct prevention of screwworm infestation can be achieved by spraying or dipping of livestock with coumaphos (0.25% aqueous suspension of 50% wettable powder) or other organophosphorus insecticides at the maximum concentration prescribed for external parasite control. The effects of such treatment are twofold: firstly, the treatment kills larvae directly and provides residual protection; secondly, the treatment kills ticks and other external parasites, which means that there are fewer wounds available as sites for oviposition. Synthetic pyrethroids have potential for control of screwworm larvae in wounds, but there have been few reported trials of their effect on screwworms (e.g. Permethrin versus NWS; ref. 39). Dipping or spraying of a group of animals would be indicated if any member of the group was found to be infested, or if animals were traversing or leaving an infested area, or following wound-inducing animal husbandry practices, e.g. shearing.

A single subcutaneous injection of ivermectin (200 µg/kg) was effective against OWS in preventing navel strike of newborn calves (37) and scrotal strike of castrated calves (44). Ivermectin also prevented re-strike of treated wounds of adult cattle. Cattle treated with a sustained-release bolus of ivermectin developed no OWS myiasis from 14 to 102 days after treatment (54). However, because of the negative effects on dung-breeding fauna, it was recommended that boluses be reserved for use in containing outbreaks of OWS. Early results suggested that ivermectin may be ineffective against NWS (Mackley & Brown, in ref. 17), but more recent studies demonstrated that it can produce a significant reduction in the incidence of navel and scrotal myiasis due to NWS (7, 28). Although results of ivermectin trials show variation, results of doramectin trials are overwhelmingly positive (18). There has been an increasing number of publications reporting that a subcutaneous injection of doramectin (200 µg/kg) was up to 100% effective as a NWS prophylactic, preventing infestation of artificial wounds, umbilical or castration wounds of calves, and infestation of post-parturient cows, for up to 12–14 days post-treatment (4, 32, 33). This doramectin treatment does not reduce egg-laying and, therefore, is efficient because gravid adults are not repelled and driven towards untreated animals. Effectiveness depended on factors such as cattle breed and degree of challenge. In one comparative trial, doramectin and ivermectin, both at 200 µg/kg subcutaneous injection, gave 100% and 50% protection, respectively, against NWS myiasis, experimentally induced 2 hours after treatment (31). Doramectin also provided complete protection for 21 days and partial protection (56%) at 28 days post-treatment (31). In another, larger, comparative trial, doramectin had a mean efficacy of 94.6% (range 53.3–100%) compared with 43.7% (range 0–100%) for ivermectin (10). Abamectin (subcutaneous injection, 200 µg/kg) gave good, but not 100%, prevention of post-castration myiasis by NWS (3). Pour-on formulations of moxidectin, eprinomectin and doramectin gave poor protection against OWS myiasis (54) when compared with injectable formulations of doramectin against NWS. There are early indications that fipronil (a phenyl-pyrazole) might be effective as a preventive of post-castration myiasis of cattle. Topical application of 10 mg/kg bodyweight of a 1% fipronil solution did not prevent oviposition by NWS, but it reduced the proportion of bulls developing active myiasis over the critical 10-day post-castration period, when most ovipositions occurred, from 65% in untreated controls to just 3% in treated animals (25). Similarly, topical application of an insect growth regulator (IGR), dicyclanil, to castration wounds in cattle gave good protection (>90%) against NWS myiasis (5). IGRs are very specific to insects and, therefore, are less hazardous in the environment than many other groups of insecticides. Spinosad, a formulation of products derived from the fermentation of a bacterium with low mammalian and avian toxicity, was effective in treating and preventing myiasis due to NWS and OWS when applied as an aerosol spray (41).

Indirect prevention of screwworm flies infestation includes the avoidance of wounding procedures at the times of year when screwworm are numerous, the careful handling of livestock to minimise wounding, the removal of sharp objects (e.g. wire strands) from livestock pens, and the use of measures to reduce other wound-causing parasites, in particular ticks, e.g. by dipping and by insecticide impregnated ear-tags.

To prevent the spread of the screwworms beyond present limits, strict observation of the requirements for international trade, as set out in the OIE *Terrestrial Animal Health Code*, is necessary.
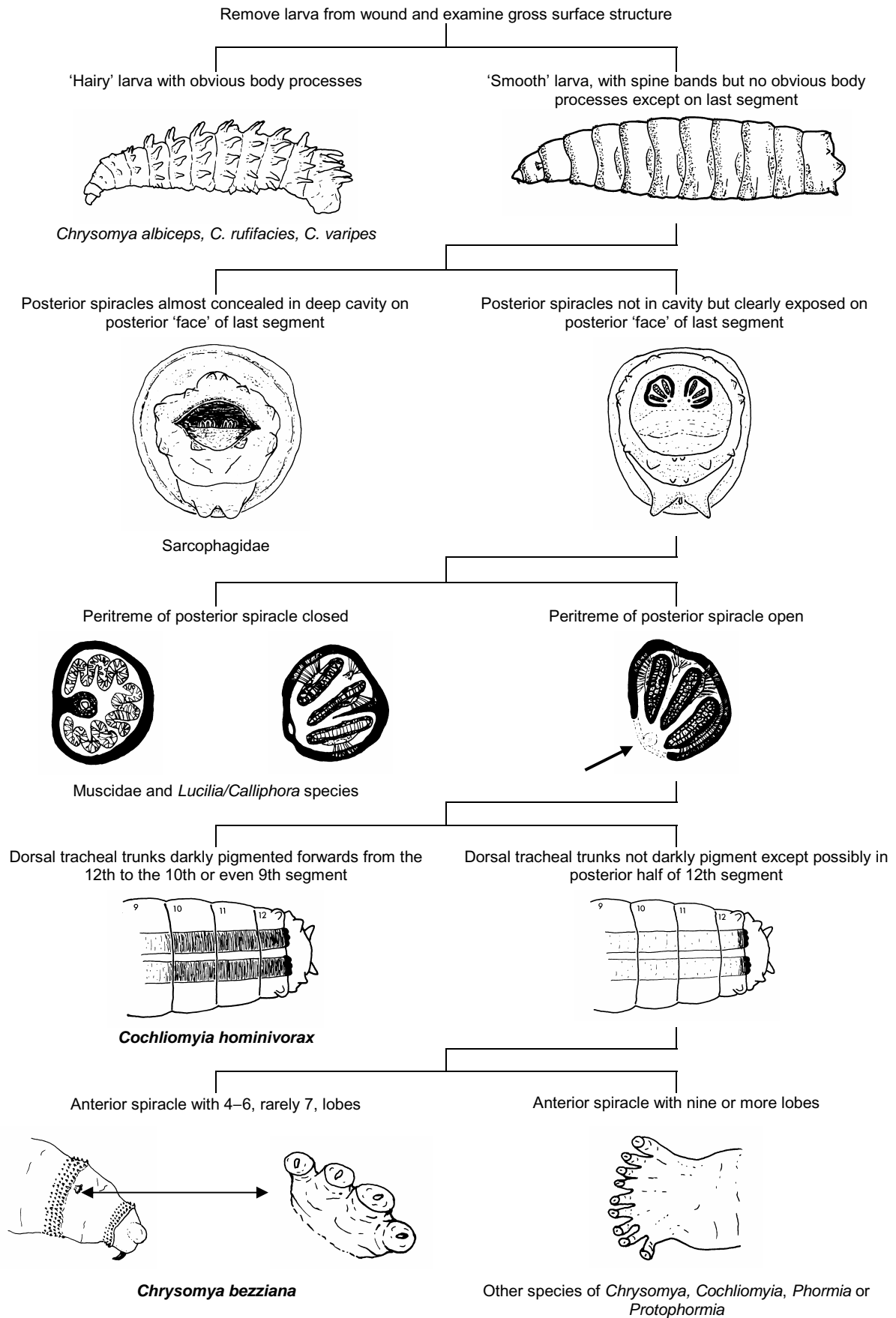
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Identification of the eggs and first instar larvae of the agents of myiasis based on morphology is difficult, and, because these stages are relatively short lived and seldom encountered during the collection of specimens from infested wounds, they will not be considered further here.

Larvae collected for diagnosis should be removed from the deepest part of the wound to reduce the possibility of collecting non-screwworm species, which may infest the shallower parts of the wound. Living specimens should first be examined for pigmentation of the dorsal tracheal trunks (Figures 1 and 4) and then be preserved in 80% ethanol and returned to the laboratory for examination under a dissecting microscope at up to ×50 magnification (for further techniques see references 13, 21, 42, 56). If larvae are placed directly into most preservative solutions they contract and darken. However, optimal preservation of larvae, in their natural extended state, can be made by killing them in boiling water (15–30 seconds immersion) before storage in 80% ethanol. This killing method had no negative effect on subsequent extraction of mitochondrial DNA, amplified by polymerase chain reaction (PCR) (20), but it might impact other molecular techniques and this should be borne in mind.

Second instar larvae: Second instars have only two spiracular slits in each of the posterior spiracular plates compared with the three slits of third instars (Figures 2 and 3). Second instars of NWS can be diagnosed by the presence of dark pigmentation of the dorsal tracheal trunks, for over half their length in the terminal segment. Other species have less extensive pigmentation of the dorsal tracheal trunks, for example, these trunks are pigmented for no more than one-third of their length in the twelfth segment of OWS. The anterior spiracles of second instar NWS have from seven to nine branches compared with about four branches in OWS (23). More positive identification may be gained by rearing living, immature larvae to third instars. This can be done on the standard meat medium used for large-scale rearing of NWS before the introduction of gel diets, i.e. in the proportion of 1 litre water, 1.3 kg ground horse or beef meat, 50 g dried bovine blood, and 1.5 ml formalin (49), mixed and maintained at 35–38°C and 70% relative humidity. For simply rearing up larvae for identification, the exact meat and blood types are not essential, and more readily available fresh blood could be used instead of dried blood.

Third instar larvae: Third instars of both NWS and OWS have a robust, typical maggot shape, with a cylindrical body from 6 to 17 mm long and from 1.1 to 3.6 mm in diameter, pointed at the anterior end (24, 42). Fully mature larvae of both NWS and OWS develop a reddish-pink tinge over the creamy white colour of younger larvae. Both screwworm species have prominent rings of spines around the body and these spines appear large and conspicuous under a microscope when compared with most non-screwworm species, the longest averaging 130 µm. In NWS the spines can be either single or double pointed, but in OWS they are always single pointed and thorn-like (Figure 2). The anterior spiracles of NWS each have from six to eleven well separated branches, but usually from seven to nine (Figure 2). In OWS, the anterior spiracles each have from three to seven branches, but usually from four to six (Figure 2). The latter character should not be used on its own to identify OWS, because third instars of the obligate myiasis-causing species *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), whose distribution overlaps that of OWS in the Middle East, have similarly branched anterior spiracles. Hence, in using any identification key, such as that in Figure 1, it is essential that each specimen be taken through the whole key to avoid misidentifications. On the posterior face of the terminal segment of both NWS and OWS, the posterior spiracular plates all have a darkly pigmented, incomplete peritreme partially enclosing three straight, slightly oval-shaped slits, which point towards the break in the peritreme. These diagnostic features are illustrated in Figure 3. Of greatest diagnostic value are the dorsal tracheal trunks, which extend forwards from the posterior spiracular plates and are darkly pigmented up to the tenth or ninth segment in NWS (Figure 1; see also refs 13, 15, 18, 21, 22, 42, 56 for identification keys). This feature is seen most easily in living larvae. Those in preservative may need dissection to remove opaque tissues covering the trunks. The dorsal tracheal trunks of OWS are darkly pigmented only in the twelfth segment. However, in OWS the secondary trachea branching off the dorsal tracheal trunks are pigmented from the twelfth segment forwards to at least the tenth segment (confirmed in specimens throughout the range, from Malaysia, Bahrain and Zimbabwe; M.J.R. Hall, unpublished). Conversely, in NWS these secondary trachea are not pigmented, only the dorsal trachea are. Hence, the tracheal pigmentation appears almost reversed between the two screwworm species (Figure 4).

Remove larva from wound and examine gross surface structure

'Hairy' larva with obvious body processes

'Smooth' larva, with spine bands but no obvious body processes except on last segment

Chrysomya albiceps, C. rufifacies, C. varipes

Posterior spiracles almost concealed in deep cavity on posterior 'face' of last segment

Posterior spiracles not in cavity but clearly exposed on posterior 'face' of last segment

Sarcophagidae

Peritreme of posterior spiracle closed

Peritreme of posterior spiracle open

Muscidae and *Lucilia/Calliphora* species

Dorsal tracheal trunks darkly pigmented forwards from the 12th to the 10th or even 9th segment

Dorsal tracheal trunks not darkly pigment except possibly in posterior half of 12th segment

Cochliomyia hominivorax

Anterior spiracle with 4–6, rarely 7, lobes

Anterior spiracle with nine or more lobes

Chrysomya bezziana

Other species of *Chrysomya, Cochliomyia, Phormia* or *Protophormia*

Fig. 1. *Identification key for the diagnosis of third instar larvae of* Cochliomyia hominivorax *and* Chrysomya bezziana *from cases of wound myiasis. To avoid misidentifications, it is essential that the key is worked through from the first step for each specimen.*

Fig. 2. Head and first two thoracic segments of third instar larvae of Cochliomyia hominivorax (*left, viewed by scanning electron microscopy, inset is the anterior spiracle of* Chrysomya bezziana) *and of* Chrysomya bezziana (*right, viewed by compound light microscopy, note the thorn-like spines and that this slide preparation has been cleared using 10% KOH so that the anterior spiracles on both sides of the first thoracic segment are visible*); *as = anterior spiracle.*
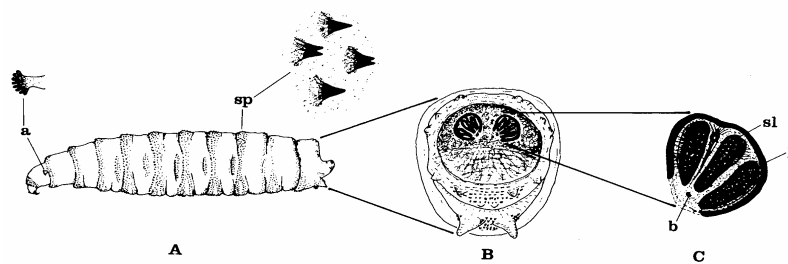

Fig. 3. Characteristics of third instar larvae of Cochliomyia hominivorax*: (A) whole larva, lateral aspect; (B) posterior face of terminal segment; (C) posterior spiracular plate; a = anterior spiracle; b = button adjacent to opening in peritreme; p = peritreme; sl = spiracular slit; sp = spines. (After Laake* et al. *[24].*)

Adult: Adult flies needed for identification purposes are often collected using wind-oriented traps (8) and sticky traps (42) baited with a synthetic odour, swormlure-4 (29). A modified bucket-trap and newly developed attractant ('Bezzilure') is being developed for surveillance of OWS in Australia (Rudolf Urech, pers. comm.). Alternative sampling systems, using electrocuting grids or sticky surfaces at odour-baited visual targets, have been used for research purposes (19). Identification of adult flies is seldom required for the diagnosis of myiasis, because the larval stages are those most apparent to livestock owners and veterinary personnel. However, a brief description follows.

i) **NWS**: The body length is usually 8–10 mm long and has a deep blue to blue-green metallic colour, with three dark longitudinal stripes on the dorsal surface of the thorax. This combination of colour and pattern is not shared by any other species commonly involved in wound myiasis except the secondary screwworm of the New World, *Cochliomyia macellaria* (Fabricius). These two *Cochliomyia* species can be separated by the presence of black setulae on the fronto-orbital plates of the head of NWS compared with only light yellow hairs on the fronto-orbital plates of *C. macellaria*. The fifth (=fourth visible) abdominal tergite of NWS has only a very slight lateral pollinose dusting, whereas that of *C. macellaria* has a dense dusting, producing a pair of distinct, lateral, silvery-white spots. In addition, females of NWS have a dark brown-black basicosta, whereas those of *C. macellaria* have a yellow basicosta (Figure 5; see also refs 11, 15, 24, 42).

ii) **OWS**: The body is up to 10 mm long and has a metallic blue, bluish-purple or blue-green colour, i.e. it is very similar to NWS, but without the thoracic stripes. The lower squama (s in Figure 5) also differs from

NWS, being distinctly covered with fine hairs over its entire upper surface in OWS and other *Chrysomya* species, whereas in NWS it is hairless above, except near the base. Adults of OWS can be distinguished from other *Chrysomya* found in cases of myiasis by the combination of black-brown to dark-orange-coloured anterior thoracic spiracles (rather than pale yellow, creamy, or white), with waxy-white, lower squamae (rather than blackish-brown to dirty-grey) (42, 56).
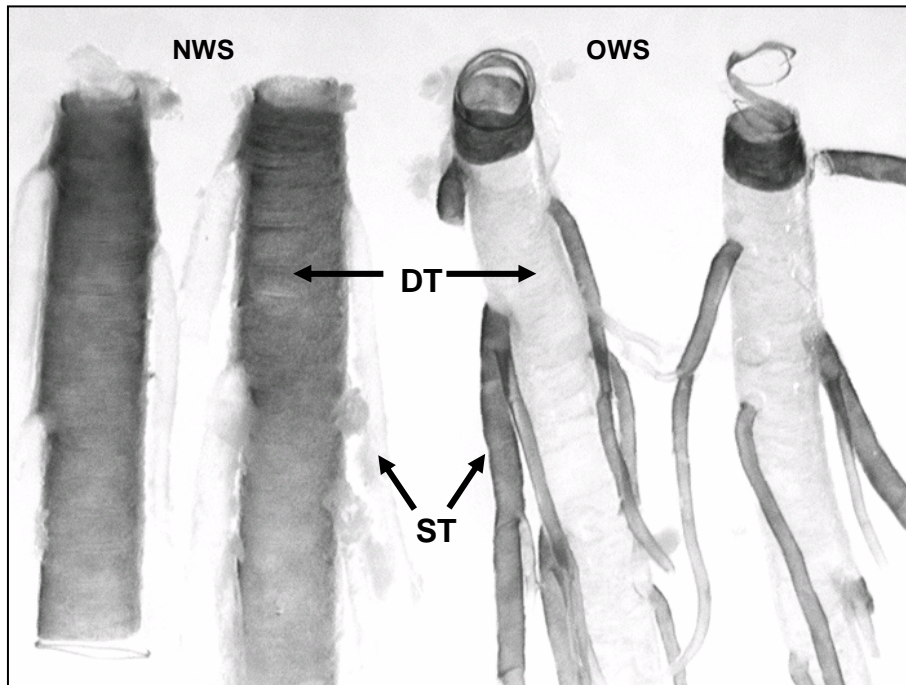
Fig. 4. *Dorsal tracheal trunks of third instar larvae of* Cochliomyia hominivorax (*left*) *and* Chrysomya bezziana (*right*) *dissected forwards from the posterior spiracles (top) to ninth abdominal segment (bottom).*
Note that the pigmentation of the main dorsal trunks (DT) and the smaller secondary trunks (ST) is almost reversed between the species.
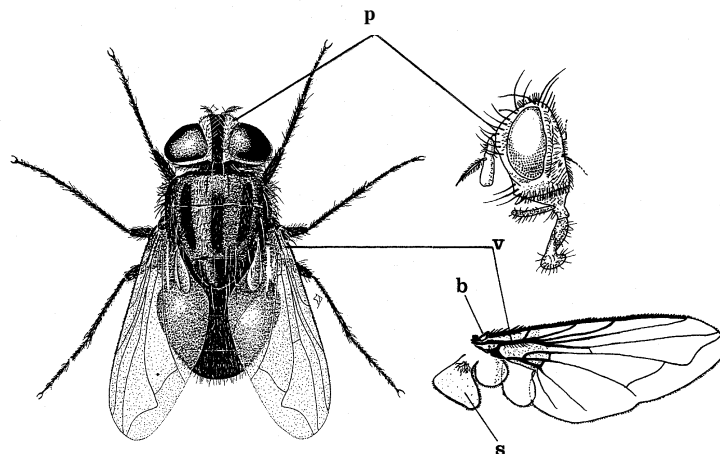
Fig. 5. *Characteristics of adult* Cochliomyia hominivorax*; note longitudinal thoracic stripes; b = basicosta; p = fronto-orbital plate, indicated from above on whole* Cochliomyia hominivorax *and laterally on head of typical calliphorid fly; s = lower squama, surface hairless except at base; v = stem vein with hairs on dorsal posterior surface.*

In addition to the standard morphological techniques discussed previously, more recent techniques for identification of screwworms and their geographical origins include cuticular hydrocarbon analysis (9), analysis of mitochondrial DNA (20, 27, 50), the complete 16,022 base-pair sequence of which is known for NWS, and use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (40). Problems with identification of larvae or adults from cases of myiasis can be referred to the Food and Agriculture Organisation of the United Nations Collaborating Centre on Myiasis-Causing Insects and Their Identification[2].

2. Serological tests

No standardised serological tests are presently available, nor are they indicated for diagnosis of this disease. However, experimental studies have shown that serological techniques have potential value in future investigations of the prevalence of screwworm infestations in animal populations to detect antibodies to screwworm post-infestation (52).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products such as vaccines, available currently. However, research towards development of potential vaccines is being conducted (48). The only proven method of eradication of NWS relies on a biological technique, the sterile insect technique, SIT (17, 26), which has also been applied experimentally to OWS (46). In this technique, male flies sterilised in their late pupal stage by gamma or x-ray irradiation are sequentially released into the wild in vast numbers. Any of their matings with wild females result in infertile eggs only, leading to a progressive population reduction and, eventually, eradication. In operational situations, SIT is supported by the insecticide treatment of screwworm-infested wounds in livestock, by strict control of livestock movement, by the quarantining of infested animals and by an active publicity campaign. SIT is very expensive because of the cost of continuous production and aerial dispersion of sterile flies. Historically, it has been considered cost effective only when used as an eradication strategy in situations where the geography would favour such a programme (e.g. references 14, 26). For many years there was only one New World sterile screwworm production facility, located at Tuxtla Gutiérrez in the south of Mexico. However, a second facility opened in Panama[3] in late 2006. An experimental facility to produce sterile OWS opened in Malaysia in 1998[4].

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NB: There is an OIE Reference Laboratory for New world screwworm (*Cochliomyia hominivorax*) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.1.11.

PARATUBERCULOSIS
(Johne's disease)

SUMMARY

Paratuberculosis (*Johne's disease*) *is a chronic enteritis of ruminants caused by* Mycobacterium avium *subsp.* paratuberculosis (M. paratuberculosis) (*49*).

Identification of the agent: *The diagnosis of paratuberculosis is divided into two parts: the diagnosis of clinical disease and the detection of subclinical infection. The latter is essential for control of the disease at the farm, national or international level.*

Diagnosis of paratuberculosis is made on clinical grounds confirmed by the demonstration of M. paratuberculosis *in the faeces by microscopy, culture, or by the use of DNA probes and the polymerase chain reaction. Diagnosis is made at necropsy by the finding of the pathognomonic lesions of the disease in the intestines, either grossly with the demonstration of typical acid-fast organisms in impression smears of the lesions or histologically, and by isolation of* M. paratuberculosis *in culture.*

The detection of subclinical infection depends on the detection of specific antibodies by serology, or culture of M. paratuberculosis *from faeces or tissues collected at necropsy, or the demonstration of cell-mediated responses. The choice of test depends on the circumstances and the degree of sensitivity required at individual animal or herd level.*

Cultures of M. paratuberculosis *may be obtained from faeces or tissues, after treatment to eliminate contaminants, by inoculation into artificial media with and without the specific growth factor – mycobactin – that is essential for the growth of* M. paratuberculosis*.*

Serological tests: *Control of paratuberculosis is difficult because of the prolonged course of infection, the predominantly subclinical nature of the disease and lack of tests for accurate detection of subclinically infected animals.*

*The serological tests commonly used for paratuberculosis in cattle are complement fixation (*CF*), absorbed enzyme-linked immunosorbent assay (*ELISA*) and agar gel immunodiffusion (*AGID*). Sensitivity and specificity are often determined by reference to results of faecal culture, which itself has unknown sensitivity in subclinically infected cattle. When used to confirm diagnosis of paratuberculosis in cows with typical clinical signs, some tests, for example CF and absorbed ELISA, perform very well.*

Requirements for vaccines and diagnostic biologicals: *Vaccines for paratuberculosis may be live attenuated or killed bacteria either incorporated with an adjuvant or lyophilised and adjuvanted on reconstitution. Bacterial counting is difficult and bacterial content of vaccines may be based on weight, while vaccine potency may be judged by batch tests for sensitising ability in guinea-pigs.*

Vaccine safety or abnormal toxicity may also be tested in guinea-pigs.

*For diagnostic skin tests, Johnin and avian tuberculin are purified protein derivatives (*PPD*) of a heat-treated culture of* M. paratuberculosis *or* M. avium*, respectively. Johnin is standardised for content of PPD by chemical assay and its biological activity is identified in guinea-pigs sensitised with* M. paratuberculosis*. Avian tuberculin activity is determined in guinea-pigs sensitised with* M. avium *by comparison with a reference preparation calibrated in international units.*

A. INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (*M. paratuberculosis*) is an organism first observed by Johne & Frothingham in 1895. *Mycobacterium avium* subsp. *paratuberculosis* causes paratuberculosis or Johne's disease, an intestinal granulomatous infection. First recognised in cattle, then in sheep and later in goats, paratuberculosis is found most often among domestic and wild ruminants and has a global distribution. The disease has also been reported in horses, pigs, deer and alpaca, and recently in rabbits, stoat, fox and weasel (3, 14). Under natural conditions, the disease in cattle spreads by ingestion of *M. paratuberculosis* from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus (25) and semen can be infected with the organism (46). The primary source of infection in calves is milk from infected cows or milk that is contaminated with the faeces of diseased cattle.

The identification of *M. paratuberculosis* is based on its mycobactin requirement and its pathogenicity in the host. Mycobactin dependence has long been used as a taxonomic characteristic for *M. paratuberculosis* because most mycobacteria are able to make mycobactin for themselves. *Mycobacterium avium* subsp. *paratuberculosis, M. silvaticum* and some primary isolates of *M. avium* lack this capacity, however, and require mycobactin to grow in the laboratory. Thus, the mycobactin requirement is not confined to *M. paratuberculosis;* this characteristic exists to various degrees within the *M. avium* group (48).

Clinical signs of paratuberculosis are a slowly progressive wasting and diarrhoea, which is intermittent at first, becoming progressively more severe until it is constantly present in bovines (13). Diarrhoea is less common in small ruminants.

Early lesions occur in the walls of the small intestine and the draining mesenteric lymph nodes, and infection is confined to these sites at this stage. As the disease progresses, gross lesions occur in the ileum, jejunum, terminal small intestine, caecum and colon, and in the mesenteric lymph nodes. *Mycobacterium avium* subsp. *paratuberculosis* is present in the lesions and, terminally, throughout the body. The intestinal lesions are responsible for a protein leak and a protein malabsorption syndrome, which lead to muscular wasting. Clinical signs usually first appear in young adulthood, but the disease can occur in animals at any age over 1–2 years.

Within a few weeks of infection, a phase of multiplication of *M. paratuberculosis* begins in the walls of the small intestine. Depending on the resistance of the individual, this infection is eliminated or the animal remains infected as a healthy carrier. The proportion of animals in these categories is unknown. A later phase of multiplication of the organisms in a proportion of carriers leads to the extension of lesions, interference with gut metabolism and clinical signs of disease. Subclinical carriers excrete variable numbers of *M. paratuberculosis* in the faeces. In most cases larger numbers of organisms are excreted as clinical disease develops.

Delayed-type hypersensitivity (DTH) is detectable early in the infection and remains present in a proportion of the subclinically infected carriers, but as the disease progresses, DTH wanes and may be absent in clinical cases. Serum antibodies are detectable later than DTH. They may also be present in carriers that have recovered from infection. Serum antibodies are present more constantly and are of higher titre as lesions become more extensive, reflecting the amount of antigen present. In sheep, there may be a serological response that is more likely to be detected in multibacillary than in the paucibacillary form of the disease.

Other mycobacterial diseases and infections, including mammalian and avian tuberculosis, cause DTH and the presence of serum antibodies. It follows therefore that these diseases need to be differentiated from paratuberculosis, both clinically and by the use of specific diagnostic tests. Exposure to environmental saprophytic mycobacteria may also sensitise livestock, resulting in nonspecific DTH reactions.

Animals vaccinated against paratuberculosis develop both DTH and serum antibodies. Vaccination is an aid to the prevention of clinical disease, but does not necessarily prevent infection. It also interferes with programmes for the diagnosis and control of bovine tuberculosis. Thus, if it is necessary to attempt a diagnosis of infection in vaccinates, only tests to detect *M. paratuberculosis* in the faeces can be used (21).

In individual animals, especially from a farm in which the disease has not previously been diagnosed, a tentative clinical diagnosis must be confirmed by laboratory tests. However, a definitive diagnosis may be warranted on clinical grounds alone if the clinical signs are typical and the disease is known to be present in the herd. Confirmation of paratuberculosis depends on the finding of either gross lesions with the demonstration of typical acid-fast organisms in impression smears or microscopic pathognomonic lesions and the isolation in culture of *M. paratuberculosis.*

B. DIAGNOSTIC TECHNIQUES

To diagnose the presence of paratuberculosis in an individual clinically suspect animal, a number of laboratory tests can be used including: faecal smears, faecal and tissue culture, DNA probes using faeces or tissues, serology, necropsy and histology.

Herd tests to detect subclinical infection are carried out to determine the prevalence of the infection, usually so that control measures can be instituted. As no test is 100% sensitive or specific, control of the disease by the disposal of positive reactors depends on repeated tests at 6-month or yearly intervals over a number of years and the elimination of reactors to serological tests or faecal shedders; the removal of offspring from female reactors is also considered to be prudent. Even these procedures are not always successful without changes in hygiene and livestock management to reduce the transmission of infection within a herd (2).

1. Identification of the agent

a) Necropsy

Paratuberculosis cannot be diagnosed on superficial examination of the intestines for signs of thickening. The intestines should be opened from the duodenum to the rectum to expose the mucosa. There is not always a close correlation between the severity of clinical signs and the extent of intestinal lesions. The mucosa, especially of the terminal ileum, is inspected for pathognomonic thickening and corrugation. Early lesions are seen by holding the intestine up to the light, when discrete plaques can be visualised. Mucosal hyperaemia, erosions and petechiation have been observed in deer with paratubeculosis. The earliest lesions are thickening and cording of lymphatic. The mesenteric lymph nodes are usually enlarged and oedematous. Smears from the affected mucosa and cut surfaces of lymph nodes should be stained by Ziehl–Neelsen's method and examined microscopically for acid-fast organisms that have the morphological characteristics of *M. paratuberculosis.* However, acid-fast organisms are not present in all cases. Diagnosis is therefore best confirmed by the collection of multiple intestinal wall and mesenteric lymph node samples into fixative (10% formol saline) for subsequent histology. Both haematoxylin-and-eosin-stained sections and Ziehl–Neelsen-stained sections should be examined. The typical lesions of paratuberculosis consist of infiltration of the intestinal mucosa, submucosa, Peyer's patches and the cortex of the mesenteric lymph nodes with large macrophages, also known as epithelioid cells, and multinucleate giant cells, in both of which clumps or singly disposed acid-fast bacilli are usually, but not invariably, found.

b) Bacteriology (microscopy)

Ziehl–Neelsen-stained smears of faeces or intestinal mucosa are examined microscopically. A presumptive diagnosis of paratuberculosis can be made if clumps (three or more organisms) of small (0.5–1.5 µm), strongly acid-fast bacilli are found. The presence of single acid-fast bacilli in the absence of clumps indicates an inconclusive result. The disadvantages of this test are that it does not differentiate among other mycobacterial species and only a small proportion of cases can be confirmed on microscopic examination of a single faecal sample.

c) Bacteriology (culture)

The isolation of *M. paratuberculosis* from an animal provides the definitive diagnosis of infection with the organism. Although culture is technically difficult and time-consuming to carry out, it is the only test that does not produce false-positive results (100% specificity).

The faecal culture is the best test available for the diagnosis of paratuberculosis in live animals. It is believed that the faecal culture method involving the double incubation method for decontamination of samples and cultivation on solid media detects about 30–40% of infected cattle (56). The faecal culture is able to detect most animals in advanced stages of the disease but identifies only a few animals in early stages of infection (56). It will detect infected animals 6 months or more before they develop clinical signs, and during the clinical stage its sensitivity approaches 100%. The culture of bovine and caprine tissues for *M. paratuberculosi*on is more sensitive than histopathological examination.

There are several culture methods, which vary with respect to media and sample processing protocols. The cultivation of *M. paratuberculosis* is always performed using special media supplemented with mycobactin J[1],

Mycobacterium avium subsp. *paratuberculosis* organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens. The successful isolation of *M. paratuberculosi*s from such samples depends on efficient inactivation of these undesirable organisms. The optimal method of decontamination must have the least inhibitory effect on growth of *M. paratuberculosis*. Routine decontamination protocols were shown to decrease the number of *M. paratuberculosis* organisms isolated per sample by about 2.7 $\log_{10}$ and 3.1 $\log_{10}$ for faeces and tissues, respectively (34).

There are two basic methods in use for the conventional culture of *M. paratuberculosis* on solid media: the method using oxalic acid and NaOH for decontamination and Löwenstein–Jensen (LJ) medium for growth, and the method using hexadecylpyridinium chloride (HPC) for decontamination in combination with Herrolds's egg yolk medium (HEYM) for growth. Both media contain mycobactin. Although it has been

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1   Mycobactin can be obtained commercially (mycobactin J) from Allied Monitor, P.O. Box 71, 201 Golden Drive, Fayette, MO 65248, United States of America, or Symbiotic Society, 299 av. Jean Jaurès, 69007 Lyon, France.

published that HEYM supports growth of bovine isolates of *M. paratuberculosis* significantly better than LJ (33), recent studies have shown that certain strains grow better on LJ or Middlebrook media (9).

In addition, there is a technique of radiometric culture where growth in liquid medium BACTEC™ 12B (Middlebrook 7H12) supplemented with egg yolk and mycobactin is measured by the release of radioactive $^{14}CO_2$ from palmitate, as a consequence of bacterial metabolism. This method reduces the time required for results and is considered more sensitive than the conventional culture methods on solid media for the detection of both ovine and bovine stains of *M. paratuberculosis* (11, 58). The decontamination protocol involving double incubation of faecal samples in HPC and mixture of antibiotics may further improve culture sensitivity (11). However, as the BACTEC™ system is radiometrically based, it is not feasible for use in some laboratories and has been phased out in others. The evaluation of the usefulness of alternative culture systems based on liquid media such as MGIT (Becton Dickinson) ESPII (Difco) and MB/BacT Alert (Organon Teknika) that do not use radioactive material for the detection of *M. paratuberculosis* is currently in progress.

Primary colonies of *M. paratuberculosis* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation (7). Sheep strains, including the uncommon, bright yellow pigmented types, grow less well than cattle strains on commonly used media such as HEYM or LJ, and primary cultures should not be discarded as negative without prolonged incubation. The solid medium Middlebrook 7H10 and liquid medium BACTEC™ 12B both supplemented with egg yolk and mycobactin are excellent for cultivation of ovine strains of *M. paratuberculosis* (58).

Primary colonies of the cattle strain of *M. paratuberculosis* on HEYM are very small, convex (hemispherical), soft, non-mucoid and initially colourless and translucent. Colony size is initially pinpoint, It may remain at 0.25–1 mm, and tend to remain small when colonies are numerous on a slope. Colony margins are round and even, and their surfaces are smooth and glistening. The colonies become bigger more raised, opaque, off-white cream to buff or beige coloured as incubation continues. Older isolated colonies may reach 2 mm. The colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate (7, 47).

On modified 7H10 medium, colonies of the cattle strain are less convex than those on HEYM, especially in aged cultures, They are pinpoint to approximately 1 mm in diameter and, being buff coloured, are only slightly lighter than the media. Compared with colonies of cattle strains on HEYM, those on 7H10 are more difficult to detect. Colonies of the sheep strain of *M. paratuberculosis* on modified 7H10 are convex, soft, moist, glistening, off-white to buff, and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope (7).

Saprophytic mycobacteria may have a similar appearance on either medium but are often evident after 5–7 days (7).

For identification of *M. paratuberculosis,* <u>small</u> inocululum of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. Mycobactin is present in the cell wall of the organism, and heavy inoculum may contain enough mycobactin to support the growth of a mycobactin-independent mycobacterium on medium that contains no mycobactin.

- **Media**

Examples of suitable media are:

i) *Herrold's egg yolk medium with mycobactin* (*28*)

For 1 litre of medium: 9 g peptone; 4.5 g sodium chloride; 2.7 g beef extract; 27 ml glycerol; 4.1 g sodium pyruvate; 15.3 g agar; 2 mg mycobactin; 870 ml distilled water; six egg yolks (120 ml); and 5.1 ml of a 2% aqueous solution of malachite green. Measure the first six ingredients and dissolve by heating in distilled water. Adjust the pH of the liquid medium to 6.9–7.0 using 4% NaOH, and test to ensure the pH of the solid phase is 7.2–7.3. Add the mycobactin dissolved in 4 ml ethyl alcohol. Autoclave at 121°C for 25 minutes. Cool to 56°C and aseptically add six sterile egg yolks[2] and sterile malachite green solution. Blend gently and dispense into sterile tubes.

It is permissible to add 50 mg chloramphenicol, 100,000 U penicillin and 50 mg amphotericin B.

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2   Use fresh eggs not more than 2 days old from a flock that is not receiving antibiotics. With a brush, scrub the eggs with water containing a detergent. Rinse with water and place the eggs in 70° alcohol for 30 minutes. Dry by inserting between two sterile towels. With sterile rat-tooth forceps, crack one end of the eggshell, making a hole of approximately 10 mm, and remove the egg white with the forceps and gravity. Make the hole larger and break the yolk. Mix the egg yolk by twirling the forceps, and remove the yolk sac. Pour the mixed egg yolk into media.

ii) *Modified Dubos's medium (41)*

For 1 litre of medium: 2.5 g Difco casamino acids; 0.3 g asparagine; 2.5 g anhydrous disodium hydrogen phosphate; 1 g potassium dihydrogen phosphate; 1.5 g sodium citrate; 0.6 g crystalline magnesium sulphate; 25 ml glycerol; 50 ml of a 1% solution of Tween 80; and 15 g agar. Dissolve each salt in distilled water with minimum heat and make up to 800 ml. Add mycobactin in alcoholic solution at 0.05% (2 mg dissolved in 4 ml ethyl alcohol), heat the medium to 100°C by free-steaming, and then sterilise by autoclaving at 115°C for 15 minutes. Cool to 56°C in a water bath, add antibiotics (100,000 U penicillin; 50 mg chloramphenicol; and 50 mg amphotericin B) and serum (200 ml of bovine serum sterilised by filtering through a Seitz 'EX' pad and inactivated by heat at 56°C for 1 hour). The medium is kept thoroughly mixed and then dispensed into sterile tubes. An advantage of this medium is that it is transparent, which facilitates the early detection of colonies.

iii) *Modified Middlebrook 7H10* (7)

To prepare this medium 19 g Middlebrook 7H10 agar (Difco), 1g Casitone and 5 ml Glycerol are resuspended in 900 ml water, autoclaved at 121°C for 15 minutes and cooled to 58°C. Using an aseptic technique, the following additional ingredients are combined adding the egg yolk last: 50 ml PANTA PLUS (Becton Dickinson), 25 ml Mycobactin J solution (50 µg/ml), 100 ml ADC enrichment (Difco), 250 ml egg yolk. The mixture is thoroughly mixed using a slow swirling action and 10-ml volumes are dispensed into sterile tubes to form slopes. After a sterility check, media are stored at 4°C.

iv) *BACTEC 12B vials* (7)

The following supplements are added to each vial to give final concentrations of 0.8–1 µg/ml Mycobactin J and a minimum of 16–17% egg yolk in a final volume of 5–6 ml. For the 6-ml volume, 0.1 ml Mycobactin J (50 µg/ml), 0.1 ml PANTA PLUS, 1 ml egg yolk and 0.8 ml water are added. For the 5-ml volume, 0.1 ml Mycobactin J (50 %g/ml), 0.1 ml PANTA PLUS and 0.8 ml egg yolk are added.

v) *Middlebrook 7H9, 7H10 and 7H11 media (Difco)*

This media enhanced with mycobactin in the same proportion as for Herrold's medium can also be used. The advantage of this media is that it is transparent, which facilitates the early detection of colonies.

vi) *Löwenstein–Jensen medium with or without mycobactin (20).*

• **Sample preparation**

• *Processing tissue specimens*

Chemical preservatives should not be used. The tissues can be frozen at –20°C.

To avoid contamination, the faeces should be rinsed from portions of intestinal tract before shipment to the laboratory.

i) *Digestion/sedimentation method for decontamination of tissues*

Approximately 4 g of mucosa from the ileocaecal valve or 4 g of mesenteric node are placed in a sterile blender jar containing 50 ml of trypsin (2.5%). The mixture is adjusted to neutrality using 4% NaOH and pH paper, and stirred for 30 minutes at room temperature on a magnetic mixer. The digested mixture is filtered through gauze. The filtrate is centrifuged at approximately 2000–3000 *g* for 30 minutes. The supernatant fluid is poured off and discarded. The sediment is resuspended in 20 ml of 0.75% HPC and allowed to stand undisturbed for 18 hours at room temperature. The particles that settle to the bottom of the tube are to be used as the inoculum and are removed by pipette without disturbing the supernatant fluid. Alternatively, other methods of decontamination can be used, such as treatment with 5% oxalic acid.

ii) *Double incubation method for decontamination of tissues* (7)

About 2 g of tissue sample (trimmed of fat) is finely chopped using a sterile scalpel blade or scissors and homogenised in a stomacher for 1 minute in 25 ml 0.75% HPC. Allow the sample to stand so that foam dissipates and larger pieces of tissue settle. Pour tissue homogenate into a centrifuge tube taking care to avoid carry over of fat or large tissue pieces. Allow to settle for 30 minutes then take 10 ml of the suspension from just above the sediment to a 30 ml tube and incubate for 3 hours at 37°C. Centrifuge for 30 minutes at 900 *g*, discard supernatant fluid and resuspend pellet in 1 ml antibiotic cocktail containing 100 µg of each of vancomycin, amphotericin and nalidixic acid (VAN). Incubate overnight at 37°C. Use the suspension to inoculate media as described below.

iii) *Inoculation of culture media and incubation*

Approximately 0.1 ml of inoculum is transferred to each of three slants of Herrold's medium containing mycobactin and to one slant of Herrold's medium without mycobactin. The inoculum is distributed evenly over the surface of the slants. The tubes are allowed to remain in a slanted position at 37°C for

approximately 1 week with screw caps loose. The tubes are returned to a vertical position when the free moisture has evaporated from the slants. The lids are tightened and the tubes are placed in baskets in an incubator at 37°C.

The egg in Herrold's medium contributes sufficient phospholipids to neutralise the bactericidal activity of residual HPC in the inoculum. The other media (Modified Dubos and Middlebrook) do not have this property. Other treatments can be used for sample decontamination, for example oxalic acid at 5%.

HPC is relatively ineffective in controlling the growth of contaminating fungi. Amphotericin B (fungizone) was found to control effectively fungal overgrowth of inoculated media (31). Fungizone may be incorporated in the Herrold's medium at a final concentration of 50 µg per ml of medium. Due to loss of antifungal activity, storage of Herrold's medium containing fungizone should be limited to 1 month at 4°C.

The slants are incubated for at least 4 months and observed weekly from the sixth week onwards.

- *Processing faecal specimens*

  No chemical preservative is used. The faecal specimens can be frozen at –70°C.

i) *Suspension and decontamination of faeces*

  1 g of faeces is transferred to a 50 ml tube containing 20 ml of sterile distilled water. The mixture is shaken for 30 minutes at room temperature. The larger particles are allowed to settle for 30 minutes. The uppermost 5 ml of faeces suspension is transferred to a 50 ml tube containing 20 ml of HPC. The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 18 hours at room temperature.

ii) *Inoculation of culture media*

  0.1 ml of the undisturbed sediment is transferred to each of four slants of Herrold's medium, three with mycobactin and one without mycobactin. A smear may be made from the sediment and stained by the Ziehl–Neelsen method.

iii) *Incubation and observation of slants*

  The same as for tissue specimens.

Variations in the above methods have been described (4, 24, 30, 35, 40, 54, 55). The sensitivity of culture may be enhanced using liquid media and with centrifugation rather than sedimentation techniques. The double incubation method described by Whitlock *et al.* (55) assists with decontamination of the inoculum (43) and offers higher sensitivity than the sedimentation or filtration protocols (11). The double incubation method involves mixing 2 g faeces with 15 ml saline or water followed by sedimentation for 30 minutes and transferring (avoiding fibrous matter) the top 5ml of the suspension to 25 ml of 0.9% HPC in half-strength brain–heart infusion (Difco). After incubating at 37°C for 16–24 hours, the mixture is centrifuged at 900 g for 30 minutes (room temperature), the supernatant is discarded and pellet resuspended in 1 ml VAN. The mixture is incubated for 24–72 hours at 37°C and used to inoculate media as described above (7).

d) DNA probes and polymerase chain reaction

DNA probes are being developed that offer a means of detecting *M. paratuberculosis* in diagnostic samples and of rapidly identifying bacterial isolates (12, 29). They have been used to distinguish between *M. paratuberculosis* and other mycobacteria.

McFadden *et al.* have identified a sequence (26, 27), termed IS*900*, which is an insertion sequence specific for *M. paratuberculosis*. It has been reported that a small number of isolates other than *M. paratuberculosis* have produced amplified products the same size as expected from *M. paratuberculosis*. A restriction enzyme digest may be applied to positive IS*900* products to confirm that their sequence is consistent with *M. paratuberculosis* (6).

The identifications of new DNA sequences considered to be unique to *M. paratuberculosis*; ISMav2, f57, and ISMap02 sequences, offer additional tools for rapid identification of this organism using the polymerase chain reaction (PCR) technology (44, 45, 51). The restriction enzyme analysis of IS*1311*, an insertion sequence common to *M. avium* and *M. paratuberculosis* can be used to distinguish between these species and for typing of ovine, bovine and bison strains of *M. paratuberculosis* (38, 57).

The use of IS*900* as a DNA probe for specific identification of *M. paratuberculosis* in faecal samples from cattle by PCR has been reported (52). Commercial diagnostic PCR tests for the detection of *M. paratuberculosis* in milk and faecal samples have been developed.

2.   Serological tests

The serological tests commonly used for paratuberculosis in cattle are complement fixation (CF), enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) (42) corresponding to humoral immunity, and the gamma interferon assay corresponding to cellular immunity.

a)   Complement fixation test

The CF test has been the standard test used for cattle for many years. The CF test works well on clinically suspect animals, but does not have sufficient specificity to enable its use in the general population for control purposes. Nevertheless, it is often demanded by countries that import cattle. A variety of CF test procedures are used internationally. An example of a microtitre method for performing the CF test is as follows:

i)   The antigen is an aqueous extract of bacteria from which lipid has been removed (strain *M. paratuberculosis* 316F). *Mycobacterium avium* D9 may also be used.

ii)   All sera are inactivated in the water bath at 60°C for 30 minutes and diluted at 1/4, 1/8 and 1/16. A positive control serum and a negative control serum should be included on each plate. The following controls are also prepared: antigen control, complement control and haemolytic system control.

iii)   Reconstituted, freeze-dried complement is diluted to contain six times H_{50} (50% haemolysing dose) as calculated by titration against the antigen.

iv)   Sheep erythrocytes, 2.5%, are sensitised with 2 units of H_{100} haemolysin.

v)   All dilutions and reagents are prepared in calcium/magnesium veronal buffer; 25 µl volumes of each reagent are used in 96-well round-bottom microtitration plates.

vi)   Primary incubation is at 4°C overnight and secondary incubation is at 37°C for 30 minutes.

vii)   *Reading and interpreting the results:* Plates may be left to settle or centrifuged and read as follows: 4+ = 100% fixation, 3+ = 75% fixation, 2+ = 50% fixation, 1+ = 25% fixation and 0 = complete haemolysis. The titre of test sera is given as the reciprocal of the highest dilution of serum giving 50% fixation. A reaction of 2+ at 1/8 is regarded as positive. Results should be interpreted in relation to clinical signs and other laboratory findings.

b)   Enzyme-linked immunosorbent assay

The ELISA is, at present, the most sensitive and specific test for serum antibodies to *M. paratuberculosis* in cattle. Its sensitivity is comparable with that of the CF test in clinical cases, but is greater than that of the CF test in subclinically infected carriers. The specificity of the ELISA is increased by *M. phlei* absorption of sera. The absorbed ELISA, designed by Yokomizo *et al.* (61, 62) and modified by Milner *et al.* (32), was developed into a commercial kit by Cox *et al.* (8).

The ELISA detects about 30–40% cattle identified as infected by culture of faeces on solid media (56). Similarly to the culture methods, the sensitivity of the ELISA depends on the level of *M. paratuberculosis* shedding in faeces and the age of animals. A large study recently performed in Australia showed that the actual sensitivity of the ELISA in 2-, 3- and 4-year-old cows was 1.2%, 8.9% and 11.6%, respectively, but remained between 20 and 30% in older age-groups (22). The overall actual sensitivity for all age-groups was calculated to be about 15% (22, 56).

In small ruminants the commercially available ELISA had a specificity of 98.2–99.5% (95% confidence intervals [CI]) and detected 35–54% (95% CI) of animals with histological evidence of infection (18). In another study the estimated specificity of an in-house ELISA was 99% and its sensitivity measured against histological results was 21.9% (37).

The absorbed ELISA combines the sensitivity of ELISA with the added specificity of an absorption step. Sera to be tested are diluted with buffer containing soluble *M. phlei* antigen prior to testing in an indirect ELISA. This procedure eliminates nonspecific cross-reacting antibodies. In early versions, sera were absorbed with whole *M. phlei*, which were removed by centrifugation prior to testing.

A microtitre plate format has been developed in which *M. paratuberculosis* antigen is coated on to 96-well plates. Samples are diluted in sample diluent containing *M. phlei* to remove cross-reacting antibodies. On incubation of the diluted sample in the coated well, antibody specific to *M. paratuberculosis* forms a complex with the coated antigens. After washing away unbound materials from the wells, horseradish peroxidase (HPRO)-labelled anti-bovine immunoglobulin is added. This reacts with immunoglobulins bound to the solid-phase antigen. The rate of conversion of substrate is proportional to the amount of bound immunoglobulin. Subsequent colour, measured (at 450 nm) spectrophotometrically is proportional to the amount of antibody present in the test sample.

The antigen used to coat the ELISA plates is available commercially.

An anti-bovine IgG labelled with HRPO is used as conjugate. The substrate chromogen solution is hydrogen peroxide tetramethyl benzidine. A solution of 0.5 M H_2SO_4 is used to stop the reaction when the absorbance of the positive control serum reaches a predetermined point.

Several absorbed ELISA kits are commercially available. The method and test materials needed, the interpretation of the results and calculations are fully described in the instructions accompanying the commercial kit. It has recently been reported that several commercially available ELISAs have similar sensitivities and specificities (5). Some commercial kits offer an option of testing milk samples. The ELISA on bovine and caprine milk has been found to have specificity similar to that of the serum ELISA, but less sensitive than the blood test (17, 36).

c) Agar gel immunodiffusion test

The AGID test is useful for the confirmation of the disease in clinically suspect cattle, sheep and goats (39). It has been reported that in small ruminants in New Zealand and Australia the AGID offers slightly higher sensitivity and specificity than that obtained by the ELISAs (15, 18, 37). The reported specificity and sensitivity of the AGID measured against histological results were 99–100% (95%CI) and 38–56% (95% CI), respectively (18).

The antigen employed is a crude protoplasmic extract of laboratory strain *M. avium* 18 (formally *M. paratuberculosis* 18) prepared by disruption of cells in a hydraulic press cell fractionator. Disrupted cells are centrifuged at 40,000 ***g*** for 2 hours to remove cell wall debris, and the supernatant fraction is retained and lyophilised. This antigen is resuspended in water at a concentration of 10 mg/ml.

Agarose is dissolved in barbital buffer, pH 8.6, containing sodium azide, to give a final agarose concentration of 0.75%. Agarose may be poured into Petri dishes or on to glass slides. Wells are cut in a hexagonal pattern. Wells are 4 mm in diameter, 4 mm apart, and the agar should be 3–4 mm deep. Antigen is added to centre wells. Test, positive and negative control sera are added to alternate peripheral wells.

Plates are incubated in a humid chamber at room temperature. Gels are examined for precipitation lines after 24 and 48 hours' incubation. The appearance of one or more clearly definable precipitation line(s), showing identity with that of a control positive serum, before or at 48 hours, constitutes a positive test result. Absence of any precipitation lines is recorded as a negative test result. Nonspecific lines may occur.

Several variations of the method are in use.

3. Tests for cell-mediated immunity

The detection of a systemic cell-mediated response precedes detectable antibody production. Animals that are minimally infected frequently fail to react on serological testing but may react positively to tests that measure cell-mediated immunity.

a) Gamma interferon assay

The assay is based on the release of gamma interferon from sensitised lymphocytes during an 18–36-hour incubation period with specific antigen (avian purified protein derivative [PPD] tuberculin, bovine PPD tuberculin or johnin) (60). The quantitative detection of bovine gamma interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma interferon. A commercial diagnostic test based on the detection of gamma interferon has been developed for the diagnosis of bovine tuberculosis. The method and test materials needed are fully described in the instructions accompanying the commercial kit. This test has not been validated by the manufacturer (Prionics, Switzerland) for the diagnosis of paratuberculosis. As such, results derived from this assay are frequently difficult to interpret because there is no agreement with respect to the interpretation criteria and types and amounts of antigens used to stimulate blood lymphocytes. In cattle the reported specificity of the test varied from 94% to 67% depending on the interpretation criteria (23).

b) Delayed-type hypersensitivity

The skin test for delayed-type hypersensitivity (DTH) is a measure of cell-mediated immunity, but has limited value. The test is carried out by the intradermal inoculation of 0.1 ml of antigen into a clipped or

shaven site, usually on the side of the middle third of the neck. In the past, avian PPD tuberculin[3] or johnin was used for this purpose as it was believed that avian tuberculin and johnin are of comparable sensitivity and specificity. The skin thickness is measured with calipers before and 72 hours after inoculation. Increases in skin thickness of over 2 mm should be regarded as indicating the presence of DTH. It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. However, sensitisation to the *M. avium* complex is widespread in animals, and neither avian tuberculin nor johnin are highly specific (19). Furthermore the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria. In a recent study in which johnin (ID-Lelystad, Lelystad, The Netherlands) was used to test cattle, the skin test specificity was 88.8% at the cut-off value of ≥2 mm, 91.3% at the cut-off value of ≥3 mm and 93.5% at the cut-off value of ≥4 mm (23). The effect of these cut-off values on the sensitivity has not been determined. The performance of this test may also be significantly affected by minor antigenic differences that occur in different batches of antigen (23). Further research is required to increase the value of the skin test.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccines: Vaccines used against paratuberculosis are: live, attenuated, incorporated with oil and pumice; lyophilised, live, attenuated, which may be adjuvanted with, for example, oil after reconstitution; and heat-killed bacterins. Vaccines may be prepared from one strain of *M. paratuberculosis* 316F or 2E (Weybridge) or *M. paratuberculosis* 3 and 5 or II (Canadian strains), or as many as three strains may be used. The information below applies to a live, attenuated vaccine adjuvanted with oil and pumice (10, 50, 59). Vaccination may cause a reaction at the site of injection. Vaccination may also interfere with eradication programmes based on immunological testing and elimination of animals identified as infected and can interfere with the interpretation of DTH skin tests for bovine tuberculosis.

Diagnostic products: Johnin PPD is a preparation of the heat-treated products of growth and lysis of *M. paratuberculosis.* Avian tuberculin PPD is a preparation of heat-treated products of growth and lysis of *M. avium* D4ER or TB 56. Details of avian tuberculin PPD are in Chapter 2.3.6 Avian tuberculosis. These two preparations are used, by intradermal injection, to reveal DTH as a means of identifying animals infected or sensitised with *M. paratuberculosis.*

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8. Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Vaccine: Seed strains should be of a prevalent type, which may be checked by biotyping or genetic analysis. They should have been demonstrated to be innocuous when administered by the recommended route of vaccination to intended target species.

Johnin: Strains of *M. paratuberculosis* used to prepare seed cultures should be identified by biotyping or genetic tests. They should be shown to be free from contaminating organisms.

b) Method of culture

Vaccine: Seed cultures may be made on potato slants partly immersed in a suitable medium, such as Reid's synthetic medium[4] (53). Cultures may be stored lyophilised. Active cultures are normally incubated at 37°C.

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3 Avian tuberculin can be obtained from VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom, or from Symbiotic Society, 299 av. Jean Jaurès, 69007 Lyon, France.

4 L-Asparagine, 5.0 g
Potassium dihydrogen phosphate (KH_2PO_4, anhydrous), 2.0 g
Magnesium sulphate ($MgSO_4$-$7H_2O$), 1.0 g
Ammonium citrate ($[NH_4]_3 C_6H_5O_7$), 2.0 g
Sodium chloride, 2.0 g
Ferric ammonium citrate, 0.075 g
Dextrose monohydrate B.P., 10 g
Glycerol B.P. (48 ml), 60 g
Distilled water to 1000 ml

Johnin: The culture substrate should be shown to be capable of producing a product free from substances known to cause toxic or allergic reactions. A suitable medium for seed culture is that of Reid, solidified with 1.75% agar, in screw cap tubes. Cultures may also be stored lyophilised.

c)   Validation as a vaccine

Vaccine: Purity tests should be carried out on seed cultures and final harvest by stained smears.

The vaccine should be used as part of a control programme and will not on its own provide complete protection against disease caused by *M. paratuberculosis* (59). There is usually good control of clinical disease, but subclinical infection persists in vaccinated herds, albeit at a reduced level. Vaccine should be administered to animals in early life only, e.g. calves in their first month of life. It should be inoculated subcutaneously and causes a small inflammatory swelling. This is gradually replaced by a cold, painless, fibro-caseous nodule, which varies in size and which may persist for years. Vaccination has been used to control the disease in sheep and goats, including older animals. In order to get the best results from vaccination, management practices to control the disease should also be in place.

The use of vaccines may interfere with the outcome of diagnostic skin tests for tuberculosis, and this should be remembered when planning a control programme (16).

Johnin: Cultures should be checked by staining smears for the presence of contaminating organisms.

To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test, are each injected intradermally on each of three occasions at 5-day intervals, with 0.01 mg of the preparation under test in a volume of 0.1 ml. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same johnin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–48 hours later.

2.   Method of manufacture

Vaccine: For vaccine batches, the organisms may be grown on a liquid synthetic medium, such as Reid's synthetic medium. The organisms grow as a pellicle on the liquid surface. To ensure a good surface area, it is convenient to use vessels such as conical flasks containing one-third of their nominal volume of liquid medium. These flasks may be seeded directly from potato slant cultures, but with some strains, one or more passages on liquid medium may be necessary to ensure adequate pellicle growth for the final, vaccine batch passage. Such passaging should usually take place at 2-week intervals as longer periods may result in over-maturation and sinking of the pellicle. Incubation is at 37°C.

To prepare the vaccine, the pellicle growth from 2-week-old cultures of each strain to be included may be separated from the liquid medium by decantation, filtration and pressing between filter paper pads. The moist *M. paratuberculosis* culture is blended with an adjuvant, such as liquid paraffin, olive oil and pumice (10).

Johnin: Johnin for skin test diagnosis is a PPD prepared from one or more strains of *M. paratuberculosis* (available from VLA Weybridge or CDI, Lelystad, the Netherlands). It may be prepared by the following method.

Mycobacterium paratuberculosis strains are grown as a pellicle on liquid Reid's medium. Production cultures are usually inoculated from liquid seeding cultures rather than directly from seed on solid medium (Reid's synthetic medium). Production cultures are incubated at 37°C for 10 weeks.

At the end of the incubation period, the culture medium has a pH of about 5 and little or no johnin will be obtained unless the pH is raised, using sodium hydroxide, to about 7.3 before steaming. After thorough mixing, the cultures are free steamed for 3 hours. The bulk of the killed organisms is removed by coarse filtration and the filtrate is clarified by further filtration. Protein in the filtrate is precipitated chemically with 40% trichloroacetic acid, washed and redissolved (alkaline solvent). The product is sterilised by filtration. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol (not more than 10% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile glass containers, which are then sealed.

3.   In-process control

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pH (not adjusted) 5.6–5.8
When required, the above medium is solidified by the addition of 1.5% granulated agar (Difco). Sterilised at 121°C for 15 minutes.

Vaccine: Adequate growth of culture and cultural purity need to be checked. Presence of contaminating organisms may be detected by conventional sterility tests on harvests. Tests for pathogenic mycobacteria are carried out by injection of moist culture, taken prior to blending with adjuvant and diluted tenfold in saline, into two guinea-pigs, each receiving 1 ml. These are observed for 8 weeks, killed humanely, and examined for any abnormal lesions.

Johnin: After final filtration the sterility of each filtrate of the PPD solution is checked.

Sterile filtrates are tested for protein content by a Kjeldahl method (1). The protein content is adjusted to give between 0.475 and 0.525 mg/ml of protein in the final product. The pH is adjusted to the range 6.5–7.5.

4.   Batch control

a)   Sterility

Tests for sterility and freedom from contamination may be found in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials. The vaccine organism will not normally grow to a detectable level in conventional sterility tests.

b)   Safety

Vaccine: These tests are normally performed in laboratory animals, although multidose tests in target animals would also be satisfactory. A typical laboratory animal test would be as follows. Each of two guinea-pigs is inoculated, subcutaneously, with an acceptable batch of vaccine at a fraction of the cattle dose previously determined to give a nodule but no overt necrosis at the injection site. Animals are observed for 8 weeks, killed humanely and examined for any abnormal lesions.

Johnin: Two guinea-pigs should each be injected subcutaneously with 0.5 ml of the johnin under test. No significant local or systemic lesions should be seen within 7 days (1).

Tests on johnin for living mycobacteria may be performed either on the material immediately before it is dispensed into final containers or on samples taken from final containers themselves. A sample of at least 10 ml should be taken, and this should be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and post-mortem examinations are carried out. Any macroscopic lesions are examined microscopically and culturally.

c)   Potency

Vaccine: As protection tests appear to be impractical, a test of sensitising ability may be used. This may then be related to bacterial content based on weight. A typical test would be as follows: guinea-pigs are sensitised by intramuscular injection of 0.5 ml of a 100-fold dilution in liquid paraffin of the vaccine under test. Skin tests are performed 6 weeks after sensitisation using intradermal inoculations of 0.2 ml of at least three serial dilutions of an *M. paratuberculosis* antigen, such as johnin PPD, the dilutions being chosen to give expected skin reactions of from 8 mm to 25 mm diameter. Each guinea-pig receives several dilutions per flank, their distribution being chosen by a Latin square design. After 24–48 hours, skin reactions are measured. A reference preparation for tests of this type has not yet been fully established. Avian tuberculin PPD of known international unitage may be used as a skin test antigen in tests of this type to ensure that the vaccine is capable of producing adequate sensitisation (corresponding to the vaccination).

Johnin: The potency of johnin is currently determined by chemical assay for protein using a Kjeldahl method. A PPD content of 0.5 ± 0.025 mg/ml of final product is recommended (1).

The identity of the material should be confirmed by injecting intradermally into guinea-pigs sensitised by injections of killed *M. paratuberculosis* (100 mg powder mycobacteria + 25 ml vaseline + 100 mg pumice stone) 6 weeks previously.

It is possible to perform a potency test using dilutions of johnin in guinea-pigs sensitised with *M. paratuberculosis*, similar to such tests for the potency of bovine and avian tuberculin, but a standard preparation for this type of test has not yet been fully established.

d)   Duration of immunity

Vaccine: After vaccination at the age of 14–30 days, the vaccination effect is expressed as the reduction in the rate of excretors among vaccinated animals as compared with nonvaccinated bovines (21).

There is usually good control of clinical disease, but a reduced level of subclinical infection persists. The favourable results probably reflect a diminishing exposure to infection resulting from a reduction in the number of heavy excretors in the herd.

e) Stability

Vaccine: The vaccine may be stored at 2–8°C for 9–12 months without loss of potency. It should not be frozen.

Johnin: Johnin should be protected from light and stored at 2–8°C. Under these conditions it should retain its potency for at least 5 years.

f) Preservatives

A preservative is normally included for vaccine in multidose containers. For johnin, the phenol used is no more than 0.5% (w/v). The concentration of the preservative in the final product and its persistence through shelf life should be checked.

g) Precautions (hazards)

Vaccine: The vaccine causes some side-effects, nodule formation and sensitisation of animals to the tuberculin test (15). In humans, accidental injection of vaccine has resulted in chronic inflammatory reactions requiring surgical treatment (21).

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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NB: There are OIE Reference Laboratories for Paratuberculosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

Q FEVER

SUMMARY

Query (*Q*) *fever is a zoonosis that occurs in most countries. Humans acquire infection from animal reservoirs, especially from domestic ruminants. Q fever is a highly infectious disease, which is due to the proliferation of* Coxiella burnetii*, a small and pleomorphic bacterium measuring 0.3–1.5 µm long × 0.2–0.4 µm wide. As an obligate intracellular bacterium,* C. burnetii *can be grown only in embryonated eggs or cell cultures or, when necessary, in inoculated laboratory animals. It occurs in two antigenic forms: the pathogenic phase I, found in infected animals or humans, and the avirulent phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Because this microorganism is extremely hazardous, handling viable* C. burnetii *must be done in facilities that meet the OIE requirements for Containment Group 3 pathogens.*

In humans, Q fever occurs in either an acute form (self-limiting febrile episode, pneumonia, hepatitis) or a severe chronic form (endocarditis) following an early infection that may be passed unnoticed. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In some countries, a vaccine is available for professionally exposed population groups.

The signs of Q fever in cattle include abortion, dead or weak offspring, retained placenta, metritis, and infertility. In small ruminants, Q fever is often associated with sporadic abortions or outbreaks of abortions followed by recovery without complications. Coxiella burnetii *infection persists for several years, and is probably life long. Sheep, goats and cows are mainly asymptomatic carriers, but can shed massive numbers of bacteria at parturition, and intermittently in various secretions and excreta. Domestic animals, such as dogs, cats, rabbits, birds, etc., are also susceptible to infection and should be considered as possible sources of infection for animals and humans.*

Identification of the agent: *For laboratory diagnosis, samples can be taken from the placenta, vaginal discharges, and liver, lung or stomach contents of aborted fetuses, and from milk, colostrum and faeces.*

The bacteria can be visualised in stained tissue smears using a microscope with an oil-immersion objective lens. Because it is acid resistant, the bacteria can be stained by several methods: Stamp, modified Ziehl–Neelsen, Gimenez, Giemsa and modified Koster. This finding is presumptive evidence of Q fever, but coupled with serological tests, clinical findings and no other infectious abortive agents, it may be sufficient to establish a diagnosis of the disease at the flock or herd level.

To date, demonstration of the agent by immunohistology using specific antibodies or by polymerase chain reaction (PCR) has proven to be more specific and sensitive than classical staining methods. No specific antibodies for immunochemistry are commercially available, but PCR can be done in suitably equipped laboratories. PCR is considered a useful test for screening large numbers of samples and various types of samples. Furthermore, samples can be heat inactivated and therefore ensure the safety of laboratory personnel.

Coxiella burnetii *can be isolated by inoculation of specimens into conventional cell cultures or embryonated chicken yolk sacs or laboratory animals. Inoculation of laboratory animals (guinea-pig, mouse, hamster) is helpful in cases requiring isolation from tissues contaminated with various microorganisms or in order to obtain phase I* Coxiella *antigens.*

Serological tests: *The diagnosis of Q fever often relies on serology. A number of tests can be used, particularly the indirect immunofluorescence test, the enzyme-linked immunosorbent assay,*

*and the complement fixation test. Currently, commercially available tests allow the detection of anti-*C. burnetii *phase II antibodies. The presence of specific IgG antibodies provides evidence of a recent* C. burnetii *infection or a past exposure.*

Requirements for vaccines and diagnostic biologicals: *Several inactivated vaccines against Q fever have been developed, but only vaccines containing or prepared from phase I* C. burnetii *should be considered protective. Repeated annual vaccination is recommended in heavily infected areas, particularly of young animals.*

An inactivated phase I vaccine is commercially available in Slovakia. A recent study has proved its efficacy with regard to both abortion and C. burnetii *shedding in experimentally vaccinated then challenged pregnant goats, but information on its safety is lacking.*

A. INTRODUCTION

Q fever is widely distributed throughout the world with the exception of New Zealand. Although Q fever is present in virtually all 'animal kingdoms', including arthropods, the disease affects mostly humans, cattle, sheep and goats (27, 30). The aetiological agent, *Coxiella burnetii*, is a gram-negative obligate intracellular bacterium, adapted to thrive within the phagolysosome of the phagocyte. It has been historically classified in the Rickettsiaceae family; however, phylogenetic investigations, based mainly on 16S rRNA sequence analysis, have shown that the *Coxiella* genus is distant from the *Rickettsia* genus in the alpha subdivision of Proteobacteria (60). *Coxiella burnetii* has now been placed in the Coxiellaceae family in the order Legionellales of the gamma subdivision of Proteobacteria (26). The complete genome sequencing of *C. burnetii* has been achieved recently and confirms its systematic position (47). Unlike rickettsiae, *C. burnetii* produces a small, dense, highly resistant spore-like form that is highly stable in the environment, a trait that is important for transmission (31). This ability has been attributed to the existence of *C. burnetii* developmental cycle variants: large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC) (10, 17, 48). The SDC and SCV represent the forms of the bacteria likely to survive extracellularly as infectious particles. Another essential characteristic is that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the avirulent phase II, obtained *in ovo* or *in vitro*. An LPS (lipopolysaccharide) change occurs during serial passages: phase I cells, with full-length LPS O-chains, change to intermediate phases with decreasing LPS O-chain lengths and then to phase II, with truncated LPS. The LPS phase variation is accompanied by a permanent chromosomal deletion that makes impossible cell reversion from phase II to phase I (56).

Q fever is a zoonosis. In humans, the infection has an acute, chronic and subclinical form (38). The acute forms commonly include a self-limiting febrile episode, pneumonia, and granulomatous hepatitis. The main clinical manifestation of chronic Q fever is endocarditis in patients with valvulopathies. In the absence of any appropriate antibiotic treatment, complications of the chronic form may be severe to fatal (11). Moreover, *C. burnetii* infection of pregnant women can provoke placentitis and often leads to premature birth, growth restriction, spontaneous abortion or fetal death (36). The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. The epidemiology of Q fever suggests that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through contact with infected animals and their reproductive tissues (29, 30). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, and even possibly following pasteurisation (16, 38, 53). Q fever is very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission and blood transfusions, is possible (12, 32). In animals, in addition to respiratory and digestive routes, vertical transmission and sexual transmission can occur (25, 58). Arthropods, principally ticks, may be involved in Q fever transmission.

In cows, ewes and goats, Q fever has been associated mostly with late abortion and reproductive disorders such as premature birth, dead or weak offspring, metritis and infertility (27). Nevertheless, serological responses in a given species or even the isolation of *C. burnetii* do not necessarily correlate with expression of the clinical disease (18, 27, 44). Indeed, the agent may persist in infected animals, be shed intermittently in milk, faeces, urine, and may be present in blood. The agent can be recovered in very high numbers in birth products (placenta, amniotic fluid and fetus), and non-pregnant animals are less of a risk. Domestic ruminants are considered the main reservoirs for *C. burnetii*, but cats, dogs, rabbits, birds, etc., have also been reported to be implicated in human disease/infection (23, 29, 30, 51). Thus, infected domestic and wild animals usually shed the agent with no outward signs of disease, and should be regarded as possible sources of infection for humans.

Abortions in ruminants have usually been investigated to determine if Q fever is present as it may affect the health of humans or other animals. Diagnosis of Q fever, and other abortive diseases, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results, is usually adequate for this purpose (27, 41, 43). The Q fever diagnostic is also required for epidemiological surveys of 'at risk' and suspected flocks in limited areas (following recent outbreaks in humans or animals), or for export

purpose. However, identification of *C. burnetii* shedders and asymptomatic carriers is not currently practised (4, 5, 7, 21).

Generally, when several animals are seropositive, an appropriate intervention is advised. The measures could be adapted according to the seroprevalence and epidemiological context. Proper pasteurisation of milk products must be ensured (53). The amount of agent can be reduced in the environment by regular cleaning and disinfection of animal facilities, with particular care of parturition areas, using 10% sodium hypochlorite. Pregnant animals must be kept in separate pens, and placentas and aborted fetuses must be removed quickly and disposed properly to avoid being ingested by dogs, cats or wildlife. Spreading manure from contaminated farms in suburban areas and gardens should be avoided. While the efficiency of fermentation by composting or decontamination with a chemical treatment (such as 0.4% calcium cyanamid or lime) for inactivating *C. burnetii* need to be evaluated, these methods are still recommended. In order to acquire and maintain *Coxiella*-free livestock, introduction of animals, regrouping of flocks, contacts with wildlife and infestation by ticks should be minimised. These methods may be effective in controlling disease but exposed animals may remain infected. Although vaccines for animal Q fever have been developed, there are not commercially available in most countries.

Finally, it is important to remember that *C. burnetii* is extremely hazardous to humans, and laboratory infections are common. Because of its ability to cause incapacitating disease in large groups of people, its low infectious dose, resistance in the environment, and aerosol route of transmission, *C. burnetii* is considered a potential agent of bioterrorism and is classified by the CDC as a group B agent (9). Appropriate precautions must be taken with this risk group 3 agent. Live culture or contaminated material from infected animals must only be handled in facilities that meet the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Coxiella burnetii can be demonstrated in various ways, depending on the type of sample and the purpose of diagnosis (11, 41, 43). Samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. Milk from the tank, individual milk or colostrum, and faeces samples can also be taken.

a) Staining

In a case of an abortion suspected of being caused by an infection, smears are prepared on microscope slides of placental cotyledon (42). Lung, liver and abomasal contents of the aborted fetus or vaginal discharge may be used in the same manner. These could be stained according to several methods: Stamp, Gimenez, Macchiavello, Giemsa and modified Koster (14, 35, 41, 42, 44). The first three techniques give the best results. These methods are close to the modified Ziehl–Neelsen method involving basic fuchsin to stain bacteria. For example, the Stamp staining method is performed with 0.4% basic fuchsin solution, followed by rapid decolouration with 0.5% acetic acid solution, and counterstaining with 1% methylene blue or malachite green solution. The smears are examined microscopically with an oil-immersion objective lens (×500 or more). The Stamp method is preferred in veterinary laboratories while the Gimenez method is widespread in human diagnosis. Gimenez is fastest because an acidic solution is not included for differentiation. *Coxiella burnetii* are characterised by a very large number of thin, pink-stained coccobacillary bacteria against a blue or green background. They may sometimes be difficult to detect due to their small size (0.3–1.5 µm long × 0.2–0.4 µm wide), but this is compensated for by their large numbers; often inclusions within the host cells appear as red masses against the blue or green background. Attention must be taken in the interpretation of the results as, microscopically, *C. burnetii* can be confused with *Chlamydophila abortus* or *Brucella* spp. However, using the same staining procedure, *Chlamydophila* have sharper outlines, are round, small and may resemble globules. *Brucella* spp, are larger (0.6–1.5 µm long × 0.5–0.7 µm wide), more clearly defined and stain more intensely. Control positive slides of *C. burnetii*, *Chlamydophila abortus* and *Brucella* must be used for comparison. Diagnosis made on the basis of microscopy, coupled with positive serological results, is usually adequate for routine purposes (43). When biological staining is inconclusive, one of the other methods (below) may be used as a confirmatory test.

b) Specific detection methods

Detection of *C. burnetii* in samples can also be achieved by specific immunodetection (capture enzyme-linked immunosorbent assay [ELISA], immunohistochemistry), or DNA amplification (8, 11, 55). Immunohistology may be used with paraffin-embedded tissues or on acetone-fixed smears (37). The method is an indirect immunofluorescence or immunoperoxidase assay using polyclonal *C. burnetii* antibodies, either a well characterised antiserum of human origin or a specific antiserum produced in

laboratory animals (rabbit or guinea-pig). An anti-species (human, rabbit or guinea-pig) anti-IgG conjugate, labelled with fluorescein isothiocyanate (FITC) or peroxidase, is then used to visualise the bacteria. Control positive slides of *C. burnetii* antigen should be available for comparison. No specific antibodies for immunochemistry are commercially available.

Polymerase chain reaction (PCR) methods have been used successfully to detect *C. burnetii* DNA in cell cultures and biological samples. As the number of *C. burnetii* is likely to be lower in milk, colostrums and faeces than in abortion material, PCR can be used for analysis of this large diversity of samples. Before performing the PCR, biological samples can be inactivated by heating at 90°C for 30–60 minutes, depending of the samples' nature, their size or their weight. This technique can be performed in suitably equipped laboratories using primers derived from various targets, such as multicopy insertion sequence (accession number M80806), the most popular employed (4, 19). The use of these primers for the amplification of this sequence allows the sensitivity of the test to be increased and this because of the presence of several copies in the *Coxiella* genome. The level of detection of the conventional trans-PCR is related to the analysed samples (for example 1–500 bacteria/ml of milk sample or 1 bacteria/mg of faeces). The other target genes reported to be used in the PCR for specific *C. burnetii* identification are: superoxide dismutase (sodB) gene (accession number M74242); com1 encoding a 27 kDa outer membrane protein (accession number AB004712); heat shock operon encoding two heat shock proteins (htpA and htpB) (accession number M20482); isocitrate dehydrogenase (icd) (accession number AF069035); and macrophage infectivity potentiator protein (cbmip) (accession number U14170). Different primers used in PCR can be obtained on the web site (http://ifr48.timone.univ-mrs.fr/Fiches/Fievre_Q), regularly updated by the French CNR.

The real-time PCR provides an additional means of detection and quantification (21, 22, 52). As the conventional PCR, various target genes are used: IS1111 (accession number M80806); com1 (accession number AB004712); and isocitrate dehydrogenase (icd). To quantify the bacteria in biological samples using the real-time PCR, it is recommended to amplify a unique and specific sequence. Indeed, recent data show that the number of the insertion sequence (IS) varied widely (between 7 and 110) depending of the isolates (22). Whereas the use of this sequence could increase the sensitivity of the test, it may not be accurate for quantification.

Ready-to-use kits are commercially available and can detect the bacteria in several samples. However, there is an urgent need for the development of a molecular method for the assessment of bacterial viability, especially in milk samples. The development of a multiplex PCR constitutes another technique for screening all infectious abortive agents.

c) Isolation of the agent

For specific laboratory investigations, it may be necessary to isolate the agent. Where microscopic examination has revealed large numbers of *C. burnetii* combined with a low contamination rate with other bacteria, direct isolation by inoculation of embryonated chicken eggs or cell culture is possible (18). For example, a portion of placenta is homogenised in phosphate buffered saline (PBS) containing antibiotics (streptomycin 100–200 µg/ml and penicillin or gentamicin 50–100 µg/ml). After low-speed centrifugation, dilutions of the supernatant fluid are inoculated into 5-day-old embryonated chicken eggs via the yolk sac. Eggs are preferably from specific pathogen free (SPF) hens. Embryos that die during the first 5 days after inoculation are discarded. The yolk sacs are harvested after 10–15 days of incubation. Stained smears of the yolk sac wall are examined to ensure the absence of bacterial contamination and to determine the presence of *C. burnetii*. PCR analysis can be used to confirm the presence of *C. burnetii*. Further passages may be required to obtain an isolate in pure culture.

A cell microculture system from a commercially available method used for virus culture, the shell vial cell culture[1], has been adapted for isolating strictly or facultatively intracellular bacteria, including *C. burnetii*. Such a method was described for *C. burnetii* in 1990 (11, 39). Suspensions of samples are inoculated into human embryonic lung (HEL) fibroblasts grown on a 1 cm² cover-slip within a shell vial. Centrifugation for 1 hour at 700 *g* enhances the attachment and penetration of bacteria into the cells. Three shell vials are used for the same sample, and by day 3, 10 and 21, the cytopathic effect (CPE) – *C. burnetii* characteristic vacuoles in HEL cells – are examined using an inverted microscope. After 10 days, detection of growing *C. burnetii* within the cells is achieved directly on the cover-slip inside a shell vial by a direct immunofluorescence assay with polyclonal anti-*C. burnetii* antibodies and an appropriate anti-species conjugated to FITC. Cells of the remaining shell vial are harvested and transferred in a 25 cm² culture flask. Incubation can be conducted for 3 months, with a culture medium change once a week. The infection can be monitored by microscopy of Gimenez-stained cells cyto-centrifuged from the culture supernatant and by PCR analysis of the culture supernatant. When the CPE observations and Gimenez staining or PCR results are positive, a passage in a 75 cm² culture flask is performed. Culture supernatant is then inoculated on

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1   Sterilin, Bibby Sterilin Ltd, Stone, Staffordshire ST15 O5A, United Kingdom.

confluent layers of Vero cells or L929 mouse fibroblasts in a 150 cm^2 culture flask in order to establish a *C. burnetii* isolate. This method was developed for humans but could be adapted for animals (50).

With heavily multi-contaminated samples, such as placentas, vaginal discharges, faeces, or milk, the inoculation of laboratory animals may be necessary. Biocontainment level 3 requirements are recommended for holding experimentally infected rodents (see Chapter 1.1.2). Mice and guinea-pigs are the most appropriate laboratory animals for this purpose (45). Following intraperitoneal inoculation with a dose of 0.5 ml per animal, body temperature and antibody status are monitored. This method should always be performed in conjunction with serological tests on other guinea-pigs or mice that have been inoculated with the same samples. Sera are collected 21 days after inoculation. A positive result confirms a diagnosis of *C. burnetii* infection. If pyrexia develops, the animal is killed and the spleen is removed for isolation of the agent by inoculation into embryonated chicken eggs or in cell cultures. Microscopic examination of *C. burnetii* is done using impressions and staining of the collected spleens. Alternatively, PCR may be performed on spleens systematically collected 7–9 days post-inoculation (8).

Although characterisation of isolates seems necessary for understanding the varying epidemiology of Q fever in different geographical areas, no discriminatory typing methods are available currently. For this purpose, efforts should focus on genetic typing methods. High resolution molecular typing methods that are currently being developed and evaluated offer promise of mapping progression of clones and contact traceback studies. Studies using multi-spacer typing (15) and variable number tandem repeat typing (2, 54) have demonstrated high levels of resolution among *C. burnetii* isolates. Techniques for genotyping *C. burnetti* have been described.

2. Serological tests

Among the various techniques that can be employed, the three most often used are: the indirect immunofluorescence assay (IFA), the ELISA and the complement fixation (CF) test (11). Three older serological tests are no longer used in routine diagnosis: the microagglutination technique, the capillary agglutination test and the indirect haemolysis test. A high-density particle agglutination (HDPA) test has been evaluated (33). Serological assays are suitable for screening herds, but interpretation at the individual animal level can be difficult. Indeed, animals may remain seropositive for several years following an acute infection, some animals may shed *C. burnetii* and pose a risk for infection prior to the development of antibodies, and some infected animals seem not to seroconvert (1, 6, 8). Serological cut-off titres used to diagnose Q fever are given below; interpretation of the results requires at least ten animals (aborted or not). Both serological responses and bacterial evidence are necessary for establishing the presence of the infection.

a) Indirect immunofluorescence test

In human medicine, the IFA adapted as a microimmunofluorescence technique is the reference method for the serodiagnosis of Q fever (11, 57). The procedure can be adapted to perform an immunoperoxidase assay. Some commercial CF test antigens are suitable, but antigens prepared for the diagnosis in humans are preferred (11). This method of preparation has been demonstrated to yield antigens with the highest sensitivity for *C. burnetii* antibody detection. Briefly, both phase I and phase II *C. burnetii* antigens are used; phase II antigen is obtained by growing *C. burnetii* Nine Mile reference strain in cell culture, while phase I antigen is obtained from the spleens of laboratory animals inoculated with phase II *C. burnetii* in cell cultures. A few phase I cells may still be present in the phase II population and can be selected and propagated within animals. Antigen is diluted, dropped on to the wells of a glass microscope slide, allowed to dry, and fixed with acetone. The two forms of the infection, acute and chronic, have different serological profiles: during acute Q fever, IgG antibodies are elevated against phase II only whereas during chronic Q fever, high levels of IgG antibodies to both phase I and II of the bacteria are observed (57). In addition, antigen-spot slide wells may be purchased from a supplier providing the phase II form, or the phase I and II forms of *C. burnetii*. These can be adapted by replacing the human conjugate by a conjugate adapted to the animal species.

Twofold dilutions of the serum under test are placed on immunofluorescence slides with wells previously coated with one or two antigens. If specific antibodies are present, they are fixed by the antigen on the slide. The complex is then detected by examination with a fluorescence microscope following the addition of the fluorescent conjugate recognising the species immunoglobulins.

- **Antigen preparation**

Antigen should only be prepared in facilities that meet the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.2 of this *Terrestrial Manual*.

Phase II *C. burnetii* Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm^2 culture flasks with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine serum. The infection is monitored by microscopic examination of Gimenez-stained cells scraped from the bottoms

of the flasks. When a heavy *C. burnetii* infection is seen, the supernatants of 15 flasks are individually pelleted by centrifugation (5000 *g*, 15 minutes) resuspended in 1 ml of PBS with 0.1% formaldehyde and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris is removed by two successive centrifugation steps (100 *g*, 10 minutes each). The 15 ml suspension is then centrifuged through 20 ml of PBS with 25% sucrose (6000 *g*, 30 minutes, without a break). The resulting pellet is washed three times in PBS (6000 *g*, 10 minutes), resuspended in the smallest possible volume of sterile distilled water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at –20°C.

In order to obtain phase I antigen, mice are inoculated with *C. burnetii* Nine Mile grown in cells (mainly in phase II). Nine days after infection, the spleens are removed. Each one is ground in 7.5 ml MEM, and inoculated into three 75 cm^2 culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I *C. burnetii* is conducted for 4 weeks, with a culture medium change once a week. The infected cells are then harvested and the bacteria are purified as described above (mainly in phase I).

Antigen production can also be performed by culture of *C. burnetii* in SPF embryonated eggs. At 5–6 days of age, the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after death of the embryo at 12–15 days. Infected yolk sacs have a characteristic straw-yellow colour. Uninfected yolk sacs are orange in colour and have a viscous consistency. Any embryos that die between 5 and 10 days of incubation are discarded. The strain used for egg inoculation is a 1/100 homogenate of yolk sac in PBS containing penicillin (500 International Units/ml) and streptomycin (0.5 mg/ml). The yolk sacs are pooled and homogenised with three parts PBS. The suspension is inactivated with 1.6% formaldehyde for 24 hours at 37°C. The lipid supernatant fluid is discarded. The suspension is then centrifuged at moderate speed (~500 *g*) for 30 minutes. After removal of the supernatant fluid, more PBS is added and centrifugation is repeated. The final suspension is diluted with PBS. Sodium azide or thiomersal is added as an antibacterial preservative. The abundance of *C. burnetii* and the absence of bacterial contaminants in homogenates of yolk sacs suspended in PBS are verified by microscopic examination of a smear on a microscope slide, stained by Stamp's method. In order to obtain phase I antigen, *C. burnetii* recovered from spleen material of infected laboratory animals can be propagated, as ground spleen extracts are subsequently transferred in the yolk sacs, given that the amount of phase I cells is still high until the sixth yolk passage (EP6).

Titration of antigen with at least three different known sera (with high, moderate and low titres, respectively) is sufficient to recover the appropriate dilution for further immunofluorescence tests.

- **Materials and reagents**

Microscope equipped for fluorescence, humidified incubator, washing basin.

Slides suitable for the antigen are necessary. The latter may be either prepared in the laboratory or purchased from a supplier (see above). The method described is adapted from the BioMérieux kit, and is given as an example. Ready-to-use slides contain 12 wells per slide, each of 7 mm diameter, coated with phase II antigen obtained from culture on Vero cells and can be stored at 4°C or –20°C.

Concentrated fluorescent conjugate, to be diluted when required with PBS + 1% Evans blue at the dilution recommended by the manufacturer.

PBS, buffered glycerine, Evans blue dye 1% solution.

- **Test procedure**

i)   Inactivate the sera under test for 30 minutes at 56°C, then dilute serially from 1/40 to 1/640 in PBS.

ii)   Allow the previously antigen-coated slides to warm to room temperature. Do not touch the wells.

iii) Add 20 µl of each serum dilution to the wells. Add negative and positive control sera. To one well, add 20 µl of PBS to serve as antigen control.

iv)   Incubate in a humid chamber for 30 minutes at 37°C. Wash the slide twice with PBS for 10 minutes each. Rinse with distilled water and air dry.

v)   Add to the wells, including the controls, 20 µl of the conjugate directed against the appropriate species (e.g. FITC-labelled rabbit anti-goat or anti-sheep IgG[H+L]), freshly diluted in PBS + Evans blue. Incubate in a humid chamber for 30 minutes at 37°C. Rinse with distilled water and air-dry. Add a few drops of buffered glycerine and cover with a cover-slip. Examine under a fluorescence microscope at magnification ×400 or more.

• **Interpretation of the results**

A positive reaction will consist of small brilliant points against a dark background. Verify that the conjugate by itself and the negative control serum give a negative result (absence of small brilliant points). Nonspecific fluorescence usually takes the form of spots of irregular shape. The positive control must give the known titre with ± one dilution.

The reaction is considered to be positive if there is obvious immunofluorescence at the 1/160 dilution and upwards. In human medicine, this method is used to determine antibodies against phases I and II in the IgG, IgM, and IgA fractions allowing acute and chronic Q fever to be differentiated. Rheumatoid factor absorbant is used for remove IgG before the determination of IgM and IgA. Screening of the sera is performed with phase II antigen, and positive sera are tested subsequently for the presence of the different classes of Ig directed against phases I and II antigens. However, neither phases I and II antibody responses nor Ig classes responses have been well studied in domestic animals.

b) Complement fixation test

This cold fixation micromethod of the type developed by Kolmer is performed with 96-well U-bottomed microtitre plates. The test detects complement-fixing antibodies present in the serum. The CF test is specific but less sensitive than the ELISA or IFA (11, 34). Seroconversion is detected later by the CF test than by the IFA or ELISA, but CF antibodies can persist for long periods after illness, and the CF test gives excellent results for routine diagnosis at the flock level for abortive diseases (41, 43). The CF test is still largely used by many laboratories in many countries. This method often uses antigen in phase II prepared from a mixture of two strains (Nine Mile and Henzerling)[2] or antigen in phase I and II mixture prepared from Nine Mile strain[3]. In France, this method has been standardised (AFNOR NFU47-006).

The reaction is done in two stages. Antigen and complement-fixing antibodies are first mixed, and sheep erythrocytes, sensitised by the anti-sheep erythrocyte serum, are added. Fixation of the complement by the antigen/antibody complex during the first step does not permit lysis of erythrocytes; in contrast, if there are no complement-fixing antibodies, the complement induces the lysis of the sensitised erythrocytes. Then the haemolysis rate is inversely proportional to the level of specific antibodies present in the sample serum.

• **Reagents**

Veronal/calcium/magnesium buffer (VB), pH 7.2.

The haemolytic system: a mixture of equal parts of a 2% suspension of sheep erythrocytes in VB; and haemolytic serum diluted to a specified titre in VB.

Complement: commercial freeze-dried preparation or fresh guinea-pig serum.

Antigen: use commercial antigens at the titre recommended by the manufacturer if the antigen titration is performed with this method.

Positive and negative control sera.

• **Pretitrations**

i) Dilute the sheep erythrocytes to a final concentration of 2% in VB.

ii) Titrate the haemolytic serum on a microplate: 25 µl of complement at a known haemolytic concentration (e.g. 1/30); 25 µl of increasing dilutions of haemolytic serum + 2% sheep erythrocytes. Include controls without complement. Incubate for 30 minutes at 37°C. Establish the dilution equivalent to 2 haemolytic units.

iii) Dilute the antigen as recommended by the manufacturer. The antigen may also be titrated: make increasing dilutions of antigen (25 µl horizontally) and a positive serum of known titre (25 µl, vertically). Add 25 µl of the suspension of sensitised erythrocytes and incubate for 30 minutes at 37°C. The antigen titre is the highest dilution producing a positive reaction with the highest serum dilution. Verify the absence of anticomplementary activity of the antigen at different dilutions.

iv) Titrate the complement on a microplate: serially dilute the complement or guinea-pig serum in VB, for example from 1/15 to 1/200. To each well containing 25 µl of this dilution, add 25 µl of antigen and 25 µl of the haemolytic system. Incubate for 30 minutes at 37°C and establish the dilution equivalent to 2 haemolytic units of complement.

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2   Dade Behring, Marburg, Germany.
3   Virion, Zürich, Switzerland.

- **Test procedure**

i) Make twofold dilutions of decomplemented sample sera from 1/10 to 1/320 in six wells and in four additional wells at dilutions from 1/10 to 1/80 to detect anticomplementary activity (25 µl per well).

ii) Add 25 µl of diluted antigen or 25 µl of VB to control serum wells.

iii) Add 25 µl diluted complement to all wells. Cover the plate with plastic adhesive film and incubate for 18 hours at 4°C.

iv) Remove the plates from the refrigerator, allow them to reach room temperature, and add 25 µl of freshly prepared haemolytic system. Incubate at 37°C for 30 minutes. Centrifuge the plates at 500 ***g*** for 5 minutes at 4°C. Examine the controls and read the results.

- **Interpretation of the results**

Titres between 1/10 and 1/40 are characteristic of a latent infection. Titres of 1/80 or above in one or more sera from a group of from five to ten animals reveal an evolutive phase of the infection.

c) Enzyme-linked immunosorbent assay

This technique has a high sensitivity and a good specificity (11, 40, 59). It is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. The ELISA tends to replace the IFA and CF tests as the test of choice because it is convenient for large-scale screening and, particularly for veterinary diagnosis, as it is a reliable technique for demonstrating *C. burnetii* antibody in various animal species (20, 49). It requires a relatively pure antigen. Antigens prepared for the CF test may be used for coating the plates. Ready-to-use kits are commercially available and can detect anti-phase II antibodies or both anti-phase I and II antibodies.

Wells of the microplate are coated with *C. burnetii* whole-cell inactivated antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically.

- **Materials and reagents**

Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with Q fever antigen; microplate reader (spectrophotometer; 405 and/or 450 and/or 492 nm filters); 37°C humidified incubator; 8- and 12-channel pipettes with disposable plastic tips; microplate shaker (optional).

Positive and negative control sera; conjugate (ruminant anti-immunoglobulin labelled with peroxidase); tenfold concentration of diluent (PBS–Tween); distilled water; substrate or chromogen (TMB [tetramethylbenzidine], ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

- **Test procedure**

i) Dilute the serum samples, including control sera, to the appropriated dilution (usually 1/100) and distribute 0.1 ml per well in duplicate. Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.

ii) Cover the plate with a lid and incubate at room temperature for 30–90 minutes. Empty out the contents and wash three times in washing solution at room temperature.

iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well).

iv) Cover each plate and incubate as in step ii. Wash again three times.

v) Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example: TMB in 0.1 M acetic acid and 30% H_2O_2 solution [0.2 µl/ml]; or 0.25 mM ABTS in citrate phosphate buffer, pH 5.0, and 30% H_2O_2 solution [0.1 µl/ml]).

vi) Shake the plate; incubate according to the manufacturer recommendations, stop the reaction by adding stopping solution to each well, e.g. 0.05 ml 2 M sulphuric acid for TMB or 10% sodium dodecyl sulphate for ABTS.

vii) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450 nm (TMB). The absorbance values will be used to calculate the results.

- **Interpretation of the results**

For commercial kits, interpretations and values are provided with the kit.

For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab_{pos}) and negative (Ab_{neg}) control sera, and for each serum, calculate the percentage:

$$\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100$$

Interpret the results as follows:
Ab <30%   negative serum
Ab 30–40%   doubtful serum
Ab >40%   positive serum

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccine

Vaccination is the most logical strategy for preventing Q fever in exposed subjects and livestock. A *C. burnetii* vaccine can only be prepared by trained staff only working in adequate conditions of protection (at a minimum in a biosafety level 3 laboratory). It is recommended to obtain the vaccine from manufacturers capable of completing and certifying tests for safety, inactivation and sterility.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

In some countries, vaccination is practised for occupationally exposed people, such as abattoir workers, veterinarians and laboratory personnel. A vaccine inactivated by formaldehyde (Q-VAX, CSL Ltd, Australia), prepared from the Henzerling strain of phase I *C. burnetii*, received the approval of the Australian authorities in 1989 (28). Phase I vaccines are more effective, but vaccination is contraindicated for individuals who had seroconverted or had been exposed to *C. burnetii* prior to immunisation.

Several vaccines have been developed against animal Q fever. Results converge today towards the use of a phase I vaccine, as the phase II vaccines are 100 times less effective against the colonisation of mouse spleen than phase I vaccines (13). An inactivated phase I vaccine is commercially available in Slovakia for vaccination of cattle. A review on Q fever in Slovakia suggests that the decrease in the occurrence of human and animal Q fever could be the result of the large-scale vaccination of cattle that was carried out there during 10 years, together with improved veterinary control of domestic animal transport within the country (46).

This vaccine consists of highly purified antigen prepared from Nine Mile strain in the phase I (egg passage 2 to egg passage 6) and inactivated by formaldehyde. Recently, a French study demonstrated the efficacy of this vaccine through experimental vaccination and challenge of pregnant goats: the vaccine prevented abortion and shedding in milk, and decreased considerably the shedding in the vaginal secretions and faeces (3). Ideally, vaccine efficacy must be demonstrated by tests on all the target species.

In the case of vaccination on already infected animals, there is a lack of information on possible adverse effects and on the shedding of the Q fever agent. Consequently, some authors believe that it is preferable to select seronegative herds or animals for immunisation, and to continue vaccination over several years in young animals (24). To date, no data are available for comparing the cost–benefit of this strategy with a nonselective strategy in the control of Q fever.

2. Diagnostic biologicals

See Section B.2.a (Antigen preparation).

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RABIES

SUMMARY

Rabies is a major zoonosis for which diagnostic techniques have been standardised inter-nationally. As there is no gross pathognomonic lesion for rabies, diagnosis can only be made in the laboratory. Laboratory techniques are preferably conducted on central nervous system (CNS) tissue removed from the cranium. A composite of CNS samples should be tested and the brain stem is the most important component of the sample.

Identification of the agent: *Agent identification is preferably done using the fluorescent antibody test (FAT). A drop of purified immunoglobulin previously conjugated with fluorescein isothiocyanate is added to an acetone-fixed brain tissue smear, preferably made from several parts of the brain stem. FAT provides a reliable diagnosis in 98–100% of cases for all serotypes if a potent conjugate is use. For a large number of samples, as in an epidemiological survey, polymerase chain reaction (PCR) or immunoenzyme techniques can provide rapid results.*

Infected neuronal cells have been demonstrated by histological tests and these procedures will reveal aggregates of viral material (the Negri bodies) in the cytoplasm of neurones. However, the sensitivity of histological techniques is much less than that of immunological methods, especially if there has been some autolysis of the specimen. Consequently, histological techniques can no longer be recommended.

As a single negative test on fresh material does not rule out the possibility of infection, cell culture or mouse inoculation tests should be carried out simultaneously. A monolayer culture of susceptible cells is inoculated with a pool of several CNS tissues, including the brain stem. FAT carried out after appropriate incubation will demonstrate the presence or absence of viral antigen. Alternatively, newborn or 3–4-week-old mice may be inoculated intracerebrally with a similar pool tissues and then kept under observation for 28 days. For any mouse that dies between 5 and 28 days post-inoculation, the cause of death should be confirmed by FAT. Wherever possible, virus isolation in cell culture should replace mouse inoculation tests.

The identification of the agent can be supplemented in specialised laboratories by identifying any variant virus strains through the use of monoclonal antibodies, specific nucleic acid probes, or the polymerase chain reaction followed by DNA sequencing of genomic areas. Such techniques can distinguish between field and vaccine strains, and possibly identify the geographical origin of the field strains. These very sensitive tests should be used by well trained personnel in specialised laboratories.

Serological tests: *Virus neutralisation (VN) assays in cell cultures are the prescribed tests for international trade. Alternatively, use may be made of a test that is known to correlate with these, notably an enzyme-linked immunosorbent assay using antibody to the G protein or the neutralisation test in mice. Results are expressed in International Units or equivalent units relative to an international standard antiserum.*

Requirements for vaccines: *Rabies vaccines for use in animals contain either live virus attenuated for the target species (such as Flury low egg passage, Flury high egg passage, Street-Alabama-Dufferin or Kelev), or virus inactivated by chemical or physical means, or recombinant vaccines. The virus is cultivated in embryonated egg, or in cell cultures.*

Rabies vaccines are usually lyophilised, but inactivated virus vaccines, preferably with an adjuvant, may be stored in liquid form.

Before newly developed vaccines can be licensed, the duration of immunity resulting from their use should be determined in vaccinated animals of the target species. Vaccines should confer protective immunity for at least 1 year.

For live virus vaccines, the minimum virus content that will elicit an adequate immune response must be established.

The potency of inactivated virus vaccines is established and controlled by mouse vaccination followed by intracerebral challenge using tests formulated by the United States Department of Agriculture in the United States of America or the European Pharmacopoeia elsewhere. The final products of both types of vaccine are subjected to tests for innocuity and absence of toxicity.

For live vaccines that are prepared for oral vaccination of wild (or domestic) animals, safety and efficacy in target animals and safety in nontarget species must be demonstrated.

A. INTRODUCTION

Rabies is caused by a neurotropic virus of the genus *Lyssavirus* of the family Rhabdoviridae, and is transmissible to all mammals. As it is transmissible to humans by inoculation or inhalation of infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organisation (WHO) (45).

Seven distinct genetic lineages can be distinguished within the genus *Lyssavirus* by cross-protection tests and molecular biological analysis (6, 16, 26), namely the classical rabies virus itself (RABV, genotype 1, serotype 1), Lagos bat virus (LBV, genotype 2, serotype 2), Mokola virus (MOKV, genotype 3, serotype 3), and Duvenhage virus (DUVV, genotype 4, serotype 4). The European bat lyssaviruses (EBLV), subdivided into two biotypes (EBLV1, genotype 5 and EBLV2, genotype 6) and the Australian bat lyssavirus (ABLV, genotype 7), isolated in Australia (30), are also members of the *Lyssavirus* genus, but are not yet classified into serotypes. Viruses of serotypes 2–4, EBLV and ABLV are known as rabies-related viruses. The use of monoclonal antibodies (MAbs) directed against viral nucleocapsid or glycoprotein antigens, and the sequencing of defined genomic areas has made possible the definition of numerous subtypes within each serotype. Lyssaviruses cause a clinical disease indistinguishable from classical rabies. Conserved antigenic sites on the nucleocapsid proteins permit recognition of all lyssaviruses with modern commercial preparations of anti-rabies antibody conjugates used for diagnostic tests on brain tissue. There exist two lyssavirus phylogroups with distinct pathogenicity and immunogenicity (5). For RABV, DUVV, EBLV and ABLV, conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination. Little or no cross-protection against infection with MOKV or LBV is elicited by rabies vaccination and most anti-rabies virus antisera do not neutralise these lyssaviruses. Four new rabies-related viruses (Aravan, Khujand, Irkut, and West Caucasian bat viruses) have been isolated recently from Eurasian bats, and are described as new putative lyssavirus species. There is a reduced protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis against all four new bat variants of rabies virus (29).

Humans working with suspect material must be vaccinated against lyssaviruses or other pathogens that may be present in diagnostic samples. The laboratory must comply with national biocontainment and biosafety regulations to protect staff from contact with pathogens; it should also comply with the guidelines in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities.

WHO recommends the preventive immunisation of exposed staff. The immunisation protocol includes three injections, e.g. at days 0, 7, and 28. The serological evaluation of immunisation is made 1–3 weeks after the last injection, and checked every 6 months in the case of laboratory workers or every 2 years for other diagnosticians. Booster vaccination must be given when the titre falls below 0.5 International Units (IU) per ml. In the absence of serological monitoring, the vaccination regimen should consist of a booster vaccination at 1 year and thereafter every 1–3 years.

As no clinical sign or gross post-mortem lesion can be considered pathognomonic in domestic or wild animals, the diagnosis of rabies has to rely on laboratory testing. Serological evidence of infection is rarely useful because of late seroconversion and the high mortality rate of host species, although such data may be used in some epidemiological surveys.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another (43). The only way to perform a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests.

As rabies virus is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the 'rabies diagnostic chain'.

Several laboratory techniques may be used, and have been detailed and standardised in the fourth edition of the WHO's *Laboratory Techniques in Rabies* (45). The methods vary in their efficiency, specificity and reliability. They are classically applied to brain tissue, but they can also be applied, though less effectively, to other organs (e.g. salivary glands). In the brain, rabies virus is particularly abundant in the thalamus, pons and medulla. The hippocampus (Ammon's horn), cerebellum and different parts of the cerebrum have been reported to be negative in 3.9–11.1% of the positive brains. The structure of choice is the thalamus as it was positive in all cases. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested (13). To reach these parts of the brain, it is necessary to remove the entire organ after having opened the skull in a necropsy room. Under some conditions (e.g. in the field or when sampling for large epidemiological studies), a simplified method of sampling through the occipital foramen (11), or through the orbital cavity (32), can be used. Precautions should be taken when handling central nervous system tissues from suspected rabies cases. Gloves should always be worn and precautions must be taken to prevent aerosols. The use of cutting tools, scissors and scalpels, should be used with care to prevent injury and contamination.

a) Shipment of samples

During the shipment of suspect material for diagnosis (animal heads, brain or other tissue samples), no risk of human contamination should arise: brains must be placed in a leak-proof rigid container (animal heads will be wrapped in absorbent material) as prescribed in the International Air Transport Association (IATA) Dangerous Goods Regulations must be followed. These regulations are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is closely linked to the tests to be used for diagnosis:

i) Formalin inactivates the virus, thus the isolation tests cannot be used and diagnosis depends on using a modified and less sensitive direct fluorescent antibody test (FAT), immunohistochemistry or histology (39, 45);

ii) Infectivity at room temperature may be extended for several days if brain material is kept in a mixture of 50% glycerol in phosphate buffered saline (PBS). Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. It does not protect against titre decline due to thermal conditions and therefore, because rabies is thermo-labile, the virus titre will decline during glycerol/PBS storage. Under normal transport conditions in the tropics, this protection may only be effective for a matter of several days. Therefore, whenever possible samples in glycerol/saline should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples.

b) Collection of samples

Usually the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected. This step may be hazardous if laboratory technicians are not fully trained, or under field conditions. In such cases, there are two possible methods of collecting some brain samples without opening the skull:

i) Occipital foramen route for brain sampling

A 5 mm drinking straw (11) or a 2 ml disposable plastic pipette (17) is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata. Bovine spongiform encephalopathy (BSE) should be considered in the differential diagnosis of most cattle that are considered to be 'rabies suspect'. Sampling of brain specimens for both diseases can be done using the 'brain scoop or tool' developed for BSE tissue sampling rather than a straw or pipette. The resulting samples are relatively easily recognised as to the area of brain sampled.

ii) Retro-orbital route for brain sampling

In this technique (32), a trocar is used to make a hole in the posterior wall of the eye socket, and a plastic pipette is then introduced through this hole. The sampled parts of the brain are the same as in the former technique, but they are taken in the opposite direction.

c) Routine laboratory tests

Laboratory diagnosis can be performed by using three kinds of procedure.

i) Immunochemical identification of rabies virus antigen

• *Fluorescent antibody test*

The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE. This test may be used directly on a smear, and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The FAT gives reliable results on fresh specimens within a few hours in more than 95–99% of cases. The

sensitivity of the FAT depends on the specimen (the degree of autolysis and how comprehensively the brain is sampled, see Section B.1) (1, 9), on the type of lyssavirus and on the proficiency of the diagnostic staff. Sensitivity may be lower in samples from vaccinated animals due to localisation of antigen, which is confined to the brainstem. For direct rabies diagnosis, smears prepared from a composite sample of brain tissue, that includes the brain stem, are fixed in high-grade cold acetone and then stained with a drop of specific conjugate. Anti-rabies fluorescent conjugates may be prepared in the laboratory. Those available commercially are either polyclonal conjugates specific to the entire virus or specific to the rabies nucleocapisid protein, or they may be prepared from a mix of different MAbs. In the FAT, the specific aggregates of nucleocapsid protein are identified by their fluorescence. The specificity and sensitivity of these anti-rabies fluorescent conjugates for locally predominant virus variants should be checked before use.

The FAT may be applied to glycerol-preserved specimens. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with a proteolytic enzyme (7, 8, 38, 39). However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue.

- *Immunochemical tests*

The antibody may be conjugated to an enzyme such as peroxidase instead of fluorescein isothiocyanate (FITC). This conjugate may be used for direct diagnosis with the same sensitivity as FAT (27), but attention should be paid to the risk of nonspecific false-positive results. This risk is considerably reduced by the thorough training of the technicians. It must also be emphasised that this technique needs one incubation step more than the FAT.

Peroxidase conjugate may be used on sections of formalin-fixed tissue for immunohistochemical tests.

ii)   Detection of the replication of rabies virus after inoculation

These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known human exposure.

- *Cell culture test*

Neuroblastoma cell lines, e.g. CCL-131 in the American Type Culture Collection (ATCC)[1], is used for routine diagnosis of rabies. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% foetal calf serum (FCS), incubated at 36°C with 5% CO_2. Its sensitivity has been compared with that of baby hamster kidney (BHK-21) cells (34). This cell line is sensitive to street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Presence of rabies virus in the cells is revealed by the FAT. The result of the test is obtained after at least 18 hours (one replication cycle of virus in the cells); generally incubation continues for 48 hours (10) or in some laboratories up to 4 days.

This test is as sensitive as the mouse inoculation test. Once a cell culture unit exists in the laboratory, this test should replace the mouse inoculation test as it avoids the use of live animals, is less expensive and gives more rapid results.

It is often advisable to carry out more than one type of test on each sample, particularly when there has been human exposure.

- *Mouse inoculation test*

Five-to-ten mice, 3–4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. The inoculum is the clarified supernatant of a 20% (w/v) homogenate of brain material (cortex, Ammon's horn, cerebellum, medulla oblongata) in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice should be anaesthetised when inoculated. The young adult mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post-inoculation. Any deaths occurring during the first 4 days are regarded as nonspecific (due to stress/bacterial infection etc.).

This *in-vivo* test should be avoided when possible on animal welfare grounds. It is also expensive, particularly if SPF mice are used, and does not give rapid results (compared with *in-vitro* inoculation tests), but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. Another advantage of this low-tech test is that it can be easily and practically applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

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1   American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, Virginia 20108, United States of America (USA).

iii) Histological identification of characteristic cell lesions

Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Immunohistochemical tests are the only histological test specific to rabies.

An unfixed tissue smear may be stained by the Seller's method, diagnosis is then obtained in under 1 hour. Generally, histological tests, such as Mann's test, are performed on fixed material after a paraffin-embedding step, and the result of the test is obtained within 3 days. These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. Whichever staining method is used, the evidence of infection is provided by intracytoplasmic acidophilic bodies. These histological methods, especially the Seller's method, can no longer be recommended because they have very low sensitivity and should be abandoned.

d) Other identification tests

An enzyme-linked immunosorbent assay (ELISA) that detects rabies antigen is a variation of the immunochemical test. It is useful for large epidemiological surveys (46). It should only be used after validation against numerous samples in different laboratories. The specificity and sensitivity of these anti-rabies enzyme conjugates for locally predominant virus variants should be checked before use. This test should be used in combination with confirmatory tests by FAT or virus isolation.

The tests above describe methods to accurately diagnose rabies and to isolate and identify the virus. Typing of the virus can provide useful epidemiological information and should be carried out in specialised laboratories (such as OIE or WHO Reference Laboratories). These techniques would include the use of MAbs, nucleic acid probes, or the polymerase chain reaction (PCR), followed by DNA sequencing of genomic areas for typing the virus (17). This characterisation enables a distinction to be made between vaccine virus and a field strain of virus, and possibly the geographical origin of the latter.

2. Serological tests

Serological tests are rarely used in epidemiological surveys, due to late seroconversion and the low percentage of animals surviving the disease and therefore having post-infection antibodies. The main application of serology is to determine responses to vaccination, either in domestic animals prior to international travel, or in wildlife populations following oral immunisation of rabies reservoirs. For follow-up investigations in oral vaccination campaigns, virus neutralisation (VN) tests in cell culture are preferred. However, if poor quality sera are submitted, the VN tests in cell culture are sensitive to cytotoxicity, which could lead to false-positive results. For such samples, the use of an indirect ELISA with rabies glycoprotein-coated plates has been shown to be as sensitive and specific as the VN test on cells (22).

a) Virus neutralisation test in cell culture: fluorescent antibody virus neutralisation test (a prescribed test for international trade)

The principle of the fluorescent antibody virus neutralisation (FAVN) test (21) is the neutralisation *in vitro* of a constant amount of rabies virus ('challenge virus standard' [CVS] strain adapted to cell culture) before inoculating cells susceptible to rabies virus: BHK-21 C13 cells (ATCC number: CCL-10).

The serum titre is the dilution at which 100% of the virus is neutralised in 50% of the wells. This titre is expressed in IU/ml by comparing it with the neutralising dilution of the OIE serum of dog origin under the same experimental conditions. The WHO standard for rabies immunoglobulin [human][2] No. 2, or an internal control calibrated against the international control may be used. The WHO standard or internal control should only be used as a control in the test and should not be used to calculate the IU/ml titre of the sera.

This microplate method uses 96-well plates, and is an adaptation of the technique of Smith *et al.* (36), modified by Zalan *et al.* (47) and by Perrin *et al.* (33). Several publications (18, 21) have shown that the FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results.

- **Essential equipment**

Humidified incubator at 35°C/37°C with 5% CO_2; dry incubator at 37°C; biocontainment cabinet; fluorescence microscope suitable for FITC fluorescence equipped with ×10 eye-piece and ×10 objective. The global magnification of the microscope ranges between ×100 and ×125 due to the extra magnification of some epi-fluorescence systems.

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2   National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom (UK).

- **Reagents and biologicals**

PBS buffer, pH 7.2, without Ca^{2+} and Mg^{2+}, stored at 4°C;

Trypsin ethylene diamine tetra-acetic acid (EDTA);

High-grade acetone 80% (diluted with deionised water), stored at 4°C;

Dulbecco's modified Eagle's medium (DMEM) + 10% heat-inactivated FCS;

FITC anti-rabies conjugate;

Cells: BHK-21 C13 (ATCC CCL-10);

Virus: CVS-11 (ATCC VR 959) strain, which is available from the ATCC or the OIE Reference Laboratory for Rabies, Nancy, France (see Table given in Part 3 of this *Terrestrial Manual*). Vials are stored at –80°C;

OIE Standard Serum of dog origin (OIE Reference Laboratory for Rabies, Nancy, France [see Table given in Part 3 of this *Terrestrial Manual*] stored at +4°C and diluted to 0.5 IU/ml with sterile deionised or distilled water according to the titre of the batch).

Naive serum: The pool of negative dog sera is stored at –20°C.

- **CVS production**

i) *Cell growth:* the BHK-21 C13 cells (ATCC CCL-10) used to produce the CVS virus (ATCC VR 959 CVS-11) are trypsinised during the rapid growth phase, i.e. cells are in the exponential phase of their kinetic growth. If the confluence of the layer is complete, a new passage should be made. The cells in the cell suspension should not be aggregated; 2×10^7 cells are used for a 75 cm^2 cell culture flask. Cells are collected within a volume of 20–30 ml in cell culture medium with 10% heat-inactivated FCS.

ii) *Infection of cells:* the multiplicity of infection (number of infective particles per cell) is adjusted to between 0.2 and 0.5. The glass bottle containing the virus/cell suspension is incubated for 60 minutes at 35–37°C. The contents of the bottle are gently stirred every 10–15 minutes.

iii) *Virus growth:* the virus/cell suspension is then centrifuged at 800-1000 ***g*** for 15 minutes and the cell pellet is resuspended in cell culture medium mixed with 10% heat-inactivated FCS. Virus is harvested 2 days later.

iv) *Harvest and storage:* the supernatant is centrifuged at 800–1000 ***g*** for 15 minutes at 4°C. If several flasks have been used, the different centrifuged supernatants are mixed and then aliquoted and frozen at –80°C. The infective titre of the harvest is established at least 3 days after freezing.

- **Titration of virus in $TCID_{50}$ (50% tissue culture infective dose)**

This titration method uses BHK-21 C13 cells (ATCC CCL-10) in microtitre plates.

Different steps in this procedure may be adapted according to the safety requirements and to the working practices of the laboratory, but the following should not be changed:

- inoculation of a 24-hour cell layer,
- tenfold dilutions prepared using 0.9 ml of diluent and 0.1 ml of virus suspension,
- six 50 µl replicates per dilution,
- incubation for 72 hours,
- qualitative reading (i.e. the well is positive or negative),
- in every titration session, a vial of a control batch of virus is titrated and this titre is integrated in a control card to validate the titration process,
- calculation according to neoprobit graphic or Spearman–Kärber methods.

i) *Cell suspension:* the day before titration, a cell suspension containing 10^5 cells/ml is prepared in cell culture medium containing 10% heat-inactivated FCS, and is distributed, 200 µl per well, into 96-well microtitre plates. The plates are then incubated for 24 hours at 35–37°C with 5% CO_2.

ii) *Dilution of the virus:* the serial dilutions are performed in 5 ml tubes using a cell culture medium without FCS as diluent. Tenfold dilutions from 10^{-1} to 10^{-12} are prepared (0.9 ml of diluent with 0.1 ml of the previous dilution).

iii) *Infection of the cells:* the medium in the microtitre plates is discarded using an aspiration system. Fifty µl of each virus dilution is distributed per well. Six replicates are used per dilution. The microtitre plate is then incubated for 1 hour at 35–37°C with 5% CO_2. Then 200 µl of cell culture medium, containing 5% FCS, is added.

iv) *Incubation:* incubate for 3 days at 35–37°C in 5% CO_2.

v) *Staining and calculation of titre:* The cells are stained using the FAT, as detailed below. Reading is qualitative, every well that shows specific fluorescence is considered to be positive. The titre calculation is made using either the neoprobit graphic method (2) or the Spearman–Kärber formula.

vi) The CVS titration must be performed by FAVN test to establish the infective dose in $TCID_{50}$.

Fig. 1. *Proposed use of microplates for the fluorescent antibody virus neutralisation test. Wells to which undiluted sera must be added are filled with the indicated '50 µl'. Wells to which 50 µl of diluted challenge virus standard must be added are shaded. Dilutions are given in log_{10}.*

Plate 1: Controls

	H	G	F	E	D	C	B	A	
Challenge virus									1
standard									2
titration									3
									4
OIE	50 µl					50 µl			5
standard serum	50 µl					50 µl			6
(0.5 IU/ml)	50 µl					50 µl			7
	50 µl					50 µl			8
	50 µl								9
Serum or internal	50 µl								10
positive control	50 µl								11
or WHO standard serum	50 µl								12
log (dilution)	0.48	0.95	1.43	1.91	2.39	2.87	CVS virus control	Cells control	

Right side labels: **Naive dog serum (negative)** (rows 5–8)

Plate 2: Sera to be tested

log dilution	0.48	0.95	1.43	1.91	2.39	*4.23*	0.48	0.95	1.43	1.91	2.39	*4.23*
	1	2	3	4	5	6	7	8	9	10	11	12
A	50 µl						50 µl					
B	50 µl						50 µl					
C	50 µl						50 µl					
D	50 µl						50 µl					
E	50 µl						50 µl					
F	50 µl						50 µl					
G	50 µl						50 µl					
H	50 µl						50 µl					

Left labels: **Serum 1** (rows A–D), **Serum 2** (rows E–H). Right labels: **Serum 3** (rows A–D), **Serum 4** (rows E–H).

- **Test procedure**

i) The microplates are used according to the pattern shown in Figure 1. Plate No. 1 is used for the titration of CVS (rows 1 to 4), and for the controls, standard sera and naive dog serum are used. All other plates are used for the sera to be tested.

ii) Medium is added to the wells as follows: plate 1, rows 1 to 4 and cells A9 to A12: add 150 µl per well; in the other plates, rows 6 and 12: add 200 µl per well; all other wells: add 100 µl.

iii) Sera to be tested are heat inactivated for 30 minutes at 56°C. As indicated in Figure 1, 50 µl of each undiluted serum to be tested is added to four adjacent wells.

iv) Dilutions of sera are conducted in the microplates as follows:

OIE serum, the internal control or WHO standard serum, and the naive dog serum: with a 50–200 µl multichannel pipette, mix the first dilution wells by sucking in and out at least eight times, transfer 50 µl from one row to the next one, until the last one is reached. Discard 50 µl from the last row.

If there is a serum to be tested on the control plate, see below for the dilution step.

A minimum of four three-fold dilutions is required.

Sera being tested (all plates): as above, transfer successively 50 µl from one row to the following one until rows 5 and 11 (dil. $10^{-2.39}$). With a 5–50 µl multichannel pipette, transfer 10 µl from rows 5 and 11 to rows 6 and 12, respectively (from dil. $10^{-2.39}$ to dil. $10^{-4.23}$). Using a multichannel pipette adjusted to 90 µl, mix rows 6 and 12 and discard 180 µl. Then add 70 µl of medium to these rows. This final step does not lend itself to high throughput testing. To attain or exceed the recommended final dilution alternative procedures may be used. These may require modifications to the plate layout.

- **Addition of challenge virus standard**

i) Stock CVS is stored in 1 ml microtubes at –80°C. One tube is thawed rapidly under cold running water, and placed in melting ice.

ii) One dilution from this tube is prepared in order to obtain 100 $TCID_{50}$ in 50 µl. Of this dilution, 50 µl is added to each serum-filled well (see Figure 1). For virus titration, 50 µl is added to wells H1 to H4 (plate 1). Next, transfer 50 µl from row to row (plate 1, lines 1–4). Discard 50 µl from the last row (plate 1, wells A1 to A4). No virus is added to wells A9 to A12 of plate 1 (controls). The range allowed for the virus dose titre must be between 30 and 300 $TCID_{50}$/50 µl.

iii) Incubate the microplates at 35°C/37°C in a humid incubator with 5% CO_2 for 1 hour.

iv) *Addition of cells:* trypsinise a subconfluent culture of 3-day-old BHK-21 cells. Resuspend the cells to obtain a 4×10^5 cells/ml suspension in DMEM supplemented with 10% heat-inactivated FCS. Add 50 µl of the cell suspension to each well.

v) Incubate the microplates for approximately 48 hours at 35–37°C in a humid incubator with 5% CO_2.

- **Fixation and staining**

i) After the 48-hour incubation period, the medium is discarded, and the microplates are rinsed once in PBS, pH 7.2, and once in 80% acetone. The microplates are then fixed in 80% acetone at room temperature for 30 minutes, and are dried at room temperature for at least 30 minutes.

ii) Add 50 µl of the FITC anti-rabies conjugate, at the working dilution, to each well, gently rock the microplates and incubate at 35–37°C for 30 minutes. Discard the fluorescent conjugate and rinse the microplates twice with PBS. Excess PBS is removed by briefly inverting the microplates on absorbent paper.

- **Reading and interpreting the results**

i) The total surface of each well is observed. The reading evaluation is qualitative (plus or minus): no fluorescent cell – a minus score is recorded for the well; fluorescent cells (one cell or more) – a plus score is recorded for the well.

ii) Cell and virus controls are read first. For titration of CVS, naïve serum, and OIE standard serum, titres are calculated according to the Spearman–Kärber method or the neoprobit graphic method (2).

iii) Results of titration of CVS ($TCID_{50}$), naive serum (D_{50} [median dose]) and positive standard (D_{50}) are reported on a control card for each of these three controls. The control results of the current test are compared with the accumulated control test results from previous tests using the same batch of control. The test is validated if the values obtained for the three controls in the current test are not statistically different from the mean (± 2 SD) of all the values obtained in the tests conducted previously according to this technique.

iv) The result of the test corresponds to the non-neutralised virus after incubation with the reference serum or with the serum to be tested. These titres are calculated with the neo-probit graphic method (2) or with the Spearman–Kärber formula (45).The comparison of the measured titre of the tested sera with that of the OIE positive standard serum of a known neutralising titre allows determination of the neutralising titre of the tested sera in IU/ml. The conversion to IU/ml can be made by using either the log D_{50} value of the day or the mean value of the OIE standard serum.

- Formula to convert the log D_{50} value in IU/ml titre:

Serum titre (IU/ml) = [($10^{(\text{serum log } D_{50} \text{ value})}$) × theoretical titre of OIE serum 0.5 IU/ml]

($10^{(\text{log } D_{50} \text{ of OIE serum 0.5 IU/ml})}$)

Example of conversion:

- log D_{50} of the serum = 2.27

- theoretical titre of OIE serum 0.5 IU/ml = 0.5 IU/ml

- log D_{50} of OIE serum = 1.43

(for the log D_{50} of OIE, the value of the day or the mean value can be considered)

Serum titre (IU/ml) = [($10^{2.27}$ × 0.5 = 3.46 IU/ml]

($10^{1.43}$)

The following parameters have to be strictly respected:

- Rabies virus: only the CVS-11 strain (ATCC number = VR 959 should be used.

- Cells culture: only BHK-21 cells (ATCC number – CCL 10) should be used.

- The FAVN test must be performed only in 96 wells microplate.

- Control charts should be used for rabies virus, naïve serum and OIE standard serum.

- The back titration of the CVS virus, as well as naïve serum and OIE serum, must be present on control plate.

- A minimum of four three-fold dilutions of sera are required. The reading method is 'all or nothing' only.

- Four replicates of each serum should be diluted.

- For the conversion of log D_{50} in IU/ml, the laboratories should use only the log D_{50} value of the OIE standard serum of dog origin.

b) The rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralising antibody (a prescribed test for international trade)

- **Standard procedure (from WHO Laboratory Techniques in Rabies, 1996; [ref. 45])**

  - **Preparation of seed virus suspension**

  i) Trypsinise one 3-day-old 150 ml flask culture of mouse neuroblastoma (MNA) cells. These cells prefer an acidic medium, supplemented with vitamins (40). A suitable cell line (CCL-131) may be obtained on request from the ATCC (see footnote 1).

  ii) Resuspend 3×10^7 cells in a 50 ml conical centrifuge tube in 2.7 ml of Eagle's minimal essential medium supplemented with 10% fetal bovine serum (EMEM-10).

  iii) Using standard rabies safety procedures, add 1×10^7 infectious units of CVS-11 rabies virus (ATCC, VR959) and vortex/mix once. Incubate the cells and virus for 15 minutes at 37°C; vortex/mix the cells once during this time.

  iv) Add 10 ml EMEM-10, vortex/mix, and centrifuge the cells at 500 *g* for 10 minutes.

  v) Discard the supernatant. Resuspend the cells in 30 ml of growth medium and transfer to a 150 ml flask.

  vi) Gently rock the flask to mix the cell suspension, and then prepare three eight-well tissue-culture chamber slides by pipetting 0.2 ml of the cell suspension into one well of each slide.

vii)   Incubate the flask and slides at 37°C in a humidified incubator with 0.5% carbon dioxide (CO_2). The flask should be incubated as a closed culture (tighten the cap).

viii) At 20, 40 and 64 hours after infection, acetone fix and stain one slide using an immunofluorescence technique (28) to determine the virus infectivity. The supernatant should be harvested 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).

ix)   Transfer the supernatant to a 50 ml centrifuge tube and centrifuge at 4000 *g* for 10 minutes.

x)   Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

- **Titration of seed virus suspension**

i)   Thaw one aliquot of the seed virus and prepare serial tenfold dilutions (from 10^{-1} to 10^{-8}) in EMEM-10.

ii)   Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration 5×10^4 cells per 0.2 ml) to each well.

iii)   Mix the cells and virus by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO_2 for 40 hours.

iv)   Acetone fix and stain the slide using an immunofluorescence technique. Evidence of virus infection should be observed at the 10^{-6} dilution of virus, indicating a virus stock suspension containing at least 1×10^6 infectious units per 0.1 ml. Prepare sufficient seed virus so that frequent serial passage of the virus is unnecessary.

- **Preparation of stock virus suspension**

i)   Infect 3×10^7 MNA cells with 1×10^7 infectious units of the seed virus preparation (see above).

ii)   Harvest the supernatant 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).

iii)   Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

- **Titration of stock virus suspension**

i)   Thaw one aliquot of the stock virus and use this to prepare serial tenfold dilutions (from 10^{-1} to 10^{-6}) in EMEM-10.

ii)   Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration 1×10^5 cells per 0.2 ml) to each well.

iii)   Mix the cells and virus suspension by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO_2 for 20 hours.

iv)   Acetone fix and stain the slide using an immunofluorescence technique.

Each well of an eight-well tissue-culture chamber slide contains 25–50 distinct microscopic fields when observed at ×160–200 magnification. One unit of virus for the RFFIT is determined as the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells (the focus-forming dose, FFD_{50}). The stock virus suspension should contain at least 1×10^4 FFD_{50} per 0.1 ml (i.e. the well with cells infected with the 10^{-4} dilution of the virus should contain at least one focus of infected cells in 50% of the observed microscopic fields). A stock virus suspension of this titre can then be diluted to $10^{-2.3}$ to obtain a challenge virus containing 50 FFD_{50}.

- **Reference sera**

A national or international reference serum standard diluted to a potency of 2.0 IU/ml should be included in each test. The reference serum used at the Centres for Disease Control and Prevention is the first international standard for rabies immunoglobulin (41), which may be obtained from the NIBSC (see footnote 2). The reference serum should be maintained as frozen aliquots in amounts sufficient for 1 week of tests. A positive serum control standard diluted to a potency of 0.5 IU/ml and a negative serum control standard with a potency of <0.1 IU/ml should also be prepared by the laboratory and included in each test.

- **Test sera**

Serum samples should be heated at 56°C for 30 minutes before testing in order to inactivate complement. If sera are frozen, they should be reheated after thawing. Serial dilutions of test sera may be prepared in an eight-well tissue-culture chamber slide. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy and may be made as follows:

i)   Prepare a 1/2.5 dilution by adding 0.1 ml of inactivated serum and 0.15 ml of EMEM-10 to one of the slides. Mix by gently rocking the slide.

ii) Transfer 0.05 ml of the 1/2.5 dilution to a second well containing 0.45 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/2.5 dilution.

iii) Mix the second well and discard all but 0.1 ml.

iv) Add 0.1 ml of the challenge virus preparation (containing 32–100 FFD_{50}) to all serum dilutions.

v) Mix and incubate at 35°C in a humidified incubator with 0.5% CO_2 for 90 minutes.

- **Addition of cells**

i) During the incubation period, trypsinise a stock culture of 3–5-day-old MNA cells.

ii) Resuspend the cells in EMEM-10 to give a final concentration of 1×10^5 cells per 0.2 ml.

iii) Distribute 0.2 ml of the cell suspension into each well of the slide and incubate at 35°C in a humidified incubator with 0.5% CO_2 for a further 20 hours.

- **Acetone fixation and staining by immunofluorescence**

i) After 20 hours, remove the slides from the incubator and pour off the medium into a virucidal solution.

ii) Rinse the slides once in PBS and then fix for 10 minutes at room temperature in cold acetone (–20°C).

iii) Leave the slides to dry for 10 minutes before adding FITC-conjugated anti-rabies serum. The conjugate may be prepared in EMEM-10 or PBS; there is no need to adsorb the conjugate with tissue or cells. The working dilution of the conjugate should be determined by titration. The slides should be stained for 20–30 minutes at 37°C and then rinsed in PBS and distilled water, respectively.

iv) Observe the slides under a fluorescence microscope.

- **Calculation of virus-neutralising antibody titres**

Residual virus is detected using a standard fluorescence microscope. The serum neutralisation end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contain one or more infected cells (i.e. a 97% reduction in the virus inoculum). This value may be obtained by mathematical interpolation. Alternatively, a 100% neutralisation titre may be determined by recording the highest serum dilution at which 100% of the challenge inoculum is neutralised and there are no infected cells in any of the observed fields. For both titration methods, the titre of antibody in the test serum (in IU/ml) can be obtained by comparison with the titre of the national reference standard included in each test. It should be noted that it is also valid to perform the RFFIT using BHK-21 cells instead of neuroblastoma cells. A modified protocol for this has been published (45).

The following parameters have to be strictly adhered to:

- Rabies virus; only the CVS-11 strain (ATCC number VR 959) should be used.

- Cells cultures: only BHK-21 cells (ATCC number CCL10) or MNA cells (ATCC number CCL131) should be used.

- The test should be performed only on Labteck chamber slides.

- Control charts should be used for rabies virus, naïve serum and OIE standard serum.

- The back titration of the CVS virus, as well as the naïve serum and OIE serum, must be present on control plate.

- Reading method for the test: each chamber slide should contain 25–50 fields and be observed at ×160–200 magnification.

- A minimum of three-to-five-fold dilutions of sera is required.

- For the conversion of log D_{50} to IU/ml, only the log D_{50} value of the OIE standard serum of dog origin should be used.

c) **Virus neutralisation in mice**

This method is no longer recommended by either OIE or WHO and should be discontinued.

d) **Enzyme-linked immunosorbent assay (a prescribed test for international trade or movement)**

Commercial kits are available for indirect ELISA that allow a qualitative detection of rabies antibodies in individual dog and cat serum samples following vaccination. In accordance with the WHO recommendations (41), 0.5 IU per ml rabies antibodies is the minimum measurable antibody titre considered to represent a level of immunity that correlates with the ability to protect against rabies infection. The ELISA provides a rapid (~ 4 hours) test that does not require handling of live rabies virus, to determine if vaccinated dogs and cats have sero-converted. The sensitivity and specificity of any kit used should be determined by comparison with virus neutralisation methods. The ELISA is acceptable as a Prescribed Test for international movement of dogs or cats provided that a kit is used that has been validated and adopted on the OIE Register as fit for such purposes (see http://www.oie.int/vcda/eng/en_VCDA_registre.htm?e1d9)]. Virus neutralisation methods may be used as confirmatory tests if desired.

ELISA methods are also useful for monitoring of vaccination campaigns in wildlife populations, provided the kit used has been validated for the wildlife species under study.

C. REQUIREMENTS FOR VACCINES

Rabies vaccines prepared from Pasteur's original 1885 strain and its derivative strains (Pasteur Virus, Challenge Virus Standard, Pitman-Moore, etc.), and strains isolated more recently (Flury, Street-Alabama-Dufferin [SAD], Vnukovo and Kelev), protect against all strains of genotype 1 isolated so far. Conventional rabies virus vaccines may not provide adequate cross-protection against other lyssaviruses, especially in phylogroup II; there is no protection provided against Mokola virus (37) and the recently identified West Caucasian Bat Virus (29). Cross neutralisation using conventional rabies virus vaccines has been demonstrated against two phylogroup I viruses: EBLV type-1 and EVLV type-2 (20). The principles governing the preparation of inactivated rabies vaccines are identical whether they are to be used in humans or animals, although an adjuvant may be added to vaccines for animal use.

Recombinant vaccine (e.g. vaccinia rabies-glycoprotein recombinant) has also proved to be effective (19, 31). The rabies-glycoprotein recombinant vaccines are not live rabies vaccines. They are prepared by inserting non-infectious rabies nucleic acid into a vector such as vaccinia or canary pox. Since these do not contain live rabies virus, animals vaccinated with rabies-glycoprotein recombinant vaccines should not be restricted from entry into countries that have restrictions on entry of animals vaccinated with live rabies vaccines.

For animals, live and recombinant vaccines are effective by the oral route and can be distributed in baits in order to immunise wild (or domestic) animals.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Different standards apply to vaccines containing live virus modified by passage in eggs or cell cultures to reduce its virulence for the target animal, and to vaccines prepared from inactivated virus. Both types of vaccine have their advantages and disadvantages (6), but they can both be used to immunise animals for periods of between 1 and 3 years. Live attenuated rabies vaccines are not accepted in some countries. They are not to be relied on to protect previously unvaccinated animals that have been exposed to infection (15). Only in humans has the efficiency of post-exposure prophylaxis with vaccine alone been proven and even in these cases there is an additional strong recommendation to administer anti-rabies immunoglobulin.

All handling of the virus during manufacture and testing of vaccines must conform to the strict safety precautions specified by WHO (43, 45), the OIE (Chapter 1.1.2) and to national guidelines and regulations.

1. Seed management

a) **Characteristics of the seed**

Any strain belonging to serotype 1, which has been proved to protect against field rabies viruses (currently found in the country where the vaccine is to be used), is suitable. The strain of virus used should have well-known biological (e.g. pathogenicity) and antigenic properties (typing by MAbs). If it is to be used as a live vaccine, the master seed virus must be shown not to cause clinical rabies. At least two animals (preferably five to six per group) of each of the species for which the vaccine is intended and, so far as possible, any species that might be in contact with vaccine or vaccinated animals, should be tested. This can be done by

inoculating in or adjacent to a major nerve, a dose equivalent to ten times the intended viral titre in one dose of the proposed final product. Animals should be observed for at least 90 days for any adverse effect attributable to the master seed.

b) Method of culture

A master cell stock of the seed virus should be prepared and kept at or below –70°C. Subculture from this stock will be used for vaccine production. Virus multiplication is verified by titration during growth of the seed virus.

c) Validation as a vaccine

Before a vaccine is licensed, evidence of efficacy should be established by the challenge of vaccinated and control animals of each target species. The challenge should be performed at the end of the period after vaccination for which the manufacturer claims maintenance of immunity. Antibody kinetics should also be determined in order to establish the correlation between antibody titre and resistance to challenge.

The efficacy of the produced vaccine is assessed by studies on every target species previously vaccinated as recommended. Protection at the end of the period of immunity is monitored by a measurement of specific neutralising antibodies and by challenge with rabies virus. The experimental conditions of this challenge should mimic the natural conditions of infection. The challenge virus should preferably be prepared from locally isolated strains. In animals vaccinated with inactivated vaccines, the percentage of seroconversion and the mean level of antibody allow a good prognosis for survival to challenge (3).

The correlation between potency in the target species and antigenic value as estimated in mice should be established (see Section C.4.c below).

For the purposes of licensing a vaccine, safety tests should be conducted in the target species. In the case of live virus vaccines used in oral vaccination campaigns (including recombinant vaccines), safety tests should also be carried out on those other species that live in the area of vaccination and could become exposed to the vaccine (6).

Vaccine stability is ascertained by testing batches after prolonged storage, usually 1–2 years. A process of accelerated ageing, by storage at 37°C for 1 week, is sometimes used. The storage life claimed by the manufacturer is checked by the national licensing authority. In general, it is 12–18 months for fluid vaccines, and possibly 24 months for lyophilised vaccines.

2. Method of manufacture

Whatever method is adopted, close attention should be paid to the quality of the substrate. Both animals and eggs should be of SPF origin, and the cell cultures, such as BHK cell lines, should conform to international standards of sterility and innocuity.

a) In cell cultures

Cultures are infected with cell-culture-adapted strains of rabies virus and incubated at 35–36°C. These may then be used as live virus vaccines (as in Flury and SAD vaccines), or as inactivated vaccines after the addition of phenol (Semple vaccine) or some other chemical, such as beta-propiolactone.

Cell culture can also be used to grow the vector viruses (e.g. vaccinia virus) harbouring the gene coding for the expression of rabies virus glycoprotein (31).

During manufacture, the multiplication of the virus in one of the substrates mentioned above is monitored, followed by harvesting at the most appropriate time, usually 4–6 days after inoculation of animals, eggs or cell cultures. The virus harvest is suspended in a buffer solution at a dilution that will provide an optimum antigenicity of the end-product. If required, the suspension is either inactivated or lyophilised. An adjuvant is recommended for vaccines prepared from inactivated virus, as well as for other vaccine antigens that may be incorporated in polyvalent vaccines.

b) In eggs

A modified egg-adapted strain of virus is inoculated into SPF-embryonated chicken eggs, which are then incubated at 38°C for 5–6 days. The virus is harvested in the form of infective embryo tissues, and is usually lyophilised and used as a live vaccine. Examples of such vaccines include those that contain the Flury low egg passage (LEP), or the more desirable high egg passage (HEP) variant strain, which is safer for some animal species such as the cat.

c) In animals

Nervous tissue vaccines prepared in animals are no longer considered safe or effective, and their use should be discontinued.

3. In-process control

This consists of monitoring virus growth to provide an optimum titre and ensure the absence of undesirable microbial contamination.

In live virus vaccines, kinetics of virus growth should be established in order to ensure a final titre of virus correlated to the desired protection in target species.

In inactivated virus vaccines, immunogenic properties of the final product may be evaluated by *in-vitro* techniques (e.g. ELISA, agar gel immunodiffusion, antibody-binding tests or infected cell staining). These evaluations will indicate the best time for harvesting the virus in cell cultures.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Safety tests for batches of inactivated virus vaccines are carried out by inoculation of cell culture or intracerebrally into mice to detect viable virus. A suitable safety test for live rabies vaccines should be carried out on each lot of vaccine, in the intended host species. At least three, preferably five to six animals of the intended host species should be given a dose equivalent to ten times the recommended field dose, by the recommended route of administration. The animals should be observed for 180 days for adverse reactions attributable to the vaccine (23).

c) Potency/biological activity

The amount of virus present in live attenuated and recombinant vaccines is determined by titration. Once a correlation has been established between the activity of the vaccine in the target species and virus titres, virus titrations become reliable indicators of vaccine efficacy. This is carried out using cell cultures or by the intracerebral inoculation of unweaned mice (in mice it is only possible with a few attenuated viruses). Recombinant vaccines should be monitored for the expressed rabies protein until assured that expression stablilty is maintained in the manufacturing process. Titre of the vector can then be used as a reliable indicator of vaccine efficacy.

For inactivated virus vaccines, correlation between potency in the target species and antigenic value as estimated in mice provides a reliable indicator of vaccine activity. The potency of the vaccine is established in the USA by the National Institutes of Health (NIH) test. Elsewhere, the European Pharmacopoeia test is widely adopted.

Groups of at least ten mice, aged 3–4 weeks, are inoculated with single, decreasing doses of vaccine in accordance with the European Pharmacopoeia (23), or with two doses, 1-week apart, according to the NIH test (45). A sufficient number of dilutions of vaccine are compared to estimate the dilution at which 50% of the mice are protected against intracerebral challenge 14 days later (23, 45).

A WHO international standard vaccine is available (see footnote 2) for calibration of national standards, so that the results of testing for antigenicity can be expressed in IUs. The test is not valid unless:

i) For both the vaccine to be examined and the standard preparation, the PD_{50} (50% protective dose) lies between the largest and smallest doses given to the mice.

ii) The titration of the challenge virus suspension shows that 0.03 ml of the suspension contained 25 mouse intra-cranial LD_{50} (MIC LD_{50}). The challenge dose should be in the range 12–50 LD_{50} for a valid test.

iii) The confidence interval ($p = 0.95$) for the test should not be less than 25% and not more than 400% of the estimated potency: statistical analysis should show a significant slope and no significant deviations from linearity or parallelism of the dose–response lines.

The vaccine passes the test if the estimated potency is not less than 1 IU per dose, in the smallest prescribed dose.

A simplified test can also be used for the purpose of anticipating which vaccines are likely to be of an antigenic value ≥1 IU per dose (4). This test used as a screening test is a good way to reduce the number of mice used in vaccine potency control tests.

d) Duration of immunity

Duration of immunity must be established for the product licence in the target species with a defined vaccination protocol.

d) Stability

The proposed shelf life must be verified by appropriate tests. These experiments include biological and physico–chemical stability tests, and should be performed on a sufficient number of batches of vaccine stored under recommended conditions.

The thermostability of live virus vaccines in liquid form is generally poor. For freeze-dried inactivated virus vaccines, stability is generally granted for 2 years at 4°C.

e) Preservatives

Inactivated virus vaccines may contain preservatives (formalin, merthiolate). The nature and quantity of these preservatives should comply with national control regulations.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

6. Oral vaccination

The concept of oral vaccination is unique: as stray or wild animals are out of physical reach, dropping vaccine baits into their environment is the only way to immunise them. In the 1980s and 1990s, the Veterinary Public Health Department of WHO organised several meetings of rabies experts to define the requirements for guaranteeing the safety and efficacy of vaccines both for the target species (red fox, raccoon dog, skunk, dog, etc.) and nontarget species, namely wild rodents and any other wild and domestic species that might be in contact with baits or a recently vaccinated animal (42, 44).

Several guidelines have been established for the quality criteria that vaccines have to satisfy before marketing; the most precise documents are those produced by WHO, the European Pharmacopoeia and the European Commission (24, 25, 44). Available oral vaccines have been extensively tested by different routes (cerebral, muscular and oral) in a variety of species: puppies and adults of carnivores, avian species, nonhuman primates, rodents and immunocompromised mice. Nonhuman primates have been added to this list since the discovery in 1992 that the original SAD Bern strain is highly pathogenic for baboons by the oral route (12).

All vaccines currently used for oral vaccination programmes are either modified live-virus vaccines or live recombinant vaccines. At the present time, two oral vaccines are recommended by WHO (44): a recombinant vaccine – VRG vaccine, and a highly attenuated vaccine – SAG2.

The production controls are closely related to the ones used for parenteral vaccines. The major differences concern three points:

i)   Safety of the vaccine for man, target and non target species.

ii)   Efficacy of the protection induced by the vaccine.

iii) Monitoring of the impact of oral vaccination campaigns in the field.

a) **Safety considerations**

For oral vaccination, either attenuated rabies strains or live-recombinant vaccines may be used. The vaccine should not induce any adverse signs in target and nontarget species. For vaccines used for dog immunisation, saliva should be checked for the absence of vaccinal virus because of possible contact with humans.

The attenuated rabies virus-based vaccines must achieve the lowest residual pathogenicity for target and nontarget species (24); this is of utmost importance in the case of oral vaccination of dogs as dogs are often in close contact with humans (44).

The recombinant vaccines cannot induce any risk of rabies; the safety controls concern only the possible residual pathogenicity of the recombined parental virus.

b) **Protection induced by the vaccine**

The protection induced by the vaccine must be tested not only with the virus itself (to determine the minimal vaccinating dose) but also with manufactured baits ready to be used in the field. For foxes for instance, the vaccine should have a minimal titre corresponding to at least ten times the 100% protective dose (obtained with the same vaccine experimentally by direct oral instillation) (14).

The protection status cannot be then checked by serology only; a virulent challenge with the homologous street rabies virus is necessary because of the important implication of cell-mediated immunity in response to oral vaccination (25).

c) **Monitoring the impact of oral vaccination**

The stability of the vaccine in the field is important. The European Commission stresses the importance of checking the 100% protective dose after 7 days of exposure at 25°C (24). Each vaccine bait should be tested for stability with a melting point above 40°C, and the blister or sachet containing the vaccine should still be covered by the bait casing after 7 days exposure at 40°C (24).

Aerial distribution of baits is the only way to perform an homogenous, rapid and sufficient distribution for wildlife vaccination. Quality control measures should be used to monitor different key points of baiting; control of vaccine titre, control of area coverage by air and of baiting density should at least be constantly monitored. The cross border cooperation between neighbouring countries is also needed to avoid any unvaccinated area along the border.

For wildlife in Europe, two campaigns are performed yearly: the spring one aims at vaccinating the young population of the target species, its period should then be fixed according to the biology of the target species. The autumn campaign concerns both adult and young animals. It is generally admitted that four campaigns (i.e. 2 years) should be conducted after the last rabies diagnosis.

The impact of vaccination on the host/vector population is monitored in two different ways:

- Directly by measuring the bait uptake by the wild target species. This supposes that a biomarker (generally tetracycline) is included in the bait casing. The same examination allows the age of animals to be determined.

- Directly by measuring the serological response of target animals. This serological control is better done using validated ELISA techniques (22, 35) as they are more robust than seroneutralisation tests when testing poor quality field specimens.

- Indirectly by measuring the incidence of rabies in the vaccinated area. Typing of field isolates should be performed (44) either by using MAbs or by sequencing positive samples from areas where the target species have been vaccinated with attenuated vaccines to possibly distinguish vaccine and field virus strains.

The first two controls should be performed on specially killed animals to collect good quality samples. Rabies monitoring is more sensitive when performed in found dead or ill animals.

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NB: There are OIE Reference Laboratories for Rabies (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

RIFT VALLEY FEVER

SUMMARY

Rift Valley fever (RVF) is a peracute or acute zoonotic disease of domestic ruminants in Africa. It is caused by a single serotype of a mosquito-borne bunyavirus of the genus Phlebovirus. *The disease occurs in climatic conditions favouring the breeding of mosquito vectors and is characterised by liver damage. The disease is most severe in sheep, goats and cattle, in which it produces abortions in pregnant animals and a high mortality rate in the newborn. Older nonpregnant animals, although susceptible to infection, are more resistant to clinical disease. There is considerable variation in the susceptibility to RVF of animals of different breeds. Those breeds or strains that are exotic to Africa or are from areas where RVF is not endemic, tend to be more susceptible. Camels suffer an inapparent infection with RVF, but abortion rates can be as high as in cattle. Among ruminant game, buffalo also abort during an inapparent RVF infection.*

Humans are susceptible to infection through contact with infected material or mosquito bites. Infection of humans by vectors is a striking feature in countries with a relatively small population of animal hosts. In such areas, RVF may be recognised first in humans. It has caused serious disease in laboratory workers and must be handled with high level biosecurity. It is recommended that laboratory workers be vaccinated.

Identification of the agent: *RVF virus consists of a single serotype of a bunyavirus of the genus* Phlebovirus *and has morphological and physicochemical properties typical of bunyaviruses.*

The virus can be isolated from blood, preferably collected in an anticoagulant, during the febrile stage of the disease, or from liver, spleen and brain tissues of animals that have died and from the organs of aborted fetuses. Primary isolations are usually made on cell cultures of various types, such as African green monkey kidney (Vero) cells, baby hamster kidney cells, chicken embryo reticulum, or primary cells of sheep or cattle origin. Alternatively, hamsters, adult or suckling mice, embryonated chicken eggs or 2-day-old lambs may be used for primary virus isolation.

A rapid diagnosis can be achieved by using the supernatant of homogenised samples as antigen in virus neutralisation (VN) tests; immunofluorescent staining of impression smears of liver, spleen, brain or infected cell cultures; or by the demonstration of virus in serum, taken during the febrile stage of the disease, by enzyme immunoassay or immunodiffusion.

The presence of characteristic histopathological lesions in the liver assists in the diagnosis.

Serological tests: *Infected animals develop specific antibodies that may become demonstrable by VN as early as 3 days following infection and after 6–7 days by enzyme-linked immunosorbent assay, and by haemagglutination inhibition. Serological tests used less often include immunofluorescence, complement fixation and immunodiffusion.*

Requirements for vaccines and diagnostic biologicals: *Live virus vaccines and antigens for use either in countries where RVF is endemic or during outbreaks, should be prepared from nonpathogenic mouse- or mutagen-attenuated strains of RVF virus grown in cell cultures. The mutagen-attenuated strain of RVF is not yet at a stage where it can be recommended for use.*

In RVF-free countries, vaccines and diagnostic tests should be limited to those using inactivated virus. Suitable virus strains can be obtained from the OIE Reference Laboratory for RVF (see Table given in Part 3 of this Terrestrial Manual).

A. INTRODUCTION

RVF virus consists of a single serotype of a bunyavirus of the genus Phlebovirus and has morphological and physicochemical properties typical of bunyaviruses. The virus is enveloped, spherical and 80–120 nm in diameter. Short glycoprotein spikes project through a bilayered lipid envelope and the virus is readily inactivated by lipid solvents and acid conditions below pH 6. The virus has a three-segmented, single-stranded, negative-sense RNA genome. and consists of the three segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The S segment is ambisense RNA, i.e. has bi-directional coding (12).

Rift Valley fever (RVF) is a peracute or acute, febrile, mosquito-borne, zoonotic disease caused by a virus of the family Bunyaviridae, genus *Phlebovirus*. It usually presents in epizootic form over large areas of a country following heavy rains and flooding, and is characterised by high rates of abortion and neonatal mortality, primarily in sheep, goats and cattle. The susceptibility of different breeds to RVF varies considerably. Some indigenous African animals may have only inapparent infections, while exotic or other breeds suffer severe clinical disease with mortality and abortion. Susceptible, older nonpregnant animals and some other species usually do not show signs of disease. Camels have been regularly involved in the RVF epidemics in East Africa and Egypt. Clinical disease is not seen in adult camels, but abortion occurs and some early post-natal deaths have been observed.

Signs of the disease tend to be nonspecific, rendering it difficult to recognise individual cases (8–11, 13, 22, 34) and during epidemics; however, the occurrence of numerous abortions and mortalities among young animals, together with disease in humans, is characteristic. RVF has a short incubation period: 12–36 hours in lambs. A biphasic fever of up to 41°C may develop, and the fever remains high until shortly before death. Affected animals are listless, disinclined to move or feed, and may show enlarged superficial lymph nodes and evidence of abdominal pain. Lambs rarely survive longer than 36 hours after the onset of signs of illness. Animals older than 2 weeks may die peracutely, acutely or may develop an inapparent infection. Some animals may regurgitate ingesta and may show melaena or bloody, foul-smelling diarrhoea and bloodstained mucopurulent nasal discharge. Icterus may sometimes be observed, particularly in cattle. In addition to these signs, adult cattle may show lachrymation, salivation and dysgalactia. In pregnant sheep, the mortality and abortion rates vary from 5% to almost 100% in different outbreaks and between different flocks. The death rate in cattle is usually less than 10%.

The hepatic lesions of RVF are very similar in all species, varying mainly with the age of the infected individual (9). The most severe lesion occurring in aborted fetuses and newborn lambs is a moderately to greatly enlarged, soft, friable liver with a yellowish-brown to dark reddish-brown colour with irregular congested patches. Numerous greyish-white necrotic foci are invariably present in the parenchyma, but may not be clearly discernible. In adult sheep, the lesions are less severe and pinpoint reddish to greyish-white necrotic foci are distributed throughout the parenchyma. Haemorrhage and oedema of the wall of the gallbladder are common. Hepatic lesions in lambs are almost invariably accompanied by numerous small haemorrhages in the mucosa of the abomasum. The contents of the small intestine and abomasum are dark chocolate-brown as a result of the presence of partially digested blood. In all animals, the spleen and peripheral lymph nodes are enlarged, oedematous and may have petechiae.

Microscopically, hepatic necrosis is the most obvious lesion of RVF in both animals and humans. In fetuses and neonates of cattle and sheep, foci of necrosis consist of dense aggregates of cellular and nuclear debris, some fibrin and a few inflammatory cells. There is a severe lytic necrosis of most hepatocytes and the normal architecture of the liver is lost. In about 50% of affected livers, intranuclear inclusion bodies that are eosinophilic and oval or rod-shaped are found. Mineralisation of necrotic hepatocytes is also seen. In adult animals, hepatic necrosis is less diffuse and in sheep, icterus is more common than in lambs (32).

In humans, RVF infections are usually inapparent or associated with a moderate to severe, nonfatal, influenza-like illness (19, 21). A minority of patients may develop ocular lesions, encephalitis, or severe hepatic disease with haemorrhagic manifestations, which is generally fatal. RVF virus has caused serious human infection in laboratory workers. Staff should either be vaccinated and work under containment level 3, work under containment level 4 conditions, or wear respiratory protection. Particular care needs to be exercised when working with infected animals or when performing post-mortem examinations (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

No significant antigenic differences have been demonstrated between RVF isolates and laboratory-passaged strains from many countries, but differences in pathogenicity have been shown (5, 33).

Infection of humans by mosquito vectors is a striking feature in countries, such as Egypt, with a relatively small population of animal hosts and a large population of mosquitoes.

RVF usually occurs in epizootics in Africa, which may involve several countries in a region at one and the same time. These follow the periodic cycles of exceptionally heavy rain, which may occur very rarely in semi-arid zones

(25–35-year cycles), or more frequently (5–15-year cycles) in higher rainfall savannah grasslands. Low level undetectable RVF activity may take place in inter-epizootic periods. RVF should be suspected when unusually heavy rains are followed by the occurrence of abortions together with fatal disease marked by necrosis and haemorrhages in the liver that particularly affect newborn lambs, kids and calves, concurrent with the occurrence of an influenza-like illness in farm workers and people handling raw meat.

Preventative measures to protect workers from infection should be employed when there are suspicions that RVF-virus-infected meat and tissue samples are to be handled.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

RVF virus may be isolated from serum and blood collected in an anticoagulant during the febrile stage of the disease, from liver, spleen and brain of animals that have died, or from aborted fetuses. Primary isolation is usually performed in hamsters, infant or adult mice, or on cell cultures of various types.

a) Culture

Approximately 5 ml of blood collected during the febrile stage of the disease or approximately 5 g of liver, spleen and brain collected after death should be presented for virus isolation. The samples should be kept at 0–4°C during transit. If transport to the laboratory is likely to take more than 24 hours, the samples should be frozen and sent on dry ice.

Approximately 1 g of homogenised tissue is suspended 1/10 in cell culture medium or buffered saline, pH 7.5, containing sodium penicillin (1000 International Units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml), or fungizone (2.5 µg/ml). The suspension is centrifuged at 1000 g for 10 minutes and the supernatant fluid is injected intracerebrally into 1–5-day-old mice or intraperitoneally into hamsters or adult mice. Infant mice will either die or be obviously ill by day 2. Adult mice are affected 1–3 days later. Although mice or hamsters are the laboratory animal of choice, lambs and embryonated chicken eggs may also be used.

A variety of cell monolayers including African green monkey kidney (Vero), baby hamster kidney (BHK), chicken embryo reticulum (CER: cells developed by Tsunemasa Motohashi at the Nippon Institute for Biological Science, Tokyo, Japan; recharacterised as a hamster line) (4) and primary kidney or testis cells of calves and lambs may be inoculated with 1 ml of clarified sample supernates and incubated at 37°C for 1 hour. It is advisable to also inoculate some cultures with a further 1/100 dilution of the inoculum. This is to avoid the production of defective particles, which follows the use of very high virus inocula. Some tubes containing flying cover-slips should also be prepared. The cultures are washed with phosphate buffered saline at room temperature and covered with medium containing 2% serum free from antibodies against RVF. The cultures are observed microscopically for 5–6 days. RVF virus induces a cytopathic effect (CPE) characterised by slight rounding of cells followed by destruction of the whole cell sheet within 12–24 hours. Specific identification of RVF virus antigen may be made 18–24 hours after infection by immunofluorescent staining of the cover-slip preparations.

The virus may also be detected by immunofluorescence carried out on impression smears of liver, spleen and brain. A rapid diagnosis can sometimes be made by demonstrating viral antigen in tissues or in serum of febrile animals by a complement fixation or agar gel immunodiffusion (AGID) test. A rapid diagnosis can also be made by detection of viral RNA using a reverse-transcription polymerase chain reaction (RT-PCR).

b) Agar gel immunodiffusion

The AGID test is useful in laboratories without tissue-culture facilities. Approximately 1 gram of tissue, preferably liver, is homogenised and made up to a 10–20% suspension in borate saline buffer, pH 9.0. The material is centrifuged at 1000 g and the supernatant is used in the test. Micro-AGIDs are performed on standard microscope slides covered with 3 ml of 1% agarose in borate saline. Patterns of six peripheral wells and a central well are prepared and filled with reagents as follows: a positive, preferably hyperimmune serum in the central well, positive control antigen in wells 1 and 4, test tissues in wells 2 and 5 and negative tissues in wells 3 and 6. A precipitin line of continuity should be formed between control antigen and positive serum that extends to include a line between test tissue and serum for a case to be considered positive.

c) **Polymerase chain reaction**

A rapid diagnosis can also be made by detection of viral RNA (30) using RT-PCR. The PCR was used, among other techniques, for antigen detection in two recent RVF virus outbreaks in Africa – one in Kenya in 1998 and a limited outbreak in South Africa in 1999. It may also be used to detect RVF virus in mosquito pools (18). RT-PCR followed by sequencing of the NS(S) protein-coding region has been used in phylogenetic analysis to characterise two distinct lineages of RVF virus – one Egyptian and the other sub-Saharan – making this technique a powerful molecular epidemiological tool (29).

d) **Histopathology**

Histopathological examination of the liver of affected animals will reveal characteristic cytopathology, and immunostaining will allow the specific identification of the RVF viral antigen in infected cells. This is an important diagnostic tool because liver or other tissue may be placed in formol saline in the field for diagnostic purposes, which facilitates handling and transport in areas remote from the laboratory.

2. Serological tests

Virus neutralisation (VN) tests including microneutralisation, plaque reduction neutralisation (PRN) and neutralisation in mice have been used to detect antibodies against RVF virus in the serum of a variety of species. Neutralisation tests are highly specific and will record the earliest response, but these tests can only be performed with live virus and are not recommended for use outside endemic areas or in laboratories without appropriate biosecurity facilities and vaccinated personnel.

Other available tests include enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HI), AGID, immunofluorescence, radioimmunoassay and complement fixation. In these tests, however, cross-reactions may occur between RVF virus and other phleboviruses. An advantage of these tests is the fact that they can be performed with inactivated antigen and can therefore be used in RVF-free countries.

The ELISA is a reliable and sensitive test that may be employed with several species to detect antibodies against RVF virus. An IgM-capture ELISA allows diagnosis of a recent infection to be made on a single serum sample.

The HI test can be employed with great confidence in nonendemic areas. However, sera from individuals that have had previous infections with phleboviruses other than RVF may react with RVF antigen to titres as high as 40 and, rarely, to titres of 320 (33). In suspected cases, the OIE Reference Laboratory for RVF (see Table given in Part 3 of this *Terrestrial Manual*) can be of assistance in carrying out neutralisation tests for specificity. The HI antibody titre after vaccination with RVF virus vaccine may be as high as 640 or, rarely, 1280, whereas titres following natural infections with RVF virus are usually significantly higher.

a) **Virus neutralisation (the prescribed test for international trade)**

The VN test may be employed to determine the presence of antibodies in naturally infected animals and in animals vaccinated with RVF vaccine. The test is highly specific and can be used to test serum of any species. It is generally used to measure vaccine efficacy. Factors other than neutralising antibodies may play a part in resistance to RVF. The Smithburn neurotropic mouse brain strain of highly attenuated RVF virus (31), also referred to as modified live virus and adapted to cell culture, is used as antigen. The antigen is stored at –80°C or 4°C in freeze-dried form. The stock is titrated to determine the dilution that will give 100 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl under the conditions of the test.

• **Test procedure**

i) Inactivate the test sera for 30 minutes in a water bath at 56°C.

ii) Add 25 µl of cell culture medium with 5% RVF-negative serum and antibiotics to each well of a 96-well cell culture plate.

iii) Add 25 µl of test serum to the first well of each row and make twofold dilutions. Titrate each serum in duplicate from 1/10 to 1/80 for screening purposes or in quadruplet and to higher dilutions for determination of end-point titres. Include known positive and negative control sera.

iv) Add 25 µl per well of RVF virus antigen (diluted in cell culture medium and calculated to provide 100 $TCID_{50}$ per well) to each well that contains diluted test serum and to wells in rows containing negative and positive control serum. In addition, make twofold dilutions of antigen in at least two rows each containing cell culture medium only.

v) Incubate for 30 minutes at 37°C.

vi) Add 50 µl per well of Vero, CER or any other suitable cell suspension at 3×10^5 cells/ml or at a dilution known to produce a confluent monolayer within 12 hours.

vii)   Incubate the plates in an atmosphere of 3–5% CO_2 for 3–5 days.

viii) Using an inverted microscope, the monolayers are examined daily for evidence of CPE. There should be no CPE in rows containing positive control serum and clear evidence of CPE in rows containing negative control serum indicating the presence of virus. Determine the results by the Spearman–Kärber method.

b)   Enzyme-linked immunosorbent assay

For the serodiagnosis of RVFV a number of ELISAs using different formats have been published and are commercially available (1, 28). The use of inactivated whole virus or mouse liver antigens has recently been replaced by recombinant nucleocapsid (N) protein as antigen.

These ELISAs are at present in an indirect format and apart from the very important safety consideration also have the advantage of antigen stability and the ability to test 40 sera in duplicate per plate instead of only 20.

An indirect ELISA with pre-coated plates using a nucleocapsid protein (NC) recombinant antigen and Protein G peroxidase conjugate is described below (17).

- **Test procedure**

Unless otherwise stated, all dilutions are made with 10% (w/v) dried milk buffer and all washes performed three times with volumes of 250–300 µl/well.

i)   Using pre-coated plates add 50 µl of diluted (1/100) serum in duplicate wells

ii)   Add control sera at predetermined dilutions in duplicate wells. Incubate for 60 minutes at 37°C. Wash the plate.

iii) Add Protein G/horseradish peroxidase conjugate at a working dilution to all wells of the plate. Incubate for 60 minutes at 37°C. Wash the plate

iv)   Add 50 µl of ready-to-use TMB Substrate to all wells of the plate. Cover the plate and incubate at room temperature in the darkness for 20–30 minutes.

v)   Add 50 µl of ready-to-use Stop solution to all wells of the plate. Tap plate gently to allow contents to mix. Wait 5 minutes and read plate using a spectrophotometer equipped with a 450 nm filter.

vi)   Suggested plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	CC	1	2	3	4	5	6	7	8	9	10
B	CC	CC	1	2	3	4	5	6	7	8	9	10
C	C++	C++	11	12	13	14	15	16	17	18	19	20
D	C++	C++	11	12	13	14	15	16	17	18	19	20
E	C+	C+	21	22	23	24	25	26	27	28	29	30
F	C+	C+	21	22	23	24	25	26	27	28	29	30
G	C–	C–	31	32	33	34	35	36	37	38	39	40
H	C–	C–	31	32	33	34	35	36	37	38	39	40

CC: Conjugate control; C++: High positive control serum; C+: low positive control serum;
C–: negative control serum; 1–40: test samples.

Newer ELISA formats are being introduced, including formats that are more specific for IgG and IgM (27).

c)   Haemagglutination inhibition

The HI test adapted to a microtechnique is based on Clarke & Casals (7). A sucrose/acetone-extracted hamster liver antigen is used in a 96-well U-bottomed plate test and antigen is diluted so that 4 haemagglutinating units are used in the test. Nonspecific inhibitors of haemagglutinin are removed by kaolin extraction of sera followed by adsorption with packed goose erythrocytes (RBC) prior to testing. Doubling dilutions of sera made in borate saline buffer, pH 9, are tested against equal volumes of antigen.

Plates are held overnight at 4°C before the addition of 50 µl of 0.5% RBC to each of the wells. Plates are read after 30 minutes at room temperature and end-points are recorded as the reciprocal of the highest serum dilution producing complete inhibition of agglutination.

Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Sera with titres below 1/40 are considered to be negative.

HI is an appropriate screening test for surveys although it is not specific. Marked cross-reactions do occur between the phleboviruses, but homologous titres exceed heterologous titres. Experimentally, African phleboviruses other than RVF have been shown to be nonpathogenic for ruminants, and antibodies that they might induce are unlikely to cause confusion in RVF diagnosis (33).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A live vaccine prepared from Smithburn's attenuated strain of RVF virus has been used for the control of RVF in nonpregnant cattle and sheep in endemic areas and during outbreaks (6), while inactivated vaccines for use in pregnant animals and in RVF-free countries are prepared from virulent field strains (2, 3). Inactivated virus vaccines should be prepared from highly immunogenic strains of RVF virus produced in cell culture. The virus should be inactivated with formaldehyde and mixed with an adjuvant to enhance immunogenicity. The inactivated vaccine should be carefully safety tested to ensure that there is no residual live virus.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

In humans, an inactivated experimental RVF vaccine has been used for 25 years with considerable success to protect persons at risk. This vaccine is currently produced on diploid cells. However, the limited availability of the vaccine precludes its use in the general population.

Two new vaccine candidates produced from human RVF virus isolates are undergoing extensive testing with a view to replacing existing vaccines.

The first, MV P12, is a mutagen-derived strain of virus passaged in the presence of 5-florouracil with serial mutagenesis resulting in attenuation for mice. Immunogenicity and pathogenicity have been tested in sheep and the virus found to be non-abortogenic in pregnant ewes (23). MV P12 was protective in young lambs (14, 20) and in cattle (24, 25). In further testing in sheep, the vaccine, when used after 28 days of pregnancy, i.e. in the first trimester, resulted in abortion and severe fetal teratology (16).

The second candidate, Clone 13, a small plaque variant that did not react with two specific monoclonal antibodies, was found to be avirulent in mice and hamsters and highly immunogenic. Immunogenicity and pathogenicity have been tested in lambs, sheep, young and adult goats (26). In further trials it was non-abortogenic in pregnant sheep and gave more than 80% protection from virulent challenge (15). Clone 13 possesses a large deletion in the portion of the sRNA segment coding for the nonstructural proteins, which should result in a stable vaccine candidate.

In the following description of vaccine production, information is given on live vaccine production adjacent to information on inactivated vaccine production. It must be stressed that live and inactivated vaccines must never be produced in the same facility at the same time, because of the risk of contaminating the attenuated live vaccine with a virulent strain of virus before it is inactivated. Staff handling live RVF virus should be vaccinated and work at containment level 3 to minimise the risk of self infection.

1. Seed management

a) Characteristics of the seed virus

Live vaccine: The stock antigen is derived from Smithburn's original neurotropic strain. This strain is not lethal to adult mice inoculated intraperitoneally and is safe for use in all breeds of cattle, sheep and goats. However, it may cause fetal abnormalities or abortion in pregnant animals.

Inactivated vaccine: For seed virus, a highly immunogenic strain of RVF virus adapted to growth in cell culture may be used. It differs from the attenuated strain in that it is lethal to adult mice when injected intraperitoneally.

b)   Method of culture

Both attenuated and inactivated virus strains are produced on BHK, Vero or CER cell cultures. The viruses are stored in a lyophilised form in vials containing 1 ml of a cell culture suspension. The virus titre (following intracerebral inoculation of infant mice) should be at least $10^{6.5}$ mouse LD_{50} (50% lethal dose) per ml.

c)   Validation as a vaccine

Seed virus must be shown to be free from adventitious agents, safe for use and able to stimulate effective immunity in species and breeds for which it is intended.

- **Tests**

The lyophilised seed virus is reconstituted in sterile cell culture medium without antibiotics and tested for freedom from bacteria and fungi. The contents of a reconstituted vial are inoculated into two tubes of thioglycollate and two tubes of soybean casein digest medium. The thioglycollate cultures are incubated at 37°C for 7 days and the soybean casein digest medium cultures at 20°C for 14 days. The cultures should remain negative.

In addition, 5 ml of reconstituted seed virus is mixed with an equal volume of specific RVF antiserum produced in rabbits. After incubation of the serum/virus mixture at 37°C for 30 minutes, the virus suspensions are tested before and after neutralisation on cell cultures, as well as in adult and infant mice, embryonated eggs, and guinea-pigs. The neutralised virus is:

i)   Seeded on to six roller tube cultures of primary lamb kidney cells and six roller tube cultures of BHK cells. The cell cultures are incubated at 37°C and observed daily for 7 days for CPE, after which they are subjected to the haemadsorption test with guinea-pig RBCs at 4°C and 37°C. There should be no evidence of CPE or haemadsorption. If cultures degenerate or show suspicious CPE, the material from these cultures should again be mixed with antiserum and subinoculated into new cell cultures, which are observed for a further period of 14 days. The presence of specific CPE or haemadsorption disqualifies the seed virus pool.

ii) Inoculated intraperitoneally (0.2 ml) into groups of at least six adult and six 2–5-day-old mice. The mice should remain healthy for 14 days. If any mice should die, appropriate tissue should be emulsified, mixed with antiserum and subinoculated into further groups of mice, which should again be observed for a further period of 14 days. If there is any evidence of specific mortality, the seed virus pool is disqualified.

iii) Inoculated into at least ten 8-day-old embryonated chicken eggs by means of the 'stab' method (combination of chorioallantoic membrane and allantoic sac route). The eggs are incubated at 37°C for 8 days and are candled daily. Embryos that die within 24 hours are discarded. However, the test should be repeated if <70% of the embryos are alive after 24 hours. The cause of embryo mortality during the subsequent observation period should be determined by setting up appropriate sterility and HI tests, and by examination of yolk-sac smears. If these tests are negative, subinoculation of embryo suspensions mixed with antiserum should be set up as before. On day 4 of incubation, at least four eggs are opened and allantoic fluid is collected. The remaining eggs are opened on day 8 of incubation. The membranes of both groups are examined for lesions and abnormalities of the embryos. The allantoic fluids are subjected to the HI test with guinea-pig and chicken RBCs at 4°C and 37°C. Specific embryo mortality, haemagglutinating activity of the allantoic fluids or any lesions on the membranes or embryo abnormalities disqualifies the seed virus pool.

iv) Injected intraperitoneally with 1.0 ml of seed virus into each of two guinea-pigs. The guinea-pigs should remain healthy over an observation period of 14 days.

Failure to pass any test disqualifies the antigen for use as seed virus.

2.   Method of manufacture

A vial of lyophilised seed virus is reconstituted and diluted 1/100 to 1/1000 with sterile Eagle's medium for the attenuated vaccine and 1/1000 for the inactivated vaccine. To prepare a working suspension, the diluted virus is seeded on to confluent BHK cell cultures in roller bottles and incubated at 37°C. When 70% of cells is affected (CPE), the medium and cells are harvested and the material is diluted 1/100 to 1/1000, after which 10 ml is again seeded on to roller bottles with confluent BHK cells and again incubated. As soon as 70% CPE is observed, the medium and cells are harvested and pooled.

Virus suspensions for both attenuated and inactivated vaccines are titrated intracerebrally in infant mice and should have a titre of at least $10^{6.5}$ mouse LD_{50}/ml. Alternatively, a plaque titration on CER cells may be performed.

Attenuated vaccine is lyophilised immediately after completion of titration and testing for bacteria and fungi.

A stabiliser should be used, such as 5% peptone in 0.3 M phosphate buffer. The volume of inactivated vaccine is adjusted so that the final vaccine will contain at least $10^{6.5}$ mouse LD_{50}/ml. The adjusted virus suspension is then inactivated at 37°C for 24 hours with formaldehyde at a final concentration of 0.2%. After inactivation, an equal volume of aluminium hydroxide gel is added to the cell suspension. The vaccine should have a final pH of 7–7.5.

3. In-process control

Prior to inoculation of cell cultures, seed virus is subjected to tests for bacteria and fungi in thioglycollate and soybean casein digest medium (see Section C.1.c and Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

A representative sample from each batch of vaccine is selected and the contents of each are reconstituted with 5 ml sterile distilled water and tested for freedom from bacteria and fungi.

For inactivated vaccines, inactivation must be checked using two passages in the same type of cell culture as used in the production of the vaccine.

4. Batch control

a) Sterility

Prior to freeze-drying or inactivation, each container of pooled vaccine, and thereafter representative samples of the batch, are tested for sterility in thioglycollate and soybean casein digest medium (see also Chapter 1.1.9 of this *Terrestrial Manual*).

b) Safety

Live vaccine: Final containers of lyophilised attenuated vaccine are selected at random, and each is reconstituted in distilled water as for vaccination. Four susceptible sheep are injected subcutaneously with one dose of vaccine. The sheep are observed daily for 14 days and the rectal temperatures are recorded. The sheep must remain healthy.

Vaccine is also injected intraperitoneally into six adult mice (0.25 ml each), two hamsters and two guinea-pigs (1 ml each). The animals are observed for a period of 14 days during which they should remain healthy. Mortality attributed to the vaccine disqualifies the batch.

Inactivated vaccine: In the case of inactivated RVF vaccine, each of four susceptible sheep is injected subcutaneously with 2.0 ml of vaccine, observed daily for 3 weeks and rectal temperatures are recorded. The sheep should remain healthy.

In addition, safety is also determined by intracerebral injection of six adult mice and two litters of at least six infant mice per litter, and by intraperitoneal injection of two guinea-pigs and two hamsters. The mice, hamsters and guinea-pigs are observed for a period of 14 days. They should remain healthy. Mortality attributed to the vaccine disqualifies the batch.

c) Potency

Live vaccine: Lyophilised attenuated vaccine from two final containers is reconstituted and titrated intracerebrally in infant mice. The final vaccine should contain at least $10^{4.4}$ mouse LD_{50}/dose. Alternatively, titrations may be done on cell cultures.

Two final containers are kept at 37°C for 1 week, reconstituted and titrated as before. Each should contain at least $10^{3.4}$ mouse LD_{50}/dose. Alternatively, titrations may be done on cell cultures.

Inoculated sheep (see Section C.4.b) are bled 2 and 3 weeks after vaccination, and their antibody response is determined by PRN. A virus neutralising antibody titre of 100 or more is regarded as satisfactory.

Inactivated vaccine: The sheep, injected subcutaneously to determine safety (Section C.4.b), are bled after 3 weeks and their antibody response is determined by VN test. A virus neutralising antibody titre of 100 or more is regarded as satisfactory.

d) Duration of immunity

Both the live attenuated and the inactivated vaccines have had extensive field use. The live vaccine is considered to induce lifelong immunity against clinical disease, although controversy exists over the immunogenicity of the Smithburn vaccine. Nevertheless, cattle can be immunised with the live virus vaccine

using this strain. Experience of the field efficacy of inactivated vaccines is limited because they are used in areas where RVF is not endemic, consequently natural field challenge of the vaccine does not occur. However, in South Africa, during the outbreak of RVF in 1976–1978, observations by State Veterinarians supported the efficacy of the vaccine. In more recent epizootics elsewhere, the inactivated vaccine failed to protect animals against abortion, following two vaccinations. When using the inactivated vaccine, a booster dose should be given 3–6 months after the initial vaccination and thereafter vaccination should be repeated yearly (2, 3).

e) Stability

When stored at 4°C, lyophilised attenuated vaccines are stable for at least 4 years, while inactivated vaccine may be stored for many years. Storage at higher temperatures is not recommended.

f) Preservatives

No preservatives are used.

g) Precautions (hazards)

Although humans can be infected by handling infected material, no case of disease is known to have occurred in humans infected with attenuated vaccine virus, but seroconversion often occurs. However, the strains used to prepare inactivated vaccine may cause disease. Therefore, all staff likely to be exposed to vaccine virus should be vaccinated with the human formalin-inactivated vaccine.

5. Tests on the final product

a) Sterility

Representative samples of the final product are collected and tested as in Section C.4.a.

b) Moisture content

The moisture content of the lyophilised attenuated vaccine should not exceed 3%.

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NB: There is an OIE Reference Laboratory for Rift Valley fever (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

RINDERPEST

SUMMARY

Classical rinderpest is an acute, viral disease of domestic cattle, buffaloes and yaks characterised by high morbidity and mortality rates. Sheep, goats, pigs and wild ungulates may also be affected. Clinically this form of the disease is characterised by pyrexia, the progressive development of shallow erosions on the gums, tongue, cheeks and hard palate, together with serous or mucopurulent ocular and nasal discharges. Alimentary tract involvement is marked by the development of diarrhoea or dysentery, leading to severe dehydration and depression. Rinderpest conforming to this description has not been seen since 2001 (Pibor, Southern Sudan). A milder form of the disease, with the potential to regain classical characteristics, used to occur in association with endemic situations, latterly in East Africa; it has not been positively diagnosed since 1997 (Tanzania) and could have died out, in which case wild-type rinderpest virus may no longer exist. Based on historical virus collections, three genetically distinct lineages of the virus have been recognised as causal agents of rinderpest disease in Africa and Asia. The Food and Agriculture Organisation of the United Nations (FAO) launched a Global Rinderpest Eradication Programme (GREP) in 1992, calling for eradication of the virus by the year 2010. The success of this programme may be judged by the fact that two of the three rinderpest lineages have now assuredly been eradicated and the third might well have joined them.

Identification of the agent: Clinical confirmation of classical rinderpest is based on the finding of individual or small groups of animals showing pyrexia, inappetance, depression, shallow erosions of the upper and lower lip and gum, erosions or blunting of the cheek papillae, serous or mucopurulent ocular discharges and/or nasal discharges, diarrhoea, recumbency and possibly death. Laboratory confirmation is based on demonstrating the presence of the virus, virus-specific RNA or precipitating antigens in samples from the spleen, lymph nodes, or ocular or nasal secretions of acutely infected animals. It is particularly important to isolate the virus if a geographical extension or significant animal health deterioration has occurred. Following the successes of global eradication, rinderpest-free countries may now confirm the presence of peste des petits ruminants (PPR) in sheep or goats based on the clinical appearance of affected animals and the presence of precipitating antigens, even though both the clinical signs and the virus-induced antigens are common to both viruses.

In cases where rinderpest is suspected, post-mortem examinations should pay particular attention to the abomasum, which may be highly engorged or show a grey discoloration; to the Peyer's patches, which may show lymphoid necrosis; and to the development of linear engorgement and blackening of the crests of the folds of the caecum, colon and rectum. The principal differential diagnoses are PPR in sheep and goats, and bovine viral diarrhoea/mucosal disease and malignant catarrhal fever in cattle; differentiation of these diseases requires the use of appropriate laboratory methods.

Serological tests: The OIE has developed a set of Recommended Standards for Epidemiological Surveillance for Rinderpest (the 'OIE Pathway') that governs the actions of Member Countries wishing to demonstrate that they have achieved freedom from infection. To this end, a competitive enzyme-linked immunosorbent assay (ELISA) has been described to determine the presence of rinderpest antibodies in animals that have been infected with field virus or received rinderpest vaccine. An indirect ELISA has also been described. Whatever test is used it should be sensitive with respect to the lineage of virus likely to be present and be highly specific. Neutralising antibody estimations may be used for the same purpose. Member Countries may wish to seek expert advice from an OIE Reference Laboratory or the GREP Secretariat with regard to the selection of the test most appropriate for their purpose.

Requirements for vaccines and diagnostic biologicals: *A live attenuated cell culture rinderpest vaccine is available. In recent years its use has been considerably curtailed, as the lifelong immunity it induces may interfere with post-campaign serological assessments aimed at a Country's efforts to gain a rinderpest-free accreditation. Given that the inadvertent use of this vaccine has given rise to confusing serosurveillance results, and that considerable quantities of it are still available, Member Countries should catalogue and secure all remaining stocks in order to safeguard the ability to undertake post-campaign serosurveillance.*

A. INTRODUCTION

In recent years the Global Rinderpest Eradication Programme (GREP) of the Food and Agriculture Organisation of the United Nations (FAO) has made enormous progress in organising and documenting the decline of rinderpest (13). Historically, the virus was widely distributed throughout Europe, Africa, and Asia; recently however, it has only occurred in Africa and Asia. Gene sequence analysis has shown that all known rinderpest isolates fall into one of three non-overlapping phylogenetic lineages, and in recent years it has been possible to describe the virus' distribution in lineage-specific terms. Thus, the so-called Asian lineage (lineage 3) was only ever recorded in Afghanistan, India, Iran, Iraq, Kuwait, Oman, Pakistan, Russia, Saudi Arabia, Turkey, Sri Lanka and Yemen. As a result of concerted and coordinated vaccination and surveillance campaigns, this virus lineage has failed to resurface since September 2000 (Pakistan). Although evaluations are not yet complete, it is almost certain that this virus has been successfully eradicated.

Rinderpest virus lineages 1 and 2 have only been recorded from Africa. Lineage 1 appears to have been distributed from Egypt to southern Sudan and eastwards into Ethiopia and into northern and western Kenya. On the other hand, lineage 2 has been recorded from both East and West Africa and at one time may have been distributed in a sub-Saharan belt running across the whole of the continent (12). Now however, as the result of further coordinated vaccination and surveillance programmes (Pan African Rinderpest Campaign in particular), neither West nor Central Africa have reported rinderpest since 1988 (Ghana/Burkina Faso). Until recently both lineages were being reported from eastern Africa, but it is now clear that lineage 1 was eliminated from southern Sudan in 2001 by intensive vaccination.

Reappearing in 1994, 1996 and 2001 in wildlife, Lineage 2 has been transmitting within the Somali pastoral ecosystem (9) where its continued presence caused considerable concern (10). In 1994, this virus reappeared in south-east Kenya where its effects were expressed most dramatically in buffaloes in Tsavo National Park (7) thereby illustrating its ability to engage in cryptic persistence for a period of at least 30 years, during which time it is likely to have been transmitted with a low level of virulence among susceptible cattle. Although this virus is now seen as having evolved to the point where it has been possible for it to escape veterinary attention in remote areas, its presence did not go unnoticed by the nomadic pastoralists whose cattle it infected. Nevertheless, it is clear that this virus has not been confirmed within this last reservoir since 2001 and although not yet an accredited achievement, it is more than likely that sporadic vaccination has broken the transmission chain of Lineage 2.

Rinderpest is caused by a negative-strand RNA virus of the Morbillivirus genus within the family Paramyxoviridae. Classic descriptions of rinderpest refer to it as a highly fatal disease of domestic cattle, buffaloes and yaks. The virus also affects some breeds of pigs and a very large variety of wildlife species within the order Artiodactyla, although not always in a clinically apparent form; a recent review views sheep and goats as susceptible but largely epidemiologically unimportant hosts of rinderpest (14).

Although in its final stages of eradication, some strains of rinderpest had evolved into a mild, nonfatal, infectious disease of cattle, all strains retained two very dangerous attributes. The first was an almost certain ability to undergo virulence modulations. The second was an ability to infect game animal species and, in buffaloes, eland, giraffe, lesser kudu and warthog, to cause an acute infection associated with high levels of mortality.

Classical rinderpest has an incubation period of between 1 and 2 weeks, the clinical disease is characterised by an acute febrile attack within which prodromal and erosive phases can be distinguished. The prodromal period lasts approximately 3 days, during which affected animals develop a pyrexia of between 40 and 41.5°C together with partial anorexia, constipation, congestion of visible mucosae, serous ocular and nasal discharges, depression and drying of the muzzle. However, it is not until the onset of the erosive phase, and the development of necrotic mouth lesions, that a tentative clinical diagnosis of rinderpest can be made. At the height of fever, flecks of necrotic epithelium appear on the lower lip and gum and in rapid succession may appear on the upper gum and dental pad, on the underside of the tongue, on the cheeks and cheek papillae and on the hard palate. Through the enlargement of existing lesions and the development of new foci, the extent of the oral necrosis can increase dramatically over the following 2–3 days. Much of the necrotic material works loose giving rise to shallow, non-haemorrhagic mucosal erosions.

Diarrhoea is another characteristic feature of rinderpest and develops 1–2 days after the onset of mouth lesions. The diarrhoea is usually copious and watery at first, but later on may contain mucus, blood and shreds of epithelium and it may be accompanied, in severe cases, by tenesmus. During the erosive phase, necrosis may be observed in the nares, in the vulva and vagina, and on the preputial sheath. Anorexia develops, the muzzle dries out completely, the animal is depressed, the breath is fetid and mucopurulent ocular and nasal discharges develop.

Deaths will occur but the mortality rate will be variable and may be expected to rise as the virus gains progressive access to large numbers of susceptible animals. Initial mortality rates will probably be in the order of 10–20% and, in the terminal stages of the illness, animals may become recumbent for 24–48 hours prior to death. Some animals die while showing severe necrotic lesions, high fever and diarrhoea, others after a sharp fall in body temperature, often to subnormal values. Alternatively, the pyrexia may remit slightly in the middle of the erosive period and then, 2–3 days later, return rapidly to normal accompanied by a quick resolution of the mouth lesions, a halt to the diarrhoea and an uncomplicated convalescence.

Typically the carcass of the dead animal is dehydrated, emaciated and soiled. The nose and cheeks bear evidence of mucopurulent discharges, the eye is sunken and the conjunctiva congested. In the oral cavity, there is often extensive desquamation of necrotic epithelium, which always appears sharply demarcated from adjacent areas of healthy mucosa. The lesions frequently extend to the soft palate and may also involve the pharynx and the upper portion of the oesophagus; the rumen, reticulum and omasum are usually unaffected, although necrotic plaques are occasionally encountered on the pillars of the rumen. The abomasum, especially the pyloric region, is severely affected and shows congestion, petaechiation and oedema of the submucosa. Epithelial necrosis gives the mucous membrane a grey colour. The small intestine is not commonly involved except for striking changes to the Peyer's patches where lymphoid necrosis and sloughing leaves the supporting architecture engorged or blackened. In the large intestine changes involve the ileocaecal valve, the caecal tonsil and the crests of the longitudinal folds of the caecal, colonic and rectal mucosae. The folds appear highly engorged in acute deaths or darkly discoloured in long-standing cases; in either event the lesions are referred to as 'zebra striping'.

Taking the lineage 2-associated form of rinderpest as an example of the mild form adopted by the virus within endemic situations, the incubation period is between 1 and 2 weeks and the ensuing clinical disease is little more than a subacute febrile attack in cattle. The fever is not invariable; it is short-lived (3–4 days) and not very high (38–40°C). The depression that characterises more acute forms of rinderpest is absent from mildly affected animals and, as a result they might not lose their appetite, they will probably continue to graze, water and trek as well as unaffected animals. These animals do not usually develop diarrhoea. On close examination there may be some slight congestion of the visible mucous membranes and small, focal areas of raised, whitish epithelial necrosis may be found on the lower gum – sometimes no larger than a pin head – along with a few eroded cheek papillae. Some animals may escape the development of such erosions, the appearance of which is fleeting. Other animals may show a slight, serous, ocular or nasal secretion but, in contrast to the more severe forms of the disease, these do not progress to become mucopurulent.

Even though infections with lineage 2 may pass unnoticed in cattle, the virus is highly infectious for wildlife species, and among those generally regarded as highly susceptible (buffalo, giraffe, eland, and lesser kudu) it causes fever, a nasal discharge, typical erosive stomatitis, gastroenteritis, and death. Kock (7) observed that in addition, buffaloes infected with lineage 2 showed enlarged peripheral lymph nodes, plaque-like keratinised skin lesions and keratoconjunctivitis. Lesser kudus were similarly affected, but whereas blindness – caused by a severe keratoconjunctivitis – was common, diarrhoea was unusual. Eland also showed necrosis and erosions of the buccal mucosa together with dehydration and emaciation. Therefore, under the present circumstances, a diagnosis of rinderpest in any of these species points to the likelihood of the simultaneous transmission of the virus, even at a subclinical level, in neighbouring cattle.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

In view of the high level of expectation surrounding the Global Rinderpest Eradication Campaign, any recent outbreak of rinderpest would be of immense epidemiological significance, not only as a threat of pandemic eruption but as a indicator of a lacuna in global surveillance. Consequently, until a country has reached an accredited rinderpest infection free status (based on serosurveillance), samples from all outbreaks considered rinderpest-like on clinical or pathological grounds must be routinely submitted for laboratory examination. For rinderpest, a variety of suitable laboratory tests is available, but under the circumstances outlined above it is of paramount importance to isolate the virus, identify its lineage and assess its virulence in experimental cattle (1). Blood in anticoagulant is the preferred sample wherever possible. On average, the onset of viraemia slightly precedes the onset of pyrexia, and may continue for 1–2 days after pyrexia begins to wane. Consequently, animals showing a pyrexia are probably viraemic and therefore the best source of blood with which to attempt

virus isolation. However, as occasional febrile animals may no longer be viraemic, samples from several febrile animals should be collected for submission. It is important to ensure that there is adequate tissue available for at least two virus isolation attempts from the initial submission of a suspected outbreak. The other procedures described should only be attempted if there is extra tissue available.

a)   Virus isolation

Rinderpest virus can be cultured from the leukocyte fraction of whole blood that has been collected into heparin or EDTA (ethylene diamine tetra-acetic acid) at final concentrations of 10 international units (IU)/ml and 0.5 mg/ml, respectively. Samples should be thoroughly mixed and transferred to the laboratory on ice, but never frozen. Virus can also be isolated from samples of the spleen, prescapular or mesenteric lymph nodes of dead animals; these samples may be frozen for transportation.

To isolate the virus from blood, uncoagulated blood is centrifuged at 2500 **g** for 15 minutes to produce a buffy coat layer at the boundary between the plasma and erythrocytes. This is removed as cleanly as possible, mixed in 20 ml physiological saline and recentrifuged in a washing procedure designed to remove any neutralising antibody present in the plasma. The resulting cell pellet is suspended in cell culture maintenance medium and 2 ml aliquots are distributed on to established roller tube monolayers of primary calf kidney, B95a marmoset lymphoblastoid or African green monkey kidney (Vero) cells. The culture maintenance medium should be decanted and replaced every 2 or 3 days and the monolayer observed microscopically for the development of cytopathic effects (CPE). These are characterised by refractility, cell rounding, cell retraction with elongated cytoplasmic bridges (stellate cells) and/or syncytial formation. The speed with which the CPE develops varies by substrate and probably by strain of virus also. Up to 12 days should be allowed in primary cells, a week in Vero and 2–4 days in B95a cells. Blind passages may be attempted before declaring an important sample negative, but a preferable technique would be to inoculate the cell suspension, and any residue of the original sample, intravenously into a rinderpest-susceptible ox and attempt to re-isolate the virus from its blood. Isolates of virus can be partially identified by the demonstration of morbillivirus-specific precipitinogens in infected cell debris, or completely identified by the demonstration of specific immunofluorescence using a conjugated monoclonal antibody (MAb).

Alternatively, 20% suspensions (w/v) of lymph node or spleen may be used. These should be made by macerating the solid tissues in serum-free culture maintenance medium using standard grinding or shearing techniques and inoculating monolayers as before. The release of virus from solid tissue can be achieved in several ways. Perhaps the easiest is with a pestle and mortar, but this technique requires the use of sterile sand as an abrasive. Alternatively, tissue may be ground without an abrasive using all-glass grinders, for example, a Ten Broeck grinder. Shearing techniques are equally applicable using, for example, Silverson or Waring blenders. Virus-containing suspensions are clarified by low-speed centrifugation. The volume of the inoculum is not critical; a working volume is between 1 and 2 ml. Commonly used antibiotics are penicillin and streptomycin in combination, each at a concentration of 100 IU/ml. A similar broad-spectrum cover can be obtained using neomycin at 50 µl/ml. Fungizone should be included at 2.5 µg/ml.

b)   Antigen detection by agar gel immunodiffusion

The agar gel immunodiffusion (AGID) tests may be conducted in Petri dishes or on glass microscope slides (5). In either instance the surface should be covered with agar to a depth of about 4 mm using a 1% aqueous solution of any high quality agar or agarose. Wells are usually cut in a hexagonal pattern of six peripheral wells around a single central well. For slides, wells should be 3 mm in diameter and 2 mm apart. For Petri dishes, the wells can be increased to 4 mm in diameter and the distance between wells to 3 mm. The closer the wells are placed from each other, the shorter the reaction time.

Using a small volume pipette, rinderpest hyperimmune rabbit serum should be placed in the central well. Similarly, control positive antigen, prepared from the macerated lymph nodes of rabbits infected with the Nakamura III lapinised strain of rinderpest, should be placed in alternate peripheral wells (i.e. one, three and five). Negative control antigen is placed in well four. Test antigens are obtained as exudates from the cut surface of spleen or lymph nodes submitted for testing; if no exudate can be obtained a small portion of the sample should be ground with a minimum of saline. Ocular exudates may be squeezed directly from the swabs or, alternatively, by compression in a microtip (the cotton wool should be cut off the swab and placed into the wide end of a plastic 50–250 µl pipette tip; the stem of the swab may then be used to compress the cotton wool and force a small volume of exudate out of the narrow end of the tip). Test samples are added to wells two and six. Tests are best developed at 4°C or low ambient temperatures. The reaction area should be inspected from 2 hours onwards for the appearance of clean, sharp lines of precipitation between the wells forming a line of identity with the controls. Tests should be discarded after 24 hours if no result has been obtained. The result is not acceptable unless precipitation reactions are also obtained giving a line of identity with the control positive antigen preparation.

Although the test is neither highly sensitive nor highly specific, it is robust and adaptable to field conditions. A positive reaction from a large domestic ruminant should be treated as if it were rinderpest. From a small

ruminant, a positive result should be treated as having been derived from a case of rinderpest or peste des petits ruminants (PPR) and requiring further differentiation.

c) Histopathology and immunohistochemistry

At post-mortem examination, tissues should be collected and placed in 10% neutral buffered formalin for histopathology and immunohistochemistry; the base of the tongue, retropharyngeal lymph node and third eyelid are suitable tissues. Sections stained with haematoxylin and eosin should be examined for the presence of syncytial cell formation, and cells with intranuclear viral inclusion bodies. The presence of rinderpest antigens can be demonstrated in the same formalin-fixed tissues by immunoperoxidase staining following the quenching of endogenous peroxidase activity. If a polyclonal antiserum is used, this test will fail to differentiate between rinderpest and PPR. However, this problem can be circumvented by using monoclonal antibodies specific for rinderpest and PPR in duplicate tests (3).

d) Lineage identification using the reverse-transcription polymerase chain reaction

The reverse-transcription polymerase chain reaction (RT-PCR) (6) produces DNA suitable for gene sequence analysis. Viral RNA can be purified from spleen (not ideal due to its high blood content), lymph node and tonsil (ideal), peripheral blood lymphocytes (PBLs), or swabs from eyes or mouth lesions (contingent). Solid tissues (0.5–1.0 g) are minced and homogenised with 4.0 ml denaturing solution, eye and mouth swabs are treated with 1.0 ml, and purified PBLs (from 5 to 10 ml whole blood) are treated with 0.4 ml according to the published procedure. Solution D (disruption solution): the procedure is that recommended to minimise the hazard of handling poisonous guanidium thiocyanate. It should be carried out in a chemical safety hood. The following are the amounts of guanidium thiocyanate for a 250 g bottle, but the volumes can be adjusted for other quantities. Do not attempt to weigh out the guanidium thiocyanate, but dissolve it in the manufacturer's bottle by adding 293 ml sterile distilled water, 17.6 ml 0.75 M sodium citrate, pH 7.0, and 26.4 ml 10% sarcosyl, then heat to 65°C in a water bath to dissolve. This solution can be kept for several months in the dark at room temperature in a chemical safety cabinet. The final solution D is made by the addition of 0.36 ml 2-mercaptoethanol to 50 ml of the stock solution. This solution should not be kept for more than 1 month. In the past few years, RNA extraction spin columns have become widely used for fast purification of high quality RNA (RNeasy kit, Qiagen) The resulting RNA is precipitated with 2.5 volumes of ethanol, washed in 70% ethanol, dissolved in sterile water, or TE buffer (Tris/EDTA, 10 mM, pH 7.5, 1 mM EDTA) and stored at –70°C or –20°C until required. The cDNA synthesis is carried out using random hexanucleotide primers to enable several different specific primer sets to be used in the PCR amplification step. Aliquots of the resulting cDNA are amplified using at least three primer sets that can detect and differentiate between the two morbilliviruses. These primer sets include two 'universal' sets based on highly conserved regions in the phosphoprotein and nucleoprotein genes that should detect all morbilliviruses, and rinderpest virus-specific sets based on sequences in the fusion protein genes of the virus. The PCR products are analysed on a 1.5% (w/v) agarose gel along with a suitable DNA marker to identify the specific DNA product. A positive control such as measles or canine distemper virus RNA, and a negative control using sterile distilled water instead of RNA, must be included in each RT-PCR. Positive reactions should be confirmed either by using 'nested' primer sets based on the F gene sequences or by sequence analysis of the DNA product. It is important to use more than one set of primers for the PCR step when testing for the presence of RNA viruses, as their nucleotide sequences can vary significantly and one change at the 3'-end of the primer sequence may result in failure of the primers to amplify the DNA. The World Reference Laboratory in the United Kingdom (UK), which is also an OIE Reference Laboratory for rinderpest, and the OIE Reference Laboratory in France (see Table given in Part 3 of this *Terrestrial Manual*), can advise on use of the technique for field sample analysis.

Most recently, a simple Taqman real-time RT-PCR assay for RPV diagnostic has been described.This real-time RT-PCR assay for rinderpest virus has been validated to be highly sensitive in infected tissue culture supernatant and clinical samples from experimentally infected cattle. The assay has proved to be able to detect isolates representative of all known phylogenetic lineages of the virus and clearly differentiate from PPR virus and other look-alike diseases (foot and mouth disease virus, bovine viral diarrhoea virus, bovine herpesvirus, vesicular stomatitis virus). The analytical sensitivity of the L10 primer-probe system exceeded 1–100 $TCID_{50}$ (50% tissue culture infective dose)/ml, depending on the rinderpest virus strain. Comparison of samples from experimentally infected animals showed that white blood cells and conjunctival swabs are the sample of choice for epidemiological surveillance of the disease, allowing the preclinical detection of the disease by 2–4 days. In the event of a rinderpest virus outbreak, this portable, single-tube format, real-time RT-PCR has the capability of preclinical diagnosis, thus aiding efforts to prevent further transmission of disease.

e) Differential immunocapture ELISA

Neither clinical observations nor AGID tests can differentiate between rinderpest and PPR; consequently, if either disease is suspected in sheep or goats in countries where both diseases occur, other tests like the real-time PCR must be used. Rapid differentiation can be achieved using a differential immunocapture ELISA test (8). This test employs MAbs directed against the N protein of the two viruses. One MAb, with a

reactivity against both viruses, is used as a capture antibody, while a second biotinylated MAb specific for a nonoverlapping antigenic N protein site, and specific against either rinderpest or PPR, is used to determine which N protein has been captured.

High protein-binding ELISA plates (or strips) are coated with 100 µl/well of capture antibody. After three washes, the wells are loaded with 50 µl of test sample diluted 1/10 in a lysis buffer, 25 µl of the manufacturer's recommended dilution of the virus-specific MAb and 25 µl of streptavidin peroxidase at a final dilution of 1/3000. The wells are then placed on an orbital shaker for 1 hour at 37°C, after which time they are again washed; following the addition of 100 µl of ortho-phenylenediamine (OPD), the wells are re-incubated at room temperature for 10 minutes. Reactions are halted by the addition of 100 µl of 1 N sulphuric acid, and the results, measured at 492 nm with an automated ELISA reader, are expressed as absorbance values.

f) Chromatographic strip test

While not a definitive diagnostic test, a rapid chromatographic strip test (penside test; ref. 4) has proved a useful tool for assisting field personnel in investigating suspected outbreaks of rinderpest.

2. Serological tests

a) The competitive enzyme-linked immunosorbent assay (the prescribed test for international trade)

A competitive ELISA is available for the detection of rinderpest antibodies in the serum of animals of any species previously exposed to the virus. The test is based on the ability of positive test sera to compete with a rinderpest anti-H protein MAb for binding to rinderpest antigen. The presence of such antibodies in the test sample will block binding of the MAb, producing a reduction in the expected colour reaction following the addition of enzyme-labelled anti-mouse IgG conjugate and a substrate/chromogen solution. As this is a solid-phase assay, wash steps are required to ensure the removal of unbound reagents.

The rinderpest antigen is prepared from Madin–Darby bovine kidney cell cultures infected with the attenuated Kabete 'O' strain of rinderpest virus. The viral antigen is extracted from the infected cells by repeated cycles of sonication and centrifugation. The MAb was obtained by fusing the splenocytes of hyperimmunised mice with the NSO myeloma cell line, and then shown to be rinderpest H protein specific (2); this MAb has now been designated as C1. Both C1 and standardised rinderpest antigen are directly available from the OIE Reference Laboratory for Rinderpest in the UK (see Table given in Part 3 of this *Terrestrial Manual*). Kits are available commercially.

• **Test procedure**

i) Reconstitute the freeze dried rinderpest antigen with 1 ml of sterile water and further dilute it to the manufacturer's recommended working dilution using 0.01 M phosphate buffered saline (PBS), pH 7.4.

ii) Immediately dispense 50 µl volumes of the diluted antigen into an appropriate number of wells of a flat-bottomed, high protein-binding ELISA microplate using two wells per test serum. Tap the sides of the microplate to ensure that the antigen is evenly distributed over the bottom of each well and, having sealed the plate, incubate it on an orbital shaker for 1 hour at 37°C. Wash the wells three times with 0.002 M PBS, pH 7.4.

iii) Add 40 µl of blocking buffer (0.01 M PBS, 0.1% [v/v] Tween 20 and 0.3% [v/v] normal bovine serum) to each test well followed by 10 µl volumes of all test sera.

iv) Follow the manufacturer's recommendations to prepare a working dilution of the MAb in blocking buffer, and add 50 µl of this to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.

v) Follow the manufacturer's recommendations to prepare a working dilution of rabbit anti-mouse immunoglobulin horseradish peroxidase conjugate in blocking buffer and add 50 µl to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.

vi) At the end of this period the plates are washed as before and immediately refilled with 50 µl volumes of substrate/chromogen mixture (1 part 3% H_2O_2 to 250 parts OPD), and incubate at room temperature for 10 minutes without shaking. Then add 50 µl of a stopping solution consisting of 1 M sulphuric acid.

vii) The test system must include known rinderpest positive and negative serum samples, a MAb control and a conjugate control.

viii) Measure the resulting absorbance values on an ELISA reader with a 492 nm interference filter and express the test results as percentage inhibition values compared with the value obtained using the

Lowering the positive/negative threshold to 40% or less increases the sensitivity of the test, but inevitably affects specificity by increasing the proportion of false-positive test results encountered. In practise, the 50% value is recommended by GREP at which level sensitivity is at least 70% and specificity exceeds 99%. The sensitivity needs to be taken into account when designing sampling frames for serosurveillance.

An indirect ELISA method has been developed and might be useful for rinderpest surveillance programmes, especially in areas in which lineage 2 rinderpest virus could be present (17). However, the performance characteristics of the test indicate a problem with specificity and therefore its use will require confirmatory testing.

b) Virus neutralisation

The 'gold standard' virus neutralisation (VN) test is performed in roller-tube cultures of primary calf kidney cells following the method of Plowright & Ferris (11); the test has been validated in experimentally infected cattle. In the roller tube procedure, sera, that has not been inactivated, are diluted at intervals of 1 in 10 and then, starting with undiluted serum, mixed with an equal volume of $10^{3.0}$ TCID$_{50}$ per ml of the attenuated Kabete 'O' vaccine strain virus. Mixtures are held overnight at 4°C, after which 0.2 ml volumes are inoculated into each of five roller tubes, immediately followed by 1 ml of dispersed indicator cells suspended in growth medium at a rate of 2×10^5 cells per ml. Tubes are incubated at 37°C, sloped for the first 3 days, after which they are replenished with maintenance medium and placed on a roller apparatus. They are examined regularly for virus-specific cytopathology and positive tubes recorded and discarded; the final examination takes place on day 10.

For calculating end-points, the virus dose is regarded as satisfactory if the final dilution falls within the range $10^{1.8}$ to $10^{2.8}$ TCID$_{50}$/tube. This test should be used to examine the sera of ELISA reactors during national serosurveillance programmes designed to demonstrate freedom from infection, or to qualify susceptible cattle for vaccine testing. Under these circumstances, the presence of any detectable antibody in the 1/2 final serum dilution is considered to indicate previous infection with rinderpest virus. The VN test is the test of choice for the examination of wildlife serum samples.

A microplate method may be used as a screening test. In this procedure, an initial serum dilution of 1/5 is further diluted at twofold intervals. Thereafter, 50 µl volumes of serum are incubated with 50 µl volumes of virus diluted to contain between $10^{1.8}$ and $10^{2.8}$ TCID$_{50}$ (15). Following a 45-minute or an overnight incubation period, between 1 and 2×10^5 calf kidney, lamb kidney or Vero cells are added as indicators. Tests are terminated after 6 or 7 days. Such tests may give indications of non-specific neutralisation at high serum concentrations. There appear to be factors in some normal (with respect to prior rinderpest exposure) sera that bring about the failure of the virus to penetrate and replicate in indicator cells. In the tube test, these factors were probably removed during changes in maintenance medium; in the microplate method, they remain present the whole time. If the most concentrated final serum dilution is limited to 1/10, the effect disappears.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Many countries have used rinderpest vaccine to reduce the incidence of rinderpest to zero, and then followed the OIE Pathway in order to have their rinderpest-free status internationally recognised. To obtain this status, the process of annual rinderpest vaccination has largely been replaced by active and passive clinical and serological surveillance. Intensive focal vaccination with homologous vaccine (immunosterilisation) was retained for dealing with emergency campaign management (16).

The live attenuated tissue culture rinderpest vaccine (TCRV) described in previous editions of this *Terrestrial Manual* was developed by Plowright by the serial passage of the virulent bovine rinderpest strain Kabete 'O' (RBOK) in primary bovine calf kidney cells. Due to the success of the Global Rinderpest Eradication Programme, it is believed that few vaccine manufacturers continue to make this product, although a number of them may be storing considerable stocks. However, the description published in the previous edition of the *Terrestrial Manual* will be repeated here so that it is available if conditions change.

1. Seed management

a) Characteristics of the seed

Seed lots used in the manufacture of TCRV must produce a cell-culture vaccine that is safe, that confers an immunity in cattle lasting at least 5 years, that retains its attenuated characteristics during at least five back

passages in cattle, and that lacks the ability to spread by contact. Substrains of RBOK used in the manufacture of TCRV must be identifiable by written historical records, which must include information on the origin of the strain and of its subsequent manipulations.

b)   Method of culture

Vaccine seed must be maintained in a seed-lot system between passage levels 90 and 120. Seed-lot virus must be preserved in a freeze-dried state at a temperature of –20°C or lower. The virus must be cultured in Vero cells or primary or serially cultivated kidney cells derived from a normal bovine foetus or a very young calf. Serially cultivated cells may not be more than ten passages removed from the primary cultivation.

c)   Validation as a vaccine

Seed lots must be shown to be:

i)   *Pure:* Free from contamination with viruses, bacteria, fungi or mycoplasmas.

ii)   *Safe:* Inducing no abnormal clinical reaction on inoculation into rinderpest-susceptible cattle.

iii) *Efficacious:* Inducing an immunity to rinderpest in rinderpest-susceptible cattle.

2.   Method of manufacture

Individual vaccine batches are prepared by infecting cell cultures and, after an appropriate incubation period, harvesting the overlying media into which large numbers of live virus particles have been released. To facilitate long-term storage and cold-chain distribution, this fluid is freeze dried in the presence of a cryoprotectant consisting of 5% lactalbumin hydrolysate and 10% sucrose. Virus may be grown in primary kidney cells from bovine embryos or calves, or cells derived in a homogeneous manner by up to ten serial subcultures from either of these sources. In addition, vaccine may be manufactured in approved continuous cell lines provided the cells are known to be non-infected with bovine viral diarrhoea (BVD) virus and are maintained in a seed lot system; Vero cells have been used for this purpose. To constitute a batch, infected cultures must have been inoculated with the same seed virus and incubated and harvested together. Two harvests are permissible from the same set of cultures and may be pooled to form a bulk suspension. Written records must accompany all stages of vaccine manufacture.

3.   In-process control

Cells: Primary cells, serially cultivated primary cells or continuous cell lines must have been derived from normal looking animals or embryos, and must retain a normal morphology during cultivation. They must be shown to be free of contamination with adventitious viruses, particularly BVD virus. Whatever cells are committed for vaccine production, uninfected control cultures must be maintained using the same media and incubation conditions as the rinderpest-infected cells. They must be subjected to frequent microscopic observations. After harvesting the vaccine, the control cultures should be washed to remove ox serum and re-incubated for 10 days in media containing ox serum substitutes. They are again subject to frequent microscopic observations for evidence of cytopathic change. Simultaneously a sample of the cultures should be examined for the presence of noncytopathic BVD virus using an immunofluorescence or immunoperoxidase test or RT-PCR. The serum used in the culture media must come from rinderpest-susceptible animals.

Virus: A virus titration must be undertaken on the seed lot using tenfold virus dilutions in a microplate or roller tube system and employing ten replicates per dilution. A similar titration must be undertaken on the final bulk. Virus should be derived from cultures maintained in roller bottles and may not be harvested more than 10 days after the date that these cultures were infected. The harvest should be clarified by low-speed centrifugation before mixing with cryoprotectant. Prior to lyophilisation it may be held for not more than 5 days at 4°C but for considerably longer if frozen at –20°C to –60°C. As adventitious viral contamination may arise during a manufacturer's manipulations or from the use of contaminated media, rabbit hyperimmune serum should be used to neutralise the rinderpest content of the bulk suspension, after which the mixture should be used to infect calf kidney or Vero cells, which are handled as described above. The final bulk must be tested for freedom from bacteria, fungi and mycoplasmas.

4.   Batch control

a)   Identity

The contents of one container from each filling lot must be exposed to neutralisation by rabbit rinderpest antiserum, using a varying virus/constant serum method, and inoculated into bovine kidney cells. The identity of the product is established if no rinderpest-specific CPE develop.

b) **Sterility**

Tests for sterility and freedom of contamination of biological materials may be found in Chapter 1.1.9.

c) **Safety and efficacy**

Using rinderpest susceptible cattle, the contents of five randomly selected vials are pooled and used to inoculate one ox with a volume equivalent to 100 cattle field doses and one ox with a volume equivalent to 1/10th of a cattle field dose. These animals are maintained in close contact with an uninoculated ox for the following 3 weeks. During this period the animals are subjected to daily temperature recording and frequent clinical inspections. At the end of the 3 weeks, the cattle are examined for rinderpest neutralising antibodies and challenged with a strain of rinderpest capable of inducing a pyrexia. The vaccine is considered safe and efficacious if it does not induce any abnormal clinical reaction, if both animals receiving vaccine are protected and if there is no evidence that the vaccine virus has been transmitted. This test is not a potency test. Each vaccine lot must also be tested for innocuity in small animals.

d) **Potency**

The close relationship between immunising potency and infectivity allows the latter to be used as the basis for potency estimations. Three infectivity titrations are undertaken using cells of an approved continuous line or cells grown from each of three different bovine calf or embryonic kidneys. For the first titration, the pool of vials used for the safety test may be employed. The second and third estimates are made on further pools, each of three final containers. The sensitivity of the cells used in each working session must be measured using a standard laboratory rinderpest virus preparation. The final titre is the geometric mean of the three estimates, each undertaken using tenfold dilutions and ten observations per dilution.

e) **Duration of immunity**

It is unnecessary to routinely establish the duration of immunity to TCRV. Reported results indicate that lifelong immunity can be expected following the successful vaccination of cattle free of all vestiges of maternal immunity.

f) **Stability**

TCRV is highly stable when correctly freeze-dried and will keep for long periods at either +4 or –20°C provided the product is stored under vacuum. Recent evidence indicates that the rate of degradation of lyophilised TCRV can be altered by the choice of stabiliser and by variations in the drying cycle. The most advantageous results were associated with the use of a 5% lactalbumin hydrolysate/10% sucrose stabiliser, a 72–74 hour drying cycle under reduced vacuum (100 milliTorr), initial drying for 16 hours at –30°C, and a final shelf temperature of 35°C. With high release titres, such vaccine can be used in the field for 30 days without refrigeration. Following reconstitution in either normal saline or 1M magnesium sulphate, the virus becomes much more thermolabile. The period for field distribution of reconstituted vaccine should not exceed its half-life, but as this parameter is temperature dependent and varies between 8 and 24 hours over a range from 4°C to 37°C, a common sense limit must be applied; this can be determined by National Control Authorities, but a universal period of 4 hours can be recommended.

g) **Preservatives**

TCRV contains lactalbumin hydrolysate and sucrose which are added as cryoprotectants; otherwise it contains no specific chemical preservative.

h) **Precautions (hazards)**

There are no known hazards associated with the manufacture or field use of TCRV.

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NB: There are OIE Reference Laboratories for Rinderpest (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

TRICHINELLOSIS

SUMMARY

Trichinellosis in humans is caused by eating raw or undercooked meat from Trichinella-*infected domestic animals or game. The adult worms survive less than 2 months in the small intestine of host species including humans, pigs, rats, bears, walruses, occasionally in horses and many other flesh-eating mammals, and birds and reptiles.* Trichinella *larvae occur in the muscles of their hosts and susceptible individuals become infected by ingestion of tissue containing these larvae.*

Identification of the agent: *Diagnostic tests for trichinellosis fall into two categories: 1) direct detection of first-stage larvae encysted or free in striated muscle tissue, and 2) indirect detection of infection by tests for specific antibodies.*

Tissue digestion and tissue compression methods have been used for the direct detection of Trichinella *larvae in tissues.* Trichinella *larvae usually localise in preferred muscle sites, particularly in low level infections, and these sites may vary by host species. It is important that preferred sites be sampled to maximise test sensitivity. For example, in pigs, the diaphragm (*crus*), tongue, masseter and abdominal muscles are preferred sites, whereas in horses, the tongue and masseter harbour the most worms, followed by the diaphragm and neck muscles.*

*The artificial digestion methods involve the enzymatic digestion of individual or pooled muscle tissue samples and incorporate mechanical homogenisation or grinding, stirring, and incubation. This is followed by filtration and sedimentation procedures to recover and concentrate any larvae that are released from muscle during digestion. Samples processed by these methods are examined under a stereomicroscope for the presence of larvae. Digestion tests can detect <1 larva per gram (*lpg*) of tissue, but at these low levels of infection, uneven distribution of larvae within tissues is a limiting factor. This is compensated for by testing larger samples per carcass, such as a minimum of 1–5 g for pigs and 5–10 g for horses and game. Digestion methods are recommended for the inspection of individual carcass of food animals such as pigs, horses and game.*

The compression method is less sensitive than artificial digestion and is not recommended as a reliable test for inspection of carcasses. Although now outdated, this method has been used widely in the past and is mentioned here for completeness. It involves visual inspection of compressed pieces of muscle tissue for the presence of larvae in situ*. This method can be performed with a stereomicroscope, or a specialised microscope, (*trichinoscope*) which has an estimated efficiency of detecting as few as three larvae/g of tissue. It has the disadvantage of requiring considerable time for the inspection of multiple samples from each carcass. It is also very difficult to detect the non-encapsulated* Trichinella *larvae (T.* pseudospiralis*, T.* papuae *and T.* zimbabwensis*). Compression techniques are only useful for detecting medium to high infections when few animals require examination and facilities are not available for testing by artificial digestion.*

Serological tests: *Serological assays are the most common tests used for indirect detection. The sensitivity and specificity of serological methods are mainly dependent upon the type and quality of antigen used. Most serological test performance (*validation*) data are from pigs. False negative serological results may occur 3 weeks or longer after muscle larvae become infective in pigs with light or moderate infections. A low rate of false-positive results has also been reported for serological tests. For the purposes of individual carcass inspection, only direct methods can be recommended. For surveillance or verification of* Trichinella-*free herds or regions, serological methods are acceptable. Pigs harbouring as few as one larva/100 g of tissue have been detected. The specificity of enzyme-linked immunosorbent assays (*ELISA*) for* Trichinella *infection is directly*

linked to the type and quality of the antigen employed in the test. Secretory antigens collected by short-term (18–20 hours) maintenance of T. spiralis *muscle larvae in vitro and synthetic carbohydrate antigens currently provide the most specific and economical source, although a low rate of false-positive results has been obtained in some studies. It is critical that appropriate positive and negative control sera be used to ensure that ELISAs are performing at a minimum acceptable level of sensitivity and specificity. The digestion of 100 g or more of tissue is recommended as a confirmatory test for serologically positive animals.*

Requirements for vaccines and diagnostic biologicals: *There are no suitable vaccines for Trichinella infection in food animals. For indirect (serological) detection methods, appropriate antigens must be used to ensure adequate test specificity and sensitivity. These antigens may be obtained from the secretory products of muscle larvae maintained* in-vitro. *There is a critical need for an international bank of reference sera to provide a common standard for* Trichinella *serological assays.*

A. INTRODUCTION

Trichinellosis in humans is caused by eating raw or undercooked meat from *Trichinella*-infected food animals or game (10). The short-lived adult worms live in the small intestine of host species including humans, pigs, rats, bears, walruses, horses, many other flesh-eating mammals, and some birds and reptiles. The parasite has a direct life cycle. Within hours following consumption of infected muscle by a suitable host, first stage muscle larvae (L1) are released by digestion and burrow into the villi of the small intestine. They develop rapidly into adults (males up to 1.8 mm long, females up to 3.7 mm long) and survive for less than 2 months. During this time, copulation takes place and the ovo-viviparous females release new-born larvae (NBL), which migrate via venules and lymphatics into the general circulation. NBL are distributed throughout the body where they invade striated muscles, showing predilection for specific muscle groups. For example, in pigs, the tongue usually contains the highest concentration of larvae, followed by the diaphragm, and in horses, the tongue followed by masseter muscle. Predilection sites vary by host species, but in general, tongue, masseter and diaphragm are optimal sites for sampling. Current knowledge on predilection sites is available for several host species (22). In cases of severe infection most voluntary muscles contain high numbers of larvae. The larvae of most *Trichinella* species become encapsulated in collagen in host musculature where they remain infective for years.

Within the genus *Trichinella* eleven genotypes have been identified, eight of which have been designated species status (10, 21, 27). *Trichinella spiralis* (T-1) is distributed in temperate regions world-wide and is commonly associated with domestic pigs. It is highly infective for domestic and sylvatic swine, mice and rats, but it can be also be detected in other mammalian carnivores. *Trichinella nativa* (T-2) occurs in mammalian carnivores of arctic and sub-arctic regions of North America, Europe and Asia. *Trichinella britovi* (T-3) is found predominantly in wild animals, and occasionally in pigs or horses. It occurs in temperate regions of Europe, Asia, and in Northern and Western Africa. *Trichinella pseudospiralis* (T-4) is cosmopolitan in distribution and has been recovered from raptorial birds, wild carnivores and omnivores, including rats and marsupials in Asia, North America, Europe and Australia. Unlike most other *Trichinella* genotypes, T-4 is not enclosed within a collagen capsule in muscle. *Trichinella murrelli* (T-5) is found in mammalian carnivores of North America. It has low infectivity for domestic pigs, but poses a risk to humans who eat game meats. *Trichinella* T-6 is cold-climate-adapted and appears to be closely associated with *T. nativa* in northern North America (27). Both *T. nativa* and T-6 are highly resistant to freezing. They have limited infectivity for pigs. *Trichinella nelsoni* (T-7) has been isolated from mammalian carnivores and sporadically from wild pigs in Eastern Africa. *Trichinella* T-8 has been detected in mammalian carnivores in Namibia and South Africa and *Trichinella* T-9 in mammalian carnivores in Japan (27). T-8 and T-9 have some intermediate characteristics with *T.* britovi and *T. murrelli*, respectively. Like *T. pseudospiralis*, *T. papuae* (T-10) and *T. zimbabwensis* (T-11) are non-encapsulated muscle parasites. *Trichinella papuae* has been reported from wild and domestic pigs, farmed crocodiles and humans in Papua New Guinea. *Trichinella zimbabwensis* has been described in farmed and wild crocodiles in Zimbabwe, Ethiopia and Mozambique and in monitor lizards in Zimbabwe. Experimentally, it shows a high infectivity for a wide spectrum of mammalian hosts including pigs and rats (27). All species and genotypes of *Trichinella* cause disease in humans.

Human trichinellosis can be a debilitating disease and may result in death. The short-lived adult worms in the intestine can cause transient gastroenteritis, but the most severe signs and symptoms result from the migration and presence of the larvae in voluntary muscle. The disease is transmitted by eating infected meat that has not been sufficiently cooked (or otherwise made safe). Prevention of human infection is accomplished by meat inspection, by processing (cooking, freezing, or curing of meat), and by preventing the exposure of food animals to infected meat including uncooked food waste, rodents and other wildlife (10, 12, 13,). Game meats should always be considered a potential source of infection, and should be tested or properly cooked. *Trichinella* found in game meats (mainly *T. nativa*, T-6 and to a lesser degree *T. britovi*) may be resistant to freezing and therefore untested, frozen game poses a public health risk.

Testing methods for the detection of *Trichinella* infection in pigs and other species either: (a) directly demonstrate the parasite in tissue samples; or (b) indirectly demonstrate the parasite by using immunological methods to detect specific antibodies to *Trichinella* spp. in blood, serum or tissue fluid samples.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (the prescribed test for international trade)

The only recommended procedures for the detection of *Trichinella* larvae in meat are digestion assays. A number of digestion assays are officially recognised in various countries for trade purposes. The International Commission on Trichinellosis (ICT) recommends several of these assays, which are documented standards in the EU, Canada or the USA. However, a number of other official methods not currently used routinely are not recommended because of their lack of efficiency or reliability. Modern diagnostic assays should meet internationally accepted standards of quality assurance, which include scientifically derived validation data and a design that allows routine monitoring and documentation of critical control points. Although there is general consensus that the digestion assay is the best procedure, a universally accepted digestion test protocol for trade and food safety purposes is not yet available. The digestion assay recommended here is based on desirable innovations inherent in some digestion assays that are accepted for international trade purposes.

a) Recommended direct procedure for testing meat

Sensitivity: the sensitivity of direct testing methods depends on the amount of tissue examined and the site from which the sample was obtained. Direct methods will identify infected pigs, horses or other animals infected with *T. spiralis* as early as 17 days after exposure, coincident with the time that muscle larvae become infective for a new host. Direct methods remain effective as long as muscle larvae remain viable. To insure the viability of the trichinae, tissue samples must not be kept for long periods of time or frozen before testing. Current methods for testing by artificial digestion employing a 1-g sample have a sensitivity of approximately three larvae/g of tissue, and testing of a 5-g sample increases sensitivity to 1 larva/g of tissue (13, 22). Where large amounts of tissue (up to 100 g) are available for digestion, the sensitivity of this test is further increased.

Sampling: tests are usually conducted on carcass samples collected post-mortem. Muscle samples are taken from predilection sites, usually the diaphragm pillars or tongue of pigs, or tongue or masseter muscles of horses. Sample sizes can vary; individual samples of 100 g may be taken from one animal, or multiple samples of lesser amounts may be collected from a number of animals to make a 100-g pool. The size of the samples that make up the pool will determine the sensitivity of the method. The ICT recommends 5-g samples per pig for testing in endemic areas. For testing horsemeat, a minimum of 5 g per carcass is required. For horses originating from endemic areas, a 10-g sample is recommended.

- **Digestion and detection**

i) Determine the volume of digestive solution required for the digestion (2000 ml of the solution for 100 g of meat, and 1000 ml for 50 g or less).

ii) Digestive solution: Prepare the appropriate volume of 37% HCl/water solution (0.55% v/v 37% HCl) by combining the HCl with tap water (e.g. 11 ml of 37% HCl with 1989 ml water). Do not add pepsin to the solution at this time. This solution should be preheated to 45°C before use.

iii) Remove as much fat and fascia as possible from each sample of meat.

iv) Weigh the appropriate amount of trimmed meat from each sample. Cut each sample into 1–2 g pieces and pool with other samples into a 100-g amount.

v) Place the pooled meat sample into a blender. Add 50–100 ml of the water/HCl solution for a 100 g sample pool.

vi) Chop the meat in a blender until it is homogeneous (no chunks of meat should be present; the sample should be the consistency of pureed baby food). This is usually achieved with several 1–3-second pulses. Add approximately 100 ml of the prepared water/HCl solution and blend until the mixture is uniformly liquid. This may take 5–10 seconds (additional solution may be needed).

vii) Sprinkle 10 g of pepsin (1:10,000 NF/1:12500 BP/2000 FIP; granular preferred) on to the homogenate, add about 200 ml of water/HCl solution, and blend for about 5 seconds.

viii) Transfer the homogenised sample to a 3-litre beaker containing a stir bar. Add the remainder of the 2 litres of water/HCl solution by pouring the water/HCl into the blender and rinsing all residual homogenate into the 3-litre beaker. Rinse any adhering material from the blender lid into the beaker using 10–20 ml of digestive solution from a squirt bottle.

ix) Place the beaker on a preheated magnetic stirrer hotplate or in an incubation chamber set at 45±2°C. Cover the beaker with aluminium foil. Activate the stirrer at a sufficiently high speed to create a deep vortex without splashing. <u>Note</u>: If the digest temperature at the beginning of digestion is not 45±2°C, the sample should be allowed to warm to this temperature before the timing of the digestion is started.

x) Allow the digestion to proceed for 30 minutes. If the temperature of the digest has fallen below 45±2°C, additional digestion time may be required to complete the digestion. This can be determined by observing the digestion mixture. If pieces of undigested muscle tissue are present, the digestion should be continued for an additional 30 minutes or until the pieces are digested. Care should be taken to ensure that the digestion temperature range is not exceeded. Alternatively, the digestion may be performed at 37°C for a longer period of time.

xi) Within 5 minutes of removal from the magnetic stirrer hot plate pour the digestion fluid through a 177–180-µm sieve and into a 2-litre separatory funnel. Rinse the beaker with room temperature tap water from a squirt bottle and pour this through the sieve into the 2-litre separatory funnel.

xii) Rinse the sieve into the 2-litre separatory funnel by squirting a small volume of room temperature tap water through the top of the sieve. There should be no undigested pieces of muscle remaining on the sieve, although small remnants of fat, fascia and other tissues may be present. Allow the fluid in the separatory funnel to settle undisturbed for 30 minutes.

xiii) Drain 40 ml of digestion fluid from the separatory funnel into a 50 ml conical tube or measuring cylinder (Pilsner flask) and allow to stand for 10 minutes.

xiv) At the end of 10 minutes use a pipette to remove 30 ml of the upper part of the fluid (supernatant), leaving the bottom 10 ml in the tube (do not pour off the upper 30 ml, as this will disturb the sediment).

xv) Gently swirl the remaining 10 ml of fluid and quickly transfer it into a gridded Petri dish or larval-counting basin. Rinse the tube or cylinder into the Petri dish twice using 5 ml of tap water each time. The layer of fluid in the petri dish should not be more than a few millimetres deep.

xvi) Wait a minimum of 1 minute to allow larvae to settle to the bottom, then use a stereomicroscope at ×10–16 magnification to systematically examine each grid of the Petri dish for the presence of *Trichinella* larvae. The detection of any suspect larvae on the systematic examination must be confirmed by the identification of morphological details at a higher magnification such as ×40. If the sediment is cloudy or otherwise difficult to examine, it will require further clarification as described below.

xvii) Digests should be examined soon after they are ready. Under no circumstance should examination of digests be postponed until the following day.

xviii) If digests are not examined within 30 minutes of their preparation they may require clarification as described below.

xix) Sample clarification: transfer the contents of the Petri dish into a 50 ml conical tube using a pipette. Rinse the Petri dish thoroughly with tap water, adding the rinse water to the conical tube. Add additional tap water to bring the volume to 45 ml. Let the tube settle undisturbed for 10 minutes.

At the end of 10 minutes use a pipette to withdraw the supernatant, leaving the bottom 10 ml (do not pour off the supernatant, as this will disturb the sediment). Save the removed fluid for disposal or decontamination after the sample has been read.

Repeat steps xv and xvi.

xx) In the event of a positive or doubtful result, a further sample should be collected from each carcass making up the pooled sample. These should be tested individually or in successive smaller pools until the individual infected animals are identified.

Identification of the larvae: first stage larvae, digested free from muscle cells, are approximately 1 mm in length and 0.03 mm in width. The most distinguishing feature of *Trichinella* larvae is the stichosome, which consists of a series of discoid cells lining the oesophagus and occupying the anterior half of the worm's body. *Trichinella* larvae may appear coiled (when cold), motile (when warm) or C-shaped (when dead). In case of doubt, larvae should be viewed at higher magnification and further tissues should be examined. If the counts are high, appropriate dilutions must first be made.

Quality assurance: laboratories using artificial digestion methods should maintain a suitable quality assurance system to ensure test sensitivity. Components of a quality assurance system for digestion testing are described by the ICT (13) and elsewhere (9) and should include regular use of proficiency testing (7, 8).

b) Other tests

- ### Other direct detection methods

i) *The double separatory funnel method:* this assay is recommended as an alternative to the commonly used digestion procedure described above, and is approved by the EU for export use. The method

was designed to operate under strict conditions of quality control, minimise technical error, and has been extensively validated for use on pork and horse meat (5). It includes a spin-bar digestion technique and sequential separatory funnels for sedimentation of the larvae. The procedure has fewer steps, requires less time and seldom needs further clarification steps. An incubation chamber equipped with transparent glass doors and set at 45°C is used to perform the digestion. The digestion is conducted in 3 litres of digest fluid on a magnetic stirrer. Following digestion the suspension is poured into a 4-litre separatory funnel through a 177–180-µm sieve, which is rinsed thoroughly into the separatory funnel with tap water. The suspension is allowed to settle for 30 minutes and 125 ml is drained into a 500-ml separatory funnel. The volume is increased to 500 ml by adding 375 ml of tap water, and the resultant suspension is allowed to settle for an additional 10 minutes. Finally, 22–27 ml of sediment is drained into a Petri dish and observed for larvae as previously described.

ii) *The mechanically assisted pooled sample digestion method/sedimentation technique* (*Method 4: 84/319/EEC*): this method uses a heated Stomacher blender for the digestion phase, and a separatory funnel for sedimentation of the larvae (3).

iii) *Polymerase chain reaction:* limited studies have shown that PCR can be used to detect larvae in the nucleic acid of larvae in the musculature of infected animals. However, this method lacks sensitivity and is not practical for routine testing of food animals. Identification of the species or genotype of *Trichinella* recovered from muscle tissue is useful in understanding the epidemiology of the parasite in animals, in assessing the relative risk of human exposure and to trace back the infection to the farm of origin. Specific primers have been developed that allow the identification of single larva collected from muscle tissues at the species and genotype by PCR (25). Requests for speciation or genotyping of *Trichinella* larvae can be made through the OIE Reference Laboratories in Rome, Italy or Saskatoon, Canada (see Table given in Part 3 of this *Terrestrial Manual;* and www.iss.it/Trichinella/index/asp).

- **Direct detection methods not recommended for meat inspection**

    i) *Trichinoscopy:* This method involves the compression of multiple 2 × 10 mm pieces of muscle tissue between two glass plates (compressorium) until they become translucent, followed by examination using a microscopic technique (2). Although this method has been in use for many decades, it is labour intensive and there are good comparative data available indicating that it is not as sensitive as digestion assays (6). Increasing the sample size to compensate is not practical for testing large numbers of animals, and the non-encapsulated *Trichinella* spp. (*T. pseudospiralis, T. papuae and T. zimbabwensis*) may occur uncoiled outside of muscle cells, making them difficult to detect using trichinoscopy. Because of these limitations, trichinoscopy and similar compression methods are not recommended for the routine examination of carcasses.

    ii) *Trichomatic 35*: this method involves an automated digestion chamber and a membrane filter for the recovery and examination of larvae. The critical steps of the digestion and recovery process are difficult to monitor in this system, and test capacity is only 35 g.

- **Immunological methods**

    A variety of immunological assays have been described for the diagnosis of *trichinellosis* in domestic and wild animals (17). Methods include immunofluorescence assay (IFA), immuno-electrotransfer blot (IEBT), western blot, enzyme immunohistochemical assays, and enzyme-linked immunosorbent assays (ELISA). Except for the ELISA, these tests have not been standardised, and reagents are not available for routine use. Nevertheless, the ICT has provided a uniform set of recommendations for the development and use of serological tests for the detection of circulating antibodies (17). The ELISA is the only immunological assay endorsed by the ICT. It is only approved as a epidemiological surveillance tool to detect anti-*Trichinella* antibodies in pigs; it is not reliable for the detection of *Trichinella* infection in individual animals.

2. Serological tests

Although other serological tests may have some practical applications, the ELISA is generally acknowledged as the test of choice based on economy, reliability, adaptability to good quality assurance practices, increasing body of validation data and good sensitivity and specificity when conducted under appropriate conditions. It is a useful tool for testing populations and is routinely used for surveillance programmes and disease outbreak investigations. Nevertheless, for reasons given below, the ELISA is not recommended for the testing of individual pigs for food safety purposes.

a) Enzyme-linked immunosorbent assay (ELISA)

- **Sensitivity and specificity**

Infection levels as low as one larva/100 g of tissue are detectable by ELISA in pigs (17). This high level of sensitivity makes serological testing by ELISA a useful method for detecting ongoing transmission of *Trichinella* infection at the farm or for more broadly based surveillance programmes. A disadvantage of

serology for the detection of *trichinellosis* is the low rate of false-negative results observed in infected animals. This is primarily due to the lag time of the immune response following the ingestion of infective larvae. Detectable levels of antibody are not usually present in pigs until 3–5 weeks or more following exposure (11, 14). For this reason, serological methods are not recommended for individual carcass testing. Serological responses in pigs persist for a long time after infection with no decline in titre, however, antibody has been reported to decline in horses within a few months following infection (22). Serological tests may be of little practical use in horses as antibody titres eventually drop below diagnostic levels despite the presence of infective larvae in muscle (19, 26). Little is known of antibody responses to *Trichinella* infection in game animals, but high quality serum samples should be obtained to reduce the likelihood of false positive reactions.

- **Samples**

The use of ELISA to detect the presence of parasite-specific antibodies provides a rapid method that can be performed on serum, blood or tissue fluid collected before or after slaughter (15). The dilution used is different for serum than for tissue fluid (23).

- **Antigens**

The specificity and sensitivity of ELISA is largely dependent on the quality of the antigen used in the test. Antigens that are specifically secreted from the stichocyte cells of living L1 larvae and bear the TSL-1 carbohydrate epitope are recognised by *Trichinella*-infected animals. The antigens recognised in worm ES products consist of a group of structurally related glycoproteins with molecular weights of 45–55 kDa (24). A synthetic carbohydrate antigen (Tyvelose) has also been used in ELISA. Studies in swine indicate that Tyvelose may be as good as ES antigen for surveillance testing in pigs however, the sensibility of the ELISA using this synthetic antigen is lower than that using ES antigens (4, 18). Antigen preparations have been developed that provide a high degree of specificity for *Trichinella* infection in pigs (16). The *T. spiralis* ES antigens used in the ELISA are conserved in all species and genotypes of *Trichinella* (24), and therefore infection may be detected in pigs or other animals harbouring any of the eight species.

- **Antigen production**

Diagnosis of *Trichinella* infection by ELISA can be accomplished by using stichosome antigens collected from the ES products of *Trichinella* larvae in culture (16). For purposes of standardisation, it is recommended that *T. spiralis* be used for antigen production for food animal testing. However, it has been demonstrated that antigen prepared from any of the *Trichinella* species can be used for detection of antibodies in infected animals regardless of the infecting species (20). Parasites to be used for antigen preparation may be maintained by serial passage in mice or rats.

To prepare antigen for use in the ELISA (16), *T. spiralis* (T-1) muscle-stage larvae are recovered from skinned, eviscerated, ground mouse or rat carcasses by digestion in 1% pepsin with 1% HCl for 30 minutes at 37°C (as described above). These larvae are washed (three times for 20 minutes each) in Dulbecco's modified Eagle's medium (DMEM) with penicillin (500 units/ml) and streptomycin (500 units/ml), and then placed (at a density of 5000 L1/ml) into DMEM supplemented with HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) (10 mM), glutamine (2 mM), pyruvate (1 mM), and penicillin (250 units/ml)/ streptomycin (250 µg/ml) (complete DMEM) at 37°C in 10% CO_2 in air. Culture medium is recovered after 18–20 hours, worms are removed by filtration, and the fluid is concentrated under pressure with a 5000 Da molecular weight retention membrane. ES antigens thus recovered may be stored frozen for short periods at –20°C or for longer at –70°C; they consist of approximately 25 protein components as determined by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis), many of which bear the diagnostic TSL-1 carbohydrate antigen epitope.

Antigen purity is critical to the specificity of the ELISA. Steps should be taken to monitor growth of bacteria either visually, by phase microscopy, or by plating a sample of media. Cultures showing any bacterial growth should be discarded. Larvae should not be maintained longer than 18 hours; worm deterioration after this time contributes to leaking of somatic antigens that reduce test specificity. Antigen, produced as described, should have a 280:260 nm absorbance ratio of >1.0. The antigens obtained from *in-vitro* maintenance of *Trichinella* larvae, should be tested against a panel of known negative and positive sera before use.

- **Test procedure**

An example of an ELISA for detecting *Trichinella* infection in pigs is given below. It is essential that all reagents used in the assay be standardised for optimal concentration to obtain reliable results. Typical values are indicated in the example.

i) Coat 96-well microtitre plates with 100 µl/well of *T. spiralis* ES antigens diluted to 5 µg/ml in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6). Coating is performed for 60 minutes at 37°C or overnight at 4°C.

ii) Wash antigen-coated wells three times with wash buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5.0% non-fat milk powder and 1.0% Triton X-100. Following each washing, plates are blotted dry.

iii) Dilute pig sera 1/50 or 1/100 in wash buffer. Alternative sources of antibodies that may be used in place of sera include whole blood or tissue fluids at the dilution of 1/5 or 1/10 (23). Add 100 µl of diluted sera to antigen-coated wells. A known positive and known negative serum sample should be used on each plate at the same dilution as the test sera. Incubate at room temperature for 30 minutes.

iv) Wash wells three times as in step ii.

v) Add 100 µl/well of an affinity-purified rabbit anti-swine IgG–peroxidase conjugate at an appropriate dilution in wash buffer, e.g. a 1/1000 dilution of rabbit anti-swine IgG (0.1 mg/ml) produced by Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA. Following the addition of the second antibody, incubate the plates for 30 minutes at room temperature.

vi) Wash wells three times as in step ii. Rinse once with distilled water.

vii) Add 100 µl of a suitable peroxidase substrate (e.g. 5'-aminosalicylic acid [0.8 mg/ml] with 0.005% hydrogen peroxide, pH 5.6–6.0).

viii) After 5–15 minutes, read plates for colour density at 450 nm on an automated microplate reader. Values obtained in the ELISA four times that of normal serum pool controls are considered to be positive. Values three times higher than normal are classified as suspect.

Commercial adaptations of the ELISA are available. The manufacturer must validate the kit prior to licensure and the user should also evaluate the performance of the kit, prior to use, by using selected negative and positive reference samples.

The test should be conducted within an environment in which internationally accepted standards of quality management, such as ISO 17025, have been implemented.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines for *trichinellosis* in food animals or game. There are no biological reagents required for direct observation of the parasite. For applicable immunological methods, TSL-1 antigens are recommended to maximise test specificity. These antigens may be obtained as ES products recovered from in-vitro maintenance of muscle larvae as described above.

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NB: There are OIE Reference Laboratories for Trichinellosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

TRYPANOSOMA EVANSI INFECTIONS (INCLUDING SURRA)

SUMMARY

Definition of the disease: Trypanosoma evansi *causes a disease known as Trypanosomosis[1]* ('*surra*'). *It affects a number of species of domesticated animals in Asia, Africa and Central and South America. The principal host species affected varies geographically, but buffalo, cattle, camels and horses are particularly affected, although other animals, including wildlife, are also susceptible. It is an arthropod-borne disease. Several species of haematophagous flies, including* Tabanus *spp. and* Musca *spp., are implicated in transferring infection from host to host mechanically vectors. In Brazil, vampire bats have been implicated in transmission.*

Description of the disease: *The disease in susceptible animals is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are often observed. Abortions have been reported in buffaloes and camels. There are indications that the disease causes immunodeficiencies.*

Identification of the agent*: The general clinical signs of* T. evansi *infection are not sufficiently pathognomonic for diagnosis. Laboratory methods for detecting the parasite are required. Examination of the host blood is problematic as trypanosomes can be detected only when there is a high parasitaemia. Under these circumstances examination of wet blood films, stained blood smears or lymph node material might reveal the trypanosomes. In other, more chronic cases, such as the carrier state, the examination of thick blood smears, as well as methods of parasite concentration and the inoculation of laboratory animals are required.*

Serological tests: *Infection gives rise to specific antibody responses and a variety of antibody detection tests have been introduced for laboratory and field use. Some have been partially validated, but await large-scale evaluation and standardisation. Among those that are used regularly in the laboratory are immunoenzyme assays, card agglutination tests and latex agglutination tests. For field use both card agglutination tests (*CATT*) for* T. evansi *and latex can be applied, yet an individual test format (*pen side test*) is currently unavailable. Assays for detection of circulating antibodies have high measures of validity. Estimates of predictive values of different serological tests indicate that enzyme linked immunosorbent assays (*ELISA*) for detecting IgG antibodies are more likely to classify correctly uninfected animals, and are more likely to classify correctly truly infected animals. An IgG ELISA would thus be suitable for verifying that animals are free from infection, prior to movement or during quarantine. In situations where there is overt disease, CATTs can be used to target individual animals for treatment with trypanocidal drugs. For declaring a disease-free status, serial testing – ELISA followed by re-testing of suspect samples by CATT – is recommended. It must be stressed however, that there are considerable antigenic similarities among the different species of pathogenic trypanosomes, hence in areas where tsetse-transmitted trypanosomoses occur cross-reactions may occur with any serological test employed.*

Requirements for vaccines and diagnostic biologicals: *No vaccines are available for the disease.*

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1   Nomenclature of parasitic diseases: see the note in Chapter. 2.4.18 Trypanosomosis (Tsetse-borne).

A. INTRODUCTION

The clinical signs of surra, the disease caused by *Trypansoma evansi,* are indicative but are not sufficiently pathognomonic and diagnosis must be confirmed by laboratory methods. The disease in susceptible animals, including cattle, buffalo, camels (dromedary and bactrian), horses, pigs, sheep and goats, is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are often observed. Abortions have been reported in buffaloes and camels (8, 17) and there are indications that the disease causes immunodeficiency (5, 21).

There is considerable variation in the pathogenicity of different strains and the susceptibility of different host species to disease. Disease may manifest as an acute or chronic condition, and in the latter case may persist for many months. The disease is often rapidly fatal in camels, buffaloes, horses, cattle, llama and dogs, but mild and subclinical infections can also occur in these species. Wild animals such as deer and capybara can become infected. Animals subjected to stress – malnutrition, pregnancy, work – are more susceptible to disease.

Biologically *T. evansi* is very similar to *T. equiperdum*, the causative agent of dourine (2), and morphologically resembles the slender forms of the tsetse-transmitted trypanosomes, *T. brucei, T. gambiense* and *T. rhodesiense.* Molecular characterisation indicates that various strains of *T. evansi* isolated from Asia, Africa and South America have a single origin. Molecular characterisation using random amplified polymorphic DNA techniques and endonuclease fingerprinting showed that isolates of *T. evansi* and *T. equiperdum* formed a closely homogeneous group. One possibility of this finding is that *T. equiperdum* is not a genuine species *per se* and that the clinical outcome of disease is related primarily to the hosts' immune response. Like all pathogenic trypanosomes, *T. evansi* is covered by a dense protein layer consisting of a single protein called the variable surface glycoprotein. This acts as a major immunogen and elicits the formation of specific antibodies. The parasites are able to evade the consequences of these immune reactions by switching the variant surface glycoprotein, the phenomenon known as antigenic variation.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The classical direct parasitological methods for the diagnosis of trypanosomosis, namely examining blood or lymph node material, are not highly sensitive. In regions where other *Trypanozoon spp.* occur in addition to *T. evansi*, specific identification by microscopy is not possible. Specific DNA probes (19, 24) may enable identification of trypanosome species by nonradioactive DNA hybridisation. A species-specific polymerase chain reaction (PCR) based on *T. evansi* specific antigen (RoTat 1.2 VSG) has been developed, but has not been validated in the field (3).

- **Direct methods**

a) Usual field methods

   i) *Blood sampling*

     Trypansoma evansi is a parasite of the blood and tissues often inhabiting the deep blood vessels in cases of low parasitaemia. For this reason, it is recommended that blood for diagnosis be obtained from both the peripheral and deep blood vessels. However it should be realised that less than 50% of infected animals may be identified by examination of peripheral blood.

     Peripheral blood is obtained by puncturing a small vein in the ear or tail. Deeper samples are taken from a larger vein by syringe. Cleanse an area of the ear margin or tip of the tail with alcohol and, when dry, puncture a vein with a suitable instrument. Ensure that instruments are sterilised or disposable instruments are used between individual animals, so that infection cannot be transmitted by residual blood.

   ii) *Wet blood films*

     Place a small drop of blood on to a clean glass slide and cover with a cover-slip to spread the blood as a monolayer of cells. Examine by light microscopy (×200) to detect any motile trypanosomes.

   iii) *Stained thick smears*

     Place a large drop of blood on the centre of a microscope slide and spread with a toothpick or the corner of another slide so that an area of approximately 1.0–1.25 cm in diameter is covered. Air-dry for

1 hour or longer, while protecting the slide from insects. Stain the unfixed smear with Giemsa's Stain (one drop of commercial Giemsa + 1 ml of phosphate buffered saline [PBS, 2.4 g $Na_2HPO_4.2H_2O$, 0.54 g $NaH_2PO_4.2H_2O$, 0.34 g NaCl], pH 7.2), for 25 minutes. After washing, examine the smears by light microscopy at high magnification (×500–1000). The advantage of the thick smear technique is that it concentrates the drop of blood into a small area, and thus less time is required to detect the parasites. The disadvantage is that the trypanosomes may be damaged in the process, and the method is therefore not suited for species identification in case of mixed infections.

iv)   *Stained thin smears*

Place a drop of blood 20 mm from one end of a clean microscope slide and draw out a thin film in the usual way. Air-dry briefly and fix in methyl alcohol for 2 minutes and allow to dry. Stain the smears in Giemsa (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. Pour off, stain and wash the slide in tap water and dry. Unfixed smears can be stained by covering them with May–Grünwald stain for 2 minutes, then adding an equal volume of PBS, pH 7.2, and leaving the slides for a further 3 minutes. Pour off and add diluted Giemsa for 25 minutes. Pour off, wash the slides with tap water, and dry. Examine at high magnification (×400–1000). This technique permits detailed morphological studies and identification of the trypanosome species. Rapid staining techniques also exist (Field's stain, Diff Quick®).

v)   *Lymph node biopsies*

Samples are usually obtained from the prescapular or precrural (subiliac) lymph nodes. Select a suitable node by palpation and cleanse the site with alcohol. Puncture the node with a suitable gauge needle, and draw lymph node material into a syringe attached to the needle. Expel lymph on to a slide, cover with a cover-slip and examine as for the fresh blood preparations. Fixed thin or thick smears can also be stored for later examination.

b)   Concentration methods

In most hosts *T. evansi* can induce mild clinical or subclinical carrier state infections with low parasitaemia in which it is difficult to demonstrate the parasites. In these circumstances, concentration methods become necessary.

i)   *Haematocrit centrifugation*

Collect blood (70 µl) into at least two heparinised capillary tubes (75 × 1.5 mm). Seal at the dry end by heat and centrifuge, sealed end down, at 3000 ***g*** for 10 minutes. Place the capillary tube between two pieces of glass (25 × 10 × 1.2 mm) glued to a slide. Place a cover-slip on top at the level of the buffy coat junction where the trypanosomes will be concentrated. Flood the space around this part of the tube with water or immersion oil, and examine the buffy coat area under the microscope (×100–200). This technique can detect around 400 trypanosomes/ml. A simpler alternative is to examine the centrifuged capillary tube by placing a drop of immersion oil on the tube and ensuring that there is contact between the objective lens and the immersion oil.

ii)   *Dark-ground/phase-contrast buffy coat technique*

Collect blood into heparinised capillary tubes and centrifuge as above. Scratch the tube with a glass-cutting diamond and break it 1 mm below the buffy coat layer – the upper part thus contains the top layer of red blood cells (RBCs), the buffy coat (white blood cells) and some plasma.

Partially expel the contents of this piece on to a slide, cover with a cover-slip and examine under dark-ground, phase-contrast or ordinary illumination.

As an alternative to the electrically powered haematocrit centrifuge, hand-powered micro-centrifuges have been used successfully for detection of trypanosomosis in cattle and camels (9, 14).

iii)   *Haemolysis techniques*

Sodium dodecyl sulphate (SDS) can be used as a reagent to haemolyse RBCs to facilitate detection of motile trypanosomes in parasitised blood samples. As SDS is toxic, contact with skin, inhalation and ingestion should be avoided. SDS solution can be stored for several months at ambient temperature. Both the SDS solution and the blood samples should be used at a temperature above 15°C. At lower temperatures the trypanosomes may be destroyed.

Two general procedures, namely wet blood film clarification and haemolysis centrifugation, can be used[2].

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2   All necessary materials and instructions can be obtained from the Institute of Tropical Medicine, Laboratory of Serology, Nationalestraat 155, B-2000 Antwerp, Belgium.

- *Wet blood film clarification method*

  This method uses the partial lysis of RBCs to facilitate the detection of motile trypanosomes. The method requires an SDS solution: 1% SDS dissolved in Tris/glucose/saline, pH 7.5, inoculating loops (10 µl), slides and cover-slips (24 × 24 mm), and a drop of fresh meat or heparinised blood. Dissolve 100 mg of SDS in 100 ml of isotonic Tris/NaCl/glucose buffer, pH 7.5 (Trizma base 14.0 g, NaCl 3.8 g, glucose 10.8 g; dissolve chemicals in 750 ml distilled H_2O, add 90–100 ml 1 N HCl and adjust pH to 7.5 then make up to final volume of 1000 ml with distilled H_2O). This buffer can be stored in small vials at ambient temperature for several months. The test does not give a significantly higher sensitivity than wet film technique. In addition, the SDS can cause problems when focusing the microscope and the movements of trypanosomes can be severely curtailed owing to the high viscosity of the SDS.

  Put approximately 10 µl of blood on a slide. Add 10 µl of SDS solution using a dip inoculation loop and mix gently. Apply a cover-slip and examine the preparation without delay at low magnification (×100 or ×200).

- *Haemolysis centrifugation technique*

  Nearly complete lysis of RBCs is required for this procedure. The materials needed include: SDS solution (0.1% SDS dissolved in Tris/glucose/saline, pH 7.5), conical centrifuge tubes, ordinary test tubes, large and fine plastic tapering pipettes with attached bulb, slides, cover-slips (24 × 24 mm or 24 × 32 mm), and heparinised blood.

  Using a pipette or syringe, transfer nine volumes (maximum 6.3 ml) of SDS solution into an ordinary test tube. Aspirate one volume (maximum 0.7 ml) of heparinised blood into a bulb pipette and expel it just above the surface of the SDS solution; mix quickly and thoroughly. Avoid foam formation, which may result in destruction of the trypanosomes. Wait for 10 minutes so as to achieve complete haemolysis.

  Pour the haemolysed suspension into a conical centrifuge tube and spin at approximately 500 *g* for 10 minutes. With a clean bulb pipette, remove as much supernatant as possible without disturbing the sediment. Using a fine tapering bulb pipette, remove more supernatant, leaving 10–20 µl of undisturbed sediment at the bottom.

  Very carefully collect the entire sediment and put on to a microscope slide. Apply a cover-slip and examine the preparation without delay at low magnification (×100 or ×200).

iv) *Mini-anion exchange centrifugation technique*

  When a blood sample from animals infected with salivarian trypanosomes is passed through an appropriate anion-exchange column, the host blood cells, being more negatively charged than trypanosomes, are adsorbed onto the anion-exchanger, while the trypanosomes are eluted, retaining viability and infectivity (15). A simplified field method for detection of low parasitaemia has been developed (26). The sensitivity of this technique can be increased by approximately tenfold by the use of buffy coat preparations rather than whole blood (25).

- *Preparation of phosphate buffered saline glucose, pH 8*

  Na_2HPO_4 (anhydrous) (13.48 g); $NaH_2PO_4.2H_2O$ (0.78 g); NaCl (4.25 g); distilled water (1 litre).

  Solutions of different ionic strength are made by diluting the stock PBS, pH 8, and adding glucose to maintain a suitable concentration. For blood of mice, domestic and wild ruminants and dog, add four parts PBS to six parts distilled water and adjust the final glucose concentration to 1%. For blood of pigs and rabbits, add three parts PBS to seven parts distilled water and adjust the final glucose concentration to 1.5%. The PBS/glucose solution (PSG) must be sterile.

- *Equilibration of DEAE-cellulose*

  Suspend 500 g of DEAE-cellulose (diethylamino-ethylcellulose) in 2 litres of distilled water. Adjust the pH to 8 with phosphoric acid. Allow to settle for 30 minutes. Discard the supernatant fluid containing the fine granules. Repeat the procedure three times. Store the equilibrated concentrated suspension of DEAE-cellulose (slurry) at 4°C or in small aliquots at –20°C.

- *Packing of equilibrated DEAE-cellulose*

  Place a 2 ml syringe without the plunger on a test-tube rack complete with needle (20 G × 1.5 inch). Put a disc of Whatman No. 41 filter paper at the bottom of the syringe and moisten by adding a few drops of PSG. Pour 2–2.5 ml of the slurry of equilibrated cellulose into the syringe and allow to pack by elution of the buffer. The height of the sediment should be approximately 3 cm. Wash and equilibrate the column with 2 ml of PSG without disturbing the surface.

- *Adsorption of blood eluate of the trypanosomes*

  Gently place 100–300 µl of heparinised blood above the surface of the cellulose column. Add ten drops of PSG and discard the first ten drops of the eluate. Progressively add 1.5 ml of PSG and start collecting the eluate into a finely tapered Pasteur pipette with a sealed end. Put the filled pipette, protected by a conical plastic pipette tip, in a tube and centrifuge at 525 *g* (or up to 1000 *g*) for 10 minutes. Examine the bottom of the pipette under the microscope (×100 or ×200) using a special mounting device. Alternatively, the eluate could be collected into 50 ml plastic tubes, with conical bottoms, centrifuged at 1000 *g* and the sediment examined by dark-ground microscopy.

  The cellulose column should remain wet throughout the procedure.

c) Animal inoculation

Laboratory animals may be used to reveal subclinical (nonpatent) infections in domesticated animals. *Trypanosoma evansi* has a broad spectrum of infectivity for small rodents, and so rats and mice are often used. Rodent inoculation is not 100% sensitive (18) but further improvement in its efficacy can be obtained by the use of buffy coat material. Such a procedure was able to detect as few as 1.25 *T. evansi*/ ml blood (25).

Inoculate heparinised blood intraperitoneally into rats (1–2 ml) or mice (0.25–0.5 ml). Inoculate a minimum of two animals. Bleed animals from the tail three times a week to detect parasitaemia. The incubation period before appearance of the parasites and their virulence depends on the strain of trypanosomes, their concentration in the inoculum, and the strain of laboratory animal used. Sensitivity of this *in-vivo* culture system may perhaps be increased by use of immunosuppressed laboratory animals. Drugs such as cyclophosphamide or hydrocortisone acetate, or X-ray irradiation or splenectomy are used for this purpose.

d) Recombinant DNA probes

Specific DNA probes have been used to detect trypanosomes in infected blood or tissue but are not routinely applied (28, 31). Although molecular methods have a potentially high analytical sensitivity there have been few convincing studies to critically evaluate the diagnostic sensitivity of these tests as compared with other techniques, such as serology.

e) Detection of trypanosomal DNA

Detection of minute amounts of trypanosomal DNA using a PCR procedure is a possible means of identifying animals with active infections, and could have the sensitivity and specificity required (3, 20, 32). Experimental studies in buffalo (10) showed the diagnostic sensitivity of a PCR was only 78%, which is similar to mouse inoculation.

- **Indirect methods**

These methods involve tests that demonstrate the effects of the parasite on its host rather than directly detecting the parasite itself.

a) Haematology

Anaemia is usually a reliable indicator of trypanosome infection, although it is not in itself pathognomonic. However, animals with a mild subclinical infection can have parasitaemia without evidence of anaemia (4).

Anaemia can be estimated by measuring the packed cell volume and may be used in surveys of herds at risk. The technique is identical to that of haematocrit centrifugation. The capillary tube is examined and the results are expressed as a percentage of packed RBCs to total blood volume.

2. Serological tests

Historically, many different methods have been used to detect specific humoral antibodies to trypanosomal antigens but, more recently, there has been a tendency to concentrate on more easily standardised techniques such as ELISA (6, 7, 12, 22, 23, 24, 27) card agglutination tests (CATT) (1, 20, 24) and latex agglutination tests (LAT) (11, 16). Extensive evaluation of ELISA and CATT has been carried out in buffaloes in Indonesia and Vietnam (6, 11, 29). The collection of samples can be simplified by using filter paper blood spots for later use in the ELISA, while for the CATT whole blood can be substituted for serum (11). Other innovative modifications that might be developed in the future are the use of a colloidal-dye dipstick test (13) that could enable the tests to be carried out under field conditions. It is vitally important that serological tests are validated and standardised if they are to be suitable for correctly identifying infected animals. This means that standard criteria for interpreting the tests might have to be developed for each animal species as well as each laboratory operating the procedure.

a) **Enzyme-linked immunosorbent assay**

The principle of this technique is that specific antibodies to trypanosomes can be detected by enzyme-linked anti-immunoglobulins using solid-phase polystyrene plates coated with soluble antigen. The enzyme may be peroxidase, alkaline phosphatase or any other suitable enzyme. The enzyme conjugate binds to the antigen/antibody complex and then reacts with a suitable substrate to yield a characteristic colour change either of the substrate itself or of an added indicator (the chromogen).

The antigen for coating the plates is derived from the blood of a heavily parasitaemic rat. The trypanosomes are separated on a DEAE-cellulose column and washed three times by centrifugation in cold PSG, pH 8 (PBS with 1% glucose). The final pellet is suspended in cold PSG to a concentration of 3–5%, and briefly ultrasonicated on ice for 30–120 seconds until disintegration of the organisms is complete. This preparation is centrifuged at 4°C and 40,000 g for 60 minutes. The supernatant is diluted in water so as to obtain a protein concentration of 1 mg/ml. The reagent thus obtained can be stored in small aliquots at –70°C for several months. It can also be freeze-dried and stored at –20°C. Various treatments of the antigen preparations have been applied to improve the accuracy of antibody detection (32).

- **Test procedure**

i) Dilute or reconstitute the frozen or freeze-dried antigen with freshly prepared 0.01 M carbonate/bicarbonate buffer, pH 9.6. Add 100 µl to each well of a 96-well microtitre plate and incubate overnight at 4°C or for 1 hour at 37°C.

ii) Remove excess antigen by washing plate with 0.01 M PBS containing 0.05% Tween 20 (PBST). Add test serum dilutions in PBST (100 µl). Include control negative and positive sera. Dilutions must be determined empirically, but are usually between 1/100 and 1/1000.

iii) Incubate plates at 37°C for 30 minutes. Eject contents and wash three times with PBST.

iv) Add a specific peroxidase conjugated species-specific anti-globulin (100 µl) appropriately diluted in PBST (usually between 1/1000 and 1/50,000).

v) Incubate the plates at 37°C for 30 minutes, eject contents and wash three times with PBST.

vi) For peroxidase conjugates a number of substrate/chromogen solutions can be used, consisting of hydrogen peroxide with a chromogen, such as tetramethylbenzidene (TMB), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ortho-diphenylenediamine (OPD). A suitable substrate/chromogen solution for peroxidase conjugates is 30% hydrogen peroxide (0.167 ml and 35 mg) in citrate buffer (100 ml), pH 6.0. The citrate buffer is made up as follows: Solution A (36.85 ml): (0.1 M citric acid [21.01 g/litre]); Solution B (65.15 ml): (0.2 M, Na_2HPO_4 [35.59 g/litre]); and distilled water (100 ml). Dissolve 10 mg TMB in 1 ml dimethyl sulphoxide and add to 99 ml of the citrate buffer. A number of these combinations are available commercially in ready-to-use formulations that remain stable at 4°C for up to 1 year.

vii) Add the substrate chromogen (100 µl) to the plates and incubate at room temperature for 15–20 minutes.

viii) Stop the reaction by adding 50 µl 1 M sulphuric acid. Read the absorbance of each well at 450 nm for TMB chromogen. Other chromogens may require the use of a different wavelength. All tests should include known high and medium positive control sera, a negative control serum, and a buffer control.

A large variety of other test procedures exists. For closely related animal species, cross-reacting reagents may often be used (e.g. anti-bovine immunoglobulin for buffaloes and the use of monospecific anti-IgG conjugates is generally recommended. There are a number of methods that can be used to determine a cut-off point to discriminate between positive and negative results. The simplest method is to base the cut-off on visual inspection of the test results from known positive and negative populations. These results are likely to show some overlap. The operator can choose the most appropriate point to modify the false negative or false positive results depending on the required application of the assay. An alternative is to base the cut-off on the mean +2 standard deviations (SD) or +3 SD values from a large sample of negative animals. Finally, if no suitable negative/positive samples are available a cut-off can be based on the analysis of the data from animals in an endemic situation. If a bimodal distribution separates infected from uninfected animals, then an appropriate value can be selected. The ELISA is likely to correctly identify uninfected animals. Equivocal results can be re-tested using CATT. The Institute of Tropical Medicine in Antwerp developed an ELISA using the VSG from a *T. evansi* RoTat 1.2 clone. It was shown (30) that this antigen is a predominant antigen in *T. evansi* and absent in *T.b brucel.* This ELISA/RoTat 1.2 was successfully used in the field in Vietnam (11, 29). Protocols are available at ITM Antwerp for use in equines, camelidae and water buffaloes.

b) **Card agglutination tests**

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. This finding was used as a basis for a test

for the diagnosis of *T. evansi*, the card agglutination test – CATT/*T.evansi* – was developed at the Laboratory of Serology, Institute of Tropical Medicine, Antwerp[3]. The test makes use of fixed and stained trypanosomes of a defined VAT known as RoTat 1.2. Both variable and invariable surface antigens take part in the agglutination reaction. The CATT is available in kit form from ITM, Antwerp. It consists of lyophilised antigen, PBS, pH 7.4, plastic-coated cards, spatulas, positive and negative control sera and a rotator. The lyophilised antigen can be stored at 2–8°C for up to 1 year. Reconstituted antigen can be stored at 2–8°C for 2 days, but preferably should be used within 8 hours.

For screening, dilute test sera 1/4 or 1/8 in PBS on to circles inscribed on the plastic cards. Add 45 µl of the prepared antigen suspension onto circles inscribed on the plastic cards. Add 25 µl of each test serum. Mix and spread the reagents with a spatula and rotate the card for 5 minutes using the rotator provided in the kit. Compare the pattern of agglutination with the illustrations of different reactions provided in the kit. Blue granular deposits reveal a positive reaction visible to the naked eye.

c) **Latex agglutination tests**

A kit is available from ITM, Antwerp. It comprises a lyophilised latex suspension coated with *T. evansi* RoTat 1.2 variable antigens, PBS, positive and negative controls, test cards, plastic spatulas and a rotator.

Reconstitute the antigen-coated latex particles using distilled, deionised water. Mix gently. Add 20 µl of latex suspension onto each black spot on the test cards.

Dilute test sera with PBS (1/2 to 1/4) and add 20 µl to the latex suspension. Include appropriate controls. Mix the reagents carefully with a plastic spatula.

Rotate test cards at 70 rotations/minutes for 5 minutes and view cards under a good light source at the end of the incubation. Positive sera will cause agglutination of the latex particles.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available for this disease.

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3   CATT/*T. evansi* kits are available at the Laboratory of Parasite Diagnostic, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. (pbuscher@itg.be ; fclaes@tg.be)

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NB: There is an OIE Reference Laboratory for Trypanosoma evansi *infections* (*including surra*) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

2 TULAREMIA

3 **SUMMARY**

4 *Tularemia is a zoonosis caused by* Francisella tularensis. *The causative bacterium is a Gram-*
5 *negative coccoid rod, 0.2–0.5 µm × 0.7–1.0 µm, non-motile and non-spore-forming organism that is*
6 *an obligate aerobe with optimal growth at 37°C. It is oxidase-negative, weakly catalase-positive*
7 *and cysteine is required for growth. It occurs naturally in lagomorphs (*rabbits and hares*), and in*
8 *rodents, especially microtine rodents (*such as voles, vole rats and muskrats*), and beavers. A wide*
9 *range of other mammals and several species of birds have also been reported to be infected.*
10 *Among domestic animals, the cat seems to be able to act as a carrier of the bacterium.*

11 *Two types of* F. tularensis *are recognised on the basis of cultural characteristics, epidemiology,*
12 *and virulence in some hosts. Tularemia is largely confined to the Northern Hemisphere and is not*
13 *normally found in the tropics or the Southern Hemisphere.* Francisella tularensis *subsp.* tularensis
14 (*Type A*) *is associated with lagomorphs in North America. It is transmitted primarily by ticks and*
15 *biting flies, is highly virulent for humans and domestic rabbits, and most of the isolates ferment*
16 *glycerol.* Francisella tularensis *subsp.* palaearctica (*Type B*) *occurs mainly in aquatic rodents*
17 (*beavers, muskrats*) *in northern North America, and in hares and small rodents in Eurasia. It may*
18 *be water- or arthropod-borne, is less virulent to humans and rabbits, and does not ferment glycerol.*
19 *In addition to vector transmission, tularemia may be spread contact with infected animals or*
20 *environmental fomites by inhalation, or by ingestion of the poorly cooked flesh of infected animals*
21 *or contaminated water.*

22 *The disease is characterised by fever, depression and septicaemia. In humans, there may be*
23 *ulcers or abscesses at the site of inoculation (*this is rarely seen in animals*), and swelling of the*
24 *regional lymph nodes. On post-mortem examination, lesions may include caseous necrosis of*
25 *lymph nodes and multiple greyish-white foci of necrosis in the spleen, liver, bone marrow and*
26 *lungs. The spleen is usually enlarged.*

27 *It is important to understand that there is a high risk of direct infection of humans by direct contact*
28 *with this organism. Special precautions, including the wearing of gloves, masks and eyeshields,*
29 *are therefore recommended when handling infective materials. The facility should meet the*
30 *requirements for Containment Group 3 pathogens (*see Chapter 1.1.2 Biosafety and Biosecurity in
31 *the veterinary microbiological laboratory and animal facilities*).

32 **Identification of the agent:** *The bacterium can be demonstrated in impression smears or in fixed*
33 *specimens of organs, such as liver, spleen, bone marrow, kidney and lung, as well as in blood*
34 *smears. Immunological methods, such as the fluorescent antibody test (*FAT*) are the most reliable*
35 *way to identify the bacterium. With Gram staining, the bacteria appear as very small punctiform*
36 *Gram-negative rods, often difficult to distinguish as bacteria. They can also be stained with May–*
37 *Grunwald–Giemsa or phenol thionin.*

38 *The organism is highly fastidious. For growth it is necessary to use Francis medium, McCoy and*
39 *Chapin medium, or Modified Thayer-Martin agar. The colonies are small, round and transparent,*
40 *and do not appear before 48 hours incubation at 37°C. On Francis medium, the colonies may be*
41 *confluent and have a milky appearance. If transportation is necessary, samples should be*
42 *inoculated into sterile nutrient broth and stored at 4–10°C for a few hours or at –70°C if transit is*
43 *likely to be prolonged.*

44 *In the past mice or guinea-pigs were experimentally inoculated with infected tissue material or with*
45 *cultures to aid in the diagnosis of tularemia. Animal inoculation has been replaced by polymerase*

46   *chain reaction (PCR) protocols for the identification of* F. tularensis. *The FAT demonstrates*
47   F. tularensis *in pathological specimens.*

48   ***Serological tests:*** *Serological tests are useful diagnostic aids in human infection, but are of*
49   *limited value in the more susceptible animal species that usually die before developing antibodies.*
50   *Epidemiological surveys can be conducted in domestic animals in relatively resistant species that*
51   *survive the infection, such as sheep, cattle, pigs, dogs, cats and wild ungulates, as these species*
52   *develop antibodies. Also relatively resistant species of rodents and lagomorphs can also be used*
53   *in epidemiological surveys. Serological surveys can and are conducted in a number of wildlife*
54   *species, such as moose, that are exposed to* F. tularensis.

55   ***Requirements for vaccines and diagnostic biologicals:*** *A live attenuated vaccine strain is*
56   *available for vaccination of humans at high risk of exposure to virulent F. tularensis. However, the*
57   *vaccine is only available for restricted use. The outcome of vaccination can be estimated by the*
58   *demonstration of specific antibodies and the ability of lymphocytes to proliferate in the presence of*
59   F. tularensis *antigen.*

A. INTRODUCTION

61   Tularemia is a zoonosis caused by *Francisella tularensis.* It occurs naturally in lagomorphs (rabbits and hares)
62   and rodents, especially microtine rodents such as voles, vole rats and muskrats, as well as in beavers. In
63   addition, a wide variety of other mammals, birds, amphibians and invertebrates have been reported to be
64   infected (19, 20). Tularemia occurs endemically in the Northern Hemisphere. The disease can occur as epizootic
65   outbreaks in many countries in North America and Europe, while it occurs only as sporadic cases in some other
66   countries in Europe and Asia. It is rarely reported from the tropics or the Southern Hemisphere. Several epizootic
67   outbreaks have been reported as a result of importation of subclinically infected lagomorphs.

68   The clinically most relevant two types of *F. tularensis* are recognised on the basis of culture characteristics,
69   epidemiology, and virulence. *Francisella tularensis* subsp. *tularensis* (Type A) is mainly associated with
70   lagomorphs in North America. It is primarily transmitted by ticks or biting flies, or by direct contact with infected
71   lagomorphs. It is highly virulent for humans and domestic rabbits, and most isolates ferment glycerol. *Francisella*
72   *tularensis* subsp. *palaearctica* (Type B) occurs mainly in aquatic rodents (beavers, muskrats) and voles in
73   northern North America, and in lagomorphs (hares) and rodents in Eurasia. It is primarily transmitted by direct
74   contact or by mosquitoes, but may be transmitted through inhalation or through infected water or food. It is less
75   virulent for humans and domestic rabbits, and does not ferment glycerol (1, 7, 14, 15, 17, 22).

76   In sensitive animals, clinical signs of severe depression are followed by a fatal septicaemia. The course of the
77   disease is approximately 2–10 days in susceptible species, and animals are usually dead when presented for
78   diagnosis. Most domestic species do not usually manifest signs of tularemia infection, but they do develop
79   specific antibodies to the organism following infection. Outbreaks with high mortality caused by the Type A
80   organism have occurred in sheep (1, 17). Among domestic animals, the cat has been reported to be able to act
81   as a carrier of the bacterium (6) and the disease is occasionally spread from cats to humans (8).

82   At necropsy, animals that have died from acute tularemia are usually in good body condition. There are signs of
83   septicaemia characterised by whitish foci of necrosis randomly distributed in the liver, bone marrow and spleen.
84   In addition, the spleen is usually enlarged. Necrotic foci vary in size, and in some cases may be barely visible to
85   the naked eye. The lungs are usually congested and oedematous, and there may be areas of consolidation and
86   fibrinous pneumonia or pleuritis. Fibrin may be present in the abdominal cavity. Foci of caseous necrosis are
87   often present in one or more lymph node(s). The lymph nodes that are most often affected are those in the
88   abdominal and pleural cavities and lymph nodes draining the extremities. In less sensitive species, the
89   histological picture can resemble that of tuberculosis with chronic granulomas in liver, spleen, lungs and kidneys.

90   There is a high risk of human infection from *F. tularensis,* as the infective dose is extremely low and infected
91   animals excrete bacteria in urine and faeces. Infection can occur by simple contact. Suitable precautions, such
92   as the wearing of gloves, masks and eyeshields during any manipulation of pathological specimens or cultures,
93   must be taken in order to avoid human infection. The facility should meet at least the requirements for
94   Containment Group 3 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary
95   microbiological laboratory and animal facilities. Countries lacking access to such a specialised national or
96   regional laboratory should send specimens to the OIE Reference Laboratory. Experimentally inoculated animals
97   and their excreta are especially hazardous to humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Francisella tularensis can be demonstrated in smear preparations or in histological sections. It can also be identified by culture or animal inoculation. However, *F. tularensis* may be difficult to isolate from dead animals and carcasses due to overgrowth of other bacteria. As the post-mortem picture is variable, diagnosis is sometimes difficult and immunological or immunohistochemical methods are preferable, although reagents may be difficult to obtain. It can sometimes be recommended, therefore, that fixed specimens be analysed at laboratories equipped with proper reagents or methods, such as the OIE Reference Laboratory (see Part 3 of this *Terrestrial Manual*).

a) Smear preparations

Smear preparations are made on microscope slides as impression smears of organs such as the liver, spleen, bone marrow, kidney, lung or blood. The bacteria are abundant in such smears, but may be overlooked because of their very small size (0.2–0.7 µm). The bacteria can be demonstrated by direct or indirect fluorescent antibody staining. This is a safe, rapid and specific diagnostic tool (13, 16, 18).

Gram staining of smears reveals a scattering of small, punctiform Gram-negative bacteria near the limit of visibility. The use of oil microscopy increases the visibility of the bacteria. The bacteria may be difficult to distinguish from precipitates of stain.

b) Histological sections

Bacteria can be demonstrated in sections using immunohistochemical methods, such as the fluorescent antibody test (FAT) (16). The test is normally performed on specimens from liver, spleen or bone marrow, fixed in neutral buffered formalin and paraffin embedded. Slides are treated with rabbit anti-tularemia serum, washed and thereafter treated with sheep fluorescein-isothiocyanate-conjugated anti-rabbit serum. The samples are examined under a fluorescence microscope. Large numbers of bacteria can be seen in necrotic lesions and in the blood.

c) Culture

Francisella tularensis will not grow on ordinary media, although an occasional strain can sometimes, on initial isolation, grow on blood agar. Incubation is at 37°C in ambient air or in 5% CO_2. Heart blood, liver, spleen and bone marrow from moribund animals should be used for culture. It is necessary to use special culture media, such as:

i) *Francis medium:* Peptone agar containing 0.1% cystine (or cysteine) and 1% glucose, to which is added, before solidification, 8–10% defibrinated rabbit, horse or human blood.

ii) *McCoy and Chapin medium:* This consists of 60 g egg yolk and 40 ml normal saline solution, carefully mixed and coagulated by heating to 75°C.

iii) *Modified Thayer–Martin agar:* Glucose cysteine agar (GCA)-medium base supplemented with haemoglobin and Iso VitaleX.

Media can be stored for up to 8–10 days at 4°C. Colonies that form on McCoy medium are small, prominent, round and transparent. A more abundant growth is obtained on Francis medium and modified Thayer–Martin agar, with confluent colonies that have a milky appearance and a mucoid consistency. On either medium, colonies do not appear until after 48 hours' incubation at 37°C.

iv) *GCA agar with thiamine* (*BBL*): When used with added blood, the medium is commonly referred to as GBCA and can be substituted for the original, noncommercial medium described by Down *et al.* (5). Suspend 58 g of the dry material in 1 litre of distilled or demineralised water, and mix thoroughly. Heat with frequent agitation and boil for 1 minute. Dispense into tubes and sterilise by autoclaving at 118–121°C for 15 minutes.

For larger volumes (up to several litres) of culture medium, autoclave at the same temperatures for 30 minutes. Cool to 45–48°C. Aseptically add 25 ml of packed human blood cells or 50 ml of defibrinated rabbit or sheep blood. Mix thoroughly and pour into plates. Incubate at 37°C for 24 hours before use to decrease surface moisture and to test for sterility (5).

The following selective medium can be used in addition to the non-selective media: *Cystine heart agar* (DIFCO) with 5% rabbit blood, and penicillin (100,000 units), Polymyxin B sulphate (100, 000 units), and cycloheximide (0.1 ml of a 1% stock solution) per litre.

Differential criteria for the identification of *F. tularensis* include absence of growth on ordinary media, distinctive cellular morphology, and specific fluorescent antibody and slide agglutination reactions. The

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bacteria are nonmotile, nonsporulating, bipolar staining, and of uniform appearance in 24-hour cultures, but pleomorphic in older cultures.

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Francisella tularensis can be identified in stained smears, by agglutination with tularemia hyperimmune antiserum, or by animal inoculation. In areas of North America where both types of *F. tularensis* may occur, Type A may be distinguished from Type B by the fact that most Type A ferment glycerol.

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The bacteria can also be identified by hybridisation with probes specific to the 16S rRNA of *F. tularensis*, *F. tularensis* Type A, and *F. tularensis* Type B (10), or by polymerase chain reaction (PCR) with primers targeting specific regions of the 16 rDNA molecules. The PCR will allow identification at the genus, species and subspecies level (11).

160 **d) Capillary tube precipitation test on pathological samples**

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Tissues, such as spleen, liver or bone marrow, are ground with sterile sand in three-to-five times their volume of normal saline. The suspension is transferred to a tube and two volumes of ethyl ether are added. After shaking, the mixture is allowed to stand for 4–5 hours at room temperature. It is again shaken and then allowed to stand overnight.

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The aqueous phase is drawn off and centrifuged at 2000 **g** for 30 minutes. The supernatant fluid, containing the antigen, is drawn off and distributed into capillary tubes to which tularemia antiserum is added.

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The tubes are incubated at 37°C for 3 hours, then kept at 4°C overnight. A positive result is the formation of a ring of precipitate.

169 **e) Animal inoculation**

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Animal inoculation is extremely hazardous and is only recommended for agent identification in cases when culture is negative and agent identification is needed for epidemiological reasons. It should only be undertaken where proper biosafety facilities and cages are available (see Chapter 1.1.2). PCR techniques should be used for identification of *F. tularmsis.*

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Laboratory animals (preferably mice) are inoculated with culture material to confirm the nature of an isolate. Pathological specimens may be inoculated for the direct detection of *F. tularensis*. Inoculated mice usually die before lesions can form.

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Intraperitoneal injection is sufficient for passage of pure cultures. All routes of administration in mice, such as subcutaneous, percutaneous, or intravenous, will lead to an infection that is invariably fatal within 2–10 days.

180 **f) Molecular techniques**

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The PCR has recently been developed for the diagnosis of *F. tularensis* and is an excellent method for identifying the agent in wound specimens from humans (12).

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A real-time PCR has proven to be a highly sensitive and specific assay and is a major diagnostic improvement, as all other methods for the specific identification of *F. tularensis* subsp. *tularensis* are very time-consuming (23).

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A DNA microarray has also been developed that is capable of distinguishing the highly virulent subspecies *F. tularensis* subsp. *tularensis* and the moderately virulent subspecies *F. tularensis* subsp. *holarctica* (3).

188 ## 2. Serological tests

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Serology is currently carried out for diagnosis of tularemia in humans, but is of limited value in sensitive animal species, which usually die before specific antibodies can develop. Serology may be employed, either on sera or on lung extracts (18), in epidemiological surveys of animals that are resistant to infection, such as sheep, cattle, pigs, moose, dogs or birds (18, 20). As there is no antigenic difference between Type A and Type B, the less virulent *F. tularensis palaearctica* could be used as antigen in all serological tests.

194 **a) Tube agglutination**

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The most commonly used serological test is the tube agglutination test. The antigen is a culture of *F. tularensis* on Francis medium. The culture is harvested after 5–6 days. Younger cultures yield a poorer antigen. The colonies are suspended in 96% alcohol, giving a thick suspension that can be stored for 1–7 days at room temperature. The sediment is washed with normal saline and resuspended in an equal

199 volume of normal saline. Crystal violet powder is added to a final concentration of 0.25%. The bacteria are
200 stained by adding crystal violet and incubating at 37°C for at least 24 hours and at most 7 days.

201 After the supernatant fluid has been discarded, the deposit is suspended in normal saline with or without
202 thimerosal (merthiolate) at a final concentration of 1/10,000, or formaldehyde at a final concentration of
203 0.5%. The suspension is calibrated with positive and negative sera, and adjusted by adding normal saline to
204 provide an antigen that when tested on a slide gives readily visible stained agglutination reactions against a
205 clear fluid background.

206 The test is performed in tubes containing a fixed amount of antigen (0.9 ml) and different dilutions of serum
207 commencing with 1/10, 1/20, 1/40, etc. The results are read after 20 minutes of shaking, or after 1 hour in a
208 water bath at 37°C followed by overnight storage at room temperature. The agglutinated sediment is visible
209 to the naked eye or, preferably, by using a hand lens. The positive tubes are those that have a clear
210 supernatant fluid. Possible cross-reactions with *Brucella abortus, B. melitensis* and *Legionella* sp. have to
211 be taken into consideration.

212 **b) Enzyme-linked immunosorbent assay**

213 Another serological test, the enzyme-linked immunosorbent assay (ELISA), also allows an early diagnosis
214 of tularemia (4). This method is now widely used for clinical purposes. Different antigens, whole bacteria as
215 well as subcellular components (9), have been used as recall antigens against immunoglobulines IgA, IgM
216 and IgG; 2 weeks after the onset of tularemia, specific antibodies can be detected in the serum. IgM is
217 sustained for a long period and cannot be used as an indication of a recent infection (2). For routine
218 diagnosis, whole heat-killed (65°C for 30 minutes) bacteria can be used as antigen. Bacteria can be coated
219 to plastic plates, using the usual procedures (4) followed by serial dilutions of serum to be tested. Positive
220 reactions can be visualised by anti-antibodies labelled with enzyme. The test should also be read in a
221 photometer with positive and negative sera as controls.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

223 Vaccines have been produced with the aim of protecting humans, but as all vaccine development involves testing
224 in animals, it is clear that some of the vaccines could be used to protect animals. However, in most countries,
225 there is no vaccine licensed for use in animals.

226 Before 1940, attempts to vaccinate against tularemia were performed by use of whole killed bacteria or bacterial
227 extracts. None of these vaccines induced protection against highly virulent strains of *F. tularensis*. Instead, viable
228 attenuated vaccines were developed. Attenuation was performed by repeated culture of bacteria on various
229 media with or without antiserum. Such live attenuated vaccines have been used in mass vaccinations of people
230 in the former Soviet Union since 1946, either as monocultures or as a mixture of strains.

231 An attenuated live vaccine strain of *F. tularensis* biovar palaearctica is available and can be used for restricted
232 vaccination of individuals at high risk (21).

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286   *

287   * *

288 **NB:** There is an OIE Reference Laboratory for Tularemia (see Table in Part 3 of this *Terrestrial Manual* or consult
289   the OIE Web site for the most up-to-date list: www.oie.int).

VESICULAR STOMATITIS

SUMMARY

Vesicular stomatitis (VS) is a vesicular disease of horses, cattle and pigs caused by vesiculoviruses of the family Rhabdoviridae. This disease is clinically indistinguishable from foot and mouth disease (FMD), vesicular exanthema of swine (VES), or swine vesicular disease (SVD) when horses are not involved. Sheep, goats and many other wild species can be infected. Humans are also susceptible. The disease is limited to the Americas; however, it was previously described in France and in South Africa.

Although virus is transmitted directly by the transcutaneous or transmucosal route, it has been isolated from sandflies and mosquitoes, suggesting that it could be insect-borne. There is, therefore, seasonal variation in the occurrence of VS: it disappears at the end of the rainy season in tropical areas, and at the first frosts in temperate zones. There is also some evidence that it could be a plant virus and that animals are the end of the epidemiological chain. The pathogenesis of the disease is unclear, and it has been observed that the humoral-specific antibodies do not always prevent infection with VS serogroup viruses.

Although VS may be suspected when horses are involved as well as pigs and cattle, prompt differential diagnosis is essential because the clinical signs of VS are indistinguishable from FMD when cattle and pigs are affected, and from SVD or VES when only pigs are affected.

Identification of the agent: *Virus can be readily isolated by the inoculation of several tissue culture systems, unweaned mice or embryonated chicken eggs. Viral RNA can be detected from epithelial tissue and vesicular fluid by conventional and real-time reverse transcriptase polymerase chain reaction (PCR). Viral antigen can be identified by an indirect sandwich enzyme-linked immunosorbent assay (IS-ELISA) – this is the least expensive and most rapid test. The complement fixation (CF) test is also a good alternative. The virus neutralisation (VN) test may be used, but it is elaborate and time-consuming.*

Serological tests: *Convalescent animals develop serotype-specific antibodies within 4–8 days of infection that are demonstrated by a liquid-phase blocking ELISA (LP-ELISA), a competitive ELISA (C-ELISA) and VN. Other described tests are CF, agar gel immunodiffusion and counter immunoelectrophoresis.*

Requirements for vaccines and diagnostic biologicals: *Inactivated virus vaccines with aluminium hydroxide or oil as adjuvants have been tested in the United States of America and in Colombia, respectively. Both vaccines generated high levels of specific antibodies in the sera of vaccinated cattle. However, it is not yet clear if serum antibodies would prevent the disease. An attenuated virus vaccine has been used in the field with unknown efficacy.*

A. INTRODUCTION

Vesicular stomatitis (VS) was described in the United States of America (USA) in 1926 (18) and 1927 (7) as a vesicular disease of horses, and subsequently of cattle and pigs. Vesicles are caused by virus on the tongue, lips, buccal mucosa, teats and in the coronary band epithelium of cattle, horses, pigs, and many other species of domestic and wild animals. Natural disease in sheep and goats is rare, although both species can be experimentally infected. Mixed infections of foot and mouth disease (FMD) and VS viruses have occurred in the same herds of cattle and can be induced experimentally. Many species of laboratory animals are also susceptible. The disease is limited to the Americas; however, it was described in France (1915 and 1917) and in South Africa (1886 and 1897) (11).

Influenza-like signs, normally without vesicles, have been observed in humans who are in contact with animals with VS or who handle infective virus. All manipulations involving virus, including infective materials from animals, should be undertaken with using proper biosafety procedures.

There are two distinct immunological classes of vesicular stomatitis virus (VSV) that have been recognised: New Jersey (NJ) and Indiana (IND). Both viruses are members of the genus *Vesiculovirus*, family Rhabdoviridae and have been extensively studied at the molecular level. Several other closely related rhabdoviruses have been isolated from sick animals over the past decades. There are three subtypes of the IND serogroup based on serological relationships: IND-1 IND-2 and IND-3; they are also known as classical IND virus (VSIV), cocal virus (COCV), and alagoas virus (VSAV), respectively (8). Strains of the serotype NJ and subtype IND-1 are endemic in livestock in areas of southern Mexico, Central America, Venezuela, Colombia, Ecuador and Peru, with VSV NJ causing the vast majority (>80%) of the clinical cases. Sporadic activity of NJ and IND-1 VSV has been reported in northern Mexico and western United States. IND-2 has only been isolated in Argentina and Brazil and only from horses (Salto-Argentina/63, Maipú-Argentina/86, Rancharia-Brazil/66, Riberao-Brazil/79) (2, 3). Cattle living together with the affected horses did not develop antibodies against VSV (2). The IND-3 subtype, (Alagoas-Brazil/64), has been identified, sporadically only in Brazil and only in horses until 1977. However, in 1977 the IND-3 serotype (Espinosa-Brazil/77 strain) was first isolated from cattle in Brazil; it has been observed that this serotype affects cattle to a lesser degree than horses (2, 3). This finding confirms the first descriptions, in 1926 and 1927 (7, 18), of the NJ and IND serotypes in horses, and subsequently in cattle and pigs; this same predilection has been observed in other VS outbreaks.

The mechanism of transmission of the virus is unclear. The fact that viruses have been isolated from sandflies, mosquitoes, and other insects tends to substantiate the hypothesis that it could be transmitted by insects (6, 10, 17). There are also hypotheses that the VS virus is a plant virus present in pasture (17) and that animals are the end of the epidemiological chain and, in special circumstances, the virus could undergo an adaptation process to infect animals, followed by direct transmission between susceptible animals. During the 1982 epizootic in western USA, there were a number of cases where there was direct transmission from animal to animal (20). While VS is not diagnosed in livestock every year in the USA, it is considered to be endemic in feral pigs on Ossabaw Island, Georgia (5).

The incidence of disease can vary widely among affected herds. Usually 10–15% of the animals show clinical signs. Clinical cases are mainly seen in adult animals. Cattle and horses under 1 year of age are rarely affected. Mortality is close to zero in both species. However, high mortality rates in pigs affected by the NJ virus have been observed. Sick animals recover in about 2 weeks. The most common complications of economic importance are mastitis and loss of production in dairy herds (16). Both NJ and IND-1 serotypes in the 1995, 1997 and 1998 US outbreaks primarily caused clinical disease in horses. Although some clinical signs have been observed in cattle, the primary finding in cattle was seroconversion.

B. DIAGNOSTIC TECHNIQUES

VS cannot be reliably clinically differentiated from the other vesicular diseases, such as foot and mouth disease (FMD), vesicular exanthema of swine (VES), and swine vesicular disease (SVD) when horses are not involved. An early laboratory diagnosis of any suspected VS case is therefore a matter of urgency.

The sample collection and technology used for the diagnosis of VS must be in concordance with the methodology used for the diagnosis of FMD, VES and SVD, in order to facilitate the differential diagnosis of these vesicular diseases. Note: VS serogroup viruses can be human pathogens and appropriate precautions should be taken when working with potentially infected tissues or virus (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

Vesicle fluid, epithelium covering unruptured vesicles, epithelial flaps of freshly ruptured vesicles, or swabs of the ruptured vesicles are the best diagnostic samples. These samples can be collected from mouth lesions, as well as from the feet and any other sites of vesicle development. It is recommended that animals should be sedated before samples are collected to avoid injury to helpers and for reasons of animal welfare. Samples from all species should be placed in containers of Tris-buffered tryptose broth with phenol red, pH 7.6. If complement fixation (CF) is to be carried out for antigen detection, samples from all species can be collected in glycerol/phosphate buffer, pH 7.2–7.6. (Note: glycerol is toxic to virus and decreases the sensitivity of virus isolation; it is therefore only recommended for collection of samples for CF test.) Samples should be kept refrigerated and if they can arrive at the laboratory within 48 hours after collection, they should be sent refrigerated. If samples are sent frozen with dry ice, precautions should be taken to protect the sample from contact with any CO_2. There are special packaging requirements for shipping samples with dry ice (see Chapter 1.1.1 Collection and shipment of diagnostic specimens, for further information on shipping of diagnostic samples).

When epithelial tissue is not available from cattle, samples of oesophageal–pharyngeal (OP) fluid can be collected by means of a probang (sputum) cup. In pigs, throat swabs can be taken for submission to a laboratory

for virus isolation. This material should be sent to the laboratory refrigerated in Tris-buffered tryptose broth. If the samples will be in shipment for more than 48 hours after collection, they should be sent frozen with dry ice as described previously. Probang samples for isolation of virus should not be treated with solvents such as chloroform. Virus can be isolated from oral and nasal specimens up to 7 days post-infection.

When it is not possible to collect samples for identification of the agent, serum samples from recovered animals can be used for detecting and quantifying specific antibodies. Paired sera from the same animals, collected 1–2 weeks apart, are preferred for checking the change in antibody titre.

Specific reagents for VS diagnosis are not commercially available and each laboratory must produce its own or obtain them from a Reference Laboratory. The two OIE Reference Laboratories for vesicular stomatitis (see Table given in Part 3 of this *Terrestrial Manual*), and the Institute for Animal Health[1], produce and distribute diagnostic reagents on request.

1. Identification of the agent

For identification of VS serogroup viruses and the differential diagnosis of vesicular diseases, clarified suspensions of field samples suspected to contain virus should be submitted for immunological testing. For virus isolation, the same samples are inoculated into appropriate cell cultures. The inoculation of African green monkey kidney (Vero), baby hamster kidney (BHK-21) and IB-RS-2 cell cultures with the same sample permits differentiation of the vesicular diseases: VS serogroup viruses cause a cytopathic effect (CPE) in all three cell lines; FMD virus causes a CPE in BHK-21 and in IB-RS-2, while SVD virus causes a CPE in IB-RS-2 only. Many other cell lines, as well as most primary cell cultures of animal origin, are susceptible to VS serogroup viruses.

Virus replicates and can be isolated in 8–10-day-old chicken embryos by inoculation into the allantoic sac, in 2–7-day-old unweaned mice by inoculation using any route, or in 3-week-old mice by intracerebral inoculation. In all three cases, virus causes death in between 2 and 5 days after inoculation.

The most susceptible route for horses and cattle is intradermalingual administration. Pigs are inoculated in the coronary band or on the snout. Vesicular lesions may be observed in the epithelial tissues of the mouth, teats and feet, 2–4 days after inoculation. The presence of secondary vesicles after inoculation of cattle and horses depends mainly on the VS virus isolate used. The snout is normally affected in pigs.

If a CPE develops in the cultures, the suspension fluids can be used for identification of the agent by different immunological tests and the cell culture can be stained with VS-specific fluorescent antibody conjugate and viral antigen detected by enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR). Similar tests can be performed on homogenate suspensions of the dissected musculo-skeletal tissues of dead mice and chicken embryos and with suspensions of epithelial samples. The brain tissue from mice is an excellent source of virus.

Due to the different morphological characteristics of the rhabdovirus (VS serogroup viruses), picornavirus (FMD virus and SVD virus), calicivirus (VES) and the large number of virus particles present in vesicular fluids and epithelial tissues, electron microscopy can be a useful diagnostic tool for differentiating the virus family involved.

The preferred immunological methods for the identification of the viral antigens in the laboratory are the ELISA (2, 9), the CF test (2, 13) and fluorescent antibody staining. The virus neutralisation (VN) test, with known positive antisera against the VS virus NJ and IND serotypes, may be used in tissue cultures, unweaned mice or embryonated eggs, but it is more time-consuming.

a) Virus isolation

 i)   Inoculate cell culture in Leighton tubes and 25 cm^2 flasks with the clarified suspension of tissues or vesicular fluid.

 ii) Incubate inoculated cell cultures at 37°C for 1 hour.

 iii) Discard inoculum and wash cell cultures three times with cell culture medium and replace with cell culture medium containing 2.5% fetal bovine serum (FBS).

 Iv) Incubate Leighton tube cell cultures at 33–35°C and observe for CPE.

 v)   After 18–24 hours of incubation, the cover-slip from one Leighton tube culture per specimen inoculated is stained with New Jersey and Indiana VS virus-specific fluorescent antibody (FA) conjugate.

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1   Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, United Kingdom.

vi) Remaining Leighton tube cultures and 25 cm^2 flask cultures are incubated at 35–37°C for 6 more days and observed daily for CPE.

vii) At 7 days post-inoculation, the remaining Leighton tube cover-slips are stained with FA conjugate.

viii) If CPE is observed and the FA staining is negative, a second passage as is made, as described above, using the cells from the 25 cm^2 flask. Note: First passage cultures with significant CPE may yield false-negative immunofluorescence results. Serial tenfold dilutions may be prepared and inoculated to provide distinct plaques of fluorescing cells.

ix) *Interpretation of the results:* If no fluorescence is observed and no CPE evident in the flask culture, the sample is negative for virus isolation. If specific fluorescence is observed, the sample is positive for virus isolation.

x) Alternatively cell culture in flasks can be inoculated with field samples, incubated at 35–37°C for 48 hours and observed daily for CPE. If no CPE is observed after 48 hours, the flask cultures are frozen and thawed and a sample of the supernatant is inoculated into fresh cell culture. Up to three passages are made, of 48 hours each. To detect the presence of VSV antigen, clarified supernatants of each passage are tested by ELSA or CF test.

b) **Enzyme-linked immunosorbent assay**

The indirect sandwich ELISA (IS-ELISA) (2, 9) is currently the diagnostic method of choice for identification of viral serotypes of VS and other vesicular diseases. Specifically, the ELISA procedure with a set of polyvalent rabbit/guinea-pig antisera, prepared against virions of the representative strains of the three subtypes of the IND serotype, identifies all strains of the VS virus IND serotype (2). For detection of VS virus NJ strains, a monovalent set of rabbit/guinea-pig antisera is suitable (2, 9).

- **Test procedure**

i) *Solid phase:* ELISA plates are coated either for 1 hour at 37°C or overnight at 4°C with rabbit antisera and normal rabbit serum (as described in refs 2 and 4), and optimally diluted in carbonate/bicarbonate buffer, pH 9.6. Subsequently, the plates are washed once with phosphate buffered saline (PBS) and blocked for 1 hour at room temperature with 1% ovalbumin in PBS. The plates are used immediately or are washed three times and stored at –20°C for future use.

ii) *Test samples:* Antigen suspensions of test samples (10–20% epithelial tissue suspension, musculo-skeletal tissue of chicken embryo or mice in PBS or undiluted clarified cell culture supernatant fluid) are deposited in the corresponding wells and the plates are incubated for 1 hour at 37°C on an orbital shaker.

iii) *Detector:* Monovalent and polyvalent guinea-pig antisera to VS virus NJ and IND serotypes, respectively, that are homologous to coated rabbit serum and that have been diluted appropriately in PBS containing 0.05% Tween 20, 1% ovalbumin, 2% normal rabbit serum, and 2% normal bovine serum (PBSTB) are added to the corresponding wells and left to react for 1 hour at 37°C on an orbital shaker.

iv) *Conjugate:* Peroxidase/rabbit or goat IgG anti-guinea-pig Ig conjugate, diluted in PBSTB, is added and left to react for 1 hour at 37°C on an orbital shaker.

v) *Substrate:* H_2O_2-activated substrate is added and left to react at room temperature for 15 minutes, followed by the addition of sulphuric acid to stop the reaction. Absorbance values are measured using an ELISA reader.

Throughout the test, 50 µl reagent volumes are used. The plates are washed five times between each stage with PBS containing 0.05% Tween 20. Controls for the reagents used are included.

vi) *Interpretation of the results:* An antiserum giving an absorbance more than 20% greater than the other antisera, negative serum and controls is considered to be positive for the corresponding virus subtype.

c) **Complement fixation test**

The ELISA is preferable to the CF test because it is more sensitive and it is not affected by pro- or anti-complementary factors. When ELISA reagents are not available, however, the CF test may be performed. The CF test in U-bottomed microtitre plates, using the reagents titrated by CF50% test, is described.

- **Test procedure**

i) *Antisera:* Guinea-pig monovalent anti-NJ VS virus and polyvalent anti-IND VS virus, diluted in veronal buffer (VB) at a dilution containing 2.5 CFU$_{50}$ (50% complement fixation units) against homologous virus, are deposited in plate wells. Those antisera are the detectors used in ELISA.

ii)   *Test samples:* The antigen suspension of test samples, prepared as described for IS-ELISA, is added to the wells with serum.

iii) *Complement:* 4 CHU_{50} (50% complement haemolytic units) are added to the serum and antigen. (An alternative is to use 7.5, 10 and 20 CHU_{50} with the goal of reaching 4 CHU_{50} in the test.) The mixture of antisera, test samples and complement is incubated at 37°C for 30 minutes.

iv)   *Haemolytic system:* A suspension of sheep red blood cells (SRBC) in VB, sensitised with 10 HU_{50} (50% haemolytic units) of rabbit anti-SRBC serum, is added to the wells. The haemolytic system has an absorbance of 0.66 read at 545 nm, in the proportion of two volumes of haemolytic system + three volumes of distilled water. The mixture is incubated for 30 minutes at 37°C. Subsequently, the plates are centrifuged and the reaction is observed visually.

Volumes of 25 µl for antisera, test samples and complement, and 50 µl of haemolytic system, are required. Appropriate controls for the antisera, antigens, complement and haemolytic system are included.

It is possible to perform the CF50% test in tubes (2) using reagent volumes eight times greater than those indicated for the CF in microtitre plates. With the CF50% test, the reaction can be expressed as absorbance read spectrophotometrically at 545 nm.

v)   *Interpretation of the results:* When controls are as expected, samples with haemolysis <20% for one antiserum in comparison with the other antiserum and controls are considered to be positive for the corresponding type.

Field samples that are negative on the ELISA or CF test should be inoculated into cell culture or unweaned mice. If there is no evidence of viral infection after three passages, the specimen is considered to be negative for virus.

d)   Nucleic acid recognition methods

The PCR can be used to amplify small genomic areas of the VS virus (12, 19). This technique will detect the presence of virus RNA in tissue and vesicular fluid samples and cell culture, but cannot determine if the virus is infectious. In general, PCR techniques have not been routinely used for screening diagnostic cases for viruses causing VS.

2.   Serological tests

For the identification and quantification of specific antibodies in serum, the ELISA and the VN test are preferable. The CF test may be used for quantification of early antibodies. Antibody can usually be detected between 5 and 8 days post-infection; the length of time antibody persists has not been accurately determined for the three tests but is thought to be relatively short for the CF and for extended periods for the VN and ELISA (14).

a)   Enzyme-linked immunosorbent assay (a prescribed test for international trade)

The liquid-phase blocking ELISA (LP-ELISA) is the method of choice for the detection and quantification of antibodies to VS serogroup viruses. The use of viral glycoproteins as antigen is recommended because they are not infectious, allow the detection of neutralising antibodies, and give fewer false-positive results than the VN (4).

*   **Test procedure**

i)   *Solid phase:* As described above in Section B.1.a for the IS-ELISA.

ii) *Liquid phase:* Duplicate, twofold dilution series of each test serum, starting at 1/4, are prepared in U-bottomed microtitre plates. An equal volume of VS virus NJ or IND glycoprotein, in a dilution providing 70% reaction, is added to each well and the plates are incubated for 1 hour at 37°C. 50 µl of these mixtures is then transferred to the ELISA plates with the solid phase and left to react for 30 minutes at 37°C on an orbital shaker.

iii) *Detector, conjugate and substrate:* The same reagents and methods are used as those indicated for the IS-ELISA.

iv) *Interpretation of the results:* 50% end-point titres are expressed in $\log_{10}$ in reference to the 50% reduction of negative serum control, according to the Spearmann–Kärber method. Titres of >1.3 (1/20) are considered to be positive.

*   **Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)**

A competitive ELISA for detection of antibodies has also been developed. The procedure described here is based on a procedure described by Afshar *et al.* (1). It uses vesicular stomatitis NJ and IND-1 recombinant antigens as described by Katz *et al.* (15).

- **Test procedure**

i) *Solid phase:* Antigens are diluted in carbonate/bicarbonate buffer, pH 9.6, and 50 µl is added to each well of a 96-well ELISA plate. The plates are incubated overnight at 4°C; coated plates can be frozen at –70°C for up to 60 days. The plates are thawed, antigen is decanted, and 100 µl of blocking solution (5% nonfat dry milk powder solution in PBS [for example, 5 g dry milk powder dissolved in 95 ml PBS) is added. The plates are then incubated at 25°C for 30 minutes and blocking solution is decanted. The plates are washed three times with PBS/0.05% Tween 20 solution.

ii) *Liquid phase:* 50 µl of serum diluted 1/8 in 1% nonfat dry milk in PBS is added to each of the duplicate wells for each sample. A positive and negative control serum for each serotype should be included on each ELISA plate. The plates are incubated at 37°C for 30 minutes. Without washing, 50 µl of polyclonal ascites fluid is added to each well and plates are incubated at 37°C for 30 minutes.

iii) *Detector:* The plates are washed three times, and 50 µl of goat anti-mouse horseradish-peroxidase conjugate diluted in 1% nonfat dry milk with 10% normal goat serum is added to each well. The plates are incubated at 37°C for 30 minutes, washed three times, and 50 µl of tetramethyl-benzidine (TMB) substrate solution is added to each well. The plates are incubated at 25°C for 5–10 minutes and then 50 µl of 0.05 M sulphuric acid is added to each well. The plates are read at 450 nm and the optical density of the diluent control wells must be > 1.0.

iv) *Interpretation of the results:* A sample is positive if the absorbance is ≤50% of the absorbance of the diluent control.

b) **Virus neutralisation (a prescribed test for international trade)**

The VN test is carried out in tissue culture microtitre plates with flat-bottomed wells using inactivated serum as test sample, 1000 $TCID_{50}$ (50% tissue culture infective dose) of VS NJ or IND virus, and Vero M cells, or preformed monolayer (4) or a suspension IB-RS-2 cells to test for the presence of unneutralised virus.

- **Test procedure**

i) *Virus:* VS NJ or IND virus is grown in Vero cell monolayers and stored in liquid nitrogen or frozen at –70°C.

ii) *Test samples:* Sera are inactivated at 56°C for 30 minutes before testing. Positive and negative control standard sera are included in the test.

iii) *Virus neutralisation:* Sera are diluted in a twofold or four-fold dilution series across the plates, starting from 1/4 dilution. Two rows of wells are used per serum. The same volume of NJ or IND VS virus suspension containing about 1000 $TCID_{50}$/25 µl is added and incubated at 37°C for 60 minutes to allow neutralisation to take place. Subsequently, 50 µl of the mixtures is deposited on preformed cell monolayers in microtitre plates or 150 µl of 300,000/ml IB-RS-2 or Vero cell suspension is added to each well with the serum/virus mixtures. The plates are covered with loosely fitting lids and incubated for 48–72 hours at 37°C in an atmosphere of 5% CO_2 or sealed with pressure-sensitive tape and incubated in a normal atmosphere. (It has been determined that a virus titre of 1000 $TCID_{50}$ will decrease the nonspecific reactions and maintain a high test sensitivity.)

iv) *Interpretation of the results:* Wells without CPE are considered to be positive. End-point titres of test serum titres are determined by the Spearmann–Kärber method when the virus titres are between 750 and 1330 $TCID_{50}$ and when titres of positive and negative standard sera are within twofold of their mean values as estimated from previous titration. The 100% neutralisation titres of each serum are expressed at log 10. Sera with values of 1/32 or greater are considered to be positive for antibodies against VSV. In an alternative protocol, the end-point titre of the test serum is determined when the virus doses are between $10^{2±0.5}$/100 µl and when titres of positive and negative standard sera are within twofold of their mean values as estimated from the previous titration. The 50% neutralisation titre of each serum is expressed as log 10. Sera with values of 1.3 (1/20) or greater are considered to be positive for VS antibodies (4).

c) **Complement fixation (a prescribed test for international trade)**

A detailed description of this test is given in Section B.1.b. This is modified as follows. The CF test may be used for quantification of early antibodies, mostly IgM. For this purpose, twofold serum dilutions are mixed with 2 CFU_{50} of known antigen and with 5% normal bovine or calf sera included in 4 CHU_{50} of complement. The mixture is incubated for 3 hours at 37°C or overnight at 4°C. Subsequently, the haemolytic system is added followed by incubation for 30 minutes at 37°C. The serum titre is the highest dilution in which no haemolysis is observed. Titres of 1/5 or greater are considered to be positive. This CF has low sensitivity and is frequently affected by anticomplementary or nonspecific factors.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Attenuated virus vaccines have been tested in the field in the USA, Panama, Guatemala, Peru and Venezuela (16, 17) with unknown efficacy. Killed vaccines for the Indiana and New Jersey serotypes are manufactured in Colombia and Venezuela (2002 OIE vaccine survey).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Identity of the seed and the source of the serum used in growth and passage of the virus should be well documented, including the source and passage history of the organism.

b) Method of culture

Virus seed can be grown in cell culture. Selection of a cell type for culture is dependent on the degree of virus adaptation, growth in medium, and viral yield in the specific culture system. Vaccine products should be limited to the number of passages from the master seed virus (MSV) that can be demonstrated to be effective.

c) Validation of culture

The purity of the seed and cells to be used for vaccine production must be demonstrated. The MSV should be free from adventitious agents, bacteria, or *Mycoplasma*, using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against the seed virus and the virus/antibody mixture is cultured on several types of cell line monolayers. A cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended as one of the cell lines chosen for evaluation of the MSV. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems. Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for adventitious viruses that may have infected the cells or seed during previous passages.

d) Validation as a vaccine

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

Virus(es) used in vaccine production should be antigenically relevant to virus(es) circulating in the field. A vaccination/challenge study in the species for which the vaccine will be used will indicate the degree of protection afforded by the vaccine. Species used in vaccination/challenge studies should be free of antibodies against vesicular stomatitis. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using animal species of the minimum recommended age listed on the label. Initially, lots are formulated to contain varying amounts of viral antigen. The test lot containing the least amount of antigen that demonstrates protection becomes the standard against which future production lots are measured. For vaccines containing more than one virus (for example, New Jersey and Indiana-1), the efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different viruses exists.

2. Method of manufacture

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. When the virus has reached its appropriate titre, as determined by CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated. An inactivation kinetics study should be conducted using the approved inactivating agent on a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line, should indicate a linear and complete loss of titre by the end of the inactivation process. Typically, adjuvant is added to enhance the immune response.

3. In-process control

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. Virus concentration can be assessed using antigenic mass or infectivity assays.

4. Batch control

a) Sterility

During production, tests for bacteria, *Mycoplasma*, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

The completeness of viral inactivation in a killed product can be determined by multiple passes in cell culture of the post-inactivation, pre-adjuvant production fluids, followed by testing for the presence of virus.

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended. If the vaccine is to be used in horses, swine, cattle, or other ruminants destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

c) Potency

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation and prior to further processing. Relative potency can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

d) Duration of immunity

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label.

e) Stability

Vaccines should be stored at 4°C ± 2°C, with minimal exposure to light. The shelf life should be determined by use of the approved potency test (Section C.5.b) over the proposed period of viability.

f) Preservatives

Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible.

g) Precautions

Inactivated vesicular stomatitis vaccines probably present no special danger to the user, although accidental inoculation may result in an adverse reaction due to the adjuvant and secondary components of the vaccine.

5. Tests on the final product

a) Safety

Final container samples of completed product from inactivated vaccines should be tested.

b) Potency

The potency assay established at the time of the minimum antigen protection study should be used to evaluate new lots for release. The assay needs to be specific and reproducible. It must reliably detect vaccines that are not sufficiently potent.

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NB: There are OIE Reference Laboratories for Vesicular stomatitis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

WEST NILE FEVER

SUMMARY

*West Nile virus (*WNV*) is a member of the genus* Flavivirus *in the family* Flaviviridae*. The arbovirus is maintained in nature by cycling through birds and mosquitoes; numerous avian and mosquito species support virus replication. For many avian species, WNV infection causes no overt signs while other birds, such as American crows (*Corvus brachyrhynchos*) and Blue Jays (*Cyanocitta cristata*), often succumb to fatal systemic illness. Among mammals, clinical disease is primarily exhibited in horses and humans.*

Clinical signs of WNV infection in horses arise from viral-induced encephalitis or encephalomyelitis. Infections are dependent on mosquito transmission and are seasonal in temperate climates, peaking in the early autumn in the Northern Hemisphere. Affected horses frequently demonstrate mild to severe ataxia. Signs can range from slight incoordination to recumbency. Some horses exhibit weakness, muscle fasciculation, and cranial nerve deficits. Fever is not a consistently recognised feature of the disease in horses.

Identification of the agent: *Bird tissues generally contain higher concentrations of virus than equine tissues. Brain and spinal cord are the preferred tissues for virus isolation from horses. In birds, kidney, heart, brain, liver or intestine can yield virus isolates. Cell cultures (*using, for example, rabbit kidney or Vero cells*) are used most commonly for virus isolation. WNV is cytopathic in susceptible culture systems. Viral nucleic acid and viral antigens can be demonstrated in tissues of infected animals by reverse-transcription polymerase chain reaction (*RT-PCR*) and immuno-histochemistry, respectively. The most sensitive method for identifying WNV in equine tissues is a nested format of the RT-PCR procedure.*

Serological tests: *Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (*IgM capture ELISA*), haemagglutination inhibition (*HI*), IgG ELISA or plaque reduction neutralisation (*PRN*). The ELISA and PRN methods are most commonly used for identifying antibody against WNV in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus Japanese encephalitis virus, or tick-borne encephalitis (*TBE*) virus may be encountered.*

Requirements for vaccines and diagnostic biologicals: *A formalin-inactivated WNV vaccine derived from tissue culture, WNV live canarypoxvirus vectored vaccine, a WNV DNA vaccine and a chimeric vaccine are licensed for use in horses.*

A. INTRODUCTION

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae* (26). The genus *Flavivirus* also includes Japanese encephalitis virus (see Chapter 2.1.7), St Louis encephalitis virus, Murray Valley virus, Usutu virus, and Kunjin virus, among others (6). WNV has a wide geographical range that includes portions of Europe, Asia, Africa, Australia (Kunjin virus) and in North, Central and South America. Migratory birds are thought to be primarily responsible for virus dispersal, including reintroduction of WNV from endemic areas into regions that experience sporadic outbreaks (6). WNV is maintained in a mosquito–bird–mosquito transmission cycle, whereas humans and horses are considered dead end hosts. Genetic analysis of WN isolates separates strains into two clades. Lineage 1 isolates are found in northern and central Africa, Israel, Europe, India, Australia (Kunjin virus) and in North and Central America, and Columbia and Argentina in South America (18). Lineage 2 strains are endemic in central and southern Africa and Madagascar, with co-circulation of both virus lineages in central Africa (3, 7). There has been a recent report of linage 2 from Hungary. While recent human and equine outbreaks have been due to lineage 1 viruses, strains from each lineage have been implicated in human and animal disease.

WNV was recognised as a human pathogen in Africa during the first half of the 20th century. Although several WNV fever epidemics were described, encephalitis as a consequence of human WN infection was rarely encountered prior to 1996, but since then, outbreaks of human West Nile encephalitis have been reported from Romania, Russia, Israel, North America, France, and Tunisia (4, 11, 13, 15, 33). During the 1960s, West Nile viral encephalitis of horses was reported from Egypt and France (23, 25). Since 1998, outbreaks of equine WNV encephalitis have been reported from France, Italy, Canada, United States of America, Israel and Morocco (8, 14, 19, 21). In the Western Hemisphere, the virus range has dramatically expanded from a discrete region along the East Coast of New York State to include the contiguous States of the United States of America (USA), Canada, Mexico, the Caribbean islands, Central America, Argentina, Columbia and Venezuela (10, 18, 21, 30). Other than in the United States and Canada, the introduction of West Nile virus in the Western Hemisphere has not been characterised by large disease outbreaks or significant mortality in any species, possibly because of exposure to indigenous flaviviruses circulating in these regions

The incubation period for equine WN encephalitis following mosquito transmission is estimated to be 3–15 days. A fleeting viraemia of low virus titre precedes clinical onset (5, 25). WN viral encephalitis occurs in only a small per cent of infected horses; the majority of infected horses do not display clinical signs (21). The disease in horses is frequently characterised by mild to severe ataxia. Additionally, horses may exhibit weakness, muscle fasciculation and cranial nerve deficits (8, 21, 22, 27). Fever is an inconsistently recognised feature. Treatment is supportive and signs may resolve or progress to terminal recumbency. The mortality rate is approximately one in three clinically affected horses. Differential diagnoses in horses include other arboviral encephalidites (e.g. eastern, western or Venezuelan equine encephalomyelitis, Japanese encephalitis), equine protozoal myelitis (*Sarcocystis neurona*), equine herpesvirus-1, Borna disease and rabies.

Most species of birds can become infected with WNV; the clinical outcome of infection is variable. Chickens and turkeys, are resistant to disease, Outbreaks of fatal neurologic disease have been reported in zoo birds in the USA and in domestic geese in Israel and Canada (1, 28, 33). WNV has been associated with sporadic disease in small numbers of other species, including squirrels, chipmunks, bats, dogs, cats, white-tailed deer, reindeer, sheep, alpacas, alligator and a harbour seal during intense periods of local viral activity. Most human infections occur by natural transmission from mosquitoes, but laboratory acquired infections have been reported. In clinically suspicious cases, diagnostic specimens from all animals, particularly birds, should be handled at containment level 3 following appropriate laboratory procedures (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities) (24). There has been confirmed transmission of WNV in humans by blood transfusion, organ transfer and breast milk.

Due to the occurrence of inapparent WNV infections, diagnostic criteria must include a combination of clinical assessment and laboratory tests.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Culture

Specimens for virus isolation include brain and spinal cord from encephalitic horses (21, 22); a variety of bird tissues including brain, heart or liver may be used with success (28); WHV has been isolated from kidney but the tissue may be toxic to cell culture. In general, virus isolates are obtained more easily from avian specimens. Virus may be propagated in susceptible cell cultures, such as rabbit kidney (RK-13) and African green monkey kidney (Vero) cells, or embryonating chicken eggs. Intracerebral inoculations of newborn mice are less likely to yield virus isolates from mammalian tissues than cell culture methods. More than one cell culture passage may be required to observe cytopathic effect (CPE). Confirmation of WNV isolates is achieved by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods (see below).

b) Immunological methods

Immunohistochemical (IHC) staining of formalin-fixed avian tissues is a reliable method for identification of WNV infection in birds. Brain, heart, kidney, spleen, liver, intestine, and lung are often IHC-positive tissues in infected birds. The success rate of IHC detection in positive birds is enhanced by the examination of multiple tissues. The specificity of identification (e.g. flavivirus specific or WNV specific) depends on the selection of detector antibody. The brain and spinal cord tissues of horses with WN viral encephalitis are inconsistently positive in IHC tests; approximately 50% of equine encephalitis cases yield false-negative results. Failure to identify WNV antigen in equine central nervous system does not rule out infection.

c) Nucleic acid recognition methods

Nucleic acid detection by reverse-transcription polymerase chain reaction (RT-PCR) significantly enhances the identification of WNV-infected tissues, particularly when a nested PCR approach is applied to fresh,

unfixed, equine brain and spinal cord specimens (16). The RT-nested PCR method to detect WNV nucleic acid encoding a portion of the E gene is described below. This method was developed using a 1999 North American isolate and has been successful in detecting WNV RNA in animal tissues during recent North American outbreaks. St Louis encephalitis virus is not detected by this method. Lineage 1 West Nile viruses from China (People's Rep. of), France, Egypt, Israel, Italy, Kenya, Mexico and Russia demonstrate a highly conserved nucleotide sequence in the target region, regardless of species of origin (17). Analysis of sequence information for the Uganda 1937 Lineage 2 strain (GenBank M12294) in the region targeted by the PCR primers indicate that amplification of lineage 2 strains of WNV would not be expected. Other viruses from, the Japanese encephalitis serogroup have not been examined. Non-nested methods, including real-time PCR, pose less risk of laboratory cross-contamination and may be applied successfully to avian tissue samples (17). A real-time RT-PCR has been described for the detection of WNV nucleic acid (29). In order to standardise WNV molecular techniques a proficiency study based on formalin-fixed tissues was developed, administered and reported (20). Tissues selected for PCR are the same as those selected for virus isolation attempts.

- **Reverse-transcription nested polymerase chain reaction (RT-nPCR) procedure**

The RT-nPCR described here includes several procedures: extraction of RNA, reverse transcription to generate DNA from RNA and first stage PCR, second stage PCR using 'nested' primers and, finally, detection of the appropriately sized amplicon by gel electrophoresis. WNV E protein gene regions of 445 bp (base pairs) and 248 bp are amplified in the first-stage and nested procedures, respectively. The kits and reagents described below are provided as examples. Equivalent products may be available from other sources. Extreme care in handling all materials and inclusion of proper controls are essential to ensure accurate results. The precautions to be taken have been covered in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases. Duplicate samples of each diagnostic specimen should be processed and tested to increase confidence in test results. Use appropriate precautions when handling hazardous reagents such as ethidium bromide.

- **Extraction of viral RNA**

From 50 to 100 mg of tissue, extract total RNA using Trizol® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Also extract total RNA from WNV control stock virus containing 10–100 tissue culture infective dose ($TCID_{50}$) per 100 µl volume.

- **Reverse transcription and first stage PCR**

  First stage primers:
  1401: 5'-ACC-AAC-TAC-TGT-GGA-GTC-3'
  1845: 5'-TTC-CAT-CTT-CAC-TCT-ACA-CT-3'

i) Suspend extracted RNA samples in 12 µl RNase-free water.

ii) Incubate at 70°C for 10 minutes.

iii) Add 2 µl of each RNA sample to 48 µl of RT-PCR mixture containing a final composition of:
  10 mM Tris/HCl, pH 8.3
  50 mM KCl
  2.0 mM $MgCl_2$
  0.8 mM deoxynucleoside triphosphate (dNTP) pool
  25 units M-MLV (Moloney murine leukaemia virus) RT
  1.25 units RNase inhibitor
  1.25 units AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA)
  37.5 pmol of the first stage primers.

  Include 'no RNA' controls using 2 µl RNase-free water in place of denatured RNA.

iv) Incubate reaction tubes at 45°C for 45 minutes.

v) Incubate reaction tubes at 95°C for 11 minutes.

vi) PCR amplification through 35 cycles:
  Denaturation at 95°C for 30 seconds,
  Primer annealing at 55°C for 45 seconds,
  Primer extension at 72°C for 60 seconds (for the 35th cycle, primer extension at 72°C for 5 minutes).

vii) Hold samples at 4°C until second stage PCR.

- **Second stage (nested) PCR**

  Second stage primers:
  1485: 5'-GCC-TTC-ATA-CAC-ACT-AAA-G-3'
  1732: 5'-CCA-ATG-CTA-TCA-CAG-ACT-3'

i)   For each sample and control, add 1.5 µl of the first-stage amplification product to 48.5 µl of PCR mixture with a final composition of:
10 mM Tris/HCl, pH 8.3
50 mM KCl
2.0 mM MgCl$_2$
0.8 mM deoxynucleoside triphosphate (dNTP) pool
1.25 units AmpliTaq Gold^{TM} (Applied Biosystems, Foster City, CA, USA)
37.5 pmol each of the nested primers.

ii)   Incubate reactions tubes at 95°C for 11 minutes.

iii) PCR amplification through 35 cycles:
Denaturation at 95°C for 30 seconds,
Primer annealing at 55°C for 45 seconds,
Primer extension at 72°C for 60 seconds (for the 35th cycle, primer extension at 72°C for 5 minutes).

iv)   Hold samples at 4°C or –20°C until electrophoresis.

•   **Analysis of PCR products by gel electrophoresis**

i)   Prepare a 2.5% NuSieve$^®$ 3/1 (FMC Bioproducts, Rockland, Maine, USA) agarose solution in 0.045 mM Tris/borate, pH 8.6, 1.5 mM EDTA (ethylene diamine tetra-acetic acid) (1 × TBE buffer). Boil the agarose on a hotplate or in a microwave oven until completely dissolved. Cool the agarose to 45–55°C. Add 5.0 µl ethidium bromide solution (10 mg/ml) per 100 ml warm agarose and pour agarose gel with comb. Allow to solidify then remove comb.

ii)   Add 30 µl ethidium bromide solution (10 mg/ml) per 600 ml 1 × TBE tank buffer. Position gel in apparatus and fill buffer tanks.

iii) Mix 15 µl of each sample and control with 5 µl gel loading solution (e.g. Sigma product G-2526, St Louis, MO, USA). Include 100 bp DNA ladder (e.g. Life Technologies, Grand Island, NY, USA product 15268-019, range 100–1500 bp) in at least one gel well. Load samples into agar wells and electrophorese at 65–75 volts until the gel loading dye has travelled approximately two-thirds the length of the gel.

iv)   Visualise and photograph gel under ultraviolet illumination.

•   **Interpretation of the test**

For the PCR test to be valid, the positive controls must show the appropriate size band (248 bp). The 'no RNA' controls should have no bands. Samples are considered to be positive if there is a band of the same size as the positive control. Duplicate samples should both show the same reaction. If there is a disparity, the test should be repeated, starting with extraction from tissue. If further validation is required, the final nested PCR product can be sequenced and compared with the published sequences of WNV from GenBank.

2.   Serological tests

Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), hemagglutination inhibition (HI), IgG ELISA or plaque reduction neutralisation (PRN) (2, 12). The IgM capture ELISA described below is particularly useful to detect antibodies resulting from recent natural exposure to WNV. Equine WNV-specific IgM antibodies are usually detectable from 7–10 days post-infection to 1–2 months post-infection. Most horses with WN encephalitis test positive in the IgM capture ELISA at the time that clinical signs are first observed. WNV neutralising antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The HI and PRN methods are most commonly used for identifying WNV antibody in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus or Japanese encephalitis virus, will be encountered. The PRN test is the most specific among WNV serological tests; when needed, serum antibody titres against related flaviviruses can be tested in parallel. Finally, WN vaccination history must be considered in interpretation of serology results, particularly in the PRN test and IgG ELISA. IgM capture ELISA may be used to test avian or other species provided that species-specific capture antibody is available (e.g. anti-chicken IgM). The PRN test is applicable to any species, including birds.

a)   Equine IgM capture ELISA

WNV and negative control antigens for the IgM capture ELISA may be prepared from mouse brain (see Chapter 2.5.5), tissue culture or recombinant cell lines (9). Commercial sources of WNV testing reagents are available in North America. Characterised equine control serum, although not an international standard, can be obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA. Virus and negative control antigens should be prepared in parallel for use in the ELISA. Antigen preparations must be titrated

with control sera to optimise sensitivity and specificity of the assay. Equine serum samples are tested at a dilution of 1/400 and equine cerebrospinal fluid samples are tested at a dilution of 1/2 in the assay. To ensure specificity, each serum sample is tested for reactivity with both virus antigen and control antigen.

- **Test procedure**

i)   Coat flat-bottom 96-well ELISA plates (e.g. Immulon 2HB, Dynex Technologies, Chantilly, VA, USA) with 100 µl/well anti-equine IgM diluted in 0.5 M carbonate buffer, pH 9.6, according to the manufacturer's suggested dilution for use as a capture antibody.

ii)   Incubate plates overnight at 4°C in a humid chamber. Coated plates may be stored for several weeks.

iii) Prior to use, wash plates twice with 200–300 µl/well 0.01 M phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST).

iv)   Block plates by adding 300 µl/well freshly prepared 5% nonfat dry milk in PBST and incubate 60 minutes at room temperature. After incubation, remove blocking solution and wash plates three times with PBST.

v)   Test and control sera are diluted 1/400 (cerebrospinal fluid is diluted 1/2) in PBST and 50 µl/well of each sample is added to duplicate sets of wells (total of four wells per sample) on the plate. Include control positive and negative sera prepared in the same manner as samples.

vi)   Cover the plates and incubate 75 minutes at 37°C in a humid chamber.

vii) Remove serum and wash plates three times in PBST.

viii) Dilute virus and negative control antigens in PBST and add 50 µl of virus antigen to one set of wells per test and control sera and add 50 µl normal antigen to the second set of wells per test and control sera.

ix)   Cover the plates and incubate overnight at 4°C in a humid chamber.

x)   Remove antigens from the wells and wash the plates three times in PBST.

xi)   Dilute horseradish peroxidase conjugated anti-*Flavivirus* monoclonal antibody[1] in PBST according to manufacturer's directions and add 50 µl per well.

xii) Cover the plates and incubate at 37°C for 60 minutes.

xiii) Remove conjugate and wash plates six times in PBST.

xiv) Add 50 µl/well freshly prepared ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate with hydrogen peroxide (0.1%) and incubate at room temperature for 30 minutes.

xv)   Measure absorbance at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing control antigen.

b)   **Plaque reduction neutralisation (applicable to serum from any species)**

The PRN test is performed in Vero cell cultures in either 25 cm^2 flasks or 6-well plates. The sera can be screened at a 1/10 and 1/100 final dilution or may be titrated to establish an endpoint. A description of the test as performed in 25 cm^2 flasks using 100 plaque-forming units (PFU) of virus is as follows.

Prior to testing, serum is heat inactivated at 56°C for 30 minutes and diluted (e.g. 1/5 and 1/50) in media. Virus (200 pfu per 0.1 ml) working dilution is prepared in media containing 10% guinea-pig complement. Equal volumes of virus and serum are mixed and incubated at 37°C for 75 minutes before inoculation of 0.1 ml on to confluent cell culture monolayers. The inoculum is adsorbed for 1 hour at 37°C, followed by the addition of 4.0 ml of primary overlay medium. The primary overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle's Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin and 0.45% sodium bicarbonate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 72 hours at 37°C. A second 4.0 ml overlay prepared as above, but also containing 0.003% neutral red is applied to each flask. Following a further overnight incubation at 37°C, the number of virus plaques per flask is assessed. Endpoint titres are based on 90% reduction compared with the virus control flasks, which should have about 100 plaques.

Standard microneutralisation or microtitre plaque reduction neutralisation assays may be more suitable when small volumes of samples are available (32).

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1   Available from the Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia, 30333, USA.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In February 2003, the United States Department of Agriculture (USDA) issued a license for a formalin-inactivated WNV vaccine derived from tissue culture for use in horses. This was followed in December 2003, by a USDA licensed live canarypoxvirus vectored WNV vaccine for use in horses. In 2004, an inactivated human cell line-derived WNV vaccine developed by Crucell NV (the Netherlands) and Kimron Veterinary Institute (Israel) obtained a market authorisation in Israel as a veterinary vaccine for geese. In July 2005, the USDA issued the first fully licensed WNV DNA vaccine for animals in the USA. The vaccine contains genes for two WNV proteins, and therefore, does not contain any whole WNV, live or killed. In late 2006, a chimeric vaccine, based on a yellow fever virus vector, was licensed by USDA for use in horses. These vaccines have demonstrated sufficient efficacy and safety in adequately vaccinated horses. Vaccination may be helpful in preventing neurological signs associated with WNV infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

The isolate of WNV used for vaccine production must be accompanied by documentation describing its origin and passage history. The isolate must be safe in host animals at the intended age of vaccination and provide protection after challenge.

b) Method of culture

The WNV should be propagated in cell lines known to support the growth of WNV. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the master seed virus (MSV), unless further passages prove to provide protection in the host animal.

c) Validation as a vaccine

The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and be free of extraneous viruses, including equine herpesvirus, equine adenovirus, equine viral arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic equine cell type.

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible horses against a virulent challenge strain. A statistically significant number of vaccinated horses must be protected from viraemia when compared with the controls. Field trial studies should be conducted to determine the safety of the vaccine.

2. Method of manufacture

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), is used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with WN working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant.

The DNA vaccine expression cassette is amplified in *Escherichia coli* using a plamid vector cutting out plasmid backbone and purified for formulation into a vaccine.

3. In-process control

Production lots of WNV must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Production lots of DNA are quantified by analytical methods and characterised before standardisation and blending at the correct DNA content. The highest level of lipopolysaccharide (LPS) contamination of the DNA vaccine is 100 EU/dose (EU = endotoxin units).

4. Batch control

Final container samples are tested for purity, safety and potency.

a) Purity

Samples are examined for bacterial and fungal contamination. To test for bacteria, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth.

b) Safety

Safety tests can be conducted in a combination of guinea-pigs, mice or horses. Field safety studies should be conducted before the vaccine receives final approval. Generally, two serials should be used, in three different geographical locations, and a minimum of 600 animals. About one-third of the animals should be at the minimum age recommended for vaccination (correlated to efficacy). If the final product is a modified-live vaccine, additional safety testing of the MSV is required to demonstrate a lack of virulence.

c) Potency

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal (31). Live viral products are titred in cell cultures to determine the potency of the final product. The final release potency titre should include an additional 0.7 $\log_{10}$ for test variability and 0.5 $\log_{10}$ for end-of-dating stability than the minimum protective dose established in the immunogenicity trial.

DNA vaccines are tested for bioactivity and DNA content using parallel-line direct quantification methods that compare a standard preparation to the final product.

d) Duration of immunity

Duration of immunity studies are conducted before the vaccine receives final approval. The duration should be for the length of the mosquito season in the infected areas. For animals at higher risk and in infected areas with year-round mosquito activity, more frequent vaccine boosters may be advised.

e) Stability

All vaccines are initially given 24 months before expiry. Real-time stability studies are conducted to confirm the appropriateness of all expiration dating.

f) Preservatives

Antibiotics are added during production, generally gentamicin sulphate or neomycin not to exceed 30 µg/ml.

g) Precautions (hazards)

Vaccination is only recommended for horses in WN-positive areas. Vaccinated horses may develop a serological titre that may interfere with the ability to export the horse.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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NB: There is an OIE Reference Laboratory for West Nile fever (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

APIDAE

INTRODUCTORY NOTE ON BEE DISEASES

Bees are insects that are closely related to ants and wasps. There are many thousands of species of bee, most of which are not social insects, living solitary lives. The honey bee, Apis *species, lives as a colony, which is a family of social insects. There are many species, subspecies, races and subraces of honey bees that are adapted to their environment.*

Two species are important for bee keeping – the western honey bee Apis mellifera*, and the eastern honey bee* A. cerana*. The Africanised bee, which is found in South and Central America and some states of the United States of America, is a cross between two subspecies of the western honey bee, the European bees and the South African bee.* Apis cerana *is important in South and South-East Asia. The colonies are small and docile, but the honey yields are low. In a suitable climate, the western honey bee,* A. mellifera*, is sometimes preferred for its greater honey production.*

It is thought that all bees are susceptible to the known diseases of bees, but different races may have varying susceptibility. For example, A. cerana *is less susceptible to varroosis. When sampling a colony of bees for diagnosis of diseases, live bees must first be killed with diethyl ether or in a deep freezer (–20°C) overnight. Bees may also be killed by submersion in 70% ethyl alcohol, e.g., when collected for diagnosis of acariosis* (Acarapis)*. Larval and pupal smears must be made when testing for brood diseases or a piece of comb containing brood showing visible signs of disease may be sent to the laboratory.*

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* *

CHAPTER 2.2.1.

ACARAPISOSIS OF HONEY BEES

SUMMARY

Acarapisosis or acariosis or acarine disease is a disease of the adult honey bee Apis mellifera *L. and other* Apis *species. It is caused by the Tarsonemid mite, known as the tracheal mite,* Acarapis woodi (*Rennie*). *The mite is approximately 150 µm in size, and is an internal parasite of the respiratory system, living and reproducing mainly in the large prothoracic trachea of the bee. Sometimes they are also found in the head, thoracic and abdominal air sacs. Mites feed on the haemolymph of their host.*

The pathogenic effects found in infected bees depend on the number of parasites within the trachea and are attributable both to mechanical injuries and to physiological disorders consequent to the obstruction of air ducts, lesions in the tracheal walls, and the depletion of haemolymph. As the parasite population increases, the tracheal walls, normally white and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts.

The mortality rate may range from moderate to high. Early manifestations of infection normally go unnoticed, and only when infection is heavy does it become apparent. This is usually in the early spring. The infection spreads by direct contact. Generally, only newly hatched bees under 10 days old are susceptible. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8–20 eggs. There are 2–4 times as many females as males. Development takes 11–12 days for males and 14–15 days for females.

Identification of the agent: *The parasites are demonstrated only by laboratory methods and under the microscope. The mites need to be observed inside the tracheae or removed from them to be observed microscopically. Several techniques are available for demonstrating the mites, such as dissection, grinding and staining.*

The thoraces of suspect bees are dissected to expose the trachea. Each trachea is examined under a dissecting microscope (×18–20), where the mites will be seen through the transparent wall as small oval bodies.

Alternatively, larger samples of suspect bees can be ground or homogenised in water, followed by coarse filtration of the suspension, and centrifugation. The deposit is treated with undiluted lactic acid for 10 minutes. This is then mounted for microscopic examination.

The parasites may be stained by histological techniques so that they can be observed within the bee trachea. The tracheae are separated out, cleared with 8% potassium hydroxide, and stained with 1% methylene blue. This is the best method for large numbers of samples.

Serological tests: *Serological tests are not available.*

Requirements for vaccines and diagnostic biologicals: *There are no biological products available. Menthol crystals or oil patties made with vegetable oil (*not animal fat*) and white granulated sugar will keep mite levels under control.*

A. INTRODUCTION

Acarapisosis is a disease of the adult honey bee *Apis mellifera* L*.* and other *Apis* species, caused by the microscopic Tarsonemid mite *Acarapis woodi* (Rennie). The mite is approximately 150 µm in size and is an

internal parasite of the respiratory system (Figure 1). These tracheal mites enter, live and reproduce mainly in the large prothoracic tracheae of all bees, feeding on the haemolymph of their host (Figure 2). Sometimes they are also found in the head, thoracic and abdominal air sacs (6, 18).
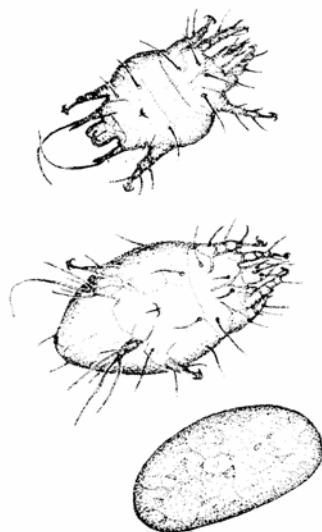
Fig. 1. Acarapis woodi *(Rennie). Top: Adult male, Centre: Adult Female, Bottom: Egg.*
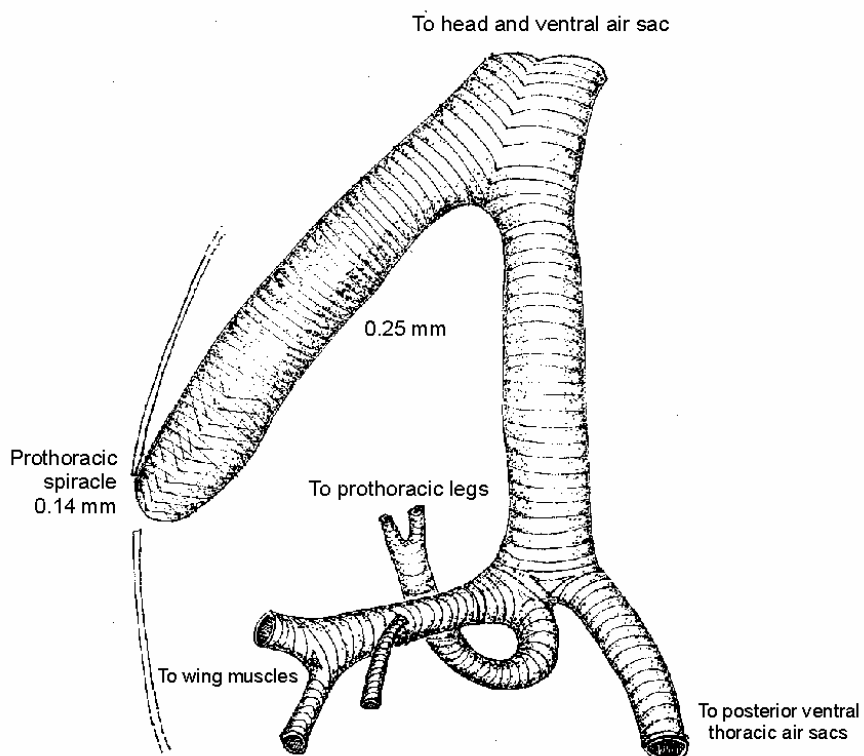
Fig. 2. *Main thoracic tracheae of a honey bee where* Acarapis *is commonly found; light infestations are near the spiracle opening.*

The pathogenic effects on individual bees depend on the numbers of parasites within the tracheae and are attributable both to mechanical injuries and to physiological disorders consequent to the obstruction of the air ducts, lesions in the tracheal walls, and to the depletion of haemolymph. As the parasite population increases, the tracheal walls, which are normally whitish and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts (5).

The mortality rate may range from moderate to high. Early signs of infection normally go unnoticed, except for a slow dwindling in the colony size. Only when infection is heavy does it become apparent. This is generally in the early spring after the winter clustering period when the mites have bred and multiplied undisturbed into the longer-living winter bees. This applies mainly to the Northern Hemisphere where there are seasonal variations in the reproduction of bees.

Infection spreads from one bee to another by direct contact. Generally, only newly hatched bees under 10 days old, are susceptible. Attempts to rear *A. woodi* on artificial and synthetic diets have been unsuccessful, while culturing them on immature stages of the honey bee itself has been only partially successful (7). The life span of the mites in dead bees is approximately 1 week. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8–20 eggs. There are 2–4 times as many females as males; development takes 11–12 days for males and 14–15 days for females.

There are no reliable clinical signs for the diagnosis of acarapisosis as the signs of infection are not specific and the bees behave in much the same way as do bees affected by other diseases or disorders. They crawl around in the front of the hive and climb blades of grass, unable to fly. Dysentery may be present.

# B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Acarapisosis can be detected only in the laboratory using microscopic examination or an enzyme-linked immunosorbent assay (ELISA). There is no reliable method for detection of very low levels of infection. The number of bees sampled determines the detection threshold of the method. It has been shown that a 1 to 2% rate of infection can be detected by sampling 50 bees Sequential sampling data are available (4, 17). The best time to take bee samples is in the early spring or late autumn (Northern hemisphere), when *Acarapis* populations are high. Visualisation of mites is easier in older bees, which have more mites. Samples of queens, drones or workers can be used, but *Acarapis* prefer drones.

a) Dissection (8)

A sample of 50 bees (see above) is collected at random from the suspected colony. These are mainly bees crawling and unable to fly, found within about 3 metres of the front of the hive. This is preferable to random collection from within the colony. The bees may be living, dying, or dead. Live bees must first be killed with ethyl alcohol or in a deep freezer (–20°C); bees must not have been dead for over 2–3 days unless kept at 4°C for up to 4 weeks or –20°C for several months. They may be preserved indefinitely in a preservative such as Oudemann solution: glacial acetic acid (80 ml); glycerol (50 ml); 70% ethanol (870 ml).

- **Test procedure: direct preparation (15, 18)**

i)   Remove the abdomen at the thorax of the bees (see Figure 3).

ii) Pick up the thorax with the beginning of the head and examine it under the binocular magnifying glass at 20–30-fold magnification.

Iii) Remove the pleural sclerite of the first thoracic segment with the first pair of legs, by means of a pair of tweezers. In the circular opening the main strains of the thoracic tracheae and the branches of the head tracheae can be seen.

iv) By means of a fine pair of tweezers, remove the thoracic tergite of the first thoracic segment and part of the second thoracic tergite. After removing the overlying musculature, the two thoracic tracheae are exposed. Positive diagnosis consists of either the presence of melanisation of one or both tracheae or, in light infestation, of the presence of oval translucent bodies (eggs etc.) easily seen within the tracheae.

v)   For further microscopis examination (e.g. confirmation of light infestation), remove the tracheae and put them onto a slide, with a drop of water. Under the microscope at 100-fold magnification the adult mites as well as their individual stages of development can be recognised.
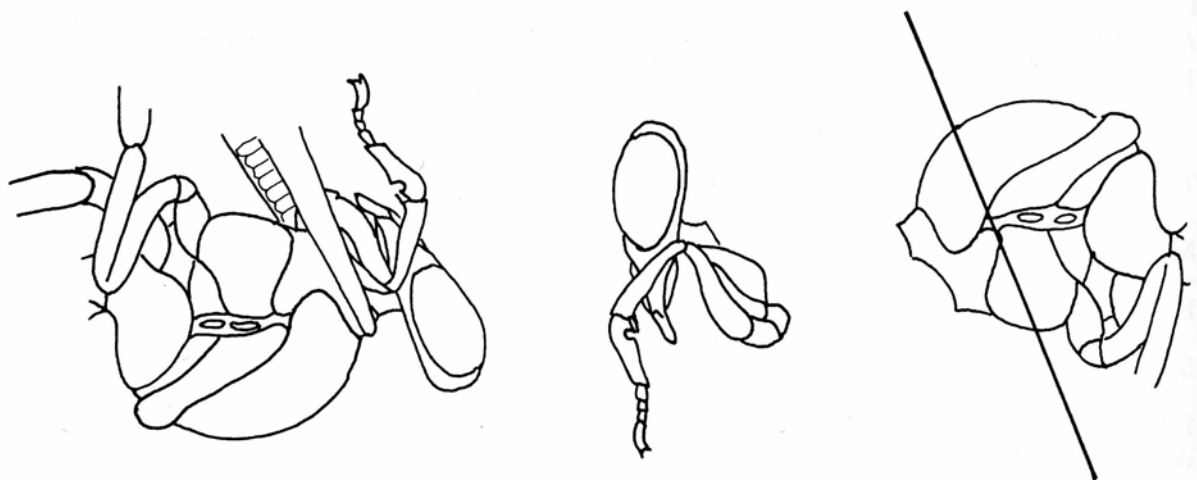
***Fig. 3**. Preparation of bees to reveal* Acarapis woodi *in the first thoracic pair of tracheae.*

- **Test procedure: maceration (15)**

i) Lay and secure bees on their backs or hold with thumb and first finger.

ii) Remove the heads and forelegs using a small forceps and remove the collar surrounding the neck opening to expose the tracheae (Figure 4). Check the tracheae nearest to the spiracle (as mites enter through the spiracle) to see light infestations. Heavy infestations are easily visible as shadows or dark objects in clear to dark brown tracheae. Old and heavy infestations will make the tracheae brown to black.

iii) Cut through the thorax in front of the middle pair of legs and the base of the forewings with a sharp razorblade. These thin disks can be further treated to clear muscle tissue.

iv) Macerate either by gentle heating in an 8% solution of potassium hydroxide for approximately 20 minutes or by leaving them to stand overnight without heating.

v) Examine the first pair of tracheae, which are covered by muscle tissue, under a dissecting microscope at a magnification of ×18–20, or transfer the tracheae to another slide, add glycerin or water and observe at higher magnification.

vi) Mites are easily seen through the transparent wall as small, oval bodies.
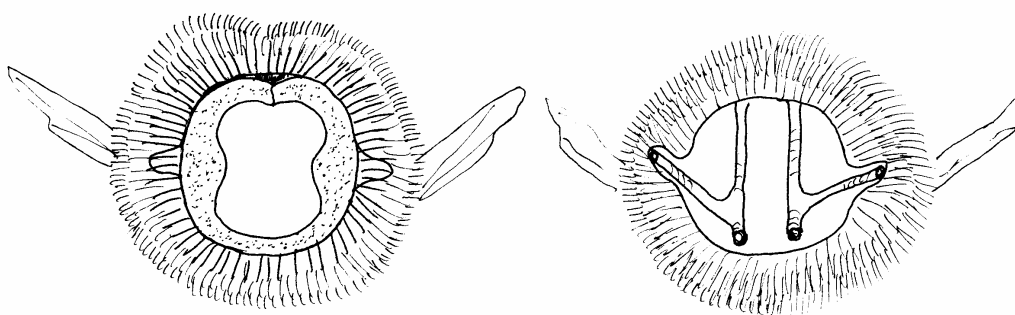
***Fig. 3**. Left: front view of bee thorax with head removed and collar intact.*
Right: Collar removed and tracheae exposed to spiracle openings.

This is the simplest and most reliable technique for the laboratory diagnosis of acarapisosis, allowing the detection of early infections and enabling the infection rate to be established. Even light infections can be detected by using a dissecting microscope with this technique. Only in very exceptional instances will it be necessary to employ higher magnifications in order to make a diagnosis. However, this is a demanding technique, especially when a large number of acarapisosis diagnoses have to be made. If it is necessary only to distinguish between heavily infected and lightly or non-infected colonies, dissection can be stopped at step ii and the colour of the tracheae observed.

b)   **Grinding (3)**

A sample of about 200 bees is collected at random from the suspect colony. The wings and legs of each bee are removed from the thorax, and the bodies are pooled in a 100 ml container that has been one-quarter filled with water. This suspension is homogenised three times, each time for several seconds, in a homogeniser at 10,000 rpm with the addition of more water. The resulting suspension is strained through a sieve (mesh 0.8 mm) and the sieve is rinsed with water to a final volume of approximately 50 ml. The filtrate is centrifuged at 1500 *g* for 5 minutes and the supernatant fluid is discarded. A few drops of undiluted lactic acid solution are added to the debris of the deposit, which will contain the mites. This is left for 10 minutes to allow the muscle fibres to dissolve, and is then mounted under a cover-slip for microscopic examination. This technique is quicker than dissection, but may be less accurate. External mites *A. externus, A. vagans* and *A. dorsalis*, all of which are morphologically similar to *A. woodi*, are often found on the thorax of healthy bees and can very easily be mistaken for *A. woodi* (Table 1). It seems, however, that they do not cause any serious threat to bees or beekeeping. This method should therefore only be chosen if all that is required is a rough estimation of the degree of infection in a region. It is not suitable for determining a first outbreak.

Table 1. *Differential diagnosis of* Acarapis *species* (*15*)

Character	A. dorsalis	A. externus	A. woodi
Notch of the coxal plate	Deep	Short	Flat
Space between stigmata	16.7 µm	16.8 µm	13.9 µm
Length of tarsal limb (IV leg pair)	7.6 µm	11.4 µm	7.5 µm

c)   **Staining (11)**

The mites and trachea can be stained specifically, rendering them easily visible by microscopy.

*   **Test procedure**

i)   Remove the head and forelegs.

ii)   Make a transverse cut through the membranous areas behind the forelegs.

iii)   Make a second transverse cut in front of the middle pair of legs at the base of the forewings.

iv)   To clear the sections (1–1.5 mm thick), place them in an 8% solution of potassium hydroxide.

v)   Stir gently and heat near to boiling point for approximately 10 minutes until the soft internal tissues are dissolved and cleared, leaving the chitinous tissues intact.

vi)   Retrieve sections by filtration and wash with tap water.

vii)   Stain and mount the sections.

viii)   Examine for mites by low-power microscopy.

Permanent mounts are prepared by the usual histological techniques.

Cationic stains are the most suitable and specific as they stain the mites intensely but the tracheae only weakly. A solution of 1% aqueous methylene blue is the most suitable, prepared by dissolving the methylene blue first and then adding sodium chloride to make a 0.85% NaCl solution.

*   **Test procedure**

i)   Stain in 1% aqueous methylene blue.

ii)   Differentiate sections in distilled water for 2–5 minutes.

iii)   Rinse the sections in 70% alcohol.

When kept in 95% ethanol, the mites will retain the stain for 6 hours (1). It is essential with this technique to macerate the tissues effectively in the potassium hydroxide solution. Using this method, it is possible to process a large number of samples rapidly and conveniently.

d) Enzyme-linked immunosorbent assay

An ELISA for trachea mites has been developed (9, 13, 14). This test may produce false-positive results, and is therefore only recommended for survey examinations. Another method is the visualisation of guanine, a nitrogenous waste product of mites (12).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available. Menthol crystals (50 g for a two story colony) control mites if left in the colony for 28 days, providing the ambient temperature is at least 18°C. The optimum temperature range for the vapours to work is 27–29°C. Small cakes made with vegetable shortening (e.g. margarine, not animal fat) and white granulated sugar will keep mite levels to 10%. The cake (about 100 g in weight) should be placed on the top bars of the frames in the brood nest in the autumn and early spring (16). Formic acid may be used to treat infected colonies (10).

Some races of bees, such as Buckfast bees (2) and some hygienic strains, are less susceptible to attack by *Acarapis*.

ACKNOWLEDGEMENTS

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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

AMERICAN FOULBROOD OF HONEY BEES

SUMMARY

American foulbrood (AFB) affects the larval stage of the honey bee Apis mellifera *and other* Apis *spp., and occurs throughout the world.* Paenibacillus larvae, *the causative organism, is a bacterium that can produce over one billion spores in each infected larva. The spores are extremely resistant to heat and chemical agents, and can survive for many years in scales (from diseased dead brood), hive products and equipment. Only the spores are capable of inducing the disease.*

Identification of the agent: *Combs of infected colonies have a mottled appearance due to a mixture of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. This is not a characteristic of AFB only. Cell cappings of a diseased larva appear moist and darkened, becoming concave and possibly punctured as infection progresses. The larval or pupal colour changes to creamy brown and then to a dark brown with a ropy appearance when drawn out. In some cases the larval remains are rather watery. The diseased brood eventually dries out to form characteristic brittle scales that adhere tightly to the lower sides of the cell. The formation of a pupal tongue is one of the most characteristic but rarely seen signs of the disease and precedes the formation of the scales.*

Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. Some of the identification methods require a previous culturing step, while others can be performed directly on collected samples. Four solid culture media are recommended: PLA (Paenibacillus larvae agar), MYPGP agar, BHIT agar and Columbia sheep blood agar. Two polymerase chain reaction (PCR) protocols are described in this chapter. The first protocol can be used for rapid confirmation of clinical AFB and for identification of bacterial colonies after a cultivation step. The second protocol is a so-called nested PCR that also permits direct analysis of spore solutions. The biochemical profiling of P. larvae *is based on the catalase test, the production of acid from carbohydrates and the hydrolysis of casein. Further, antibody-based techniques and the microscopic identification of the pathogenic agent are described.*

Serological tests: *There are no serological tests available.*

Requirements for vaccines and diagnostic biologicals: *Monoclonal and polyclonal antibodies produced for the development of diagnostic tests should be sufficiently specific.*

A. INTRODUCTION

American foulbrood (AFB) is an infectious disease of the larval stage of the honey bee *Apis mellifera* and other *Apis* spp., and occurs throughout the world where such bees are kept. *Paenibacillus larvae,* the causative organism, is a Gram positive bacterium that can produce over one billion spores in each infected larva. The bacterium is a round-ended, straight and sometimes curved rod, which varies greatly in size (0.5 µm wide by 1.5 to 6 µm long), occurring singly and in chains and filaments; some strains are motile. The sporangia are often sparse *in vitro*, and the ellipsoidal, central to subterminal spores, which may swell the sporangia, are often found free (16). The spores are extremely heat stable and resistant to chemical agents. Only spores are capable of inducing the disease.

The infection can be transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell. Although the larval stages of worker bees, drones and queens are susceptible to infection, infected queens and drone larvae are rarely seen under natural conditions. The susceptibility of larvae to AFB disease decreases with increasing age (35); larvae cannot be infected later than 53 hours after the egg has hatched. The mean infective dose (LD$_{50}$= spore dose at which 50% of the larvae are killed) needed to initiate infection, though very variable, is 8.49 spores in 24–48 hour-old bee larvae (14). Exchanging combs containing the remains of diseased brood is the most common way of spreading the disease from colony to colony. In addition, feeding or robbing of spore-laden honey or bee bread, package bees and the introduction of queens from infected colonies can also spread the disease. Wax contaminated with the spores of *P. larvae*, which are used in the production of combs foundation, can also spread the disease. The early detection of AFB helps to prevent further spread.

B. DIAGNOSTIC TECHNIQUES

1.   Identification of the agent

Diagnosis of AFB is based on identification of the pathogenic agent only. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. An initial overview of clinical signs of the disease will be provided in this chapter, followed by identification methods that require a previous culturing step, or that can be performed directly on collected samples. The techniques involved are microbiological characterisation, the polymerase chain reaction (PCR), biochemical profiling, antibody-based techniques and microscopy. The analyst should be aware of differences in sensitivity between the presented approaches and should select the most appropriate for a given situation.
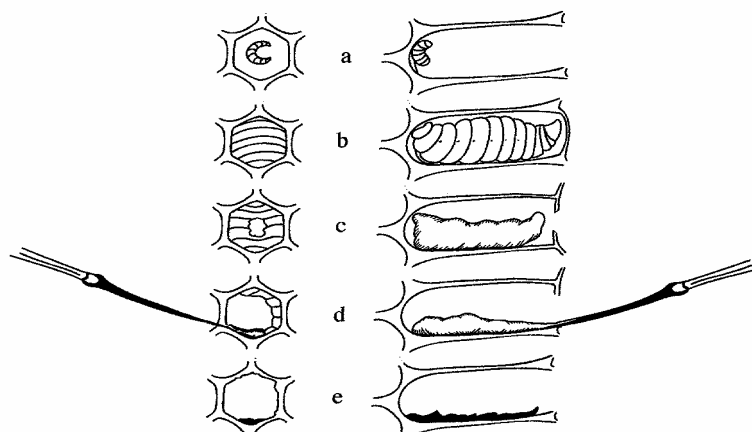
Fig. 1. *Progression of the disease:* (a) *Point of infection.* (b) *Larval development to the prepupal stage.* (c) *Cell contents reduced and capping is drawn inwards or is punctured.* (d) *Cell contents become glutinous.* (e) *Residual scale tightly adherent to bottom of cell.*

a)   Epizootology and clinical signs

Spores of *P. larvae* can survive in bee products (honey, wax, dry larval scales) and in the environment for 3 to 10 years and purified spores can survive even more than 70 years (29).

The clinical signs of AFB are very diverse and depend on the genotype involved, the stage of the disease and the strength of the bee colony (and possibly its resistance to AFB). Larvae can be killed rapidly at an early age when they are curled at the base of uncapped brood cells. Adult worker bees will remove these dead larvae leaving only an empty cell (4). Other larvae will die later on in their development, when they are in an upright position, filling most of the brood cell. Often the larvae or pupae will die after brood cell capping.

In severely infected colonies, the combs have a mottled appearance caused by a pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. The capping of a cell that contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses. Also, the larva or pupa changes colour, first to a creamy and eventually to a dark brown. The larvae can become glutinous in consistency and can be drawn out as threads when a probe is

inserted into the larval remains and removed from the cell (match-stick test). This is probably the best-known technique for field diagnosis of the disease, but in some cases the larval remains are rather watery, resulting in a negative match-stick test. Finally, 1 month or more after the larva becomes ropy, the remains of the diseased brood dry out to form typical hard, dark scales that are brittle and adhere strongly to the lower sides of the cell (Figure 1). If death occurs in the pupal stage, the pupal tongue protrudes from the pupal head, extending to the top of the brood cell or may angle back towards the bottom of the cell. The protruding tongue is one of the most characteristic signs of the disease, although it is rarely seen (Figure 2). The tongue may persist also on the dried scale. European foulbrood needs to be taken into consideration as a differential diagnosis.
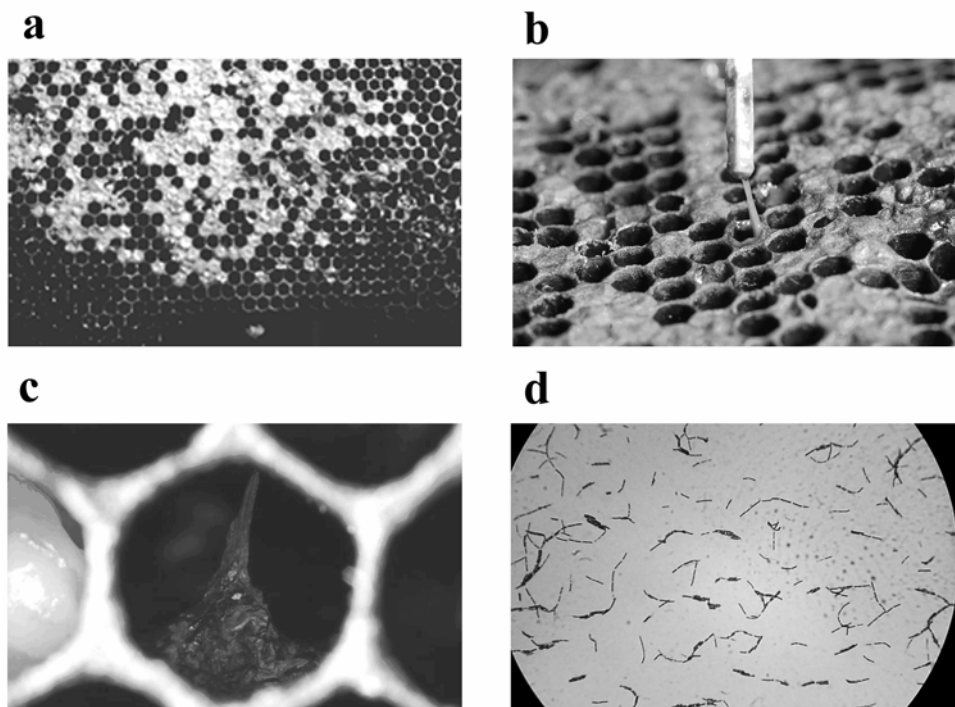
Fig. 2. *Clinical American foulbrood* (*a-c*) *and Gram staining* (*d*): (*a*) *Combs have mottled appearance.*
(*b*) *A matchstick draws out the brown, semi-fluid larval remains in a ropy thread.* (*c*) *The formation of a pupal tongue is a very characteristic sign, but rarely seen.* (*d*) *Microscopic examination reveals Gram-positive rods, occurring singly and in chains.*

b) Selection of samples

  i) Collection of samples from a suspicious or diseased colony/apiary

    While maintaining their colonies, beekeepers often find brood combs with signs of disease. In this case a brood sample can be collected for diagnosis. The brood is sampled by cutting out a piece comb of about 20 cm^2 in size, containing as much of the dead or discoloured brood as possible. An experienced person can collect infected larval/pupal remains directly from the cells with a sterile swab, significantly reducing the sample size and facilitating packaging and sample transportation to the laboratory (see below). When microscopic examination is the method of choice, smears of the remains of diseased larvae can also be made at the apiary (17). After air-drying they can be forwarded to the laboratory.

    Every bee colony in the vicinity of such a clinical case of AFB should be considered as suspicious and a broad range of samples should be taken for confirmation. Apart from brood samples, food stores (honey [27, 34], pollen [12] and royal jelly), adult workers (21) and wax debris (32) can be used to detect the presence of *P. larvae* spores. Honey samples can be collected from cells close to the brood with separate disposable spoons to prevent cross-contamination between samples; however, honey may have been sitting in the comb for months at the time of sampling. Adult bees can be shaken or brushed from the combs of the brood chamber or the honey supers into a plastic bag or container. For the most reliable picture of the actual situation, bees from the brood nest (and not the honey supers) should be analysed. Wax debris can be collected at the hive bottom all year round.

  ii) Samples for AFB monitoring/prevention programmes

    To prevent the propagation of diseased brood, honey, adult bee and debris samples can be used to detect AFB in colonies where no clinical signs are observed. Routine collection of samples from

colonies or from harvested honey can be used as part of an operational or regional AFB detection programme.

Microscopic examination of smears from larvae with no clinical signs is far less sensitive at detecting spores in colonies compared with bacteriological or PCR-based methods. In fact, bacteriological and PCR-based methods will often detect spores in colonies that never develop clinical signs of AFB. High numbers of spores cultured from honey and bee samples using bacteriological methods, however, can often predict the presence of clinical AFB signs at colony, apiary and operational levels.

b) Packaging and transportation of samples to the laboratory

Brood comb should be wrapped in a paper bag, paper towel or newspaper and placed in a wooden or heavy cardboard box for transport. Swabs with larval remains can be put into appropriate test tubes with a cap. Holders for microscope slides are commercially available. Adult bees can be kept frozen or submerged in 70% ethanol during transportation, although dried bees are adequate. Food supplies can be put into a test tube or a suitable pot, or wrapped in a plastic bag together with the spoon. Leaking and cross-contamination of the samples must be prevented. If possible, fresh material for laboratory tests should be sent refrigerated.

c) Sample preparation

i) Samples for cultivation

In general, an aqueous solution containing *P. larvae* spores should be prepared for further analysis. This spore suspension is heat-shocked at 80°C for 10 minutes or 95–96°C for 3–5 minutes in order to kill other spore-formingmicroorganisms.

Larval/pupal remains from brood comb are collected with a sterile swab and suspended in 5–10 ml of sterile water or physiological solution (phosphate buffered saline or 0.9% NaCl) in a test tube.

Honey samples to be examined for spores are heated to 45–50°C and shaken to distribute any spores that may be present. Dilution with an equal volume (25 ml) of water permits easier handling. The diluted honey is transferred into 44 mm width dialysis tubing that has been tied at one end. The open end is tied after filling. The tubes are submerged in running water for 18 hours or in a water bath with 3–4 water changes over the same time period. After dialysis, the contents are centrifuged at 2000 *g* for 20 minutes. The supernatant liquid is discarded leaving approximately 1 ml (or less) of residue in each sample. The residue is then resuspended in 9 ml of water (31).

Honey can also be prepared for cultivation without the dialysis step, however this requires longer (30 minutes) and faster (3000 *g*) centrifugation. Likewise, the volume in which the deposit is finally resuspended can be much smaller (200 µl) in order to improve the sensitivity of the test (6).

Direct plating of diluted honey (27) is widely used, but its sensitivity is inferior to that of the centrifugation method as only a fraction of the total volume will be plated out. Whatever the method of choice is, when honey is analysed quantitatively and threshold values are set, the methodology that was used to establish these values should always be strictly followed.

An aqueous filtrate of pollen can be made by thoroughly dispersing 1 g of pollen in 10 ml final volume sterile distilled water and filtering it through Whatman No. 1 paper (12).

When adult bees are dispatched in ethanol, the latter should be decanted and replaced by sterile water or physiological solution before crushing.

Debris and bee wax (1.5 g) should be dissolved in an organic solvent (10 ml): toluene (32), chloroform (19) or diethyl ether (28). The liquid part (2 ml) is then diluted in physiological solution (6 ml). After shaking roughly, this suspension can immediately be plated out (no heat-shock) (32). In another protocol, bee wax is first diluted in water (wax/water 1/10) and heated up to 90°C for 6 minutes. After cooling down, the organic solvent is added (organic solvent/water 1/9) and the mixture is shaken carefully. After 2minutes standing time, a deposit of a watery solution containing *P. larvae* spores forms (28).

ii) Samples for PCR

Cell/spore suspensions and suspensions containing only spores have to be differentiated, the latter requiring a more complex DNA extraction step (except for the nested PCR).

If the PCR is aimed at identifying bacterial colonies (= cell/spore suspension) after a cultivation step, the pre-treatment is as follows: one colony is suspended in 50 µl of distilled water and heated to 95°C for 15 minutes. Following centrifugation at 5000 *g* for 5 minutes, 1–5 µl of the supernatant is used as template DNA in a PCR 50 µl mixture (9).

For rapid confirmation of clinical AFB, the samples should be prepared as follows: the remains of two diseased honey bee larvae (= cell/spore suspension) are suspended in 1 ml of sterile distilled water

and mixed thoroughly. 100 µl of this suspension is diluted with 900 µl distilled water. This dilution is vortexed and 100 µl of it is used to extract DNA by heating and centrifugation (see above) (9).

All aqueous solutions resulting from the sampling of honey, adult bees, debris, bee wax, pollen and royal jelly should be considered as a spore suspension. Here, the extraction of DNA demands another approach. Indeed, spore suspensions are centrifuged at 6000 g and 4°C for 30 minutes. Next, the pellet is subjected to microwave treatment for 5 minutes at maximum power to break the spores, and the released DNA is suspended in 30 µl of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA (26).

When spores are to be detected from honey, DNA is serially diluted with sterile distilled water to eliminate PCR inhibition caused by honey (26). Another DNA extraction method, based on lysozyme and proteinase K treatment, has been described (3).

Good results can also be obtained by incubating a pelleted spore suspension in MYPGP broth at 37°C for 2–24 hours. Thereafter, the suspension is centrifuged at 14,500 g for 5 minutes, washed with sterile distilled water and resuspended in 200 µl of sterile distilled water. This short incubation step causes spores to germinate, making them sensitive for DNA preparation by heat treatment again (see above) (20).

When the nested PCR is chosen, the spore solution should only be boiled at 100°C for 10 minutes and thereafter centrifuged at 14,500 g for 2 minutes. The supernatant can immediately serve as template DNA sample in the nested PCR reaction (20).

d) **Culture**

Several media for cultivating *P. larvae* have been described but best results were obtained with PLA (*Paenibacillus larvae* agar) (30), MYPGP agar (the abbreviation refers to its constituents: Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate) (7), BHIT agar (Brain–Heart Infusion medium supplemented with thiamine) (11) and CSA (Columbia sheep blood agar). The formulations of the first two media are as follows:

- PLA

This selective medium combines three different media to comprise the base, to which is added antibiotics and egg yolk supplements (30). Equal quantities (100 ml) of sterile, molten *Bacillus cereus* selective agar base (Oxoid CM617), trypticase soy agar (Merck 5458) and supplemented nutrient agar (SNA) are combined and mixed. SNA is composed of (per litre): nutrient agar 23 g, yeast extract 6 g, meat extract 3 g, NaCl 10 g, Na_2HPO_4 2 g: final pH is 7.4 ± 0.2. All solid media are sterilised at 121°C/15 minutes. Nalidixic acid stock solution (18) is prepared by dissolving 0.1 g in 2 ml of 0.1 N NaOH and diluting to 100 ml with 0.01 M phosphate buffer (pH 7.2). Pipemidic acid stock (2) is prepared by dissolving 0.2 g in 2 ml of 0.1 N NaOH and then diluting to 100 ml with the same phosphate buffer. Both antibiotic solutions are filter sterilised.

After the three molten media are combined, 3 ml of stock nalidixic acid, 3 ml of stock pipemidic acid, and 30 ml of 50% egg-yolk suspension (13) is added to form the PLA medium. The PLA medium is poured (20 ml) into sterile Petri dishes and plates are dried before use (45–50°C for 15 minutes).

- MYPGP agar

MYPGP agar is composed of (per litre): Mueller-Hinton broth (Oxoid CM0405) 10 g, yeast extract 15 g, K_2PO_4 3 g, glucose 2 g, Na-pyruvate 1 g and agar 20 g (7). Addition of nalidixic acid and pipemidic acid is as above.

If cultivation of *P. larvae* is hampered by the occurrence of fungi, the addition 16.8 µg/ml medium of amphotericin B (Sigma) works very well.

A sterile cotton swab is used to transfer a portion of the sample on to the surface of the solid medium. For a quantitative evaluation, it is recommended to spread a fixed volume of the suspension on the solid agar with a sterile scraper or pipette rather than using cotton swabs.

Inoculated plates are best incubated at 34–37°C for 2–4 days in an atmosphere of 5–10% CO_2 in air, although aerobic incubation will do as well.

e) **Identification**

i)   Colony morphology

Samples from clinically diseased larvae will result in confluently grown plates after 2–4 days, leading to a subculturing step in order to isolate colonies.

On PLA, colonies of *P. larvae* are small, pale green to yellow (= the same colour as the medium), with a slightly opaque and rough surface; sometimes the centre is raised.

On MYPGP agar, colonies are small, regular, mostly rough, flat or raised and whitish to beige coloured.

On Columbia sheep blood agar, colonies are small, regular, glossy, butyrous and greyish.

Paenibacillus larvae colonies with orange to red pigmentation have been described (10, 22).

It is advised to run *P. larvae* reference strains in parallel, for instance LMG 9820 (other designation: ATCC 9545, DSM 7030) for the non-pigmented variant and DSM 16115 or DSM 16116 for the pigmented genotype.

A proven positive brood or honey sample can serve as a positive control for the entire examination.

Colony morphology is not conclusive but might serve to select the bacterial colonies for further identification.

ii) Polymerase chain reaction

PCR reactions are set up as 50 µl mixtures containing 5 µl template DNA (see sample preparation), 50 pmol forward (AFB-F) and reverse primer (AFB-R; primer sequences are given below), 10 nmol of each deoxynucleoside triphosphate and 1–2.5 U of *Taq* polymerase, in the appropriate PCR buffer (provided together with *Taq* polymerase) containing 2 mM $MgCl_2$ (ref. 9 with modifications). Reducing the volume of the PCR mixtures to 25 µl is possible. Amplification of a specific DNA fragment occurs in a thermocycler under to the following PCR conditions: a 95°C (1–15 minutes) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

Nested PCR comprises an external and an internal amplification step (20). The external amplification is performed using primers PleF and PleR (see below). Each 50 µl PCR reaction contains: 10 µl template DNA (see sample preparation), 1 × PCR buffer (with 1.5 mM $MgCl_2$), 0.5 µM PleF primer, 0.5 µM PleR primer, 0.2 mM of each dNTP, additional 0.75 mM $MgCl_2$, 1.25 U Taq polymerase. A 'touchdown' PCR protocol was performed in which annealing is lowered by 0.5 C/cycle, from 69 to 59°C, for a total of 20 cycles with each annealing step lasting 30 seconds. Twenty more cycles are then performed with the annealing temperature at 59°C for 30 seconds. Denaturation steps are all executed at 94°C (for 30 seconds) and extensions at 72°C (for 45 seconds). Following this, a final extension at 72°C for 5 minutes is performed, and then the reaction is cooled at 4°C. Internal amplification is performed using primers PliF and PliR (see below). Each 50 µl PCR reaction contains 1 µl of the external PCR amplification, 1 × PCR buffer (with 1.5 mM $MgCl_2$), 0.5 µM PliF primer, 0.5 µM PliR primer, 0.2 mM of each dNTP, additional 1 mM $MgCl_2$, 1.25 U Taq polymerase. Cycling conditions are: 94°C (30 seconds), 59°C (30 seconds), 72°C (45 seconds) for a total of 30 cycles followed by 5 minutes at 72°C and then the reaction is cooled at 4°C.

The molecular weights of the PCR products are determined by electrophoresis in a 0.8% agarose gel and staining with ethidium bromide.

Ref.	Name	Sequence	PCR-product size	Specificity level
(9)	AFB-F	5'-CTT-GTG-TTT-CTT-TCG-GGA-GAC-GCC-A-3'	1106 bp	species
	AFB-R	5'-TCT-TAG-AGT-GCC-CAC-CTC-TGC-G-3'		
(20)	PleF	5'-TCG-AGC-GGA-CCT-TGT-GTT-3'	969 bp	
	PleR	5'-CTA-TCT-CAA-AAC-CGG-TCA-GAG-3'		
	PliF	5'-CTT-CGC-ATG-AAG-TCA-TG-3'	572 bp	species
	PliR	5'-TCA-GTT-ATA-GGC-CAG-AAA-GC-3'		

iii) Biochemical tests

Paenibacillus larvae can be also be identified by its biochemical profile. The bacteria are catalase negative or weak delayed positive, they have a typical carbohydrate acidification profile with acid from glucose and trehalose, not from arabinose and xylose, and they can hydrolyse casein or milk. Some strains of *P. larvae* can change the biochemical signs.

- Catalase test

A drop of 3% hydrogen peroxide is placed on an actively growing culture on solid medium. Most aerobic bacteria break down the peroxide to water and oxygen, producing a bubbly foam, but *P. larvae*

is negative or weak delayed positive for this reaction (15). When Colombia sheep blood agar is used for cultivation, the test cannot be done on the solid medium, as the presence of sheep blood will cause a false-positive reaction. In this case, colonies should be transferred to a clean microscope slide for the execution of the test. Here the evaluation of the test occurs as above with the naked eye.

- Production of acid from carbohydrates (13)

Bacteria are grown in J-broth (per litre: yeast extract 15 g, tryptone 5 g and K_2HPO_4 3 g) in which 0.5% of the test substrate, separately sterilised in aqueous solution, is substituted for the glucose. The carbohydrates used are L (+)-arabinose, D (+)-glucose, D (+)-xylose and D (+)-trehalose. The cultures are tested at 14 days by aseptically removing one ml or less to a spot plate, mixing the sample with a drop of 0.04% alcoholic bromocresol purple, and observing the colour of the indicator. *Paenibacillus larvae* produces acid aerobically from glucose and trehalose. No acid is produced from arabinose and xylose (1).

The use of commercial kits, such as API 50 CHB (5), BBL CRYSTAL (8) and Biolog system (22) for the biochemical characterisation of *P. larvae* can be taken into consideration.

- Hydrolysis of casein (30)

Casein hydrolysis is assayed using milk agar plus thiamine (per litre: agar 20 g, yeast extract 10 g; sterilised at 121°C/15 minutes). Add to each 70 ml cooled medium 30 ml of UHT (ultra heat treated) skimmed milk and 1.5 ml filter sterilised 0.1% thiamine solution. Plates are streaked and examined after 5 days of incubation at 36 ± 1°C. *Paenibacillus larvae* hydrolyses casein, hence zones of clearing are observed around bacterial colonies.

iv) Antibody-based techniques

Different antibody-based techniques have been developed for the diagnosis of AFB. Most of them rely on polyclonal rabbit serum developed against pure cultures of *P. larvae*. They can be used for identification of bacterial colonies resulting from a culturing step or for direct examination of suspicious larval remains.

In an immunodiffusion test the antibodies interact with the bacterial antigen during a double diffusion process, leaving precipitation marks behind (25). In the fluorescent antibody technique these antibodies are conjugated with a fluorochrome dye. The resulting fluorescent antibody reacts with a bacterial smear on a slide. Any excess antiserum is washed off and the smear is examined by fluorescence microscopy. *Paenibacillus larvae* stains can be recognised specifically as brightly fluorescing bacteria on a dark background (24, 33, 36). An enzyme-linked immunosorbent assay using a monoclonal antibody specific to *P. larvae* exists (23). A lateral flow device for rapid confirmation of AFB has been commercialised.

v) Microscopy

Two microscopic techniques are commonly used. Gram staining is often done on smears of bacteria from isolated bacterial colonies. *Paenibacillus larvae* is Gram positive. Carbol fuchsin staining is done on larval smears and can confirm clinical AFB based on spore morphology. These techniques are outlined below:

- Gram staining of bacteria

Flood (cover completely) the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash the slide for 5 seconds with water. The specimen should appear blue-violet when observed with the naked eye. Now, flood the slide with the iodine solution. Let it stand for about a minute as well. When the time has expired, rinse the slide with water for 5 seconds and immediately proceed. At this point, the specimen should still be blue-violet. This step involves addition of the decolouriser, ethanol. This step is somewhat subjective because using too much decolouriser could result in a false Gram (–) result. Likewise, not using enough decolouriser may yield a false Gram (+) result. To be safe, add the ethanol drop-wise until the blue-violet colour is no longer emitted from the specimen. As in the previous steps, rinse with the water for 5 seconds. The final step involves applying the counter-stain, safranin. Flood the slide with the dye and let this stand for about a minute to allow the bacteria to incorporate the saffranin. Gram-positive cells will incorporate little or no counter-stain and will remain blue-violet in appearance. Gram-negative bacteria, however, take on a pink colour and are easily distinguishable from the Gram positives. Again, rinse with water for 5 seconds to remove any excess of dye. Blot the slide gently with bibulous paper or allow it to air dry before viewing it under the microscope.

- Carbol fuchsin staining of larval smears (17)

Heat-fix smears. Flood the slides with 0.2% carbol fuchsin for 30 seconds. Wash off the stain and allow to air dry or gently blot dry with absorbent material. Examine under the microscope for *P. larvae* spores, which are about 1.3 × 0.6 µm, ellipsoidal and thick rimmed.

2. Serological tests

No serological tests are available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Antibody production

VITA diagnostic kit for the early detection of AFB was developed by the Central Science Laboratory Pocket Diagnostic (UK).

When monoclonal or polyclonal *P. larvae*-specific antibodies are produced for the development of a diagnostic test, no cross-reactivity may occur with closely related bacteria or bacteria that commonly occur in beehives, for example against *Paenibacillus alvei*, often found in late phase European foulbrood.

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An FAO publication, Honey bee diseases and pests: a practical guide, W. Ritter & P. Akratanakul (eds). Agricultural and Food Engineering Technical Report No. 4. FAO, Rome, Italy, 42 pp. ISSN 1814-1137 TC/D/A0849/E, is available free of charge at:
http://www.fao.org/WAICENT/faoINFO/AGRICULT/ags/subjects/en/industFoodAg/pdf/AGST_techrep_4.pdf

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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

EUROPEAN FOULBROOD OF HONEY BEES

SUMMARY

The causal organism of European foulbrood of honey bees is the bacterium Melissococcus
plutonius. *The identification of its presence by the observation of signs of disease in the field is
unreliable. The most usual and obvious sign is the death of larvae shortly before they are due to be
sealed in their cells, but this may be for reasons other than European foulbrood. Most infected
colonies display few visible signs, which themselves often quickly abate spontaneously before the
end of each active season. Infection remains enzootic within individual colonies because of
mechanical contamination of the honeycombs by the durable organism. Recurrences of disease
can therefore be expected in subsequent years.*

Identification of the agent: *Examination, by high-power microscopy, of suitable preparations of
larval remains for the presence of numerous lanceolate cocci is adequate for most practical
purposes, especially when it is done by experienced individuals.*

*Traditionally the diagnosis of European foulbrood is done by isolating and identifying the causative
organism. This can be differentiated quite readily from all other bacteria associated with bees by its
fastidious cultural requirements.*

*The isolated bacterium can be identified and differentiated by means of simple tube agglutination
tests. A polymerase chain reaction and a hemi-nested polymerase chain reaction are also
available. The latter permits direct analysis of larvae, adult bees and honey bee products.*

Serological tests: *No tests for detecting antibodies in bees are available.*

Requirements for vaccines and diagnostic biologicals: *There are no biological products
available.*

A. INTRODUCTION

Bee larvae usually die of European foulbrood 1–2 days before being sealed in their cells, or sometimes shortly
afterwards, and always before transformation to pupae. The disease is caused by *Melissococcus plutonius* and
occurs mostly during the period when colonies are growing quickly. Most sick larvae become displaced from their
coiled position in the bottom of their cells before they die. Many are quickly detected and removed by nurse bees,
leaving empty cells scattered randomly among the remaining brood. Some infected larvae survive, successfully
pupate and emerge as adults. These surviving larvae are able to defecate and their infected-faeces contribute to
the continued propagation of the disease (2).

Infected larvae that escape detection by adult bees and then die, first become flaccid and turn a light yellow
colour that becomes increasingly brown, and at the same time they dissolve into a semi-liquid mass. They then
become dry and form a dark brown scale that can easily be removed from the cells. Severely affected brood may
have a very stale or sour odour, sometimes acidic, like vinegar, but often there is no smell.

Signs of disease usually disappear spontaneously from infected colonies before the end of the active season, but
are likely to return in subsequent years (4, 10).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Microscopy

Freshly dead larvae are best for diagnosis. Before any decomposition occurs, diseased larvae can be
smeared on a microscope slide or pulled apart by pinching the cuticle about the centre of the body with two

pairs of forceps, which are then pulled apart. The mid gut contents are left exposed on the slide, still within the gelatinous, transparent peritrophic membrane. This is partially or almost completely filled with bacteria, which are easily seen as opaque chalk-white clumps. The contents of the mid-guts of healthy larvae, which are less easily dissected, have a golden-brown colour. Apparently healthy larvae may contain a mixture of bacteria and pollen. The mid-gut of healthy larvae that contain much light-coloured pollen may resemble those that are filled with bacteria.
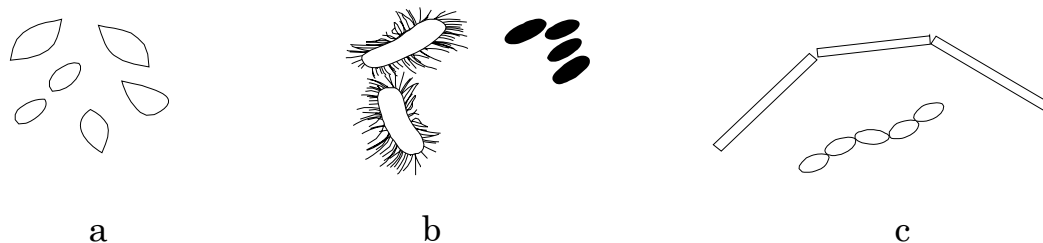
Fig. 1. *Bacteria associated with European foulbrood.*
(*a*) Melissococcus plutonius*: the cause of European foulbrood occurs singly, in longitudinal chains or in clusters. Morphologically resembles* Enterococcus faecalis*, a common secondary invader.*
(*b*) Paenibacillus alvei*: vegetative rods 2.0–7.0 ✕ 0.8–1.2 µm with flagella; sporulating with spores lying adjacently. Both rods and spores are larger than those of* Paenibacillus larvae *(see American foulbrood).*
(*c*) Bacterium eurydice*: slender, square-ended rods* in vivo *but can form chains of cocci* in vitro *in certain media.*

For a bacteriological investigation, a loopful of a dilute aqueous suspension of the midgut contents is transferred to a clean microscope slide and mixed with a loopful of 5% aqueous nigrosin. This is spread over one or two square centimetres, dried gently over a flame, and examined directly by high-power microscopy. The presence of numerous lanceolate cocci, about 0.5 × 1.0 µm in size, occurring either singly or in clusters, and arranged end to end in pairs or short chains, is almost certainly diagnostic of European foulbrood. Some very slender square-ended rod-like bacteria are also usually present (Figure 1). The cocci are Gram positive and the rods are Gram negative. Similar preparations made from aqueous suspensions of whole dead or decomposing larvae are likely to present a confusing array of bacteria in which *M. plutonius* will be difficult to distinguish.

b) Culture methods

Melissococcus plutonius (type strain NCIMB 702443) is the most abundant bacterium during the early stages of an infection (5, 6). *Melissococcus plutonius* can be cultivated on a medium (expressed in g/litre or ml/litre) comprising: yeast extract or certain peptones, 10 (5); cysteine or cystine, 0.2–2.0; glucose or fructose, 10; soluble starch, 10; 1 M KH_2PO_4, 100 at pH 6.6; and agar, 2. The medium is preferably autoclaved in 100 ml lots in screw-capped bottles at 116°C for 20 minutes and poured into Petri plates immediately before use. These plates are streaked with dilute aqueous suspensions of diseased larvae, or ideally, of diseased larval mid-gut. The latter can be prepared beforehand by allowing them to dry on a slide, which may then be kept, for years if necessary, at 4°C or –20°C. All culture media should be subjected to quality control and must support the growth of *M. plutonius* from small inocula. The reference strain should also be cultured in parallel with the suspect samples to ensure that the tests are working correctly.

The preparation and storage of dried smears also eliminates most secondary organisms after a few weeks without affecting the viability of *M. plutonius*. This organism is isolated most efficiently by inoculating decimal dilutions of the aqueous suspension into agar that has been maintained molten at 45°C and which is then poured into plates. The plates must be incubated anaerobically, such as in McIntosh and Fildes jars in an atmosphere of approximately 5–10% carbon dioxide (CO_2) at 35°C. Small white opaque colonies of *M. plutonius* usually appear within 4 days. This bacterium is somewhat pleomorphic *in vitro*, often appearing in rod-like forms. The final pH of the medium may reach 5.5. Decreasingly fastidious strains become selected *in vitro*. Simplified or modified forms of the medium then support multiplication, especially of a serologically distinct *M. plutonius* group from Brazil (1) that will multiply on chemically defined media (3). CO_2 remains essential. Inoculated slopes should be sealed when bacterial growth is apparent and may then be kept at 4°C for up to 6 months. Alternatively, the cultures can be suspended in a medium of 10% sucrose, 5% yeast extract and 0.1 M KH_2PO_4, pH 6.6, and then lyophilised.

A number of other bacteria are often associated with and may be confused with *M. plutonius*. *Bacterium eurydice* inhabits the alimentary tract of adult bees and occurs commonly in the gut of healthy larvae in small numbers. It is more numerous in larvae infected with *M. plutonius*. The incidence of *B. eurydice* in healthy bees is very low in winter and early spring, but it increases in summer. It forms thin square-ended rods, which can grow either singly or in chains. When grown in certain media, it sometimes resembles

streptococci and has been confused with *M. plutonius*. However, its cultural characteristics closely resemble those of *Corynebacterium pyogenes* (10), and it multiplies poorly in the form of thin rods, under the conditions necessary for the cultivation of *M. plutonius*.

Enterococcus (= *Streptococcus*) *faecalis* closely resembles *M. plutonius* morphologically and has often been confused with it, although they are both culturally and serologically distinct. Unlike *M. plutonius,* it does not remain viable for long when dried, or persist as mechanical contamination within bee colonies. It is probably brought into the hive by foraging adult bees, and is responsible for the sour smell sometimes encountered with European foulbrood.

Enterococcus faecalis grows well *in vitro* under the conditions suitable for *M. plutonius,* but it may be readily differentiated by its ability to grow aerobically. It forms small transparent colonies within 24 hours and is a facultative anaerobe. It multiplies on a variety of the more common media with or without carbohydrates or CO_2. The final pH in the presence of glucose is 4.0. *Enterococcus faecalis* rarely exceeds the number of *M. plutonius* in bee larvae, and can usually be diluted out. When it is not diluted out it produces sufficient acid to prevent the *in-vitro* multiplication of *M. plutonius*.

Enterococcus faecalis does not multiply in bee larvae in the absence of *M. plutonius*, so its presence in large numbers can be taken as presumptive evidence of European foulbrood.

Paenibacillus (= *Bacillus*) *alvei* is generally more common than *E. faecalis* in bee colonies affected with European foulbrood, but it is not invariably associated with the disease and so cannot act as a reliable indicator of it. In bee colonies, it multiplies only in the decomposing remains of larvae, and then its spores often predominate over all other bacteria, even to their apparent exclusion. *Paenibacillus alvei* forms very resistant spores and becomes well established in bee colonies with enzootic European foulbrood. It causes a characteristic stale odour. *Paenibacillus alvei* multiplies poorly under the conditions necessary for the *in-vitro* growth of *M. plutonius*. It produces a spreading growth of transparent colonies, some of which are motile and move in arcs over the surface of the agar. Cultures have the characteristic stale odour that is associated with European foulbrood when the bacillus is present. Spores are formed rapidly.

c) Immunological methods

For the identification of *M. plutonius*, antisera can be prepared in rabbits against washed cultures of *M. plutonius* either by intravenous injections (7) or by a single intramuscular injection of 1 ml of antigen suspension mixed with an equal volume of Freund's incomplete adjuvant.

Assays are made by agglutination tests in tubes containing suspensions of bacteria equivalent to 0.25 mg dry weight/ml. End-points are noted after tubes have been incubated for 4 hours at 37°C.

A test kit for the identification of antibodies against *M. plutonius* has recently been developed and is commercially available. It provides rapid confirmatory on-site diagnosis of European foulbrood infection in honeybee larvae.

d) Polymerase chain reaction

Polymerase chain reaction (PCR) can be done on suspicious bacterial colonies transferred to and grown in liquid medium (9). Genomic DNA is prepared according to standard methods (12). The DNA pellet is resuspended in 50 µl of 1 × TE buffer (10 mM Tris/HCl, pH 7.5; 1 mM EDTA [ethylene diamine tetra-acetic acid]). Approximately 1–3 µg of genomic DNA is amplified in a 50 µl reaction. The PCR reaction can also be done with larvae. Each larva is incubated individually in liquid medium overnight at 30°C in an anaerobic jar containing hydrogen plus 10% CO_2. Two millilitres of each sample is then centrifuged at 1000 *g* for 2 minutes, and the supernatant is centrifuged at 10,000 *g* for 5 minutes. The resultant pellet is resuspended in 100 µl of sterile H_2O and heated at 95°C for 15 minutes. One microlitre is amplified in a 50 µl PCR mixture. Besides template DNA this mixture also contains 2 mM $MgCl_2$, 50 pmol of forward (EFB-F) and reverse primer (EFB-R; primer sequences are given below) per µl, 25 mM (each) deoxynucleoside triphosphate and 1 U of *Taq* polymerase. Amplification of a specific DNA fragment occurs in a thermocycler under to the following PCR conditions: a 95°C (1 minute) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

A hemi-nested PCR was first developed by Djordjevic *et al.* (8) and thereafter improved for sensitive detection of *M. plutonius* in honey, pollen, whole larvae and adult bees (11). Here the first 50 µl reaction mixture contains 5–30 ng genomic DNA, 3 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate, 100 ng of the primers MP1 and MP2, 5 µl of 10 × PCR buffer (100 mM Tris/HCl, pH 8.3; 15 mM $MgCl_2$; 500 mM KCl) and 1 U of *Taq* polymerase. Conditions of amplification consist of an initial denaturation cycle at 95°C for 2 minutes followed by 40 cycles of denaturation (95°C, 30 seconds), primer annealing (61°C,

15 seconds), primer extension (72°C, 1 minute) followed by an additional extension step of 5 minutes at 72°C. The third primer MP3 is used in conjunction with MP1 to amplify a DNA fragment from 1 µl of the primary PCR product obtained in the previous reaction. PCR conditions for the hemi-nested PCR are exactly as described above except that the $MgCl_2$ concentration is lowered to 1.5 mM and the annealing temperature to 56°C.

The molecular weights of the PCR products are determined by electrophoresis in a 1.0–1.5 % agarose gel and staining with ethidium bromide.

Ref.	Name	Sequence	PCR-product size
(9)	EFB-F	5'-GAA-GAG-GAG-TTA-AAA-GGC-GC-3'	812 bp
	EFB-R	5'-TTA-TCT-CTA-AGG-CGT-TCA-AAG-G-3'	
(8, 11)	MP1	5'-CTT-TGA-ACG-CCT-TAG-AGA-3'	486 bp
	MP2	5'-ATC-ATC-TGT-CCC-ACC-TTA-3'	
	MP3	5'-TTA-ACC-TCG-CGG-TCT-TGC-GTC-TCT-C-3'	276 bp

2. Serological tests

No tests for detecting antibodies in bees are available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

ACKNOWLEDGMENT

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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

NOSEMOSIS OF HONEY BEES

SUMMARY

To date, two microsporidian parasites have been described from honey bees: Nosema apis (*Zander*) and Nosema ceranae (*Fries*). Nosema apis *is a parasite of the European honey bee* (Apis mellifera) *and* Nosema ceranae *of the Asian honey bee* (Apis cerana) (11) *and the European honey bees*). *The latter has recently been detected in several geographically separated populations of European honey bees in Europe* (*12*), *South and North America* (*14*) *and Asia* (*13*). *The pathological consequences of* Nosema ceranae *in* Apis mellifera *are not well known. In the following chapter, only* Nosema apis *is described. Both types are presumably very similar.* Nosema apis *is a parasite that invades the epithelial cells of the ventriculus of the adult honey bee. Infections are acquired by the uptake of spores during feeding or grooming. The disease occurs throughout the world, but treatment of bees can help to prevent the spread of infection to unaffected bee colonies.*

The parasite invades the posterior region of the ventriculus, giving rise to large numbers of spores within a short period of time. The parasite is ubiquitous. Nosema *levels generally increase when bees are confined, such as in the autumn and winter in colder climates when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. Spores are expelled with the faeces where they may retain their viability for more than 1 year. Spores may also remain infective after immersion in honey and in the cadavers of infected bees; however they may lose viability after 3 days when submerged in honey at hive temperature. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood. However, it seems likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for* N. apis *to be successfully transmitted to the next generation of bees. The spores are inactivated by acetic acid or by heating to 60°C for 15 minutes. To be effective, these treatments, which inactivate spores on hive surfaces and combs, can be combined with feeding colonies with the antibiotic fumagillin to suppress infections in live bees. The EU prohibits the use of antibiotic fumigation (*EU 3/01/081*).*

Identification of the agent: *In some acute cases, brown faecal marks are seen on the comb and the front of the hive, with sick or dead bees in the vicinity of the hive. However, the majority of colonies show no obvious signs of infection, even when the disease is sufficient to cause significant losses in honey production and pollination efficiency. During winter, there may be an increase in bee mortality. In affected bees, the ventriculus, which is normally brown, can be white and very fragile. Microscopic examinations (×400 magnification) of homogenates of the abdominal contents of affected bees will reveal the oval spores of* Nosema apis*, which are approximately 5–7 × 3–4 μm with a dark edge (*Nosema ceranae *is slightly smaller). Their internal contents can be distinguished after staining with Giemsa's stain.* Nosema apis *spores have a distinctive appearance, with a thick unstained wall and a blue-stained featureless interior. The nuclei within the spores are not visible. This method can help to distinguish* N apis *from other microbes found in bees.*

The appearance of Nosema apis *spores can be confused with yeast cells, fungal spores, fat and calciferous bodies or cysts of* Malpighamoeba mellificae*. The latter are similar in size to* Nosema *spores, being 6–7 μm in diameter, but are completely spherical instead of oval.*

Positive identifications can be made only by observation of typical spores in the ventriculus or faeces. Very mild infections may not be demonstrable. The extent of infection is determined by

counting the spores on a microscope grid and calculating the average number of spores per area and estimating from that the number of spores per bee.

Serological tests: There are no applicable serological tests.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

The microsporidium *Nosema apis* (Zander) is a protozoan parasite exclusive to the epithelial cells of the ventriculus of adult bees *and t*he disease occurs throughout the world (16). Infection occurs by the ingestion of spores in the feed (5, 19), via trophallaxis (19) or perhaps after grooming of the body hairs (6, 10, 19).

The polar tube of the spore is everted and penetrates the peritrophic matrix of the intestine, particularly in the posterior region of the ventriculus. The sporoplasm passes down the tube and enters the cytoplasm of the epithelial cells, where it reproduces. Autoinfections can occur at the same time as new infections. After a short interval, spores develop in large quantities. The parasite is ubiquitous and multiplies at a specific rate throughout the year. *Nosema* levels generally increase when bees are confined, such as in the autumn and winter in colder climates when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood (19, 20). In winter, spores are rarely to be found, or are only found in heavily infected bees.

Any inherent natural defence by a bee colony against a heavy infection with the parasite depends on the colony size as well as on the prevailing weather conditions during the early part of the autumn of the previous year (18). If these conditions are unfavourable, the overall life expectancy of the colony is reduced. This may lead to the premature death of bees during winter or early spring. In a typical case of a colony being depleted because of a *Nosema* infection, the queen can be observed surrounded by a few bees, confusedly attending to brood that is already sealed.

In faecal droppings, spores may retain their viability for more than 1 year (3). Spores may also remain viable for up to 4 months after immersion in honey (21) and for up to 4.5 years in the cadavers of infected bees (18). The spores may lose viability after only 3 days when submerged in honey at hive temperature (17). It is likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for *N. apis* to be successfully transmitted to the next generation of bees. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood and it seems that temperature may have a marked effect on the rates at which spores lose viability, regardless of their medium (17).

Spores may be killed by heating hive equipment or tools to a temperature of at least 60°C for 15 minutes. Combs may be sterilised by heating to 49°C for 24 hours (8). Fumes from a solution of at least 60% acetic acid will inactivate any spores within a few hours, depending on the concentration; higher concentrations are even more effective and will kill spores within a few minutes (2, 9). Such procedures come under the jurisdiction of national control authorities with protocols that vary from country to country. Disinfection can be carried out, for example, by putting acetic acid solution into bowls or on to sponges that can soak up the liquid. Following disinfection after an outbreak, all combs should be well ventilated for at least 14 days prior to use. Suppression of *Nosema* disease can also be achieved by feeding an antibiotic, fumagillin, in sugar syrup to the colony (8). This is forbidden in many countries and in the EU.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

In acute forms of infection, especially in early spring, brown faecal marks <u>may</u> be noted on the comb and the front of the hive (4). At the entrance to the hive, sick and dead bees <u>may</u> be seen, although other causes, such as pesticide poisoning and diseases of adult honey bees (such as acarapidosis should be eliminated first if this is the case. The detection of these infectious diseases requires microscopic examination. During winter, *Nosema apis*-infected colonies may become severely depleted of bees or die out altogether. The majority of *Nosema apis*-infected colonies will appear normal, with no obvious signs of disease even when the disease is sufficient to cause significant losses in honey production and pollination efficiency (1, 11). A proper diagnosis can be made only by microscopic examination of adult bee abdomen or ventriculus. To diagnose a *Nosema apis* infection, the posterior pair of abdominal segments is removed with a forceps to reveal the ventriculus, complete with the malpighian tubules, the small intestine and rectum. The ventriculus is normally brown but, following a *Nosema* infection, it can become white and fragile. However, this appearance is given by other causes of intestinal

disturbance, for example feeding on indigestible food stores, such as syrup containing actively growing yeast. For a reliable diagnosis, a number of bees in a sample should be examined.

a)   Microscopy

It is necessary to attempt to distinguish between a *Nosema apis* infection and an infection caused by *Malpighamoeba mellificae* (19). There is quite often an indication of dysentery in a *Nosema apis* infection. In an *M. mellificae* infection, there may be diarrhoea, often of a sulphur-yellow colour and with a distinct odour. Characteristics of *M. mellificae* cysts are described later. Secondary mixed infections may occur (17). A simple, nonquantitative method for detecting *Nosema apis* infection is as follows: sampled bees should be obtained from the hive entrance in order to avoid sampling individuals under the age of 8 days, which would lead to 'false negatives' because no spores from the protozoan in question would be determined. At least 60 bees should be collected in order to detect 5% of diseased bees with 95% confidence (10). Before sending to the laboratory, the bees should be fixed in 4% formol, 70% ethyl alcohol or frozen in a standard freezer in order to prevent them from decomposing and to improve their reception and organisation in the laboratory. The abdomens of the bees to be examined are separated and ground up in 2–3 ml of water. Three drops of the suspension are placed on a slide under a cover-slip and examined microscopically at ×400 magnification, under bright-field or phase-contrast optics. This is a slight simplification of Cantwell's original method (7). The spores are about 5–7 µm long and 3–4 µm wide (*Nosema ceranae* is slightly smaller than *Nosema apis*). They are completely oval with a dark edge. Their contents, consisting of nucleus, sporoplasm and polar tube, cannot be seen. Dyes are usually not necessary.

Nosema spores must be differentiated from yeast cells, fungal spores, fat and calciferous bodies, and from *M. mellificae* cysts, which are spherical and approximately 6–7 µm in diameter.

When air-dried, ethanol-fixed smears of infected tissue are stained with Giemsa's stain (10% in 0.02 M phosphate buffer) for 45 minutes. *Nosema apis* spores will have a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. Insect cells, fungal spores and other protozoa stained in this way will generally have thinner walls, blue/purple cytoplasm and magenta-coloured nuclei.

In order to obtain accurate, reliable and meaningful quantification of levels of *Nosema* infections in honey bees, a standardised procedure must be used. A suitable protocol is as follows:

A sample of older worker honey bees is taken, from which the abdomens of ten individuals are macerated in 5 ml of water using a mortar and pestle. When tissue pieces have become quite fine, the suspension is filtered through two layers of muslin (thin loosely woven cotton fabric) in a funnel leading to a graduated centrifuge tube. A second 5 ml of water is used to rinse the pestle, swirl around the inside of the mortar and pour through the subsample in the funnel. Water levels are equalised in the tubes and the suspensions are centrifuged for 6 minutes at 800 *g*. The supernatants are decanted and the tubes are refilled to the 10 ml level. Using disposable pipettes and a rubber bulb, the pellets are resuspended by repeated uptake and forcible ejection through the pipette tips. When the solution appears to be homogenous, a sample is taken to fill the calibrated volume under the cover-slip of a haemocytometer (blood cell counting chamber). After a few minutes the spores will have settled to the bottom of the chamber. *Nosema* spores appear transparent but with a very distinct dark edge and are 5–7 µm long and 3–4 µm wide. They are best seen using a magnification of ×400 and bright-field or phase-contrast optics. The number of spores in each square is counted. Where a spore lies over the edge of a square, count only those spores that straddle the left and upper edges of the square, not the right and bottom edges. One *Nosema apis* spore, observed in the haemocytometer's entire central square millimetre grid (25 × 16 = 400 small squares), is equal to an average of 10.000 spores per bee. If no spores are seen, the result should be designated 'not detected', but that does not mean that the bees are not infected. Regulatory agencies will decide on the level of infection useful for their purposes.

A laboratory method for the simultaneous detection of *Nosema* spores and *M. mellificae* cysts consists of the individual examination of the colonies using 30–60 bees per colony. A suspension of the abdomens of dead bees is prepared by grinding with 5–10 ml water; the volume of water depending on the number and condition of the bees. The suspension must be filtered to remove debris that would interfere with the examination, first through a 100 µm and then a 40 µm filter. Parts of the malpighian tubules pass through the 100 µm filter, but are collected on the 40 µm filter. They are placed on a slide or bacterial counting chamber and examined at ×400 magnification. Only a few tubules are filled with cysts after an *M. mellificae* infection. The normal structure of malpighian tubules is not visible in this case. Only cysts inside the malpighian tubules can be taken as a positive result, because *M. mellificae* cysts are often confused with fungal spores and yeast cells.

b)   Culture

There are no cultural methods for growing these organisms.

c) Polymerase chain reaction (PCR)

Different methods have been developed to distinguish *N. apis* from *N. ceranae*. A multiplex PCR is described below with which both pathogen types can be clearly identified at the same time (15).

- **Sample preparation for PCR**

The abdomens of 10–20 adult honey bees from each sample are macerated in 10 ml distilled water (PCR grade) and the suspension is then filtered and centrifuged at 800 *g* for 6 minutes. For DNA extraction, spore germination is induced with 200 µl freshly prepared germination buffer (0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH to 6.0 with ortophosphoric acid), and the mixture is incubated at 37°C for 15 minutes. The DNA extraction can be easily carried out using routine procedures or commercial kits, such as High Pure PCR Template Preparation Kit (No. 1796828 Roche Diagnostic).

- **Multiplex PCR**

With this technique both microsporidians (*N. apis* and *N. ceranae*) can be distinguished in just one PCR because of the use of specific primers with no interference. PCR reactions are performed in 50-µl volumes containing 5 µl of template DNA, 25 µl of High Fidelity PCR Master Mixture (catalogue no. 12140314001; Roche Diagnostic), 0.4 µM of each primer, 0.4 mM of each deoxynucleoside triphosphate, 3 mM Cl_2Mg, 0.2 mg/ml bovine serum albumin, 0.1% Triton X-100, and 5 µl of *N. apis* or *N. ceranae* DNA template. The parameters for amplification are: an initial PCR activation step of 2 minutes at 94°C, followed by 10 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 45 seconds at 72°C, and 20 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 50 seconds at 72°C plus a 5-second elongation cycle for each successive cycle and a final extension step at 72°C for 7 minutes. Negative controls (from DNA extraction) are included in all PCR experiments.

The molecular weights of PCR products are determined by electrophoresis in a 2% agarose TAE (Tris-acetate-ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with ethidium bromide, and visualised using UV illumination.

Primers selected for detection of *N. ceranae* and *N. apis* in multiplex PCR:

Primer	Sequence[a]	PCR product size (bp)	Specificity
218MITOC FOR	5'-CGGCGACGATGTGATATGAAA-ATATTAA-3'	218–219[b]	*N. ceranae*
218MITOC REV	5'-CCCGGTCATTCTCAAACAAAA-AACCG-3'		
321APIS FOR	5'-GGGGGCATGTCTTTGACGTACTATGTA-3'	321	*N. apis*
321APIS REV	5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3'		

[a]CG tails added to primers are underlined.

[b]There is a 1-bp difference in the *N. ceranae* amplicon size depending on the sequences for *N. ceranae* available in GenBank (http://www.ncbi.nlm.nih.gov).

2. Serological tests

There are no serological tests available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No biological products are available.

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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

SMALL HIVE BEETLE INFESTATION
(*Aethina tumida*)

SUMMARY

The small hive beetle Aethina tumida *Murray 1867 (Coleoptera: Nitidulidae), is a parasite and scavenger of honey bee colonies. Adults and larvae feed on honey bee brood, honey and pollen, causing death of brood, fermenting of honey and comb destruction, often resulting in the full structural collapse of the nest and absconding of the colony. The small hive beetle can be a serious problem in honey-extracting facilities where stored comb, honey and wax cappings are all potential feeding and breeding areas. Development requires 3–12 weeks, depending on temperature and food availability. The flying adult beetles actively infest colonies.*

Identification of the agent: *An infestation by* Aethina tumida *can be recognised either indirectly via colony-wide damage or directly via eggs, larvae and adults. An early diagnosis can be made after opening the colony and finding adult beetles on the bottom board or hiding in the combs. Intra- and extra-colonial acaricides and insecticides presently are used to kill the adult beetles and larvae while intra- and extra-colonial traps can be used to find the beetles.*

Serological tests: *No serological tests are applicable.*

Requirements for vaccines and diagnostic biologicals: *No biological products are available.*

A. INTRODUCTION

The small hive beetle, *Aethina tumida* Murray, Coleoptera: Nitidulidae (17), is native to sub-Saharan Africa (12) but has been introduced to the United States of America (1996), Egypt (2000) and Australia (2002) (18). It was introduced in to Canada in 2002 but did not establish; it was reintroduced in 2006 and it has not been determined if it is established. *Aethina tumida* can be spread by active flying, migratory beekeepers, or transportation of infested hive products (13, 18). Larvae and eggs of *A. tumida* have been identified in cages of imported queens in Portugal (2004), but all bee hives were immediately destroyed (pers. comm.). Within its native range, it is usually considered a minor pest, and reproduction appears more successful in weak and stressed colonies or in recently abandoned nests (18). However, within its new ranges it can cause considerable damage in colonies of European honey bee subspecies (3, 11, 13, 18).

1. Life cycle

The infesting *A. tumida* females mate in the colony (more than 1000 adult beetles may occur within a colony [11]) and oviposit several eggs in typical clutches in small cracks, in cells or within capped brood (9, 15, 18). The larvae hatch after 1–6 days and feed on pollen, honey and bee brood like the adults (15, 18, 22). Adult beetles also can be fed by worker bees via trophallaxis (8). Larval development takes 8–29 days (depending on food availability and temperature [8, 15, 18, 22]) following which they reach the wandering phase (15) and leave for pupation in the soil, mostly in close proximity to the hive (21). Pupation takes 2–12 weeks depending on temperature and soil moisture (7). Emerging adults leave the soil and can actively fly over long distances (>10 km) to search for new host colonies, thereby completing the life cycle of *A. tumida*.

The reasons for the apparent difference in the impact of the small hive beetle with its native range compared with its new ranges are not well understood (3). They may include quantitative behavioural differences between African and European honey bee subspecies, different beekeeping techniques and climatic differences (3, 13, 18).

Adult beetles can survive up to 6 months and females can oviposit about 1000 eggs in their life time (15).

While damage due to the adults is relatively minor, it nevertheless can cause absconding of the colony (6). If not prevented by the bees (9, 19), larval growth (several hundreds or thousands of individuals) is usually associated with fermentation of the honey, causes severe damage to combs and often results in the full structural collapse of the nest (12). Economic losses can also be associated with beetle infestations in the honey-extracting facility. Environmental conditions generally associated with extracting facilities, such as high temperature and humidity, provide optimal condition for beetle development. Cryptic low-level reproduction may also occur either in the debris or underneath hive inserts without any signs of damage to the beekeeper (22).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The first sign of an infestation by *A. tumida* is the occurrence of adult beetles (~5 mm length and ~3 mm width, females slightly longer than males [10]), with a dark brown to black colour (lighter shortly after eclosion) in the colony (Fig. 1). During inspections, adults avoid sunlight, hide, and can be observed while running for cover into corners or in a typical fashion over the combs. Adults can be confused with other beetles from the same family, which can also be associated with colonies (e.g. *Cychramus luteus* [20]).

Fig. 1. *Aethina tumida* adult. Photo by N. Ruppert.

a) Beetle eggs, larvae and pupae

Eggs are white and bean-shaped (~2/3 of the size of a honey bee egg) and oviposited in clutches (up to 210) in cracks, on the bottom board, on the combs and underneath sealed brood (9). Larvae are whitish, often covered with a slimy sticky coating, up to 1.2 cm long (wandering phase) and have three pairs of legs and dorsal spikes. Larvae can be found mining in combs (15) or in the debris (23). Larval infestations are typically associated with a rotten smell (e.g. rotting orange). Wandering larvae often leave smear trails inside and outside the colony. Such wandering larvae and pupae (whitish, ~5 mm long and 3 mm wide) can be found in small pupation chambers 1–20 cm deep in the soil usually in close proximity to colonies (<180 cm, [21]).

b) Colony examination

When monitoring honey bee colonies for the presence of *A. tumida,* an examination of the hive may provide an early indication of infestation. Adult beetles can be observed hiding inside cells and in the debris. Colony examinations start by removing the hive roof and placing it upside down on the ground next to the hive. Remove the hive chamber, i.e. supers and upper brood chamber (in double brood chamber colonies) and place them on the upturned roof for a few minutes. Place the hive crown board on top. A few minutes later lift the boxes out of the way and scan for beetles on the inner surface of the upturned roof. Then frames are screened one by one for the presence of adults, larvae and eggs. During cool weather, adults tend to stay close to or within the bee cluster. In warmer periods beetles are found more often on the bottom board or the outer-most frames.

c) Board examination using traps

Less labour intensive diagnosis is feasible using hive inserts. Such inserts allow for the beetles to hide in the corrugations but prevent bees from entering. They can be placed on the bottom board. To detect the beetles, place a piece of corrugated cardboard (15 cm × 15 cm), with one surface peeled to expose the ridges, on the bottom board of the bee hive with the ridged side down. Cover it with wood to fit underneath the frames on the bottom board. Leave the insert in the colony for ≤ 3 days, remove it and examine for adults and larvae. Acaricides can be used to kill adults in these inserts. The debris and all areas to which bees have no access should also be screened carefully.

2. Serological tests

No serological tests are available for routine laboratory diagnosis.

3. Treatment

In countries with infestations of *A. tumida*, control has focused on chemical treatments with acaricides and insecticides (1, 2, 11). Acaricides, non-toxic to bees, are used in traps intra-colonially (11) to kill the adults. Similarly, insecticides are used as a ground drench to kill wandering larvae and pupae (1, 2). Such treatments carry the risks of both pest resistance and residues in the hive products (18). Alternative, more sustainable controls are moderately efficient so far (3, 13, 14, 18) and currently under investigation (3–5, 13, 14, 16).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

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FURTHER READING

An FAO publication, Honey bee diseases and pests: a practical guide, W. Ritter & P. Akratanakul (eds). Agricultural and Food Engineering Technical Report No. 4. FAO, Rome, Italy, 42 pp. ISSN 1814-1137 TC/D/A0849/E, is available free of charge at:
http://www.fao.org/WAICENT/faoINFO/AGRICULT/ags/subjects/en/industFoodAg/pdf/AGST_techrep_4.pdf

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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

TROPILAELAPS INFESTATION OF HONEY BEES
(*Tropilaelaps* spp.)

SUMMARY

The mites in the genus Tropilaelaps *are parasites of honey bee brood. Feeding on bee larvae and pupae causes brood malformation, death of bees and subsequent colony decline or absconding. Development requires about 1 week, and the mites are dispersed on bees. There are at least four species in the genus* Tropilaelaps. *Each species is closely associated with a particular giant honey bee in Asia. Two species (*Tropilaelaps clareae *and* Tropilaelaps mercedesae) *are damaging pests of* Apis mellifera. *The other two species (*Tropilaelaps koenigerum *and* Tropilaelaps thaii) *appear to be harmless to* Apis mellifera (1).*

Identification of the agent: *Molecular and morphological methods are available for identifying each species (*1*). An infestation by* Tropilaelaps *can be recognised either visually on bees or by examining hive debris. Irregular brood pattern, dead or malformed immatures, bees with malformed wings that crawl at the hive's entrance, and especially the presence of fast-running, large, red-brown, elongated mites on the combs, are diagnostic for the presence of* T. clareae*. An early diagnosis can be made after opening brood cells and finding immature and adult mites therein. The hive (*colony*) may be treated with various chemicals that cause the mites to drop off combs and bees. Sticky boards on the bottom of the colony can be used to examine hive debris and mites.*

Serological tests: *No serological tests are applicable.*

Requirements for vaccines and diagnostic biologicals: *No biological products are available.*

A. INTRODUCTION

The mite species *Tropilaelaps clareae,* previously assumed to be ubiquitous in Asia, has been found to be two species. *Tropilaelaps clareae* occurs in Asia where it is a parasite of the native honey bee *Apis dorsata breviligula*. It is also a parasite of the introduced honey bee species *A. mellifera* in the Philippines and the native honey bee species *A. dorsata binghami* on Sulawesi Island in Indonesia. *Tropilaelaps mercedesae*, which until now was mistaken for *T. clareae*, together with *T. koenigerum*, parasitises the native *A. dorsata dorsata* in mainland Asia and Indonesia (except Sulawesi Island). *Tropilaelaps mercedesae* is also a parasite of the introduced *A. mellifera* in these and surrounding regions and, with another species, *T. thaii*, also parasitises *A. laboriosa* in mountainous Himalayan regions (1).

1. Life cycle

The colonising *Tropilaelaps* female (or females; as many as a dozen may occur within individual a single cells) places from one to four eggs on mature bee larvae shortly before the brood cell is capped. The drone brood is preferred by *Tropilaelaps* and may be almost 100% parasitised (4). The mite progeny, usually one male and several females feed on and seriously damage the bee brood. Development of the mite requires about 1 week. The adults, including the foundress female, emerge with the adult bee and search for new hosts.

The short life-cycle, as well as a very brief stay on adult bees, explains why populations of *T. clareae* increase faster than those of *Varroa* mites. When both *T. clareae* and *Varroa destructor* infest the same colony, the former may out-compete the *Varroa* mite (4, 13). It has been reported that when both mite species are in the same cell, the reproduction of both mites declines (12).

Phoretic survival on bees is quite short (only 1–2 days) because *Tropilaelaps* cannot pierce the integument of adult bees. The phoretic time for *Tropilaelaps* spp. is important in understanding the life cycle, and recent research suggests the period can be as long as 5–10 days (15, 16). Gravid female mites will die within 2 days unless they deposit their eggs (17).

Infestation by *Tropilaelaps* causes the death of many bee larvae (up to 50%), resulting in an irregular brood pattern and of which the cadavers that may partially protrude from the cells. Many malformed bees occur, with distorted abdomens, stubby wings and deformed or missing legs. Some of the affected bees crawl at the hive's entrance (2). In addition, perforated cappings are seen, the result of sanitation activities by the worker bees, which evict the infested bee pupae or young adults. Some infested colonies abscond, carrying the mites to a new location.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The first sign of an infestation by *Tropilaelaps* species is often the occurrence of large, red-brown, elongated mites on the combs or on adult bees (Figs 1 and 2). *Tropilaelaps clareae* (<1 mm in length), and *T. mercedesae* (< 9 mm in length) differ in body size but otherwise are alike. *Tropilaelaps koenigerum* is slightly smaller, only about 0.7 mm in length (5). The females also differ in the structure of their ventral anal plate and subapical tooth of the chelicerae (1). *Tropilaelaps* can easily be recognised and separated from the *Varroa* mite using a ×10 magnifying glass. The body of the *Varroa* mite is wider than it is long and it moves slowly, whereas the body of *Tropilaelaps* is elongated, with a heavily sclerotised holoventral or similar shield (Fig. 3), and it is a fast-running mite.
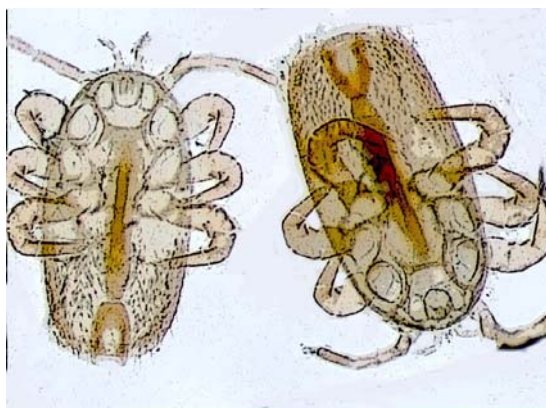

Fig. 1. Tropilaelaps clarea. Photo by J. Waddell.

Fig. 2. *Tropilaelaps* on *Apis dorsata* larvae. Photo by D. Anderson.

Fig. 3. *Tropilaelaps* offspring on *Apis mellifera* pupae. Photo by W. Ritter.

a) Mite collection

Methods to collect mites include an ether or sugar roll (13). Collect approximately 100–200 bees in a wide-mouthed jar with lid. Scrape the bees into the jar or use a modified vacuum to suck them in. Knock the bees to the bottom of the jar with a sharp blow; there should be about a 1–2 inch (2.54–5.08 cm) layer of bees on the bottom. Remove the lid and spray a 2-second burst with ether starter fluid. Alternatively, use enough 70% alcohol or soapy water to cover the bees; or add around 25 g (1 oz) powdered sugar (or flour). If using ether replace the lid and agitate or roll the jar for about 10 seconds; mites should stick to walls. If using soap or alcohol, agitate and then strain out the bees with a coarse hardware cloth or mesh strainer; mites will be in the liquid. If using sugar or other powder, put screening material (such as hardware cloth) on top of the jar and shake the mites on to white paper to count; repeat every 2 minutes. For a more accurate count, finish with an alcohol or soapy water wash to collect all the mites.

b) Colony and brood examination

When monitoring honey bee colonies for the presence of *Tropilaelaps* (or *Varroa*), an examination of both drone and worker brood may provide an early indication of infestation. Mites can be observed inside capped bee brood by using a honey scratcher (with fork-like tines) to pull up capped pupae. The mites are clearly visible. The younger mite stages are whitish and may be almost motionless while feeding on their hosts' bodies, as their mouthparts and front legs are fixed to the cuticle of the bee host (13). The extent of parasitisation can be estimated by opening a predetermined number of brood cells; infestation rates are then calculated as per cent of capped brood containing live mites (3).

c) Sticky board examination

A precise diagnosis can be made using a sticky board covered with a mesh, such as fly screen, that prevents the bees from removing the dislodged mites. The mesh must be large enough for mites to pass through. Make a sticky board with poster board, cardboard or other white, stiff paper coated with Vaseline or other sticky substance (8, 10, 14), or use a sheet of sticky shelf paper. Cut the paper to fit the bottom board of a hive. Cut a piece of hardware cloth or screen to fit on top of the sticky board. To keep the bees from cleaning off the board, fold under the outside edges of the screen to raise it off the board, and staple or tape in place. Leave the board in the colony for up to 3 days, collecting and examining the debris for mites. For faster mite diagnosis, smoke each colony adding 25 g (1 oz) pipe tobacco in the smoker. Puff the bees 6–10 times, close up the hive for 10–20 minutes. Pull out the sticky board after 10 minutes and count the mites. Acaricides are sometimes used to knock mites off bees and will appear on the sticky boards.

2. Serological tests

No serological tests are available for diagnosis.

3. Treatment

In countries with infestations of *Tropilaelaps* spp., fluvalinate in slow-release formulations controls *Tropilaelaps* (9, 11), as do monthly dustings with sulphur (2) and treatments with formic acid (6). The inability of this mite to feed on adult bees, or to survive outside sealed brood for more than a few days, such as caging the queen for a few weeks, is being used as a non-chemical control method (17, 18).

Many of the same acaricides used for *Varroa* will kill *Tropilaelaps*. Strips of plastic-impregnated fluvalinate (Apistan™) will kill mites. Alternatively, tobacco smoke in the smoker will cause mites to drop off bees. Strips of filter paper, available in some countries are prepared by soaking in an aqueous solution of 15% potassium nitrate to which two drops of amitraz (usually 12.5%) are added (9). After the paper dries, the strip is ignited and inserted into the hive. The smoke causes many mites to drop off. Another method is to use plates or pads soaked with 20 ml of 65% formic acid (very caustic and will burn hands and face). The pads are placed in the colonies, near the top (7).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

VARROOSIS OF HONEY BEES

SUMMARY

The mite Varroa destructor (*formerly* Varroa jacobsoni) *is a parasite of adult bees and their brood. It penetrates the intersegmental skin between the abdominal sclera of adult bees to ingest haemolymph. It can sometimes be found between the head and thorax. The number of parasites steadily increases with increasing brood activity and the growth of the bee population, especially late in the season when clinical signs of infestation can first be recognised. The life span of the mite depends on temperature and humidity but, in practice, it can be said to last from some days to a few months.*

Identification of the agent: *The clinical signs of varroosis can only be recognised at a late stage of infestation, so that diagnosis entails the examination of the hive debris. The debris produced during the summer is especially useful for diagnosis. The earliest and most precise diagnosis can be made only after the application of a medication that forces the mites to drop off the bees or kills them directly. Larger amounts of debris can be examined using a flotation procedure. Bees are washed in petroleum spirit, alcohol or detergent solution. However, this method is less accurate due to the unequal distribution of mites and the usually small sample sizes.*

Serological tests: *No serological tests are applicable.*

Requirements for vaccines and diagnostic biologicals: *No biological products are available.*

A. INTRODUCTION

The *Varroa* mites are parasites of adult bees and their brood. Four species have been recorded: *Varroa jacobsoni, V. destructor, V. underwoodi* and *V. rinderi.* Until recently *Varroa* mites that affect *Apis mellifera* world-wide were assumed to be *V. jacobsoni.* However it has been shown that these mites are *V. destructor* (Figure 1).
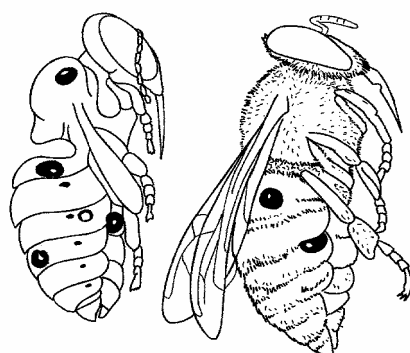
Fig. 1*.* Varroa *on pupa and adult bee. Left: pupa with four* Varroa *female mites.*
Right: worker bee with two female mites.

They are responsible for the condition of varroosis or varroatosis (1, 2). The mite inserts itself between the abdominal sclera in adult bees (10) where it penetrates the intersegmental membranes in order to ingest haemolymph. Sometimes it can also be found between the head and thorax. For reproduction, the female enters the cells with the bee brood shortly before the cells are sealed. They prefer drone brood to worker brood. After the brood cell is sealed, the mite lays after 2 to 3 days the first egg (generally male). Later up to seven eggs (generally females) are laid in intervals of about 1–2 days. These hatch into nymphs, but only two to three reach the adult stage (Figs 2 and 3).
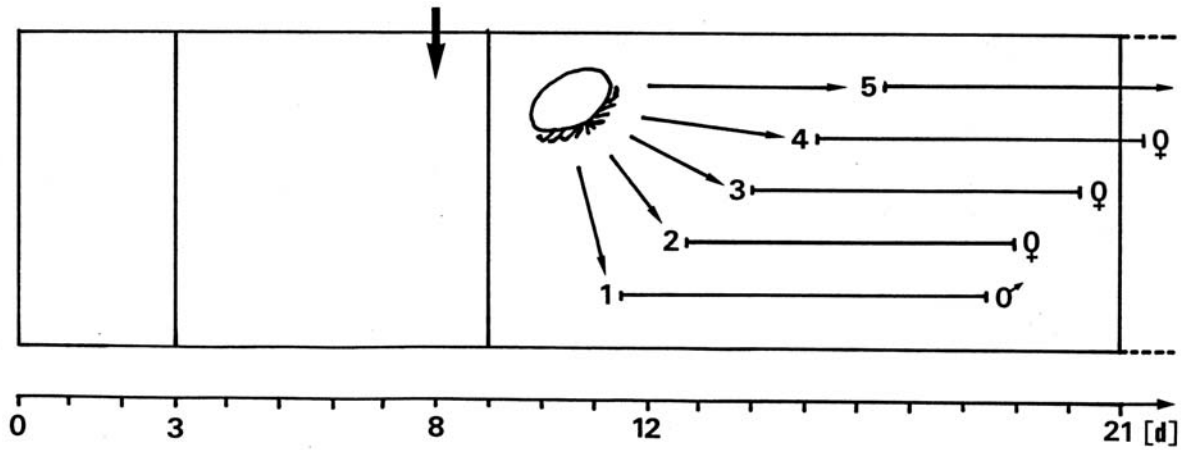
Fig. 2. *Oviposition and development of* Varroa *in brood cells of worker bee* (*until about 9th day unsealed brood, until about 21st day sealed brood*).
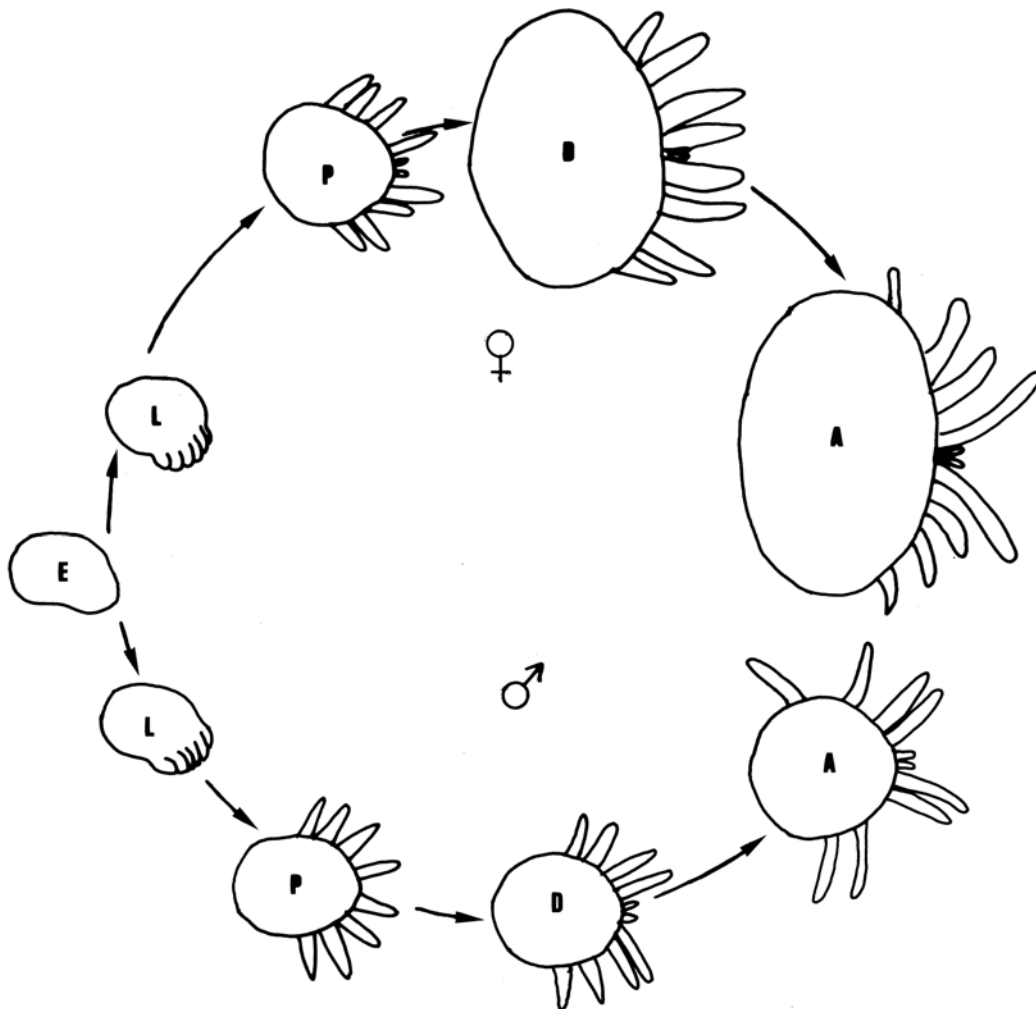

Fig. 3. *Development of Varroa: E = Egg, L = Larva, P = Protonymph, D = Deutonymph, A = Adult* (*Sex of eggs, larvae and protonymphs can only be distinguished by examining the chromosomes*).

The number of mites usually increases slowly at the beginning of the season. Clinical signs may be seen at any time during the active season, although usually maximum numbers are reached late in the season (Figure 4), when the first clinical signs of infestation can be recognised. The course of this parasitism is usually lethal, except in some areas, such as tropical Latin America (6, 12). The life span of mites on larval or adult bees depends on temperature and humidity. Under practical conditions, the life span may vary from some days to a few months.
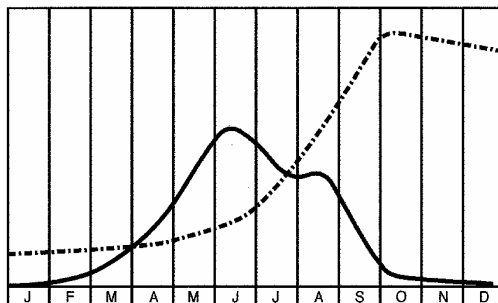
Fig. 4. *Graph of populations of bees and mites over 1 year in a temperate Northern Hemisphere climate: brood numbers (solid line); mite numbers (broken line).*

In heavily infested bee colonies, clinical signs of varroosis can often first be seen in the latter part of the season when the brood is reduced (12). Heavy infestations are usually reached 3–4 years after the primary invasion, but can occur within weeks if infested by bees from nearby colonies that are collapsing.

Essentially, the brood is damaged by the parasitic mites. Bees and their offspring that have been infected during the brood phase by only one parasitic mite show various ill effects, such as a shortened life span, changes in behaviour and an increased disease susceptibility (8). The parasitism is critical if more than one mite enters the brood cell for reproduction. Only in the lethal stage immediately before the collapse of the colonies do clinical signs, such as shrunken wings and shortened abdomen, appear (Figure 5). This is due to an increased susceptibility to deformed wing and acute paralysis virus, as well as to the infection of wounds and loss of haemolymph (3, 4). If the brood dies shortly before or after sealing, clinical signs of European foulbrood appear without the presence of the specific agent *Melissococcus pluton*. If the brood survives, the emerging bees show various behavioural changes and their life span is considerably shortened (7, 11).
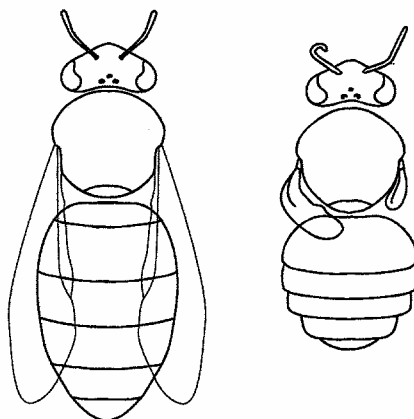
Fig. 5. *Effect of* Varroa *on bee morphology. Left: normal bee appearance. Right: bee heavily attacked by mites. This newly emerged bee has a deformed wing and reduced abdominal volume.*

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The female mite is a dark reddish/brown colour and has a flat, oval-shaped body approximately 1.1 mm × 1.5 mm. It is the only common parasite of honey bees that can be seen with the naked eye (13).

a) Debris examination

An easy method of diagnosis of varroosis is by the examination of the debris generated by bees themselves. An insert covered with a screen mesh is placed on the floor of the hive. Unless this insert is covered with such a gauze, or smeared with grease, the bees will dispose of the mites outside the hive.

The debris produced within a few days in the late season usually contains little other than visible mites (9, 11). The debris collected in winter, however, must be examined in the laboratory. An insert is placed in the hive as before, but an effective medication is used to cause the mites to fall off the bees, so that after a given time, a number of mites may be observed on the floor insert. Some countries demand the diagnostic application of certain medications for proving the absence of mites.

Large amounts of debris can be examined in the laboratory using a flotation procedure (5).

- **Test procedure**

i) Dry the debris for 24 hours.

ii) Flood the debris with industrial alcohol.

iii) Stir continuously for around 1 minute or, if debris contains wax or propolis particles, stir for 10–20 minutes.

iv) Identify and observe the mites that float to the surface.

b) Brood examination

For the second method, drone brood is examined, if available, otherwise worker brood is examined.

When a large number of samples are examined, a rough determination of the degree of infection can be obtained.

- **Test procedure**

i) Remove the cappings of the brood cells with a knife.

ii) Wash the brood cells directly into a sieve system with warm water from a hand-held shower.

iii) Collect the mites in the lower fine sieve (mesh width 1 mm) while the brood is gathered in the upper coarse sieve (mesh width 2–3 mm).

iv) Place the contents of the sieve on a bright plate, where the mites can be easily identified and counted.

When a smaller number of samples are being studied, the individual cells are examined using an appropriate source of light. After removing the cappings and the bee brood, infected cells can be identified by the presence of small white spots – the faeces of the mite – found on the cell wall. The mites themselves should be sought for confirmation, by examining the bottom of the cell and the bee brood for attached mites.

c) Bee examination

In a third method, approximately 200–250 bees are removed from unsealed brood combs. Samples should be taken from both sides of at lest three uncapped brood combs. To determine an apiary's percentage of infestation, it is necessary to collect and analyse individual samples from at least 10% of the beehives, and to determine later the average infestation rate based on these individual results.

- **Test procedure**

i) Kill the bees in a special container by submersion in alcohol.

ii) Stir the container for 10 minutes.

iii) Separate the bees from the mites by means of a sieve with a mesh size of approximately 2–3 mm.

Under some circumstances, the *Varroa* mite may be confused with the bee louse, *Braula coeca* (Figure 6). The latter is round, not oval, and being an insect, has only three pairs of legs. A number of different species of mite may be associated with *Varroa* mites on bees, but these are easily distinguished. In addition, other parasitic mites, such as those of the *Tropilaelaps* spp., are known to cause similar damage to bee colonies as the *Varroa* mites.

2. Serological tests

No serological tests are available for routine laboratory diagnosis.
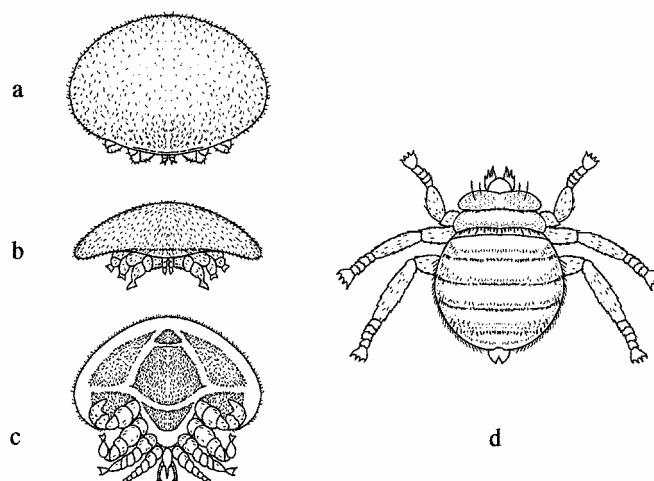
Fig. 6. *Diagram of* Varroa destructor (*formerly* Varroa jacobsoni *Oudemans*) (*female*).
a) Dorsal aspect
b) Anterior aspect   *Note the flat shell-like back and four pairs of legs.*
c) Ventral aspect
d) The bee louse (Braula coeca, *female*). *Note the lack of shell-like back and only three pairs of legs.*

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products and vaccines available. Several medicaments and substances like Formic acid, oxalic acid, lactic acid and thymol can be used to control *Varroa* mites (http://www.apis.admin.ch/english/Themes/Varroa.htm). Some hygienic honeybee strains are less susceptible to *Varroa* parasites.

* **Acknowledgement**

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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

C H A P T E R   2 . 3 . 1 .

AVIAN CHLAMYDIOSIS

SUMMARY

Avian chlamydiosis (AC) is caused by the bacterium Chlamydophila psittaci. *AC occurring in humans and all birds was originally called psittacosis, but later the term ornithosis was introduced to identify the disease contracted from or occurring in domestic and wildfowl, while the name of the disease contracted from or occurring in psittacine birds remained psittacosis. These diseases are similar when contracted by humans. The genus* Chlamydia *was recently divided into two genera,* Chlamydia *and* Chlamydophila. *All known avian strains are now in the species* Chlamydophila psittaci. *Chlamydiosis is still the term used for diseases produced by both genera. The avian strains include at least six serotypes that correlate with the avian species from which they are usually isolated. Chlamydiosis as it occurs naturally in mammalian species and not contracted from avian species is caused by distinctly different strains of the organism.*

Depending on the chlamydial serovar and the avian host, chlamydiae cause pericarditis, conjunctivitis, sinusitis, airsacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis. Generalised infections result in fever, anorexia, lethargy, diarrhoea, and occasionally shock and death. Special laboratory handling is recommended because avian chlamydial strains can cause serious illness and possibly death in humans. The disease in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds. The diagnosis of AC requires the isolation and identification of the organism, the demonstration of chlamydiae in tissues, or the demonstration of a four-fold increase in specific humoral antibody as well as typical clinical signs.

Identification of the agent: *Isolation of chlamydiae requires the inoculation of embryonated eggs or cell cultures and testing for chlamydiae by cytochemical stains or immunohistochemical methods. The direct inoculation of samples into cell cultures is preferable as they are as sensitive for the isolation of most avian strains of chlamydiae as are chicken embryos. The cell cultures are then stained by direct immunofluorescence or by other appropriate stains at appropriate times to demonstrate the presence of inclusions.*

Histochemical staining of impression smears from the liver, heart, and spleen are commonly made. The technique gives a rapid diagnosis, but requires some experience.

Enzyme-linked immunosorbent assays (ELISAs) developed for detecting trachomatis antigen in humans have been used for diagnosing chlamydiae in birds. Many of the earlier tests were developed using monoclonal or polyclonal antisera against lipopolysaccharide epitopes, some of which were shared with other Gram-negative bacteria. Their use when screening individual birds is questionable, as they lack sensitivity and specificity.

Molecular tools (polymerase chain reaction restriction length polymorphism, DNA microarray or sequencing) and immunohistochemical staining of histological sections are new techniques showing promise for the future. All of them are rapid and do not require the live agent. The current PCR tests target the MOMP gene or the ribosomal RNA genes (16S–23S), and some will amplify all chlamydial strains and allow identification at the level of the chlamydial species. Nested and

real-time PCRs can be as sensitive as isolation. There has been an increase in the use of immunohistochemical staining of histological sections because of the recent development and availability of automated staining equipment.

Serological tests: *The standard serological test for chlamydial antibodies is the complement fixation (*CF*) test. The modified direct CF test can be used with most sera. The antigen is a group-reactive lipopolysaccharide antigen present in all strains. The occurrence of high CF titres in the majority of individuals in a flock with clinical signs is presumptive evidence of active infection. The demonstration of a four-fold increase in titre in an individual bird is considered to be diagnostic of a current infection.*

Other serological tests, such as the ELISA, latex agglutination, elementary body agglutination, micro-immunofluorescence, and the agar gel immunodiffusion tests are available. These tests are of value in specific cases and may replace the CF test; however, comparisons of reliability and reproducibility are not yet available.

Requirements for vaccines and diagnostic biologicals: *There are no commercial vaccines available for chlamydiosis control in poultry. Antibiotics are the only current means of control.* Chlamydophila psittaci *is susceptible to a number of antibiotics. The drug of choice varies from country to country.*

A. INTRODUCTION

Avian chlamydiosis (AC) is caused by the bacterium *Chlamydophila psittaci*. The disease in birds was originally called psittacosis, but the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same (5). Their earlier separation was based on the assumption that in humans, ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as that contracted from psittacine birds.

Chlamydophila psittaci produces a systemic and occasionally fatal disease in birds. The clinical signs vary greatly in severity and depend on the species and age of the bird and the strain of chlamydia. AC can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds the most frequent clinical signs are conjunctivitis, anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis, biliverdinuria, nasal discharge, sneezing, lacrimation and respiratory distress (24). Many birds, especially older psittacine birds, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal multifocal hepatic necrosis, spleen and liver enlargement, fibrinous airsacculitis, pericarditis and peritonitis (5, 39, 40). Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present.

The family Chlamydiaceae was recently reclassified into two genera and nine species based on sequence analysis of its 16S and 23S rRNA genes (13). The two new genera, *Chlamydia* and *Chlamydophila*, correlate with the former species *Chlamydia trachomatis* and *C. psittaci*. The genus *Chlamydia* includes *C. trachomatis* (human), *C. suis* (swine), and *C. muridarum* (mouse, hamster). The genus *Chlamydophila* includes *C. psittaci (*avian), *C. felis* (cats), *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea-pigs), and the former species *C. pecorum* (sheep, cattle) and *C. pneumonia* (human).

The two genera and nine species have merit both molecularly and for classification of host range and clinical disease. The species show a high degree of correlation with host range, disease syndrome, and virulence, and thus provide an understanding of the epidemiology of the various species and serovars affecting livestock and birds. The terms 'chlamydiosis' and 'chlamydia(e)' are used as generic terms to refer to members of either and both genera. However, the new scientific names are used when referring to a specific chlamydial species.

The avian strains all belong to the species *Chlamydophila psittaci*. This species includes six known avian serovars and two mammalian serovars, M56 from muskrats and WC from cattle (13). M56 and WC were each isolated from a single outbreak. The six avian serovars are labelled A through F and each shows host specificity. The hosts that each serovar has been associated with are: A, psittacine birds; B, pigeons; C, ducks and geese; D, turkeys; E, pigeons and ratites; and F is a single isolate from a psittacine bird. What is not known is how many of these birds and mammals are the natural hosts of the serovars.

The strains of avian chlamydiae can infect humans and should be handled carefully under conditions of biocontainment (31). Most infections occur through inhalation of infectious aerosols. Post-mortem examinations of infected birds and handling of cultures should be done in laminar flow hoods or with proper protective

equipment. Human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common; auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Diagnosis can be difficult and is usually established through testing paired sera for antibodies to chlamydia by the complement fixation (CF) test. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The preferred method for the identification of AC is the isolation and identification of the organism. Because of the time involved, the need for high quality samples, and the hazard to laboratory personnel, other techniques are often used. These include histochemical staining of smears of exudate and faeces, and impression smears of tissues, immunohistochemical staining of cytological and histological preparations, antigen-capture enzyme-linked immunosorbent assays (ELISA), polymerase chain reaction (PCR), PCR-RFLP (restriction fragment length polymorphism), DNA microarray based detection and identification systems or sequencing.

a) Collection and treatment of samples

The samples to be collected will depend on the disease signs in evidence. They must be taken aseptically. Contaminant bacteria may interfere with the isolation of the chlamydiae. Specimens from acute cases should include inflammatory or fibrinous exudate in or around organs that display lesions, ocular and nasal exudates, impression smears of liver, whole blood and tissue samples from kidney, lung, pericardium, spleen, and liver. In cases with diarrhoea, colon contents or excrement should be cultured. In live birds, the preferred samples are pharyngeal and nasal swabs (2). Intestinal excrement, cloacal swabs, conjunctival scrapings, and peritoneal exudate can also be taken.

Proper handling of clinical samples is necessary to prevent loss of infectivity of chlamydiae during shipping and storage. A special medium consisting of sucrose/phosphate/glutamate (SPG) was developed for rickettsiae and has proven to be satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae (32) consists of SPG buffer: sucrose (74.6 g/litre); KH_2PO_4 (0.512 g/litre); K_2HPO_4 (1.237 g/litre); and L-glutamic acid (0.721 g/litre), which can be sterilised by autoclaving or filtering. Added to this are fetal calf serum (10%), vancomycin, kanamycin, and streptomycin (200–500 µg/ml), amphotericin B and gentamicin (50 µg/ml). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. This medium can also be used as a laboratory diluent and for freezing of chlamydiae.

Contaminated samples must be pre-treated before being used to inoculate animals or cell cultures. There are three basic methods: treatment with antibiotics (7, 8), treatment with antibiotics together with low-speed centrifugation (4, 5), and treatment with antibiotics with filtration (4, 7, 8). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS), pH 7.2, containing a maximum of the following: streptomycin (1 mg/ml), vancomycin (1 mg/ml), and kanamycin (1 mg/ml). Gentamicin (200 µg/ml) can be used. Amphotericin B (50 µg/ml) can be added to control yeast and fungal growth. Other antibiotic solutions are often used. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae.

When contamination is light, samples should be homogenised in an antibiotic solution prior to inoculation into chicken embryos or tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours at 5°C before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 500 *g* for 20 minutes. The surface layer and the bottom layer are discarded. The supernatant fluid is collected and recentrifuged. The final supernatant fluid is used for inoculation. Samples should be passed through a filter of 450–800 µm average pore size if contamination persists.

b) Isolation in cell culture

Cell cultures are the most convenient method for the isolation of *C. psittaci*. Cell lines are satisfactory, the more common ones being buffalo green monkey (BGM), McCoy, HeLa, African green monkey kidney (Vero), and L cells (38). The cells are grown as monolayers using standard tissue culture media containing 5–10% fetal calf serum and antibiotics that are not inhibitory to chlamydia (as described previously).

When selecting cell culture equipment, it is important to remember that:

i)   Chlamydiae can be identified by direct or indirect immunofluorescence or some other appropriate staining technique;

ii)   The inoculum is usually centrifuged on to the monolayer to enhance its infectivity;

iii) The sample may need to be blind passaged at 4–5 days to increase sensitivity of isolation;

iv)   The sample will need to be examined from two to three times during any one passage; and

v)   *Chlamydia* can be infectious to humans.

Small flat-bottomed vials, such as 1-dram (3.7 ml, 15 × 45 mm) shell vials or bottles containing cover-slips that are 12 mm in diameter, will meet these requirements (7, 8). A number of vials, often four to six, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassaging of apparently negative samples 6 days after inoculation. When testing multiple samples, 96-well multiwell dishes can also be used as they have a labour-saving advantage. However, it should be noted that cross-contamination between samples can be a problem.

Chlamydiae can be isolated from cells that are replicating normally, but the use of nonreplicating cells is preferable as these may provide increased nutrients for the growth of chlamydiae. Suppressed cells can also be observed for longer periods. Host cell division can be suppressed either by irradiation or, more commonly, by cytotoxic chemicals. The latter include 5-iodo-2-deoxyuridine, cyto-cholasin B, cycloheximide, and emetine hydrochloride (27). Cycloheximide is the most commonly used and can be added to the medium at the rate of 0.5–2.0 µg/ml at the time of inoculation of the monolayer (4, 5, 7, 8). Emetine is removed after treatment and replaced by medium. The monolayer is first treated for 5 minutes with emetine (0.5 µg/ml), after which the emetine is removed and replaced with culture medium; the monolayer is then ready for use. The growth of most chlamydial strains will be enhanced by the treatment of the monolayer by one of these drugs; the treatment will have little or no effect on the growth of other strains.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 500–1500 *g* for 30–90 minutes at 37°C. The inoculum is removed and replaced with tissue culture medium containing a cell-division inhibitor, and then incubated at 37–39°C. Cultures must be examined for chlamydiae at regular intervals using an appropriate staining method. This is usually done on day 2 or 3, as well as on day 4 or 5. Cultures that appear to be negative at the fifth day are harvested and repassaged. When repassaging chlamydiae, cells and culture media should be passaged without using freeze–thawing to disrupt cells, as this will destroy the chlamydiae.

Before staining the cultures, the medium is first removed, the cultures are washed with PBS and fixed with acetone for 2–10 minutes. The fixation time will depend on the tissue culture vessel used. As acetone will soften most plastics, the use of a mixture of 50% acetone and 50% methyl alcohol may be preferable. A number of staining methods can be employed to demonstrate chlamydial inclusions. The preferred method is direct immunofluorescence (4, 7, 25). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a humid chamber for 30 minutes at 37°C. The cover-slips are then washed three times with PBS, air-dried, mounted, and examined. Chlamydial inclusions fluoresce a green colour. Commercial conjugate preparations using monoclonal antibodies (MAbs) are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titred antisera. Polyclonal antisera can be prepared in rabbits, guinea-pigs, sheep or goats. Sheep and goats are excellent sources because of the volume and high titres that are readily obtained following infection. Conjugates are then prepared using standard techniques (4, 5, 7).

Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (4, 6, 25). Direct staining can be done with Gimenez, Giemsa, Ziehl–Neelsen, or Macchiavello's stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

c)   Isolation in eggs

Chicken embryos are still used for the primary isolation of chlamydiae. The standard procedure is to inject up to 0.5 ml of inoculum into the yolk sac of a specific pathogen free 6–7-day-old embryo (4, 5). The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydia is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer, and can be frozen to preserve the strain, or inoculated into eggs or on to cell cultures.

The organism can be identified by preparing an antigen from an infected yolk sac and testing it by direct staining of smears using appropriate stains or by using the antigen in a serological test. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence

48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic round or hat-shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm.

d) Differentiating among species/strains

All avian isolates are in the *Chlamydophila psittaci* group, as discussed earlier (13). The avian strains can be differentiated from other chlamydiae by PCR-RFLP of either the MOMP gene or the 16S–23S rDNA operon (12). A DNA microarray technology was recently developed in order to differentiate chlamydiae strains (29). A provisional *C. psittaci* determination can be made using the source of the isolate and serovar-specific MAbs.

The avian strains of *C. psittaci* contain at least six serotypes determined by serovar-specific MAbs (1, 3, 6). The syndromes caused by the various strains are quite specific; the natural host range of a particular strain may also be fairly specific. Serotypes are labelled A through F. The hosts from which they are mainly isolated are: serotype A, psittacine birds; serotype B, pigeons; serotype C, ducks; serotype D, turkeys; serotype E, pigeons and ratites; and one isolate of serotype F from a psittacine bird.

Avian strains could be differentiated by molecular tools like PCR-RFLP (3, 30, 37). Serotyping and PCR-RFLP have been compared (33, 35) and sometimes incongruent results have been observed. A new genotype, named E/B, was recently identified; sequencing clearly demonstrated that new genotypes cannot always be discovered by PCR-RFLAP or serotyping.

e) Histochemical staining

Giemsa, Gimenez, Ziehl–Neelsen and Macchiavello's stains are commonly used to detect chlamydiae in impression smears of liver and spleen. The following modified Giminez technique is used by several laboratories (4):

- **Modified Gimenez technique or (Pierce-van der Kamp) stain**

- *Reagents:*

  Solution 1: Distilled H_2O (450.0 ml) and phenol (5.0 ml) added to basic fuchsin (2.5 g) and 95% ethanol (50.0 ml). Incubate at 37°C for 48 hours. Filter and store in the dark at room temperature.

  Solution 2: Na_2HPO_4 (11.65 g); $Na_2HPO_4.H_2O$ (2.47 g); distilled H_2O, pH 7.5 (to 1.0 litre).

  Solution 3: Solution 1 (20.0 ml); and solution 2 (25.0 ml). Let stand for 10 minutes, filter and use.

  Solution 4: 0.5% citric acid.

  Solution 5: Fast green (0.2 g); distilled H_2O (100.0 ml); and glacial acetic acid (0.2 ml).

  Solution 6: Solution 5 (20.0 ml); and distilled H_2O (50.0 ml).

- *Procedure for smears is as follows:*
- i)   Fix in methanol for 5 minutes.
- ii) Stain in Solution 3 for 10 minutes and rinse in tap water.
- iii) Counterstain in Solution 6 for 2 minutes.
- iv) Rinse in tap water and air-dry.

- *Procedure for paraffin sections is as follows:*
- i)   Deparaffinise and hydrate with distilled H_2O.
- ii) Stain in Solution 3 for 10 minutes and rinse in tap water.
- iii) Dip in Solution 4 until no more red runs out of the section. Rinse in tap water.
- iv) Counterstain in Solution 6 for 20 dips.
- v)   Dip in two changes of 95% alcohol, for five dips each. Dehydrate, clear, and mount.

Chlamydiae will appear red against a green background.

f) Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations. The technique is more sensitive than histochemical staining, but some experience is necessary as cross-reactions with some bacteria and fungi require that morphology must be considered.

Most widely used immunohistochemical staining procedures can be adapted to give satisfactory results. The selection of the primary antibody is very important. Both polyclonal and monoclonal antibodies have been used. Because formalin affects chlamydial antigens, it is recommended that polyclonal antibodies be made to purified formalin-inactivated chlamydiae. The chlamydial strain used is not important, as the antibodies will be mainly to the group-reactive antigens. MAbs should also be selected for reactions to formalin-fixed chlamydia. A pool of group-reactive MAbs can be used.

g) **Enzyme-linked immunosorbent assays**

The ELISA is a relatively new technique that has been extensively promoted as kits for use in the diagnosis of human chlamydiosis. These test kits detect the lipopolysaccharide (LPS) antigen (group reactive) and will detect all species of chlamydiae. A number of these kits have been tested for use in detecting chlamydia in birds (41), but none of the kits has been licensed for detection of *C. psittaci*. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the MAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction. Most diagnosticians believe that a diagnosis of AC can be made when a strong positive ELISA reaction is obtained from birds with signs of psittacosis. Because of the number of false-positive results, a positive in an individual bird without signs of disease is not considered to be significant, but indicates the need for further testing using different methods.

h) **Polymerase chain reaction and PCR-based systems**

PCR techniques are replacing isolation for the detection of chlamydiae in animals. The sensitivity and specificity can equal or exceed isolation and the sample can be inactivated prior to testing. Current PCR tests for detection of *C. psittaci* target the MOMP gene or the 16S–23S rRNA gene (14–16, 22, 23, 28, 34, 36). The sensitivity and specificity varies on sample preparation and the PCR test. Reagents designed to stabilise the DNA should be considered when a delay in processing the sample is anticipated (11). DNA samples can be prepared using inexpensive reagents or using commercially available kits (4). Sensitivity is increased by targeting a relatively short DNA segment, using a nested procedure or using real-time PCR techniques. The nested procedure can equal isolation in sensitivity and specificity. However, the risk of contamination is increased if extreme care is not taken when manipulating the reactions (see Chapter 1.1.5 *Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases*). Some excellent nested procedures are available (23, 28, 36). The real-time PCR requires a labelled probe and special equipment, which increases costs. The sensitivity can approach that of the nested system and the contamination problems and labour are reduced as it uses one reaction in a closed system (14–16). Targeting the 16S–23S gene also increases sensitivity as multiple copies are usually present in the organism; however, cross-reactions with other bacteria can be a problem. Sequencing of PCR products will allow comparison with the sequences of reference avian chlamydia isolates and the sequence can be used in phylogenic analysis for classification and epidemiological purposes.

DNA microarray technology was recently developed in order to detect chlamydiae (29). The assay has also proved suitable for unambiguous species identification of Chlamydia in cell cultures and has demonstrated a potential for direct detection of these bacteria from clinical tissues.

2. Serological tests

a) **Modified direct complement fixation test for *Chlamydia***

The following is a widely used modified direct CF test for the detection of antibody. The reagents are relatively easy to prepare and standardise. There are other CF tests; each has advantages. The modified direct CF test is performed in 96-well round-bottom multiwell dishes. Incubation steps are usually done by floating the plates in a 37°C water bath. The chlamydial antigen can be prepared from either infected yolk sacs or cell culture preparations. The modified direct CF test differs from the direct CF test in that normal, unheated chicken serum from chickens without chlamydial antibody is added to the complement dilution. The normal serum increases the sensitivity of the CF procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea-pig complement.

• **Test procedure**

i) *Dilution of sera*

Figure 1 gives a suggested pattern for performing the test in round-bottom, 96-well multiwell dishes. All sera must be heat-inactivated at 60°C for 30 minutes prior to use. The sera are diluted in Veronal (barbiturate) buffer saline (VBS) as shown in Figure 1. The dilutions are made in the multiwell dish by adding 100 µl of VBS to each well of rows A and E, and then adding 25 µl of the undiluted sera, positive serum, or negative serum to each of three wells. This gives a starting dilution of 1/5. Then, 25 µl of VBS is added to each well in row B through to D and row F through to H. Twofold dilutions are

made, using a 25 µl micropipette, from row A through to D and row E through to H. Appropriate volumes are discarded from the starting and finishing rows to give 25 µl per well. Diluters are rinsed twice in distilled H_2O and once in VBS between each serum.

Fig. 1. Suggested test pattern for the modified direct complement fixation test when using 96-well dishes.

ii) *Addition of antigen*

To each well in columns 1, 4, 7, and 10, add 25 µl of positive chlamydial antigen. In columns 2, 5, 8, and 11, add 25 µl of VBS (anticomplementary control wells), and in columns 3, 6, 9, and 12, add 25 µl of negative antigen (normal yolk sac or cell culture prepared the same as the chlamydial antigen). The chlamydial antigens are stored undiluted at 4°C and diluted to proper concentration in VBS prior to use.

iii) *Addition of complement*

Complement (C') is stored at –70°C and should be thawed and diluted in VBS prior to the addition of the antigen. Fresh chicken serum is added before diluting the C' to give a 5% concentration in the complement. Dilutions of C' are made as in previous tests or from titrations. C' should be allowed to stand in an ice bath to stabilise for 15 minutes. The diluted C' should be stored at 4°C following stabilisation and should be used within 2 hours: 50 µl of the C' is added to each well immediately following the addition of the antigens. The plates are incubated uncovered in a 37°C water bath for 2 hours.

iv) *Addition of sheep red blood cells*

Mix 4% standardised sheep red blood cells (SRBCs) with an equal volume of VBS. To this add an equal volume of haemolysin diluted in VBS. The final dilution is incubated in a 37°C water bath for 15 minutes to sensitise the SRBCs. To each well add 50 µl of sensitised SRBCs. The plates are then incubated for 1 hour in a 37°C water bath. The plates can be centrifuged at 400 *g* for 5 minutes before reading or they can be refrigerated at 4°C overnight prior to reading.

v) *Interpretation of the results*

The wells are often scored 1+, 2+, 3+, or 4+ corresponding to reduction of haemolysis of 25, 50, 75, or 100%. A positive reaction is 2+ or higher, which is equivalent to 50% or less lysis of the SRBCs. This indicates that the C' was fixed by antibody prior to the addition of the SRBCs. Negative wells are indicated by the complete lysis of the cells: the C' remains unbound and reacts with the SRBCs and the haemolysin to produce lysis of the SRBCs.

Invalid tests occur when the serum is anticomplementary and a positive reaction occurs in the dilution with VBS as the antigen. Nonspecific serum reactions give positive reactions in both the positive and negative wells.

- **Reagents**

i) *Antigen preparation*

The simplest methods start with the growth of chlamydiae in cell culture. The two methods described below produce antigens that can be used in the micro-CF test. The procedures are quite similar: both include the growth of chlamydiae in cell culture, the inactivation of the chlamydiae, partial purification of the antigen, mechanical disruption, and dilution into the appropriate buffer. The method selected will depend on the equipment available.

The first procedure (17, 19) starts with the chlamydiae and cell culture debris harvested when cytopathic effects are noted. The culture is inactivated by the addition of phenol to a final concentration of 1.0%, incubated for 24 hours at 37°C, and concentrated by centrifugation at 10,000 ***g*** for 1 hour. The sediment is reconstituted to 10% of the original volume using VBS, pH 7.2, containing 1.0% phenol and 1.0% glycerol.

The sediment is then homogenised in an omnimixer at top speed for three 1-minute periods while cooled in ice water. The homogenate is centrifuged for 15 minutes at 100 ***g*** to remove debris. Some procedures suggest heating the antigen for 30 minutes in a boiling water bath at this time. The supernatant is saved and diluted to the desired concentration.

In the second procedure for the production of antigen for the CF test (9, 10), antigen is prepared from L cells infected with a psittacine strain. The cell culture medium is discarded, and the cells are heated for 40 minutes at 56°C. The cells are lysed in distilled water, the chlamydiae are disrupted by ultrasonication, and then made isotonic in VBS. The antigen is tested against a standard sheep convalescent serum and used at 2 units in the micro-CF test.

There are a number of procedures for preparing the antigen from infected yolk sacs, some of which are quite elaborate. However, with the following procedure it is relatively easy to prepare a crude infected yolk sac antigen that works well in the modified direct CF test. An egg-adapted strain of chlamydia is used to inoculate 6–7-day-old embryonated chicken eggs via the yolk sac. The yolk sacs are harvested from embryos that die between 3 and 7 days post-inoculation. The yolk-sac harvest is diluted 1/3 in PBS, Tris buffer, or cell culture medium, and then autoclaved for 20 minutes. The suspension is cooled and then homogenised thoroughly. The use of a high-speed tissue homogeniser for 3–5 minutes is recommended. After homogenisation, phenol is added to make a final concentration of 0.5% phenol (prepare a 5% phenol stock solution and add 1 ml for every 9 ml of antigen). The antigen preparation is prepared, held for 3 days, and then the supernatant is used after centrifugation for 20 minutes at 1000 ***g***. The antigen can be stored for long periods of time at 4°C.

ii) *Preparation of sensitised SRBCs*

Defibrinated SRBCs are preserved by mixing in an equal volume of Alsever's solution. These can be stored at 4°C for up to 4 weeks. Wash 25 ml of the stock SRBCs with 25 ml of VBS. Centrifuge at 400 ***g*** for 10 minutes. Aspirate off the VBS and resuspend in 50 ml of VBS. Repeat the wash a total of three times. Following the final wash, dilute the SRBCs at a ratio of 2.2 ml of packed SRBCs to 98 ml of VBS. The SRBCs can then be standardised by optical density: mix 1 ml of the diluted, washed SRBCs with 14 ml distilled H_2O, determine the absorbance using a spectrophotometer, and standardise to 0.25 at a wave length of 550 mm. The reading obtained can be used in the following formula to determine the dilution needed:

$$\text{Final volume of SRBCs} = \frac{(\text{absorbance reading x current volume})}{0.25 \text{ desired absorbance}}$$

The SRBCs are sensitised by rapidly adding an equal volume of VBS containing the appropriate dilution of haemolysin (dilution determined by titration). Incubate at 37°C for 15 minutes prior to use.

iii) *Veronal buffer saline*

VBS is prepared as a 5 × stock solution and diluted 1/5 with distilled H_2O prior to use. The following formula makes 4 litres. To distilled water add sodium barbital (7.5 g); barbital H_2O (dissolve in boiling H_2O) (11.5 g); $MgSO_4.7 H_2O$ (4.056 g); NaCl (170.0 g); and $CaCl_2$ (0.078 g). Add distilled H_2O to make to 4 litres.

iv) *Complement titration*

Complement (C') is unstable and will deteriorate if improperly handled. Normally it should be kept frozen at –70°C in aliquots that are used at one time to eliminate refreezing. To obtain the desired working concentration (2 units per test well) first add 5% normal chicken serum for the modification to

enhance sensitivity as described earlier. Then estimate a starting point based on previous lots. A good starting point is a dilution of 1/30 after the chicken serum has been added. Set up a series of tubes with various amounts of complement in VBS. The VBS should contain the antigen to be used in the reaction and take into account any anticomplementary properties of the antigen. A common method is to dilute 0.10 ml C' + 0.90 ml VBS; 0.12 ml complement + 0.88 ml VBS, etc. through 0.25 ml C' + 0.75 ml VBS. Incubate the tubes for 2 hours in a 37°C water bath. Add 0.5 ml of sensitised SRBCs to each tube. Incubate for 1 additional hour in the 37°C water bath. The highest dilution giving complete haemolysis equals 1 unit. Twice that amount equals 2 units. The following formula can be used to obtain 2 units/0.05 ml:

$$x = (di)\ (v)/2dh$$

where:

> x = reciprocal of C' dilution desired to yield 2 units C'/well
> di = reciprocal of C' initial dilution used in titration (1/30)
> v = volume of diluted C' to be added
> dh = twice the volume of C' giving complete haemolysis in titration

v) *Titration of haemolysin*

Haemolysin can be obtained from commercial sources. It must be standardised by titration. The following procedure is recommended:

Prepare a 1/100 dilution of the stock haemolysin in VBS. From this, prepare 1/300, 1/400, and 1/500 dilutions in tubes. From each of these dilutions, make 0.5 ml of twofold dilutions in VBS for a block titration.

To determine haemolysin concentration, add the following to 0.5 ml of each dilution: 0.5 ml of C' at 1/30 dilution, 0.5 ml of unsensitised SRBCs at 0.25 optical density, and 1.5 ml of VBS. Incubate for 1 hour at 37°C, and then centrifuge at 400 **g** for 5 minutes. One unit of the haemolysin is the dilution that gives complete lysis of the SRBCs. The haemolysin solution is prepared in VBS at the dilution containing 2 units of haemolysin. This is then added to an equal volume of SRBCs at the proper concentration.

vi) *Titration of antigen and positive control serum*

In order to standardise the CF test, it is also necessary to have titres of both the antigen and the positive control serum. If the titre is known for either the positive serum or antigen, the titre of the other component can be determined by performing the CF test using dilutions of the component being titred. If titres of both the positive serum and antigen are unknown, a block titration (chequerboard) can be used to determine the limiting dilutions of both the antigen and the antibody where haemolysis starts. It is very critical to obtain these titres accurately.

For both the antigen and the positive control serum, 4 units are used. A unit is the highest dilution that will give a positive test. That is, if a dilution of 1/160 gives a positive test, then a 1/40 dilution has 4 units and is used for the test.

Complement-fixing antibodies usually appear within 7–10 days of infection. For a positive diagnosis, a four-fold rise in CF antibody titre is required. A presumptive diagnosis by serological tests on a flock can only be made if typical clinical signs are present and a majority of the birds have antibody titres of >1/64.

b) Other tests

Other serological tests have been developed, but their specificity has not yet been sufficiently evaluated. The ELISA for group-specific chlamydial antibodies is more rapid and sensitive than the CF test; it can be automated. Evaluations of ELISAs for the detection of antibodies to both *C. trachomatis* and *C. psittaci* indicate that the tests are highly sensitive but lack specificity. New tests are being developed that use peptides or recombinant antigens which may correct the specificity problem.

Other tests include the agar gel immunodiffusion test (26), the latex agglutination (LA) test, the elementary body agglutination (EBA) test (18, 21) and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CF test. The LA test will detect antibodies to *C. psittaci*, and is easy and rapid to perform (20). Latex beads are coated with purified chlamydial antigen, mixed thoroughly with the test serum on a glass plate, and rotated for 2 minutes to enhance agglutination. The test is read against a dark background. Sera giving positive reactions should be retested with uncoated beads to eliminate possible nonspecific agglutination. The LA and direct CF tests correlate in 72.5% of tests with paired sera. The LA

test has a sensitivity of 39.1% and a specificity of 98.8% relative to the direct CF test (20). The test detects both IgM and IgG, but it is best at detecting IgM. It has been suggested for use in detecting recent or active infections. The EBA test detects only IgM, and it is indicative of a current infection. The MIFT is rapid and easy to perform; however, fluorescence-conjugated anti-species sera are not always available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no commercial vaccines available for chlamydiosis in poultry. Attempts to produce a vaccine have met with limited success, and most have been based on bacterins produced by formalin inactivation of concentrated suspensions of chlamydiae. There is evidence that immunity involves cell-mediated immune responses (30, 31), but vaccine manufacture has not been directed towards reactions of this type.

Antibiotics are the only current means of control. *Chlamydophila psittaci* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlortetracycline, doxycycline, and other tetracyclines are the most commonly used. Treatment needs to be maintained for extended periods of time. For pet birds, 45 days is often recommended (31, 39).

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NB: There is an OIE Reference Laboratory for avian chlamydiosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.3.2.

AVIAN INFECTIOUS BRONCHITIS

SUMMARY

Avian infectious bronchitis (IB) is caused by coronavirus infectious bronchitis virus (IBV). The virus causes infections mainly in chickens and is a significant pathogen of commercial meat and egg type birds. IB is an acute, contagious disease characterised primarily by respiratory signs in growing chickens. In hens, decreased egg production and quality are often observed. Some strains of the virus are nephropathogenic and produce interstitial nephritis and mortality. The severity of IBV-induced respiratory disease is enhanced by the presence of bacterial pathogens leading to chronic complicated airsacculitis. Diagnosis of IB requires virus isolation or demonstration of viral nucleic acid from diseased flocks. Demonstration of an ascending serum antibody response may also be useful. The widespread use of live and inactivated vaccines may complicate both the interpretation of virus isolation and serology findings. The occurrence of antigenic variant strains may overcome immunity induced by vaccination.

Diagnosis requires laboratory testing. Virus isolation and identification is preferred. Reverse-transcription polymerase chain reaction (RT-PCR) techniques are commonly used to identify the IBV genotype. Haemagglutination inhibition (HI) tests to determine serotype and enzyme-linked immunosorbent assays (ELISA) for general monitoring are often used for sero-diagnosis. Supplementary tests include electron microscopy, the use of monoclonal antibodies, virus neutralisation (VN), immunohistochemical or immunofluorescence tests, and immunisation-challenge trials in chickens.

Identification of the agent: *For the common respiratory form, IBV is most successfully isolated from tracheal mucosa and lung several days to one week following infection. For other forms of IB, kidney, oviduct, the caecal tonsils of the intestinal tract or proventriculus tissues are better sources of virus depending on the pathogenesis of the disease.*

Specific pathogen free chicken embryos or tracheal organ cultures (TOCs) from embryos may be used for virus isolation. Following inoculation of the allantoic cavity, IBV produces embryo stunting, curling, clubbing of the down, or urate deposits in the mesonephros of the kidney, usually within three serial passages. Isolation in TOCs has the advantage that IBV produces stasis of the tracheal cilia on initial inoculation. RT-PCR is increasingly being used to identify the spike (S) glycoprotein genotype of IBV field strains. Genotyping using primers specific for the S1 subunit of the S gene or sequencing of the same gene generally provides similar but not always identical findings to HI or VN serotyping. Alternatively, VN or HI tests using specific antiserum may be used to identify the serotype.

Serological tests: *Commercial ELISA kits may be used for monitoring serum antibody responses. The antigens used in the kits are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges. The HI test is used for identifying serotype-specific responses to vaccination and field challenges especially in young growing chickens. Because of multiple infections and vaccinations, the sera of breeders and layers contain cross-reactive antibodies and the results of HI testing cannot be used with a high degree of confidence.*

Requirements for vaccines and diagnostic biologicals: *Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines, attenuated by serial passage in chicken embryos or by thermal heat treatment, confer better local immunity of the respiratory tract than inactivated vaccines. The use of live vaccines carries a risk of residual pathogenicity associated with vaccine*

back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines.

Inactivated vaccines are injected and a single inoculation does not confer protection unless preceded by one or more live IBV priming vaccinations. Both types of vaccines are available in combination with Newcastle disease vaccine; in some countries inactivated multivalent vaccines are available that include Newcastle disease, infectious bursal disease, reovirus and egg-drop syndrome 76 viral antigens.

A. INTRODUCTION

Avian infectious bronchitis (IB) was first described in the United States of America (USA) in the 1930s as an acute respiratory disease mainly of young chickens. A viral aetiology was established, and the agent was termed avian infectious bronchitis virus (IBV). The virus is a member of the genus *Coronavirus*, family Coronaviridae, in the order Nidovirales. IBV and other avian coronaviruses of turkeys and pheasants are classified as group 3 coronaviruses, with mammalian coronaviruses comprising groups 1, 2 and 4 (group 4 being the more recently identified severe acute respiratory syndrome (SARS) coronavirus) (6). Coronaviruses have a nonsegmented, positive-sense, single-stranded RNA genome.

IB affects chickens of all ages, which, apart from pheasants (10) are the only species reported to be naturally affected. The disease is transmitted by the air-borne route, direct chicken to-chicken contact and indirectly through mechanical spread (contaminated poultry equipment or egg-packing materials, manure used as fertiliser, farm visits, etc.). IB occurs world-wide and assumes a variety of clinical forms, the principal one being respiratory disease that develops after infection of the respiratory tract tissues following inhalation or ingestion. Infection of the oviduct can lead to permanent damage in immature birds and, in hens, can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IB can be nephropathogenic causing acute nephritis, urolithiasis and mortality (11). After apparent recovery, chronic nephritis can produce death at a later time. IBV has also been reported to produce disease of the proventriculus (49). Vaccine and field strains of IBV may persist in the caecal tonsils of the intestinal tract and be excreted in faeces for weeks or longer in clinically normal chickens (2). For an in-depth review of IB, refer to Cavanagh & Naqi (11). A detailed discussion of IBV antigen, genome and antibody detection assays prepared by De Wit (24) is also available.

There have been no reports of human infection with IBV.

B. DIAGNOSTIC TECHNIQUES

Confirmation of diagnosis is based on virus isolation, often assisted by serology. Extensive use is made of live and inactivated vaccinations, which may complicate diagnosis by serological methods as antibodies to vaccination and field infections can not always be distinguished. Persistence of live vaccines may also confuse attempts at recovering the causative field strain.

1. Identification of the agent

a) Sampling

Samples appropriate to the form of IB observed must be obtained as soon as signs of clinical disease are evident. Samples must be placed in cold transport media and be frozen as soon as possible. The cold chain from bird to laboratory should be maintained. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from diseased birds should be harvested, placed in transport medium containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml) and kept on ice and then frozen. For birds with nephritis or egg-production problems, samples from the kidneys or oviduct, respectively, should be collected in addition to respiratory specimens. In some cases, IBV identification by reverse-transcription polymerase chain reaction (RT-PCR) may be desirable without virus isolation. In this case, swabbings from the respiratory tract or cloaca may also be submitted alone, without being placed in liquid transport media (8). In situations where IB-induced nephritis is suspected, kidney samples should also be selected from fresh carcases for histopathological examination as well as virus isolation. Blood samples from acutely affected birds as well as convalescent chickens should be submitted for serological testing. A high rate of virus recovery has been reported from the caecal tonsil or faeces (2). However, isolates from the intestinal tract may have no relevance to the latest infection or clinical disease. IBV isolation may be facilitated using sentinel specific pathogen free (SPF) chickens placed at one or more times in contact with commercial poultry (25).

b) Culture

Suspensions of tissues (10–20% w/v) are prepared in sterile phosphate buffered saline (PBS) or nutrient broth for egg inoculation, or in tissue culture medium for tracheal organ culture (TOC) inoculation (17). The suspensions are clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 µ) before inoculation of eggs or TOCs.

Embryonated chicken eggs and TOCs are used for primary isolation of IBV. Cell cultures are not recommended for primary isolation as it is often necessary to adapt IBV isolates to growth in chicken embryos before cytopathic effect (CPE) is produced in chick embryo kidney cells.

Embryonated eggs used for virus isolation should originate preferably from SPF chickens or from breeder sources that have been neither infected nor vaccinated with IBV. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Eggs are candled daily for 7 days with mortality within the first 24 hours being considered nonspecific. The initial inoculation usually has limited macroscopic effects on the embryo unless the strain is derived from a vaccine and is already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and further passaged into another set of eggs for up to a total of three to four passages. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros on the second to fourth passage. Embryo mortality in later passages may occur as the strain becomes more egg adapted. Other viruses, notably adenoviruses that are common to the respiratory tract, also produce embryo lesions indistinguishable from IBV. The IBV-laden allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by serotyping or genotyping. Infective allantoic fluids are kept at –20°C or below for short-term storage, –60°C for long-term storage or at 4°C after lyophilisation.

TOCs prepared from 20-day-old embryos can be used to isolate IBV directly from field material (17). An automatic tissue-chopper is desirable for the large-scale production of suitable transverse sections or rings of the trachea for this technique (21). The rings are about 0.5–1.0 mm thick, and are maintained in a medium consisting of Eagle's N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/hour) at 37°C. Infection of tracheal organ cultures usually produces ciliostasis within 24–48 hours. Ciliostasis may be produced by other viruses and suspect IBV cases must be confirmed by serotyping or genotyping methods.

c) Methods for identification

The initial tests performed on IBV isolates are directed at eliminating other viruses from diagnostic consideration. Chorioallantoic membranes from infected eggs are collected, homogenised, and tested for avian adenovirus group 1 by immunodiffusion or PCR. Group 1 avian adenovirus infections of commercial chickens are common, and the virus often produces stunted embryos indistinguishable from IBV-infected embryos. Furthermore, harvested allantoic fluids do not hemagglutinate (HA) chick red blood cells. Genetic based tests (RT-PCR or RT-PCR-RFLP [restriction fragment length polymorphism]) are used commonly to identify an isolate as IBV. Other techniques may be used, for example cells present in the allantoic fluid of infected eggs may be tested for IBV antigen using fluorescent antibody tests (12) and direct negative-contrast electron microscopy will reveal particles with typical coronavirus morphology in allantoic fluid or TOC fluid concentrates. The presence of IBV in infective allantoic fluid may be detected by RT-PCR amplification and use of a DNA probe in a dot-hybridisation assay (32). Direct immunofluorescence staining of infected TOCs for the rapid detection of the presence of IBV has been described (3). Immuno-histochemistry, with a group-specific monoclonal antibody (MAb), can be used to identify IBV in infected chorioallantoic membranes (43).

d) Serotype identification

Antigenic variation among IBV strains is common (11, 16, 23, 28, 31), but at present there is no agreed definitive classification system. Nevertheless, antigenic relationships and differences among strains are important, as vaccines based on one particular serotype may show little or no protection against viruses of a different antigenic group. As a result of the regular emergence of antigenic variants, the viruses, and hence the disease situation and vaccines used, may be quite different in different geographical locations. Ongoing assessment of the viruses present in the field is necessary to produce vaccines that will be efficacious in the face of antigenic variants that arise. Serotyping of IBV isolates and strains has been done using haemagglutination inhibition (HI) (1, 36) and virus neutralisation (VN) tests in chick embryos (23), TOCs (22) and cell cultures (29). Neutralisation of fluorescent foci has also been applied to strain differentiation (19).

MAbs, usually employed in enzyme-linked immunosorbent assays (ELISA), have proven useful in grouping and differentiating strains of IBV (30, 38). The limitations of MAb analysis for IB serotype definition are the lack of availability of MAbs or hybridomas and the need to produce new MAbs with appropriate specificity to keep pace with the ever-growing number of emerging IB-variant serotypes (34).

e) Genotype identification

RT-PCR genotyping methods have largely replaced HI and VN serotyping for determining the identity of a field strain. The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the S1 subunit of the S protein (5, 40) where most of the epitopes to which neutralising antibodies bind are found (39). An exact correlation with HI or VN results has not been seen, in that while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit (40), other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences (5). However, there is, in general, good agreement between data represented by the S1 sequence and the VN serotype, and it may eventually be possible to select vaccine strains on the basis of sequence data.

The primary advantages of genotyping methods are a rapid turnaround time, and the ability to detect a variety of genotypes, depending on the tests used. RFLP RT-PCR differentiates IBV serotypes based on unique electrophoresis banding patterns of restriction enzyme-digested fragments of S1 following amplification of the gene by RT-PCR (33, 41). The RFLP RT-PCR procedure may be used in conjunction with a biotin-labelled DNA probe to first detect IBV in egg fluids harvested following the inoculation of eggs with clinical samples (32). The RFLP RT-PCR test can identify all known serotypes of IBV as well as variant viruses.

S1 genotype-specific RT PCR may be used to identify specific IBV serotypes (35). S1 gene primers specific for serotypes Massachusetts (Mass), Connecticut, Arkansas, and JMK are used in conjunction with a universal primer set that amplifies all IBV serotypes. Primers for the DE/072/92 and California serotypes have also been developed. Other variant serotypes may be determined to be IBV using the general primers, but the specific serotype cannot be identified. Infections caused by multiple IBV serotypes may be identified.

Nucleotide sequencing of a diagnostically relevant fragment of the S1 gene is the most useful technique for the differentiation of IBV strains and has become the genotyping method of choice in many laboratories. Nucleotide sequencing has also produced evidence that recombination between IB strains occurs often (7, 50). RT-PCR product cycle sequencing of the hypervariable amino terminus region of S1 may be used diagnostically to identify previously recognised field isolates and variants (37). Comparison and analysis of sequences of unknown field isolates and variants with reference strains for establishing potential relatedness are significant advantages of sequencing.

Recently, it has been shown that coronaviruses isolated from turkeys and pheasants are genetically similar to IBV, having approximately 90% nucleotide identity in the highly conserved region II of the 3' untranslated region (UTR) of the IBV genome (9, 10). The potential role of these coronaviruses in IBV infections has not been determined.

The major uses of RT-PCR tests are virus identification and its application in the understanding of epidemiological investigations during IBV outbreaks. The RT-PCR tests, as they now exist however, do not provide information on viral pathogenicity.

- **RT-PCR test procedure**

i) *Extraction of viral RNA*

Any RNA extraction method can be used. There are many protocols available in journals, books and on the web. However, for extracting high quality RNA from allantoic fluid, the Qiagen Viral RNA Mini Kit (www.qiagen.com) is recommended. The Qiagen RNeasy Mini Kit is recommended for extracted IBV RNA from tissue or swabbings. All extracted RNA should be stored between –20°C and –80°C until tested. It is advised that for long-term storage, RNA be kept at –80°C.

ii) *Custom oligos*

Custom oligos can be purchased through any commercial supplier. Operon (www.operon.com) has been making quality custom oligos for years. The target gene for IBV characterisation is the S1 subunit of the spike glycoprotein gene. A commonly used primer pair for amplification of genotypically diverse IBV strains is oligo S15' mod (forward): 5'-TGA-AAA-CTG-AAC-AAA-AGA-3' and CK2 (reverse): 5'-CNG-TRT-TRT-AYT-GRC-A-3' (26). The oligo S15'mod/CK2 amplicon is approximately 700 bp in length beginning from the start of the S1 gene spanning two hypervariable regions used for genotyping.

iii) *Reverse-transcription polymerase chain reaction*

Many one and two-step RT-PCR kits are commercially available from manufacturers claiming superior enzyme sensitivity and fidelity. The recommended RT-PCR kit is the basic, two-step, RNA PCR kit from Applied Biosystems (http://www.appliedbiosystems.com). Reverse transcription is performed

according to the manufacturer's instructions. RT priming is accomplished with the use of random hexamers (supplied with the kit) or with the reverse PCR primer, in this case CK2 (35). One cycle of RT is performed with the following parameters: 25°C for 10 minutes, 42°C for 25 minutes, 95°C for 5 minutes, hold at 4°C. The full RT reaction volume is added to the PCR sample master mix. PCR is performed using the following parameters: 95°C for 2 minutes, 45 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 68°C for 30 seconds, final extension of 68°C for 12 minutes, hold at 4°C. Samples are concentrated in a desiccator overnight or by the use of a vacuum centrifuge. Dried samples are resuspended in 12 µl of DEPC-treated water and 6 µl of loading buffer prior to electrophoresis on a 1.8% agarose gel containing ethidium bromide. Gels are visualised with a UV light box. Bands are compared to a commercially available 100 bp ladder and an IBV positive control.

iv) *S1 gene sequencing*

Bands visualised in the agarose gel that are of similar size to the positive control are excised from the gel. The PCR product is separated from the agarose gel using the Qiagen Gel Extraction kit (www.qiagen.com) or any other commercial gel extraction kit. Purified PCR products are run on a second 1.8% agarose-ethidium bromide gel to determine the quantity of product present. Approximately 20 µl (10 ng/µl) of PCR product is required for sequencing. Sequencing can be performed at the University of Delaware Sequencing & Genotyping Center, Newark, DE (http://www.udel.edu/dnasequence) or another university or commercial sequencing facility. Sequence chromatagrams are edited using the DNAStar analysis software or on-line freeware 4peaks (http://www.mekentosj.com/4peaks/)
or chromas lite(http://www.technelysium.com.au/chromas_lite.html).
Edited sequences of IBV isolates are characterised using BLASTn for nucleotide or BLASTp for protein analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

2. Serological tests

A number of tests have been described. Those considered here include VN (23), agar gel immunodiffusion (AGID) (48), HI (1) and ELISA (42). Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, for routine serological testing, the VN tests are too expensive and impractical, and AGID tests lack sensitivity. ELISA and HI tests are most suitable for routine serology. ELISAs are useful for general monitoring of IBV exposure and can detect antibody responses to all serotypes. HI when used on sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine or field challenge responses. Because chicken sera from older birds contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB cannot be used with a high degree of confidence.

a) Virus neutralisation

In VN tests, all sera should first be heated to 56°C for 30 minutes. Virus is mixed with serum and incubated for 30–60 minutes at 37°C or room temperature. Chicken embryos are most often employed, but antibodies can be measured using TOC or cell culture systems. Two methods have been used to estimate neutralising antibodies. One employs a constant serum concentration reacted with varying dilutions of virus (the alpha method) and the other employs a constant amount of virus and varying dilutions of serum (the beta method).

In the alpha method, tenfold dilutions of egg-adapted virus are reacted with a fixed dilution (usually 1/5) of antiserum, and the mixtures are inoculated into groups of from five to ten eggs. The virus alone is titrated in parallel. End-points are calculated by the Kärber or the Reed and Muench methods. The results are expressed as a neutralisation index (NI) that represents the $\log_{10}$ difference in the titres of the virus alone and that of the virus/antiserum mixtures. The NI values may reach 4.5–7.0 in the case of homologous virus/serum mixtures; values of <1.5 are not specific, but a heterologous virus will give a value as low as 1.5.

The beta method is the more widely used neutralisation test for antibody assay with chicken embryos. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID_{50} (median embryo-infective doses) per 0.05 ml and 0.1 ml of each mixture inoculated into the allantoic cavity of each of from five to ten embryonated eggs. A control titration of the virus is performed simultaneously to confirm that the fixed virus dilution in the virus/serum mixtures was between $10^{1.5}$ and $10^{2.5}$ EID_{50}. End-points of the serum titres are determined by the Kärber or Reed and Muench method as before, but here are expressed as reciprocals of $\log_2$ dilutions. This fixed-virus/varying-serum method is also employed for neutralisation tests in tracheal organ cultures using five tubes per serum dilution, as is conventional with other viruses (22). The results are calculated according to Reed and Muench, and the virus titres are expressed as median ciliostatic doses per unit volume ($\log_{10} CD_{50}$). Serum titres are again expressed as $\log_2$ dilution reciprocals. This test is more sensitive than others, but technical logistics hamper its more widespread adoption.

b) Haemagglutination inhibition

A standard protocol for a HI test for IBV has been described (1), and the test procedure detailed below is based on that standard. Strains and isolates of IBV will agglutinate chicken red blood cells (RBCs) after neuraminidase treatment (44, 45). The strain selected to produce antigen may be varied, depending on the requirements of diagnosis. The antigen for the HI test is prepared from IBV-laden allantoic fluids.

For HA and HI tests, procedures are carried out at 4°C.

- **Haemagglutination test**

i)   Dispense 0.025 ml of PBS, pH 7.0–7.4, into each well of a plastic U or V-bottom microtitre plate.

ii) Place 0.025 ml of virus antigen in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.

iii) Make twofold dilutions of 0.025 ml volumes of the virus antigen across the plate.

iv) Dispense a further 0.025 ml of PBS into each well.

v)   Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and allow the RBCs to settle for about 40 minutes at 4°C, when control RBCs should be settled to a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA in which there is no streaming; this is 100% HA and represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination-inhibition test**

The HI test is used in the diagnosis and routine flock monitoring of vaccine responses.

i)   Dispense 0.025 ml of PBS into each well of a plastic U or V-bottom microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus antigen in 0.025 ml to each well and leave for 30 minutes.

v)   Add 0.025 ml of 1% (v/v) chicken RBCs to each well and, after gentle mixing, allow the RBCs to settle for about 40 minutes when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBCs 'stream' at the same rate as the control wells (containing 0.025 ml RBC and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre >2^2, and a positive control serum, for which the titre should be within one dilution of the known titre.

viii) Sera are usually regarded as positive if they have a titre of 2^4 or more. However, it should be noted that even in SPF flocks, a very small proportion of birds may show a nonspecific titre of 2^4, but usually in birds over 1 year of age.

c) Enzyme-linked immunosorbent assay

The ELISA technique is a sensitive serological method and gives earlier reactions and higher antibody titres than other tests (42). It lacks type or strain specificity, but is valuable for monitoring vaccination responses under field conditions. Commercial kits for ELISAs are available – these are based on several different strategies for the detection of IBV antibodies. Usually, such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The ELISA is widely used to identify IBV-infected flocks (broilers) based on high antibody titres. If IB reoccurs in the next flock on the farm, virus isolation attempts are performed and the virus is genotyped by RFLP or S1 sequencing.

d) Agar gel immunodiffusion

AGID can be used in diagnosis (48). The antigen is prepared from a homogenate of the chorioallantoic membranes of infected chicken embryos. The Beaudette embryo-lethal strain is often employed to produce antigen. The test lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

All live and inactivated commercial vaccines must be licensed. Strains used in live virus vaccines generally require attenuation. At present, many countries only permit live vaccines of the Massachusetts type, such as the H 120. Some countries may also have licensed vaccines to other live strains such as Connecticut, Arkansas, or Delaware 072 (USA) or the 4/91 (United Kingdom). Live vaccines may be given as aerosols, in the drinking water, or by the intraocular route (eyedrop).

The efficacy of inactivated vaccines depends heavily on proper priming with a live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders.

Live vaccines confer better local immunity in the respiratory tract and also may protect against a wider antigenic spectrum of field strains (18). However, vaccination with live vaccines may not protect layer flocks against variant serotype challenge especially common on farms with flocks of multiple ages where production drops as early as 40 weeks of age are not uncommon (27). Live vaccines carry a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application techniques (e.g. spray or drinking water) can achieve uniform distribution of the vaccine in the flock and avoid backpassage. Furthermore, the use of vaccines at manufacturer's recommended dosages will also help avoid backpassage reversion that may be caused by fractional dose application.

There are prospects for genetically engineered vaccines (4), and *in-ovo* vaccination (47).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature. National and international standards that apply in the country in which IB vaccines are manufactured must be complied with. The licensing authority should provide information and guidance on requirements. These are now often presented in general terms, as applying to all vaccines – avian and mammalian, live and inactivated, or viral and bacterial vaccines. There may also be specific requirements applying to IB vaccines, live and inactivated. As examples, references are given to the European and USA regulations (13–15, 46).

The list of extraneous agents that must be shown to be absent continues to grow. Manufacturers must be familiar with those that currently apply in their country. Recent additions are avian nephritis virus and avian pneumovirus.

For IB vaccines, important differences among countries may arise regarding the challenge virus to be used for potency tests, and its validation. Traditionally, the virulent M-41 (Mass 41) strain of the Massachusetts type has been used for challenge tests of both live and inactivated vaccines. Although this type is still common, it is often not the only or the dominant type in many countries and it may be advisable to prepare vaccines from other types. It is logical for challenges to be made by the same type as present in the vaccine. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their lower virulence in general. Inactivated vaccines are usually expected to protect against drops in egg production. The traditional M-41 challenge, as described in this chapter, should cause a drop of at least 67% in the unvaccinated controls, but when using other types much lower drops may be regarded as satisfactory, depending on published evidence of the effects of these strains in the field. There is also a tendency to relax the criteria for Massachusetts type challenges, and the European Pharmacopoeia now defines a satisfactory drop for Massachusetts types to be at least 35%, and for non-Massachusetts types to be at least 15%, provided that the drop is 'commensurate with the documented evidence' (15).

1. Seed management

a) Characteristics of the seed

The seed-lot (master seed) system should be employed for whatever type of vaccine is produced. Each virus must be designated as to strain and origin and must be free from contamination with other strains of IBV and extraneous agents. Separate storage facilities should be provided between the strains of virus intended for vaccines or for challenge.

For live virus vaccines, many countries permit only strains of the Massachusetts type. Some countries allow other strains, usually on the basis that those strains are already present in their national flocks. The antigenic type incorporated in both live and inactivated vaccines requires justification if there is doubt as to its existence in a country.

b)   Method of culture

All seed viruses are grown in the allantoic sac of developing chicken embryos or in suitable cell cultures. The eggs should be from an SPF flock.

c)   Validation as a vaccine

- **Purity**

Every seed lot must be free from bacterial, fungal, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred monospecific antiserum prepared against the strain under examination or against one of identical type. This mixture is cultured in a variety of ways, designed to confirm the absence of any viruses considered from past experience to be potential contaminants. The antiserum must not contain antibodies to adenovirus, avian encephalomyelitis virus, avian rotavirus, chicken anaemia virus, fowlpox virus, infectious laryngotracheitis virus, influenza A virus, Newcastle disease virus, infectious bursal disease virus, leukosis virus, reovirus, Marek's disease virus, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus, avian nephritis virus, avian pneumovirus or reticulo-endotheliosis virus. The inoculum given to each unit of the culture system used should contain a quantity of the neutralised IBV component under test that had an initial infectivity of at least ten times the minimum field dose. These systems include:

1.   SPF chicken embryos, incubated for 9–11 days, inoculated via both allantoic sac and chorioallantoic membrane (two passages);

2.   Chicken embryo fibroblast cultures, for leukosis virus subgroups A and B. The COFAL test (test for avian leukosis using complement fixation) or double-antibody sandwich ELISA for group-specific leukosis antigen is performed on cell extracts harvested at 14 days. An immunofluorescence test for reticulo-endotheliosis virus is done on cover-slip cultures after two passages.

3.   SPF chicken kidney cultures that are examined for CPEs, cell inclusions and haemadsorbing agents passaged at intervals of no fewer than 5 days for up to 20 days' total incubation.

4.   SPF chickens of minimum vaccination age inoculated intramuscularly with 100 field doses, and on to the conjunctiva with ten field doses; this is repeated 3 weeks later when the chickens are also inoculated both into the foot pad and intranasally with ten field doses. Observations are made for 6 weeks overall, and serum is collected for tests for avian encephalomyelitis, infectious bursal disease, Marek's disease, Newcastle disease and *Salmonella pullorum* infection.

- **Potency**

Vaccines intended to protect against loss of egg production should be tested for duration of antibody response. Mean HI titres should be >6 $\log_2$ up to at least 60 weeks of age. Serological tests should be done at intervals frequent enough to show that titres have not been boosted by extraneous IBV infection.

Vaccines intended for protection of broiler chickens or rearing chickens against the respiratory form of the disease should be similarly tested for duration of antibody responses; in the case of broilers this would be up to the normal age for slaughtering, and in the case of pullets up to the age when a booster vaccination would be administered (often at 16–18 weeks of age).

- **Safety**

Tests on seed virus should include a test for any potential ability to revert to virulence. Live and inactivated vaccine seed must be tested for safety as in Section C.4.b.

- **Efficacy**

To demonstrate efficacy, a trial vaccine must be made from the master seed and the working seed at five passages from the master seed and subjected to tests that demonstrate their protective effect.

For live vaccines, a minimum of ten SPF chickens aged 3–4 weeks are vaccinated intranasally or by eyedrop with the recommended dose. Ten unvaccinated control birds from the same age and source are retained separately. All birds of both groups are challenge inoculated intranasally or by eyedrop 3–4 weeks

later, each with $10^{3.0}$–$10^{3.5}$ EID_{50} of the virulent Massachusetts M-41 strain. A swab of the trachea is taken from each bird 4–5 days after challenge and placed in 3 ml of antibiotic broth. Each fluid is tested for IBV by the inoculation (0.2 ml) of five embryonated eggs after 9–11 days of incubation. An alternative test to that of taking swabs is to kill birds at 4–6 days after challenge and examine microscopically the tracheal rings for ciliary activity (20). Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 90% of the challenge vaccinated birds show no evidence of IBV in their trachea, while 90% or more of the control birds should have evidence of the presence of the virus.

To assess an inactivated vaccine intended to protect laying birds, 30 or more SPF chickens are vaccinated as recommended at the earliest permitted age. If a primary vaccination with live vaccine is first undertaken, an additional group of birds is given only the primary vaccination. In both cases, these primary vaccinations should be done at no later than 3 weeks of age. The inactivated vaccine is given 4–6 weeks after the live priming vaccination. A further group of 30 control birds are left unvaccinated. All groups are housed separately until 4 weeks before peak egg production, and then are housed together. Individual egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for a further 4 weeks. The challenge should be sufficient to ensure loss of production during the 3 weeks after challenge. The loss in the control group should be at least 67%; the group that received primary live virus vaccine followed by inactivated vaccine should remain at the previous level, and the group given only a primary vaccination should show an intermediate drop in production. Sera are collected from all birds at vaccination, 4 weeks later, and at challenge; there should be no response in the control birds.

To assess an inactivated vaccine intended to protect birds against respiratory disease, 20 SPF chickens aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with this first group. Antibody responses are determined 4 weeks later; there should be no response in the control birds. All birds are then challenged with 10^3 CID_{50} (50% chick infective dose) of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility. At least 80% of the unvaccinated controls should display complete ciliostasis, whereas the tracheal cilia of a similar percentage of the vaccinated birds should remain unaffected.

Both live and inactivated vaccines containing Newcastle disease, infectious bursal disease, reovirus and EDS76 viruses are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

2. Method of manufacture

All virus strains destined for live vaccines are cultured in the allantoic sac of SPF chicken embryos or in suitable cell cultures. For inactivated vaccines, hens' eggs from healthy non-SPF flocks may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised in vials, and for inactivated vaccines it is blended with high-grade mineral oil to form an emulsion to which a preservative is added.

3. In-process control

The required antigen content is based on initial test batches of vaccine of proven efficacy in laboratory and field trials. Infectivity titrations are done in chicken embryos.

Live vaccine should contain not less than $10^{3.5}$ EID_{50} per dose per bird until the expiry date indicated, and not less than $10^{2.5}$ EID_{50} per dose per bird after incubation at 37°C for 7 days at the time of issue. For inactivated vaccine, the inactivating agent and inactivation procedure must be shown under manufacture to be effective on both IBV and potential contaminants. With the use of beta-propiolactone or formalin, any live leukosis viruses and *Salmonella* species must be eliminated; and with other inactivating agents, the complete range of potential contaminants must be rendered ineffective. Before inactivation procedures, it is important to ensure homogeneity of suspensions, and a test of inactivation should be conducted on each batch of both bulk harvest after inactivation and the final product.

Tests of inactivation should be appropriate to the vaccine concerned and should consist of two passages in cell cultures, embryos or chickens, using inoculations of 0.2 ml and ten replicates per passage.

4. Batch control

a) Sterility

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

- **For live vaccines**

Use no fewer than ten chickens from an SPF flock that are of the minimum age stated on the label for vaccination. Administer by eyedrop to each chicken ten doses of the vaccine reconstituted so as to obtain a concentration suitable for the test. Observe the chickens for 21 days. For vaccines intended for chickens that are 2 weeks old or more, use the chickens inoculated in the 'test for extraneous agents using chickens' (see Section C.1.c.4). If during the period of observation, more than two chickens die from causes not attributable to the vaccine, repeat the test. The vaccine complies with the test if no chicken shows serious clinical signs, in particular respiratory signs, and no chicken dies from causes attributable to the vaccine.

- **For inactivated vaccines**

Inject a double dose of vaccine by the recommended route into each of ten 14–28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

c) Potency

The potency test is developed from the results of efficacy tests on the master seed virus. Live vaccines are tested for potency by titration of infectivity, and inactivated vaccines by measuring antibody production. The potency test for a batch of inactivated vaccine consists of vaccinating 20 SPF chickens, 4 weeks of age, and showing that their mean HI titre 4 weeks later is not less than 6 $\log_2$.

d) Duration of immunity

Vaccine must be shown to have the required potency to achieve the claimed duration of immunity at the end of the claimed shelf life.

e) Stability

At least three batches should be tested for stability and must give satisfactory results for 3 months beyond the claimed shelf life.

The stability of a live vaccine must be measured by maintenance of an adequate infectivity titre.

The stability of an inactivated vaccine is measured at intervals by batch potency tests. The concentration of preservative and persistence through the shelf life should be assessed. There should be no physical change in the vaccine and it should regain its former emulsion state after one quick shake.

f) Preservatives

There are maximum level requirements for the use of antibiotics, preservatives and residual inactivating agents.

g) Precautions (hazards)

IBV itself is not known to present any danger to staff employed in vaccine manufacture or testing. Extraneous agents may be harmful, however, and the initial stages of handling a new seed virus should be carried out in a safety cabinet. It is a wise precaution with all vaccine production to take steps to minimise exposure of staff to aerosols of foreign proteins. Persons allergic to egg materials must never be employed in this work.

5. Tests on the final product

a) Safety

A safety test must be carried out on each batch of final product, as in Section C.4.b.

b) Potency

A potency test must be carried out on each batch of final product, as in Section C.4.c, at manufacture and at the end of the stated shelf life.

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CHAPTER 2.3.3.

AVIAN INFECTIOUS LARYNGOTRACHEITIS

none## SUMMARY

none*Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by Herpesviridae alphaherpesvirinae gallid herpesvirus 1. It is principally a disease of chickens, although it can also affect pheasants, partridges and peafowl. The clinical signs and pathological reactions may vary from extremely severe, with some birds dying due to asphyxiation, to very mild, indistinguishable from other mild respiratory diseases of chickens. The principal lesion is tracheitis. In infected birds the virus can become latent and re-excreted at a later date without clinical signs.*

Laboratory diagnosis depends on isolation of the virus, demonstration of the presence of the virus or viral antigens, and detection of specific antibodies in the serum. Histopathological examination of the trachea for characteristic intranuclear inclusions may be of value.

Identification of the agent: Virus isolation may be done by inoculation of suspected material on to the dropped chorioallantoic membrane of embryonated eggs, or into avian embryonic cell cultures. These methods are time-consuming but sensitive. Rapid methods include direct electron microscopy on tracheal exudate, immunofluorescence on tracheal exudate or frozen sections, agar gel immunodiffusion (AGID) to detect viral antigens in tracheal samples or infected egg material, and an enzyme-linked immunosorbent assay (ELISA) to demonstrate viral antigen in mucosal scrapings. Polymerase chain reaction (PCR) methodology has been shown to be more sensitive than virus isolation for the examination of clinical material and is now widely used. Virus characterisation and differentiation of vaccine and wild-type viruses are possible using PCR followed by restriction fragment length polymorphism.

Serological tests: Antibodies to ILT virus (ILTV) can be detected by virus neutralisation (VN) tests conducted in eggs or in cell cultures, or by AGID reactions, indirect immunofluorescence, or ELISA. The latter is preferred for screening flocks.

Requirements for vaccines and diagnostic biologicals: Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection, but are not completely satisfactory. There have been recent promising studies on the efficacy of a genetically engineered vaccine.

A. INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by an alphaherpesvirus. It can also affect pheasants, partridges and peafowl. In the virulent form, the history, clinical signs and very severe tracheal lesions are highly characteristic of the disease, but the mild form may be indistinguishable from other mild respiratory diseases. Laboratory diagnosis depends on the demonstration of the presence of the virus or viral antigens or products (9, 24, 29) or specific antibodies in the serum (1, 20).

Clinically, the disease may appear in three forms, namely peracute, subacute, and chronic or mild. In the peracute form, onset of disease is sudden with a rapid spread. The morbidity is high and mortality may exceed 50%. Some birds may die in good body condition before the appearance of signs, which are characteristic and comprise difficulty in breathing with extension of the neck and gasping in an attempt to inhale. There is also gurgling, rattling and coughing when birds try to expel obstructions in the trachea. Clots of blood may be coughed up and can be found on the floor and walls of the house. Post-mortem changes are confined to the upper respiratory tract and are also characteristic, consisting of haemorrhagic tracheitis with blood clots, mucoid rhinitis, and blood-stained mucus along the length of the trachea.

In the subacute form, the onset of illness is slower and respiratory signs may extend over some days before deaths are seen. The morbidity is high but the mortality is lower than in the peracute form, between 10% and 30%. Post-mortem findings are less severe and consist of mucoid exudate with or without blood in the trachea. Yellow caseous diphtheritic membranes may be found adherent to the larynx and upper tracheal mucosa.

Chronic or mild ILT may be seen among survivors of either of the above forms of the disease, although some outbreaks themselves may be entirely mild. Incidence of chronic ILT within a flock may be only 1–2%, with most affected birds dying of suffocation. Signs include spasms of coughing and gasping, with nasal and oral discharge and reduced egg production. Infection is acquired via the upper respiratory tract and transmission occurs most readily from acutely infected birds, but clinically inapparent infection can persist for long periods with intermittent re-excretion of the virus, and these recovered carrier birds are also a potential means of transmission of the disease (12). On post-mortem examination, diptheritic and caseous necrotic plaques and plugs are found in the trachea, larynx and mouth. Outbreaks of mild ILT may affect large numbers of birds simultaneously, in which case gross lesions may consist only of conjunctivitis, sinusitis and mucoid tracheitis. Given that transmission of ILT takes place by close contact, transmission is slower in cage houses than where birds are loose-housed, and the path of infection through a cage house may be apparent. Recent work has confirmed considerable variation among ILTV strains in their tropism for trachea or conjunctiva and those with affinity for the latter site can severely affect weight gain (18).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The virus may be isolated in chick embryo liver (19), chicken embryo kidney (6) or in chicken kidney (26) cell cultures. Of these, monolayers of chicken embryo liver cells have been found to be the most sensitive (11). The virus can also be grown on the dropped chorioallantoic membrane (CAM) of 10–12-day-old specific pathogen free embryonated chicken eggs (14).

The causative herpesvirus may be demonstrated directly in tracheal exudate by electron microscopy (26). Viral antigens may be detected by immunofluorescence (4, 28), agar gel immunodiffusion (AGID) (15), or enzyme-linked immunosorbent assay (ELISA), using tracheal mucosal scrapings (30). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions may also be helpful (3, 23). Methods of detecting ILT virus (ILTV) using polymerase chain reaction (PCR) have been described and PCR has been reported to be generally more sensitive than virus isolation (2, 16, 19, 29).

a) Virus isolation

When samples are taken from live birds for virus isolation, tracheal swabs are superior to oropharyngeal or conjunctival swabs. These are placed in transport medium containing antibiotics. When selecting material for virus isolation from chronic outbreaks, it is more productive to cull a bird in the early stages of the infection, rather than to attempt to isolate virus from a bird that has died of asphyxiation after a long illness. The quality of sample is further improved if the bird is killed by barbiturate or other injection rather than by cervical dislocation. The whole head and neck from dead birds may be submitted, or only the trachea and larynx after their removal with minimal contamination. Tracheas should be transported in antibiotic broth for virus isolation, but wrapped in moist tissue paper if destined for electron microscopy. Any prolonged storage of infected tissues should be at –70°C or below to minimise loss of virus titre. Repeated freezing and thawing must be avoided as this reduces virus infectivity.

Exudate and epithelial cells are scraped from the tracheas, diluted approximately 1/5 in nutrient broth containing penicillin and streptomycin, and agitated vigorously. The resulting suspension is centrifuged at low speed to remove debris, and 0.1 ml of the supernatant fluid is inoculated on to the dropped CAM of at least three embryonated chicken eggs of 10–12 days' incubation. The eggs are sealed with paraffin wax and incubated at 37°C for up to 7 days. They are candled daily and the CAMs of dead embryos or of those surviving for 7 days are examined for typical pocks. Alternatively, at least two confluent chick embryo liver or chicken embryo kidney cell monolayers, with their medium removed, are inoculated and allowed to adsorb for 1–2 hours. Cultures are overlaid with fresh medium, incubated for up to 7 days and examined daily under the microscope for evidence of a typical syncytial cell cytopathic effect (CPE).

In each instance, up to three passages of material may be necessary before a specimen is considered to be negative. A virus isolate can be confirmed as ILTV by a neutralisation test in eggs or cell culture using hyperimmune antiserum to ILTV. Alternatively, virus particles may be identified rapidly in cell culture fluid or in pocks on CAMs by electron microscopy, viral antigens by immunofluorescence in acetone-fixed ILT-virus-infected cell cultures or in frozen sections of CAM and viral nucleic acid by PCR.

b) Electron microscopy

To demonstrate the presence of virus by electron microscopy, tracheal exudate or epithelial scrapings from the trachea are smeared on to a microscope slide and mixed with a few drops of distilled water. One drop of suspension is placed on a carbon and formvar-coated grid and left for 2 minutes, after which excess moisture is removed using filter paper. One drop of 4% phosphotungstic acid, pH 6.4, is added and the excess removed after a further 3 minutes. The grid is allowed to dry thoroughly and examined using the electron microscope at a magnification of ×30–45,000 for typical herpesvirus particles, measuring 100 nm diameter with icosahedral symmetry.

c) Immunofluorescence

In immunofluorescence tests for viral antigens, epithelial cell scrapings from the trachea are smeared on to a glass slide. Alternatively, 5 µm thick cryostat sections of trachea, snap-frozen in liquid nitrogen may be used. The preparations are fixed in acetone at room temperature for 10 minutes. These can be stained directly by applying chicken anti-ILTV immunoglobulin labelled with fluorescein isothiocyanate (FITC) for 1 hour, followed by rinsing for 15 minutes in a bath of phosphate buffered saline (PBS), pH 7.2, agitated with a magnetic stirrer. Otherwise, they can be stained indirectly by applying an appropriate dilution of chicken anti-ILT serum for 1 hour. The slide is rinsed thoroughly with PBS for 15 minutes as above, and an FITC-labelled anti-chicken immunoglobulin is applied for 30 minutes. After a final rinse, cover-slips are applied over non-fade mountant. The preparations are examined for specific intranuclear fluorescence in the epithelial cells using a microscope with epifluorescent ultraviolet illumination. Suitable controls include the use of known uninfected specimens and, for the indirect method, the application of nonimmune chicken serum. Particular care should be taken in the reading of indirect immunofluorescence preparations, as endogenous chicken IgG in the trachea may cause unwanted attachment of FITC-labelled anti-chicken IgG.

d) Agar gel immunodiffusion

ILT viral antigens may be demonstrated by AGID tests on tracheal exudate, infected CAMs and infected cell culture material using hyperimmune ILTV antiserum. The gel is made with Noble agar (1.5%) containing sodium chloride (8%) and sodium azide (0.02%) – as preservative – in distilled water. The ingredients are autoclaved at 15 lb/sq. inch (2.4 bar) for 15 minutes; 5 ml of the molten agar is poured into a 5 cm diameter Petri dish. When the agar has set, a pattern of wells is punched in the agar, consisting of a central well and six surrounding wells. The wells are usually 8 mm in diameter and 4 mm apart. The hyperimmune serum is pipetted into the central well, while the surrounding wells are filled with suspect virus samples under test, but with at least one well containing positive viral antigen. Dishes are incubated in a humid atmosphere at room temperature or at 37°C, and examined 24–48 hours later by oblique illumination for lines of precipitation (reactions of identity). Tests should include uninfected material as negative antigen and known negative antiserum as controls. For economy of materials, the test can be done on a microscale – the agar being poured in a thin layer on to a microscope slide and holes punched of 4 mm diameter and 2 mm apart.

e) Enzyme-linked immunosorbent assay

When the monoclonal antibody (MAb) ELISA is used for detecting viral antigens (19), tracheal exudate is mixed with an equal volume of PBS containing 1% (v/v) of a detergent, such as Nonidet P40 (BDH Chemicals, Poole, United Kingdom), then vortexed for 30 seconds and centrifuged at 10 *g* for 1 minute. The supernatant fluid is dropped in 50 µl volumes in wells of microtitre plates previously coated with rabbit IgG against ILTV, diluted 1/200 in 0.05 M carbonate/bicarbonate buffer, pH 9.0, and incubated for 1 hour. Next, 50 µl of MAb against major glycoproteins of ILTV, diluted 1/50 in PBS, is added to each well, followed by 50 µl of a 1/1000 dilution of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase. The substrate, 5-aminosalicylic acid (6.5 mM), is added to the wells in 100 µl volumes. After 30 minutes, the plates are read at 450 nm on a spectrophotometer and the absorbance reading for each well is corrected by subtracting the reading obtained for wells containing diluting buffer instead of tracheal exudate. The positive/negative cut-off point is taken as the mean absorbance value for several negative (i.e. tracheal material without ILTV) samples plus 3 standard deviations.

f) Histopathology

Tracheas for histopathological examination should be placed in formol saline immediately after removal from the birds and embedded in paraffin wax. Eyelids and lung are sometimes examined. Intranuclear inclusions may be seen in the epithelial cells of the trachea in longitudinal sections after staining by haematoxylin and eosin. They are the classical Cowdry type A inclusions of herpesviruses, but they may be present for only 3–5 days after infection. In severe cases where most infected cells have detached from the tracheal lining, inclusions may be seen in intact cells among the cellular debris in the lumen of the trachea. Longitudinal rather than transverse sections of trachea permit examination of the whole length of the organ.

g) Molecular methods

Several molecular methods for identifying ILTV DNA in clinical samples have been reported, but the PCR has proved he most useful. Dot-blot hybridisation assays and cloned virus DNA fragments were shown to be highly sensitive for detecting virus when isolation and ELISA were negative (16, 17). Humberd *et al*. (13),

using a nested PCR, showed that ILTV DNA could be detected in formalin-fixed, paraffin-embedded tissues independently of the presence of syncytial cells, intranuclear inclusions or both.

PCR has been found to be more sensitive than virus isolation for clinical samples, especially when other contaminant viruses such as adenoviruses are present (29). Alexander & Nagy (2) found that during the middle to the end of the infection phase, PCR and virus isolation were similar in sensitivity, but PCR was superior in the recovery phase.

A problem with the PCR for ILTV was that initially it was not able to differentiate between field and vaccine strains. However, the combination of PCR with restriction fragment length polymorphism (RFLP) analysis of single and multiple viral genes and genome regions has enabled the characterisation of different strains within a country or region (5). Several reports have shown that while some field strains are closely related to and likely to be derived from vaccines viruses, others are true 'wild types' (21). Genes commonly examined by different international authors include ICP4, TK (thymidine kinase), glycoprotein G (gG), glycoprotein E (gE) and UL47. Oldoni & Garcia (22) used 36 restriction enzymes, while others have used as few as four.

i)   *PCR protocol*

In a typical PCR protocol for ILTV, viral DNA is extracted from clinical samples (swabs, tissues pieces), chorioallantoic membrane plaques, cell culture supernatants or vaccines using DNA extraction kits. Primers used can be obtained from previously published work or designed using ILTV sequences on the Genbank international database. Amplifications are made using Taq DNA polymerase. Typical amplification reactions use an initial denaturing step of 94°C for 1 minute followed by 35 amplification cycles of 94°C for 1 minute with annealing temperatures ranging from 54–60°C for 30 seconds. Extension may be performed at 68°C, with extension times varying according to the size of the target region amplified and a final extension at 68°C for 7 minutes. The PCR products are separated by electrophoresis in 1% agarose gels, stained with ethidium bromide and exposed to UV light for visualisation.

ii)   *Real-time PCR*

Recently a real-time PCR assay had been described for ILTV (7). This has the advantage that, including amplification and melt-curve analysis, it can be conducted in less than 2 hours. It therefore provides a very rapid method of ILT diagnosis in comparison with traditional virus isolation, or even the standard PCR followed by gel electrophoresis.

iii)   *Restriction fragment length polymorphism* (*RFLP*)

A range of restriction endonucleases (RE) have been described for RFLP analysis of ILTV PCR products and several genes have been targeted for digestion. They include ICP4, TK (thymidine kinase), UL 15, UL47 glycoprotein G and ORF-BTK genes. Amplification products are digested separately with 10U RE for 3 hours. Digestion fragments are separated in 15% polyacrylamide gels. Fragments are observed after DNA silver staining and analysed under a light box. Pattern differences are recorded for each enzyme and results can be developed into dendrograms. The combination of PCR and RFLP has enabled field strains of ILTV to be distinguished from vaccine strains (7, 10, 21, 26).

2.   Serological tests

Antibodies to ILTV in chicken serum can be detected by virus neutralisation (VN), AGID, indirect immunofluorescence tests and ELISA (1).

a)   Virus neutralisation

VN tests may be conducted on the dropped CAMs of embryonating chicken eggs that have been incubated for 9–11 days, where antibody specifically neutralises pock formation due to ILTV. Alternatively, the tests can be performed in cell cultures, where antibody specifically neutralises the ILTV thus preventing CPE. Doubling dilutions of serum are added to equal volumes of a constant concentration of virus. This concentration may either be 100 median egg infectious doses (EID_{50}) for egg inoculations, or 100 median tissue culture infectious doses ($TCID_{50}$) for the inoculation of cultures. The mixtures are incubated at 37°C for 1 hour to allow any neutralisation to occur.

When the test is performed in eggs, the virus/serum mixtures are inoculated on to the dropped CAMs, using at least five eggs per dilution. Eggs are sealed and incubated at 37°C for 6–7 days. The end-point is recorded as the highest dilution of the serum where no pocks are present on the CAMs. When the tests are done in cell cultures, serum dilutions are prepared in 96-well microculture plates and virus is then added. After the period allowed for neutralisation, freshly prepared chicken embryo liver or kidney cells are added to each well. The plates are incubated at 37°C in an atmosphere of 5% CO_2 and examined daily for CPE; 50% end-points are read after approximately 4 days when the virus control titre indicates that 30–300 $TCID_{50}$ of

virus have been used in the test. For the cell culture method of testing, virus neutralisation at 1/8 (initial dilution) or greater is considered positive.

b) **Agar gel immunodiffusion**

For AGID tests, antigen is prepared from virus-infected CAMs or infected cell cultures. For the former, at least 10^4 TCID$_{50}$ of ILTV is inoculated into the allantoic cavity of a batch of 10-day-old embryonating specific pathogen free (SPF) chicken eggs. The CAMs are harvested after 4 days' incubation, and those with large pocks are homogenised and sonicated in a small amount of PBS, pH 7.1. Alternatively, heavily infected monolayers of chicken embryo liver or kidney, or chicken kidney cells are incubated at 37°C until the CPE is maximal. Any remaining attached cells are scraped from the culture vessel into the medium. Total culture harvests may be concentrated up to 100-fold by dialysis against polyethylene glycol (PEG 20,000 or PEG 30,000). For the test, the agar is prepared as described previously for antigen detection, but this time the CAM or cell culture antigen is placed in the central well with test sera in the surrounding wells. Known positive and negative antisera are incorporated in the test, which is read after 24–48 hours' incubation at room temperature or at 37°C. AGID tests are simple, economical to perform, and useful for flock screening, although they are less sensitive than the other methods.

c) **Indirect fluorescent antibody test**

For indirect fluorescent antibody tests, the antigen consists of ILT-virus-infected cell culture monolayers grown on teflon-coated multispot slides. When CPE is beginning to develop, the cultures are fixed in acetone for 10 minutes. Dilutions of test sera prepared in PBS are applied to each spot culture and the slides are incubated at 37°C for 1 hour. The slides are washed in PBS as described previously, drained and treated with an appropriate dilution of a commercial FITC-labelled rabbit anti-chicken IgG. After incubation at 37°C for 1 hour, the slides are rewashed and cover-slips are applied over a non-fade mountant. They are examined by epifluorescence with ultraviolet illumination, and end-point titres are read as the highest serum dilutions giving specific fluorescent staining. This test is more sensitive than AGID, but the interpretation of results may be subjective.

d) **Enzyme-linked immunosorbent assay**

The antigen for ELISA is obtained by sonication of heavily infected cell cultures at the time of maximum CPE, which is then absorbed on to the wells of microtitre plates. A negative antigen is provided by uninfected cell culture material treated in the same way. The test consists essentially of the addition of 0.1 ml of 1/10 dilutions of test sera to duplicate wells coated with positive or negative antigen. After incubation at 37°C for 2 hours, the plates are washed four times and a 1/4000 dilution of a rabbit anti-chicken IgG conjugated with peroxidase is added. After incubation at 37°C for 1 hour, the plates are washed again four times. Finally a substrate consisting of 5-aminosalicylic acid is added to each well followed by hydrogen peroxide to a final concentration of 0.0005%, and the absorbance of the fluid in each well is read at 450 nm on a spectrophotometer. The result for each serum is expressed as the difference between the mean absorbance produced with the positive and negative antigens. The positive/negative cut-off point is taken as the mean absorbance value for numerous negative sera plus 3 standard deviations. The test is very sensitive and possibly the best available for surveillance purposes. Antibody responses as measured by ELISA are detectable 7–10 days after infection and peak at about 2 weeks. The response to ILT vaccines may be variable and testing is not worthwhile before 14 days post-vaccination. Several laryngotracheitis antibody ELISA kits are available commercially.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

ILT is usually controlled with live vaccines, although inactivated vaccines have also been used for safety reasons. There has been some recent work with genetically engineered vaccines and the results of the initial studies look promising (8, 27). The live virus seed is a suitably attenuated or naturally avirulent strain of ILTV. Vaccines may be administered by eye-drop, spray or in the drinking water. If administered by spray and a small droplet size is produced and inhaled, clinical disese may be precipitated. Young chickens may require vaccinating in endemic areas, but show more severe reactions to the vaccine. Repeated doses may be required to afford good protection. The level of virulence of the vaccine virus is critical. Strains of low virulence may not be effective, and those of higher virulence may cause severe disease. The spray route of administration requires care over droplet size and uniformity of application. It can be more effective with low virulence strains, but may be more dangerous with high virulence strains. At present, the available vaccines attempt to make a compromise between lack of efficacy and poor safety. Because of persistence of virulent vaccine virus on a site, it may be difficult to discontinue vaccination once it has been started. Subclinical mixed infections of vaccine and field virus, in vaccinated birds, can cause severe disease in unvaccinated in-contacts.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements (e.g. ref. 25).

1. Seed management

a) Characteristics of the seed

The master seed virus (MSV) is selected and can be propagated in SPF chicken embryos or tissue cultures derived from such embryos. The MSV is tested in chicken embryos or chickens for the following: 1) purity, 2) *Mycoplasma* spp., 3) *Salmonella* spp., 4) avian leukosis virus, 5) haemagglutinating viruses, 6) virus identity, and 7) extraneous pathogens. Additionally, initial tests are performed to demonstrate the safety and efficacy of the chosen master seed. The safety test on the MSV must include tests to show lack of reversion to virulence on serial passage and also safety in birds. Evidence of shed and spread is also required. The MSV is stored in aliquots at –70°C. The MSV should not cause mortality or a severe respiratory reaction in chickens following ocular instillation, although pheasants are more susceptible. Administration by spray is convenient but may cause quite severe respiratory disease in some flocks.

b) Method of culture

In large-scale vaccine production, the virus is propagated in SPF chicken embryos or tissue culture derived from such embryos, up to the fifth passage from the MSV. The acceptable passage level is supported experimentally by the passage level used to prepare the experimental product used in the efficacy study.

c) Validation as a vaccine

A test must be carried out to establish the efficacy of the vaccine in birds of the minimum age for which the product is destined and also for each avian species. This is repeated in further batches of chickens for each of the recommended routes of administration and/or age of bird. Three weeks later (or 10–14 days in the USA), the birds, together with ten controls of the same age and source, are challenged intratracheally or in the orbital sinus with a strain of ILTV of known high virulence. To be satisfactory, only 5% of the vaccinated birds should die or show severe signs of ILT. No more than four should show mild signs of ILT. At least eight of the controls should die or show severe signs of ILT.

2. Method of manufacture

The vaccine is made by inoculation of the production seed virus into 9–11-day-old chicken embryos or tissue culture prepared from chicken embryos derived from SPF flocks. Eggs are inoculated through a hole in the shell, on to the dropped CAM. They are sealed and incubated at 37°C for 4–6 days. All eggs are candled before harvest and only those with living embryos are used. To harvest the virus, the eggs are chilled, then cleansed and opened aseptically. The CAMs and fluids are pooled in sterile, cooled containers. The CAMs should show the thick grey plaques typical of ILTV growth. Tissue culture-derived product would be prepared from virus-bearing cell culture fluids, which would also be subsequently pooled and tested.

3. In-process control

The infected tissue or tissue culture homogenate may be tested for purity, potency, and virus content, mixed with a stabiliser (usually beef peptone and sucrose) and then lyophilised and stored at 4°C.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Using the recommended route of administration, each batch of vaccine is tested in ten SPF chickens, or ten birds of other target species, using ten doses per bird. The birds are observed for at least 21 days for adverse effects attributable to the vaccine.

c) Potency

Once the *in-vivo* efficacy of the vaccine has been established, the batch potency may be determined by measuring the virus content. Serial dilutions of the vaccine are inoculated on to the dropped CAM of 9–11-day-old SPF chicken embryos, using at least seven eggs per dilution, in a volume of 100 µl. The eggs are incubated for 5 days and the virus titre is calculated by observing characteristic lesions on the CAMs. The virus content should be at or above a release value and above and expiration titre during dating of the product. Both the release and expiration titres are based on the minimum protective dose described above.

d) Duration of immunity

The results of vaccination will depend on many factors, including dose schedule and route of administration. Some degree of protection should be given, over a period of several months.

e) Stability

Stability is tested by taking samples of correctly stored vaccine at intervals and measuring virus content. Tests should be carried out on at least six batches of the vaccine or until a statistically valid number of serials have been evaluated and be continued for 3 months after the claimed shelf-life.

f) Preservatives

Preservatives may not be required, but some antibiotics may be added to the tissue harvest or at serial assembly during manufacture. For products licensed in the USA, any antibiotics added are listed on the label.

g) Precautions (hazards)

Care should be taken over diluting and administering the vaccine, and over the proper disposal of unused vaccine.

5. Tests on the final product

a) Safety

In the USA, 25 susceptible chickens are injected intratracheally and observed for 14 days. Deaths are counted as failures. Four or fewer failures are allowed for satisfactory serials. In the European Union, tests of virus content are carried out. The virus titre shall normally be no higher than one-tenth of the dose at which the vaccine has been shown to be safe.

b) Potency

The test of virus content (see above) may also be used as a measure of potency. It must be no lower than the agreed minimum release titre. Each serial or subserial shall have a virus titre of 10^7 greater than the minimum protective dose, but not less than 10^{25} EID_{50} (or $TCID_{50}$ for tissue culture prepared product)/dose.

c) Tests of final product

The lack of chicken pathogens should be confirmed in embryos or chickens. It should also be confirmed by testing for *Mycoplasma* spp., *Salmonella* spp., avian leukosis virus, and haemagglutinating viruses.

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AVIAN INFLUENZA

SUMMARY

Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenzavirus A. There are three influenza genera – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation or detection and characterisation of the virus. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, strain of virus, the host's immune status, presence of any secondary exacerbating organisms and environmental conditions.

Identification of the agent: Suspensions in antibiotic solution of oropharyngeal and cloacal swabs (or faeces) taken from live birds, or of faeces and pooled samples of organs from dead birds, are inoculated into the allantoic cavity of 9– to 11-day-old embryonated fowl eggs. The eggs are incubated at 35–37°C for 4–7 days. The allantoic fluid of any eggs containing dead or dying embryos during the incubation and all eggs at the end of the incubation period are tested for the presence of haemagglutinating activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleocapsid and/or matrix antigens, both of which are common to all influenza A viruses. Isolation in embryos has recently been replaced, under certain circumstances, by reverse-transcription polymerase chain reaction (RT-PCR).

For subtyping the virus, the laboratory must have monospecific antisera prepared against the isolated antigens of each of the 16 haemagglutinin (H1–H16) and 9 neuraminidase (N1–N9) subtypes of influenza A viruses that can be used in immunodiffusion tests. Alternatively, the newly isolated virus may be examined by haemagglutination and neuraminidase inhibition tests against a battery of polyclonal antisera to a wide range of strains covering all the subtypes.

As the term highly pathogenic avian influenza and the historical term 'fowl plague' refer to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. Any highly pathogenic avian influenza isolate is classified as notifiable avian influenza (NAI) virus. Although all virulent strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. Due to the risk of a low virulent H5 or H7 becoming virulent by mutation in poultry hosts, all H5 and H7 viruses have also been classified as NAI viruses. The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity, but still primarily involve the inoculation of a minimum of eight susceptible 4–8-week-old chickens with infectious virus; strains are considered to be highly pathogenic if they cause more than 75% mortality within 10 days or have an intravenous pathogenicity index (IVPI) of greater than 1.2. Characterisation of suspected virulent strains of the virus should be conducted in a virus-secure laboratory. All virulent AI isolates are identified as highly pathogenic notifiable avian influenza (HPNAI) viruses. Regardless of their virulence for chickens, H5 or H7 viruses with an HA0 cleavage site amino acid sequence similar to any of those that have been observed in virulent viruses are considered HPNAI viruses. H5 and H7 isolates that are not pathogenic for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in HPNAI viruses are identified as low pathogenicity notifiable avian influenza (LPNAI) viruses and non-H5 or non-H7 AI isolates that are not highly pathogenic for chickens are identified as low pathogenicity avian influenza (LPAI) viruses.

Serological tests: As all influenza A viruses have antigenically similar nucleocapsid and matrix antigens, agar gel immunodiffusion tests are used to detect antibodies to these antigens. Concentrated virus preparations containing either or both type of antigens are used in such tests. Not all species of birds develop demonstrable precipitating antibodies. Haemagglutination inhibition

49	*tests have also been employed in routine diagnostic serology, but it is possible that this technique*

49   *tests have also been employed in routine diagnostic serology, but it is possible that this technique*
50   *may miss some particular infections because the haemagglutinin is subtype specific. Enzyme-*
51   *linked immunosorbent assays have been used to detect antibodies to influenza A type-specific*
52   *antigens.*

53   **Requirements for vaccines and diagnostic biologicals:** *Historically, in most countries, vaccines*
54   *specifically designed to contain or prevent HPNAI were banned or discouraged by government*
55   *agencies because they may interfere with stamping-out control policies. During the 1990s the*
56   *prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to*
57   *control widespread outbreaks of NAI, and a recombinant fowl poxvirus vaccine expressing the*
58   *homologous HA gene was also used in Mexico, El Salvador and Guatemala. During the 1999–2001*
59   *outbreak of LPNAI in Italy, an inactivated vaccine was used with the same haemagglutinin type as*
60   *the field virus, but with a different neuraminidase. This allowed the differentiation of vaccinated*
61   *birds from birds infected with the field virus and ultimately resulted in eradication of the field virus.*
62   *Prophylactic use of H5 and H7 vaccines has been practised in parts of Italy aimed at preventing*
63   *LPNAI infections and several countries in SE Asia have used prophylactic vaccination as an aid in*
64   *controlling HPNAI H5N1 virus infections. HPNAI viruses should not be used as the seed virus for*
65   *production of vaccine.*

66   *If HPNAI is used in challenge studies, the facility should meet the OIE requirements for*
67   *Containment Group 4 pathogens.*

A. INTRODUCTION

69 Notifiable avian influenza (NAI) is caused by infection with viruses of the family Orthomyxoviridae placed in the
70 genus *influenzavirus A*. Influenza A viruses are the only orthomyxoviruses known to affect birds. Many species of
71 birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir
72 of these viruses, but the overwhelming majority of isolates have been of low pathogenicity for chickens and
73 turkeys,. Influenza A viruses have antigenically related nucleocapsid and matrix proteins, but are classified into
74 subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (80). At present, 16 H subtypes
75 (H1–H16) and 9 N subtypes (N1–N9) are recognised. To date, the highly virulent influenza A viruses that produce
76 acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only
77 with the H5 and H7 subtypes. Most viruses of H5 and H7 subtype isolated from birds, have been of low virulence
78 for poultry (2). Due to the risk of a H5 or H7 virus of low virulence becoming virulent by mutation, all H5 and H7
79 viruses have been identified as notifiable avian influenza (NAI) viruses (81).

80 Depending on the species, age and type of bird, specific characteristics of the viral strain involved, and on
81 environmental factors, the highly pathogenic disease, in fully susceptible birds, may vary from one of sudden
82 death with little or no overt clinical signs to a more characteristic disease with variable clinical presentations
83 including respiratory signs, such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of
84 the sinuses and/or head, apathy, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the
85 unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhoea. In laying birds additional
86 clinical features include a marked drop in egg production usually accompanied by an increase in numbers of poor
87 quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality.
88 However, none of these signs can be considered pathognomonic. In addition, low pathogenicity avian influenza
89 (LPAI) viruses, which normally cause only a mild or no clinical disease, may in certain circumstances produce a
90 spectrum of clinical signs the severity of which may approach that of highly pathogenic avian influenza,
91 particularly if exacerbating infections are present. Confirmatory diagnosis of the disease, therefore, depends on
92 the isolation of the causal virus and the demonstration that it fulfils one of the defined criteria described in section
93 B.2. In some specific circumstances this may be achieved by detection of the virus in the infected host; especially
94 using molecular techniques that allow the determination of virus virulence. Testing sera from suspect birds using
95 antibody detection methods may supplement diagnosis, but these methods are not suitable for a detailed
96 identification. Diagnosis for official control purposes is established on the basis of agreed official criteria for
97 pathogenicity according to *in vivo* tests or to molecular determinants (i.e. the presence of multiple basic amino
98 acids at the cleavage site of the haemagglutinin precursor protein HA0) and haemagglutinin typing. These
99 definitions evolve as scientific knowledge of the disease increases.

100 HPNAI and NAI are subject to official control and the virus has a high risk of spread from the laboratory;
101 consequently, a risk assessment should be carried out to determine the level of biosecurity needed for laboratory
102 diagnosis and chicken inoculation; characterisation of the virus should be conducted at biocontainment level 3 (at
103 least). The facility should meet the requirements for the appropriate Containment Group as determined by the risk
104 assessment and as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory
105 and animal facilities. Countries lacking access to such a specialised national or regional laboratory should send
106 specimens to an OIE Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (the prescribed test for international trade)

Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, kidney, brain, liver and heart should also be collected and processed either separately or as a pool.

Samples from live birds should include both oropharyngeal and cloacal swabs. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in humans. Where these are not available, the collection of fresh faeces may serve as an alternative.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4 with antibiotics or a solution containing protein and antibiotics. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml) for tissues and oropharyngeal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the pH of the solution to pH 7.0–7.4 following the addition of the antibiotics. It is recommended that a solution for transport of the swabs should contain protein to stabilise the virus (e.g. brain–heart infusion, cattle serum up to 5% [v/v] or bovine albumen – 0.5% [w/v]). Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days. For prolonged storage, diagnostic samples and isolates should be kept at –80°C. Repeated freezing and thawing should be avoided.

The preferred method of growing avian influenza A viruses is by the inoculation of embryonated specific pathogen free (SPF) fowl eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1000 *g* are inoculated into the allantoic sac of at least five embryonated SPF or SAN fowl eggs of 9–11 days' incubation. The eggs are incubated at 35–37°C for 4–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C and the allantoic fluids should then be tested for haemagglutination (HA) activity (see Section B.3.b). Detection of HA activity, in bacteria-free amnio-allantoic fluids, indicates a high probability of the presence of an influenza A virus or of an avian paramyxovirus. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

The presence of influenza A virus can be confirmed in agar gel immunodiffusion (AGID) tests by demonstrating the presence of the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses (see Section B.3.a). The antigens may be prepared by concentrating the virus from infective allantoic fluid or extracting the infected chorioallantoic membranes; these are tested against known positive antisera. Virus may be concentrated from infective allantoic fluid by ultracentrifugation, or by precipitation under acid conditions. The latter method consists of the addition of 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. The mixture is placed in an ice bath for 1 hour and then clarified by centrifugation at 1000 *g* at 4°C. The supernatant fluid is discarded. The virus concentrates are resuspended in glycin/sarcosyl buffer: this consists of 1% (w/v) sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 M glycine. These concentrates contain both nucleocapsid and matrix polypeptides.

Preparations of nucleocapsid-rich antigen can also be obtained from chorioallantoic membranes for use in the AGID test (7). This method involves removal of the chorioallantoic membranes from infected eggs that have allantoic fluids with HA activity. The membranes are then homogenised or ground to a paste. This is subjected to three freeze–thaw cycles, followed by centrifugation at 1000 *g* for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin.

Use of the AGID test to demonstrate nucleocapsid or matrix antigens is a satisfactory way to indicate the presence of avian influenza virus in amnioallantoic fluid, but various enzyme-linked immunosorbent assays (ELISAs) are also available. There is a sensitive and specific ELISA that demonstrates nucleoprotein of type A influenza virus using a monoclonal antibody against type A influenza nucleoprotein (47, 49, 64). This is available as a commercial kit.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be due to an influenza A virus or to an avian paramyxovirus (a few strains of avian reovirus will do this, or nonsterile fluid could contain HA of bacterial origin). There are currently nine recognised serotypes of avian paramyxoviruses. Most laboratories will have antiserum specific for Newcastle disease virus (avian paramyxovirus type 1), and in view of its widespread occurrence and almost universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests (see Chapter 2.3.14 Newcastle disease).

Alternatively, the presence of influenza virus can be confirmed by the use of reverse-transcription polymerase chain reaction (RT-PCR) using nucleoprotein-specific or matrix-specific conserved primers (3, 53). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (21, 46, 53, 79).

The method recommended for definitive antigenic subtyping of influenza A viruses by the World Health Organization (WHO) Expert Committee (80) involves the use of highly specific antisera, prepared in an animal giving minimum nonspecific reactions (e.g. goat), directed against the H and N subtypes (45). An alternative technique is the use of polyclonal antisera raised against a battery of intact influenza viruses. Subtype identification by this technique is beyond the scope of most diagnostic laboratories not specialising in influenza viruses. Assistance is available from the OIE Reference Laboratories (see Table given in Part 3 of this *Terrestrial Manual*).

2.   Assessment of pathogenicity

The term highly pathogenic avian influenza relates to the assessment of virulence in chickens and implies the involvement of virulent strains of virus. It is used to describe a disease of fully susceptible chickens with clinical signs such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, apathy, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhoea. In laying birds additional clinical features include a marked drop in egg production usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic and high mortality may occur in their absence. In addition, low pathogenicity avian influenza (LPAI) viruses that normally cause only a mild or no clinical disease, may cause a much more severe disease if exacerbating infections or adverse environmental factors are present and, in certain circumstances, the spectrum of clinical signs may mimic highly pathogenic avian influenza. At the First International Symposium on Avian Influenza held in 1981 (5), it was resolved to abandon the term 'fowl plague' and to define highly pathogenic avian influenza strains on the basis of their ability to produce not less than 75% mortality within 8 days in at least eight susceptible 4–8-week-old chickens inoculated by the intramuscular, intravenous or caudal air sac route. However, this definition proved unsatisfactory when applied to the viruses responsible for the widespread outbreaks in chickens occurring in 1983 in Pennsylvania and the surrounding states of the United States of America (USA). The problem was mainly caused by the presence of a virus of demonstrable low pathogenicity in laboratory tests, but which was shown to be fully pathogenic following a single point mutation. Further consideration of a definition to include such 'potentially pathogenic' viruses was undertaken by several international groups.

The eventual recommendations made were based on the finding that while there have been numerous isolations of strains of H5 and H7 subtypes of low pathogenicity, all the highly pathogenic avian influenza strains isolated to date have possessed either the H5 or H7 haemagglutinin. Further information concerning the pathogenicity or potential pathogenicity of H5 and H7 subtypes may be obtained by sequencing the genome, as pathogenicity is associated with the presence of multiple basic amino acids (arginine or lysine) at the cleavage site of the haemagglutinin. For example, most H7 subtype viruses of low virulence have had the amino acid motif at the HA0 cleavage site of either -PEIPKGR*GLF- or -PENPKGR*GLF-, whereas examples of amino acids motifs for highly pathogenic avian influenza H7 viruses are: -PEIPKKKKR*GLF-, -PETPKRKRKR*GLF-, -PEIPKKREKR*GLF-, -PETPKRRRR*GLF-. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza isolates of low virulence for birds should identify viruses that, like the Pennsylvania virus, have the capacity, following simple mutation, to become highly pathogenic for poultry. In 1992, the OIE adopted criteria for classifying an avian influenza virus as highly pathogenic based on pathogenicity in chickens, growth in cell culture and the amino acid sequence for the connected peptide (41). The European Union adopted similar criteria in 1992 (16).

The following criteria, which are a modification of the previous OIE procedure, have been adopted by the OIE for classifying an avian influenza virus as HPNAI:

a)   One of the two following methods to determine pathogenicity in chickens is used. A HPNAI virus is:

i)   any influenza virus that is lethal[1] for six, seven or eight of eight 4– to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid

   or

ii)   any virus that has an intravenous pathogenicity index (IVPI) greater than 1.2. The following is the IVPI procedure:

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1   When birds are too sick to eat or drink, they should be killed humanely.

- Fresh infective allantoic fluid with a HA titre >1/16 (>2^4 or >$\log_2 4$ when expressed as the reciprocal) is diluted 1/10 in sterile isotonic saline.

- 0.1 ml of the diluted virus is injected intravenously into each of ten 6-week-old SPF or SAN chickens.

- Birds are examined at 24-hour intervals for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a subjective clinical assessment. Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death[2].)

- The intravenous pathogenicity index (IVPI) is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period.

b) For all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other highly pathogenic AI isolates, the isolate being tested will be considered to be highly pathogenic.

The OIE has the following classification system to identify viruses for which disease reporting and control measures should be taken (81):

a) All AI isolates that meet the above criteria are identified as highly pathogenic notifiable avian influenza (HPNAI).

b) H5 and H7 isolates that are not virulent for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in HPNAI viruses are identified as low pathogenicity notifiable avian influenza (LPNAI).

c) Non-H5 or non-H7 AI isolates that are not virulent for chickens are identified as low pathogenicity avian influenza (LPAI).

A variety of strategies and techniques have been used successfully to sequence the nucleotides at that portion of the HA gene coding for the cleavage site region of the haemagglutinin of H5 and H7 subtypes of avian influenza, enabling the amino acids there to be deduced. The most commonly used method has been RT-PCR using oligonucleotide primers complementing areas of the gene either side of the cleavage site coding region, followed by cycle sequencing (78). Various stages in the procedure can be facilitated using commercially available kits and automatic sequencers.

Now the presence of multiple basic amino acids at the HA0 cleavage site is well-established as an accurate indicator of virulence or potential virulence for H5 and H7 influenza viruses, it appears inevitable that determination of the cleavage site by sequencing or other methods will become the method of choice for initial assessment of the virulence of these viruses and incorporated into agreed definitions. This will have the advantage of reducing the number of *in vivo* tests, although at present the inoculation of birds is still required to confirm a negative result as the possibility of virus cultures containing mixed populations of viruses of high and low virulence cannot be ruled out.

Although all the truly highly pathogenic AI viruses isolated to date have been of H5 or H7 subtypes, at least two isolates, both of H10 subtype (H10N4 and H10N5), have been reported that would have fulfilled both the OIE and EU definitions for highly pathogenic AI viruses (76) as they killed 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. These viruses did not induce death or signs of disease when inoculated intranasally and did not have multiple basic amino acids at their haemagglutinin cleavage sites. It appears that some H10 AI viruses are nephrotropic and birds that die have high titre virus in their kidneys indicating a renal pathogenic mechanism (50). Conversely, four viruses have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low virulence (IVPI <1.2) when inoculated into 6-week-old chickens intravenously (33). Other anomalies are the Chile 2002 (57) and the Canada 2004 (42) H7N3 HPAI viruses, which show distinct and unusual cleavage site amino acid sequences of PEKPKTCSPLSRCRETR*GLF and PENPKQAYRKRMTR*GLF, respectively. These viruses appear to have arisen as a result of a recombination event between the HA gene and nucleoprotein gene and matrix gene, respectively, resulting in an insertion at the HA0 cleavage site of 11 amino acids for the Chile virus and 7 amino acids for the Canadian virus. They are both extremely virulent when inoculated into 6-week-old chickens intravenously.

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2   When birds are too sick to eat or drink, they should be killed humanely and scored as dead at the next observation.

3. Serological tests

a) Agar gel immunodiffusion

All influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens. This fact enables the presence or absence of antibodies to any influenza A virus to be detected by AGID tests. Concentrated virus preparations, as described above, contain both matrix and nucleocapsid antigens; the matrix antigen diffuses more rapidly than the nucleocapsid antigen. AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection. These have generally employed nucleocapsid-enriched preparations made from the chorioallantoic membranes of embryonated fowl eggs (7) that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 *g*. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses.

Tests are usually carried out using gels of 1% (w/v) agarose or purified agar and 8% (w/v) NaCl in 0.1 M phosphate buffer, pH 7.2, poured to a thickness of 2–3 mm in Petri dishes or on microscope slides. Using a template and cutter, wells of approximately 5 mm in diameter, and 2–5 mm apart, are cut in the agar. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. This will make a continuous line of identity between the known positive, the suspect serum and the nucleocapsid antigen. Approximately 50 µl of each reagent should be added to each well.

Precipitin lines can be detected after approximately 24–48 hours, but this may be dependent on the concentrations of the antibody and the antigen. These lines are best observed against a dark background that is illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well. Crossed lines are interpreted to be due to the test serum lacking identity with the antibodies in the positive control well.

b) Haemagglutination and haemagglutination inhibition tests

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply in the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.2, and red blood cells (RBCs) taken from a minimum of three SPF or SAN chickens and pooled in an equal volume of Alsever's solution. Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

- **Haemagglutination test**

i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

ii) Place 0.025 ml of virus suspension (i.e. infective allantoic fluid) in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.

iii) Make twofold dilutions of 0.025 ml volumes of the virus suspension across the plate.

iv) Dispense a further 0.025 ml of PBS to each well.

v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should be settled to a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination inhibition test**

i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus/antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about 20°C) or 60 minutes at 4°C.

319   v)   Add 0.025 ml of 1% (v/v) chicken RBCs to each well and after gentle mixing, allow the RBCs to settle
320   for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient
321   temperatures are high, by which time control RBCs should be settled to a distinct button.

322   vi)   The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The
323   agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same
324   rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to
325   show inhibition.

326   vii)   The validity of results should be assessed against a negative control serum, which should not give a
327   titre >1/4 (>2^2 or >$\log_2 2$ when expressed as the reciprocal), and a positive control serum for which the
328   titre should be within one dilution of the known titre.

329   HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2 4$ when
330   expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI
331   tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or
332      $\log_2 3$) or more. The meaning of a minimum positive titre should not be misinterpreted; it does not imply, for
333   example, that immunised birds with that titre will be protected against challenge or that birds with lower titres
334   will be susceptible to challenge.

335   Chicken sera rarely give nonspecific positive reactions in this test and any pretreatment of the sera is
336   unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs,
337   so this property should first be determined and then removed by adsorption of the serum with chicken RBCs.
338   This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, shaking gently and
339   leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 **g** for 2–5 minutes and
340   the adsorbed sera are decanted. Alternatively, RBCs of the avian species under investigation could be used.

341   The neuraminidase-inhibition test has been used to identify the AI neuraminidase type of isolates and to
342   characterise the antibody in infected birds. The procedure requires specialised expertise and reagents;
343   consequently this testing is usually done in an OIE Reference Laboratory. The DIVA (differentiating infected
344   from vaccinated animals) strategy used in Italy also relies on a serological test to detect specific anti-N
345   antibodies; the test procedure has been described (12).

346   Commercial ELISA kits that detect antibody against the nucleocapsid protein are available. Kits with an
347   indirect and competitive format have been developed and are now being used to detect of AIV-specific
348   antibodies. The kits should be validated for specific species being tested. Several different test and antigen
349   preparation methods are used. Such tests have usually been evaluated and validated by the manufacturer,
350   and it is therefore important that the instructions specified for their use be followed carefully.

351 **4.   Antigen capture and molecular techniques**

352 At present the conventional virus isolation and characterisation techniques for the diagnosis of AI remain the
353 methods of choice, for at least the initial diagnosis of AI infections. However, conventional methods tend to be
354 costly, labour intensive and slow. There have been enormous developments and improvements in molecular and
355 other diagnostic techniques, many of these have been applied to the diagnosis of AI infections.

356 **a)   Antigen detection**

357   There are several commercially available antigen-capture kits that can detect the presence of influenza A
358   viruses in poultry (49). Most of the kits are enzyme immunoassays and use a monoclonal antibody against
359   the nucleoprotein; they should be able to detect any influenza A virus. The main advantage of these tests is
360   that they can demonstrate the presence of AI within 15 minutes. The disadvantages are that they may lack
361   sensitivity, they may not have been validated for different species of birds, subtype identification is not
362   achieved and the kits are expensive. The tests should only be interpreted on a flock basis and not as an
363   individual bird test. Oropharyngeal or tracheal samples from clinically affected or dead birds provide the best
364   sensitivity. Nevertheless, the lack of sensitivity is a major drawback to the use of available antigen detection
365   tests. Chua *et al.* (14) evaluated five detection tests and showed overall sensitivities from 36.3% to 51.4%;
366   these authors pointed out that in terms of sensitivity using cloacal and tracheal swabs, the tests performed
367   less well with samples from waterfowl or wild birds than they did with samples from chickens.

368 **b)   Direct RNA detection**

369   Although, as demonstrated by the current definitions of HPNAI, molecular techniques have been used in the
370   diagnosis of AI for some time, recently there have been developments in their application for detection and
371   characterisation of AI virus directly from clinical specimens from infected birds. It is imperative that when
372   using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for
373   confirmatory laboratory diagnosis of avian influenza infections, stringent protocols are in place to prevent the

374   risk of cross-contamination between clinical samples. In addition, RT-PCR test methodologies should be
375   validated to the OIE standard (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious
376   diseases) using clinical material to demonstrate the tests as being 'fit for purpose' for application in a field
377   diagnostic setting, which may include the use of internal test standards. For example, PCR amplification of a
378   'house-keeping gene' that aids the normalisation of results by providing information on this target gene that
379   equates quantitatively to the presence of clinical sample present on the swab. The control reactions enable
380   greater confidence in the integrity of the molecular reactions, clinical samples and results.

381   RT-PCR techniques on clinical specimens can with the correctly defined primers, result in rapid detection
382   and subtype (at least of H5 and H7) identification, plus a cDNA product that can be used for nucleotide
383   sequencing (37, 54, 55). The real application of direct RT-PCR tests may be on rapidly identifying
384   subsequent outbreaks once the primary infected premises has been detected and the virus characterised.
385   This technique was used with success during the 2003 highly pathogenic AI outbreaks in The Netherlands.
386   Ring trials conducted recently in the European Union identified H5 and H7 conventional RT-PCR protocols
387   that were sufficiently sensitive to amplify directly from swabs obtained from HPAI-infected poultry (51).

388   Modifications on the use of RT-PCR have been applied to reduce the time for both identification of virus
389   subtype and sequencing. For example Spackman *et al.* (53) used a 'real time' single-step RT-PCR
390   primer/fluorogenic hydrolysis probe system to allow detection of AI viruses and determination of subtype H5
391   or H7. The authors concluded that the test performed well relative to virus isolation and offered a cheaper
392   and much more rapid alternative with diagnosis on clinical samples in less than 3 hours. The test provides
393   high sensitivity and specificity similar to virus isolation from tracheal and oropharyngeal swabs of chickens
394   and turkeys, but may lack sensitivity for detection of influenza A virus in faecal swabs, faeces and tissues in
395   some bird species, because of the presence of PCR inhibitors resulting in false negative result (18).
396   Incorporation of a positive internal control into the test will verify a proper test run.

397   Real-time RT-PCR, usually based around the hydrolysis probe or 'TaqMan' method for generation of the
398   target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial
399   diagnosis directly from clinical specimens. The method offers rapid results, with sensitivity and specificity
400   comparable to virus isolation, and these are ideal qualities for AI outbreak management, where the speed
401   with which an unequivocal diagnosis can be obtained is crucial for decision making by the relevant
402   Veterinary Authority. In addition, RT-PCR systems can be designed to operate in a 96-well format and
403   combined with high-throughput robotic RNA extraction from specimens (1).

404   The approach to diagnosis using real-time RT-PCR adopted in most laboratories has been based on initial
405   generic detection of AIV in clinical specimens, primarily by initially targeting the matrix (M) gene, which is
406   highly conserved for all type A influenza viruses, followed by specific real-time RT-PCR testing for H5 and
407   H7 subtype viruses. For subtype identification, primers used in TaqMan real-time RT-PCRs are targeted at
408   the HA2 region as this is relatively well conserved within the haemagglutinin genes of the H5 and H7
409   subtypes, and has served as the target region for H5 and H7. Spackman *et al.* (53) demonstrated specific
410   detection of these subtypes but cautioned that their H5 and H7 primer/probe sequences had been designed
411   for the detection of North American H5 and H7 isolates and might not be suitable for all H5 and H7 isolates.
412   This proved to be the case. Slomka *et al.* (52) described modification of the H5 oligonucleotide sequences
413   used by Spackman *et al.* (53) to enable the detection of this Asian lineage HPAI H5N1 AI virus and other
414   Eurasian H5 AI viruses that have been isolated within the past decade in both poultry and wild birds. This
415   validated Eurasian H5 real-time RT-PCR has proved valuable in the investigation of many H5N1 HPAI
416   clinical specimens submitted to International Reference Laboratories from Europe, Africa and Asia since
417   autumn 2005 (52).

418   One of the problems with rapidly emerging new tests is that methods and protocols may be developed and
419   reported without the test being properly validated. This has been addressed for some of the real-time RT-
420   PCR protocols (52, 56). In the European Union, National Reference Laboratories have collaborated to define
421   and validate protocols that can be recommended for use within the European Union (51, 52).

422   Real-time RT-PCR protocols have been described that amplify regions across the cleavage site of the HA0
423   gene. This may result in useful tests for specific viruses. For example, Hoffman *et al.* (27) have described a
424   real-time RT-PCR test specific to the Asian HPAI H5N1 Quinghai clade 2.2 viruses that represents a rapid
425   means of determining the pathotype for this subgroup of H5N1 HPAI viruses without sequencing.

426   Modifications on the straightforward RT-PCR method of detection of viral RNA have been designed to
427   reduce the effect of inhibitory substances in the sample taken, the possibility of contaminating nucleic acids
428   and the time taken to produce a result. For example, nucleic acid sequence-based amplification (NASBA)
429   with electrochemiluminescent detection (NASBA/ECL) is a continuous isothermal reaction in which
430   specialised thermocycling equipment is not required. NASBA assays have been developed for the detection
431   of AI virus subtypes H7 and H5 in clinical samples within 6 hours (15, 29). The loop-mediated isothermal
432   amplification (LAMP) system for H5 detection appeared to show high sensitivity and reliable specificity (28).

433 It seems highly likely that within a very short time molecular-based technology will have developed
434 sufficiently to allow rapid 'flock-side' tests for the detection of the presence of AI virus, specific subtype and
435 virulence markers. The extent to which such tests are employed in the diagnosis of AI will depend very much
436 on the agreement on and adoption of definitions of statutory infections for control and trade purposes.

437 # C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

438 It is important that vaccination alone is not considered the solution to the control of NAI or LPAI subtypes if
439 eradication is the desired result. Without the application of monitoring systems, strict biosecurity and depopulation
440 in the face of infection, there is the possibility that these viruses could become endemic in vaccinated poultry
441 populations. Long-term circulation of the virus in a vaccinated population may result in both antigenic and genetic
442 changes in the virus and this has been reported to have occurred in Mexico (31).

443 Experimental work has shown, for both NAI and LPAI that vaccination protects against clinical signs and mortality,
444 reduces virus shedding and increases resistance to infection, protects from diverse field viruses within the same
445 hemagglutinin subtype, protects from low and high challenge exposure, and reduces excretion and thus contact
446 transmission of challenge virus (13, 19, 59, 65). However, the virus is still able to infect and replicate in clinically
447 healthy vaccinated birds. Most of the work evaluating vaccines has been done in chickens and turkeys and some
448 care must be taken in extrapolating the results obtained to other species. For example, in an experimental system
449 using HPAI H7N7 as a challenge virus it was shown for chickens and ringed teal ducks, *Callonetta leucophrys*,
450 that vaccination sufficiently reduced excretion and increased the infective dose that transmission between birds
451 was dramatically reduced, but for golden pheasants, *Chrysolophus pictus*, while giving clinical protection
452 vaccination had no effect on excretion of challenge virus and no influence on transmission (69, 70). In some
453 countries, vaccines designed to contain or prevent NAI are specifically banned or discouraged by government
454 agencies because it has been considered that they may interfere with stamping-out control policies. However,
455 most AI control regulations reserve the right to use vaccines in emergencies.

456 Live conventional influenza vaccines against any subtype are not recommended.

457 ● **Conventional vaccines**

458 Conventionally, vaccines that have been used against NAI or LPAI have been prepared from infective allantoic
459 fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil.

460 The existence of a large number of virus subtypes, together with the known variation of different strains within a
461 subtype, pose serious problems when selecting strains to produce influenza vaccines, especially for LPAI. In
462 addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly
463 prior concentration. While some vaccination strategies have been to produce autogenous vaccines, i.e. prepared
464 from isolates specifically involved in an epizootic, others have been to use vaccines prepared from viruses
465 possessing the same haemagglutinin subtype that yield high concentrations of antigen. For example, in the USA,
466 some standardisation of the latter has been carried out in that the Center for Veterinary Biologics have
467 propagated and hold influenza viruses of several subtypes for use as seed virus in the preparation of inactivated
468 vaccines (6).

469 Since the 1970s in the USA, there has been some use of inactivated vaccines produced under special licence on
470 a commercial basis (25, 35, 43). These vaccines have been used primarily in turkeys against viruses that are not
471 highly pathogenic, but which may cause serious problems, especially in exacerbating circumstances. Significant
472 quantities of vaccine have been used (26, 35). In recent years in the US, most of the special license inactivated
473 vaccine has used in breeder turkeys to protect against H1 and H3 swine influenza viruses (58). Conventional
474 vaccination against the prevailing strain of LPAI has also been used in Italy for a number of years (17).
475 Vaccination against H9N2 infections has been used in Pakistan (39), Iran (72) and the People's Republic of China
476 (32) and several countries in the Middle East.

477 Inactivated vaccine was prepared from the LPNAI virus of H7N3 subtype responsible for a series of outbreaks in
478 turkeys in Utah in 1995 and used, with other measures, to bring the outbreaks under control (26). Similarly in
479 Connecticut in 2003 vaccination of recovered hens and replacement pullets with a H7N2 or H7N3 vaccine was
480 implemented following an outbreak of LPNAI caused by a H7N2 virus (61).

481 Vaccination against HPNAI of H5N2 subtype was used in Mexico following outbreaks in 1994–1995 (21, 22, 31),
482 and against H7N3 subtype in Pakistan (38) following outbreaks in 1995. In Mexico, the HPNAI virus appears to
483 have been eradicated, but LPNAI virus of H5N2 has continued to circulate, while in Pakistan highly pathogenic AI
484 viruses genetically close to the original highly pathogenic AI virus were still being isolated in 2001 (66) and 2004.
485 Following the outbreaks of HPNAI caused by H5N1 virus in Hong Kong in 2002 (48) a vaccination policy was
486 adopted there using an H5N2 vaccine. In 2004 the widespread outbreaks of highly pathogenic AI H5N1 in some

487 countries of South-East Asia resulted in emergency and prophylactic vaccination being used in the People's
488 Republic of China, Indonesia and Vietnam. Inactivated H7N7 AI vaccine was used in North Korea during 2005 to
489 control a HPAI outbreak. Prophylactic vaccination has also been used in limited areas in Italy to aid the control of
490 H5 and H7 LPNAI viruses. Similar preventive vaccination has been allowed in outdoor poultry and in zoo birds in
491 several European Union countries in recent years.

492 • **Recombinant vaccines**

493 Recombinant vaccines for AI viruses have been produced by inserting the gene coding for the influenza virus
494 haemagglutinin into a live virus vector and using this recombinant virus to immunise poultry against AI (60).
495 Recombinant live vector vaccines have several advantages: [1] they are live vaccines able to induce both humoral
496 and cellular immunity, [2] they can be administered to young birds and induce an early protection, e.g. the fowl
497 poxvirus can be administered at 1 day of age, is compatible with the Marek's disease vaccine, and provides
498 significant protection 1 week later, [3] they enable differentiation between infected and vaccinated birds, since, for
499 example, they do not induce the production of antibodies against the nucleoprotein or matrix antigens that are
500 common to all AI viruses. Therefore, only field-infected birds will exhibit antibodies in the AGID test or ELISA tests
501 directed towards the detection of influenza group A (nucleoprotein and/or matrix) antibodies. However, these
502 vaccines have limitations in that they will replicate poorly and induce only partial protective immunity in birds that
503 have had field exposure to or vaccination with the vector virus, i.e. fowl poxvirus or infectious laryngotracheitis
504 viruses for currently available recombinant vaccines (34, 62). If used in day-old or young birds the effect of
505 maternal antibodies to the vector virus on vaccine efficacy may vary with the vector type. In the case of fowl
506 poxvirus recombinant vaccine, it has been reported that effective immunisation was achieved when given to 1-
507 day-old chicks with varying levels of maternal immunity (4). However, when very high levels of maternal
508 antibodies are anticipated due to previous infection or vaccination, the efficacy of the fowlpox vector vaccine in
509 such day-old chicks should be confirmed. In addition, because the vectors are live viruses that may have a
510 restricted host range (for example infectious laryngotracheitis virus does not replicate in turkeys) the use of these
511 vaccines must be restricted to species in which efficacy has been demonstrated.

512 The use of recombinant vaccines is restricted to countries in which they are licensed and are legally available.
513 The recombinant fowlpox-AI-H5 vaccine has been licensed in El Salvador, Guatemala, Mexico, China and the
514 USA (59, 82). Recombinant fowl poxvirus vaccines containing H5 HA have been prepared and evaluated in field
515 trials (8, 23, 44, 63), but the only field experience with this vaccine has been in Mexico, El Salvador, Guatemala
516 and China where it has been used in the vaccination campaign against the H5N2 LPAI and H5N1 HPAI viruses.
517 Between 1995 and 2006, Mexico used more than 1.788 billion doses of inactivated H5N2 vaccine in their H5N2
518 control programme (73, 74). In addition, Mexico, Guatemala and El Salvador have used over 1.6 billion doses of
519 the recombinant fowlpox-AI-H5 vaccine for control of H5N2 LPNAI from 1997 to 2005 and China used 606 million
520 doses in 2005 (82).

521 Newcastle disease virus can also be used as a vector for expressing influenza HA genes (40). A recombinant
522 Newcastle disease vaccine virus (clone 30) containing and expressing an H5 HA gene was shown to protect
523 chickens against challenge with either virulent Newcastle disease virus or an HPAI H5N2 virus (71). A similar
524 recombinant virus based on Newcastle disease virus vaccine strain La Sota and expressing the Asian lineage H5
525 HA gene was produced in China (24) and reported to be efficacious in protection studies with either virus. This
526 latter virus has been licensed in China and used widely as one of the four H5 vaccines allowed under the
527 compulsory vaccination policy currently in place that resulted in the vaccination of 8.2 billion birds between
528 January and September 2006 (36). As with other recombinant vaccines it seems doubtful that this vaccine will be
529 appropriate for use in older birds that are well-immunised against Newcastle disease and it is not clear how much
530 the efficacy will be affected by the presence of maternal immunity to either the vector or the AI HA in young
531 chicks. A baculovirus-expression system has been used to produce recombinant H5 and H7 antigens for
532 incorporation into vaccines (75). DNA encoding H5 haemagglutinin has been evaluated as a potential vaccine in
533 poultry (30).

534 • **Detection of infection in vaccinated flocks and vaccinated birds**

535 A strategy that allows differentiation of infected from vaccinated animals (DIVA), has been put forward as a
536 possible solution for the eventual eradication of NAI without involving mass culling of birds and the consequent
537 economic damage that would do, especially in developing countries (20). This strategy has the benefits of
538 vaccination (less virus in the environment), but the ability to identify infected flocks would still allow the
539 implementation of other control measures, including stamping out. DIVA strategies use two broad detection
540 strategies within the vaccinated population: 1) detection of influenza A virus, or 2) detection of antibodies against
541 influenza A virus infection. At the flock level, a simple method is to regularly monitor sentinel birds left
542 unvaccinated in each vaccinated flock, but this approach does have some management problems, particularly in
543 identifying the sentinels in large flocks. As an alternative or adjunct system, testing for field exposure may be
544 performed on the vaccinated birds either by detection of field virus or antibodies against the virus. In detection of
545 the field virus, oropharyngeal or cloacal swabs from normal daily mortality or sick birds can be tested, individually

546 or as pools, by molecular methods, such as real-time RT-PCR or antigen-capture enzyme immunoassay of the
547 vaccinated populations.

548 In order to use serological DIVA, vaccination systems that enable the detection of field exposure in vaccinated
549 populations should be used. Several systems have been developed in recent years. These include the use of a
550 vaccine containing a virus of the same haemagglutinin (H) subtype but a different neuraminidase (N) from the
551 field virus. Antibodies to the N of the field virus act as natural markers of infection. This system has been used in
552 Italy following the re-emergence of a LPNAI H7N1 virus in 2000. In order to supplement direct control measures,
553 a 'DIVA' strategy was implemented using a vaccine containing H7N3 to combat an H7N1 field infection.
554 Vaccinated and field exposed birds were differentiated using a serological test to detect specific anti-N antibodies
555 (10, 11). The same strategy was used to control LPNAI caused by H7N3 in Italy in 2002–2003 (9), in this case
556 with an H7N1 vaccine. In both cases vaccination with stamping out using this DIVA strategy resulted in
557 eradication of the field virus. Problems with this system would arise if a field virus emerges that has a different N
558 antigen to the existing field virus or if subtypes with different N antigens are already circulating in the field.

559 Alternatively the use of vaccines that contain only HA, e.g. recombinant vaccines, allows classical AGID and NP-
560 or matrix-based ELISAs to be used to detect infection in vaccinated birds. For inactivated vaccines, a test that
561 detects antibodies to the nonstructural virus protein has been described (67).This system is yet to be validated in
562 the field.

563 • **Production of conventional vaccines**

564 The information below is based primarily on the experiences in the USA and the guidance and policy for licensing
565 avian influenza vaccines in that country (68). The basic principles for producing vaccines, particularly inactivated
566 vaccines, are common to several viruses e.g. Newcastle disease (Chapter 2.3.14).

567 Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine
568 production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be
569 supplemented by national and regional requirements.

570 The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If
571 HPNAI virus is used challenge studies, that part of the facility where this work is done should meet the
572 requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.2.

573 **1. Seed management**

574 **a) Characteristics of the seed**

575 For any subtype, only well characterised influenza A virus of proven low pathogenicity, preferably obtained
576 from an international or national repository, should be used to establish a master seed for inactivated
577 vaccines. HPAI viruses should not be used as seed virus for AI vaccine.

578 **b) Method of culture**

579 A master seed is established, and from this, a working seed. The master seed and working seed are
580 produced in SPF or SAN embryonated eggs. The establishment of a master culture may only involve
581 producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised
582 aliquots (0.5 ml).

583 **c) Validation as a vaccine**

584 The master seed should be checked after preparation for sterility, safety, potency and absence of specified
585 extraneous agents.

586 **2. Method of manufacture**

587 For vaccine production, a working seed, from which batches of vaccine are produced, is first established in SPF
588 or SAN embryonated eggs by expansion of an aliquot of master seed to a sufficient volume to allow vaccine
589 production for 12–18 months. It is best to store the working seed in liquid form at below –60°C as lyophilised virus
590 does not always multiply to high titre on subsequent first passage.

591 The inactivated influenza vaccines prepared from conventional virus are produced in embryonated fowl eggs. The
592 method of production is basically that of propagating the virus aseptically; all procedures are performed under
593 sterile conditions.

594 It is usual to dilute the working seed in sterile isotonic buffer (e.g. PBS, pH 7.2), so that about 10^3–10^4 EID$_{50}$ (50%
595 egg-infective dose) in 0.1 ml are inoculated into each allantoic cavity of 9 to 11-day-old embryonated SPF or SAN
596 fowl eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be
597 discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure
598 maximum yield with the minimum number of embryo deaths.

599 The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the
600 allantoic fluids collected by suction. The inclusion of any yolk material and albumin should be avoided. All fluids
601 should be stored immediately at 4°C and tested for bacterial contamination.

602 In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a
603 typical final concentration is 1/1000) or beta-propiolactone (a typical final concentration is 1/1000–1/4000). The
604 time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are formulated with
605 non-concentrated inactivated allantoic fluid (active ingredient). However, active ingredients may be concentrated
606 for easier storage of antigen. The active ingredient is usually emulsified with mineral or vegetable oil. The exact
607 formulations are generally commercial secrets.

608 ### 3. In-process control

609 For inactivated vaccines, the completeness of the inactivation process should be tested in embryonated eggs,
610 taking at least 10 aliquots of 0.2 ml from each batch and passaging each aliquot at least two times through SPF or
611 SAN embryos.

612 ### 4. Batch control

613 Most countries have published specifications for the control of production and testing of vaccines, which include
614 the definition of the obligatory tests on vaccines during and after manufacture.

615 **a) Sterility**

616 Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

617 **b) Safety**

618 For inactivated vaccines, a double dose is administered by the recommended route to ten 3-week-old birds,
619 and these are observed for 2 weeks for absence of clinical signs of disease or local lesions.

620 **c) Potency**

621 Potency of avian influenza vaccine is generally evaluated by testing the ability of the vaccine to induce a
622 significant HI titre in SPF or SAN birds. Conventional potency testing involving the use of three diluted doses
623 and challenge with virulent virus (e.g. chapter 2.3.14) may also be used for vaccines prepared to give
624 protection against HPNAI or LPNAI subtypes. For inactivated vaccines to other subtypes where virulent
625 viruses are not available, potency tests may rely on the measurement of immune response or challenge and
626 assessment of morbidity and quantitative reduction in challenge virus replication in respiratory
627 (oropharyngeal or tracheal) and intestinal (cloaca) tracts. Assessment of haemagglutinin antigen content
628 (77) could allow *in vitro* extrapolation to potency for subsequent vaccine batches.

629 **d) Stability**

630 When stored under the recommended conditions, the final vaccine product should maintain its potency for at
631 least 1 year. Inactivated vaccines must not be frozen.

632 **e) Preservatives**

633 A preservative may be used for vaccine in multidose containers.

634 **f) Precautions (hazards)**

635 Care must be taken to avoid self-injection with oil emulsion vaccines.

636 ### 5. Tests on the final product

637 **a) Safety**

638 See Section C.4.b. above

b) Potency

See Section C.4.c. above.

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865   **NB:** There are OIE Reference Laboratories for Avian influenza (see Table in Part 3 of this *Terrestrial Manual* or
866   consult the OIE Web site for the most up-to-date list: www.oie.int).

AVIAN MYCOPLASMOSIS

(*Mycoplasma gallisepticum, M. synoviae*)

SUMMARY

Definition of the disease: *Avian mycoplasmosis is caused by several pathogenic mycoplasmas of which* Mycoplasma gallisepticum (*MG*) *and* M. synoviae (*MS*) *are the most important; they are the only ones listed by the OIE.*

Description of the disease: *MG causes chronic respiratory disease of domestic poultry, especially in the presence of management stresses and/or other respiratory pathogens. Disease is characterised by coryza, conjunctivitis, sneezing, and by sinusitis, particularly in turkeys and game birds. It can result in loss of production and downgrading of meat-type birds, and loss of egg production. MS may cause respiratory disease, synovitis, or may result in a silent infection. MG and MS strains vary in infectivity and virulence, and infections may sometimes be unapparent.*

Identification of the agent: *MG and MS can be identified by immunological methods after isolation in mycoplasma media or by detection of their DNA in field samples or cultures.*

Samples for isolation can be swabs of organs or tissues, exudates, diluted tissue homogenates, aspirates from the infraorbital sinuses or joint cavities, or material from egg yolk or embryos. Clinical signs and lesions will influence the sample selection. Broth and agar are used for isolation, but it is normally necessary to obtain mycoplasma colonies on agar before attempting identification. Basic biochemical tests can be helpful in preliminary classification of isolates but final identification is by immunological tests, the most satisfactory being fluorescent antibody and immunoperoxidase tests.

DNA detection methods based on the polymerase chain reaction are used in specialised laboratories. Once validated, they can be used on swab material or cultures.

Serological tests: *Several serological tests are used to detect MG or MS antibodies, but due to variations in specificity and sensitivity, they are recommended for flock screening rather than for testing individuals.*

*The most commonly used are the rapid serum agglutination (*RSA*) test, the enzyme-linked immunosorbent assay (*ELISA*) and the haemagglutination inhibition (*HI*) test. In the RSA test, sera are mixed with commercially produced stained antigen and sera that react within 2 minutes are heated at 56°C for 30 minutes and retested. Sera that still react, especially when diluted, are considered positive and are tested by either ELISA or HI for confirmation. Several commercial MG and MS antibody ELISA kits are available.*

Requirements for vaccines and diagnostic biologicals: *Although the preferred method of control is maintenance of MG- and MS-free flocks, both live and inactivated vaccines are used in chickens. Vaccination should be considered only on multi-age sites where infection is inevitable. The normal use is to prevent egg-production losses in commercial layers, although vaccines may also be used to reduce egg transmission in breeding stock or to aid MG eradication on multi-age sites. It is important to vaccinate before field challenge occurs.*

Available live vaccines for MG are produced from the F strain, and, more recently, strains ts-11 and 6/85, which are apathogenic strains with improved safety characteristics. Administration of the F strain by the intranasal or eyedrop route is preferred, but aerosol or drinking water administration may be used. The eyedrop method is recommended for ts-11, and a fine spray for 6/85. Pullets are generally vaccinated between 12 and 16 weeks of age. One dose is sufficient and vaccinated birds

remain permanent carriers. Long-term use of the F strain on multi-age sites results in displacement of field strains. The ts-11 strain has been successfully used to eradicate F strain in multi-age commercial layers. A live MS vaccine has been produced from the MS-H strain and should be administered by eyedrop.

Bacterins consist of a concentrated suspension of MG organisms in an oil emulsion. They are administered parenterally to pullets at 12–16 weeks of age, usually subcutaneously in the neck. Two doses are desirable. Bacterins are effective in preventing egg-production losses and respiratory disease, but they do not prevent infection with wild-type MG. A similar bacterin has been licensed in the United States of America for MS, but it is not widely used.

A. INTRODUCTION

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) belong to the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae. It should be noted, however, that *M. meleagridis* and *M. iowae* can also cause disease in poultry, but MG and MS are considered to be the most important of the pathogenic mycoplasmas, and both occur world-wide.

MG infection is particularly important in chickens and turkeys as a cause of respiratory disease and decreased meat and egg production (3, 24). It can also cause upper respiratory disease in game birds. More recently MG has been recognised in North America in house finches as a cause of conjunctivitis (27). In poultry the infection is spread vertically through infected eggs and horizontally by close contact; the MG nucleic acid has been identified in environmental samples (30). Other methods of spread are less well documented.

The clinical signs of MG in infected poultry can vary from subclinical to obvious respiratory signs including coryza, conjunctivitis, coughing and sneezing. Nasal exudate, rales and breathing through the partially open beak may occur. Unilateral or bilateral sinusitis may also be a feature, particularly in turkeys and game birds and the infraorbital sinuses may become so swollen that the eyelids are closed. Conjunctivitis, with frothy ocular exudate is also a common feature in turkeys and game birds, and sometimes in chickens. In turkeys there is often soiling of the wing feathers as the result of attempts to remove exudate from the eyes. Infected finches may reveal ocular and nasal discharge and swollen eyelids in addition to the conjunctivitis.

Mycoplasma gallisepticum may be associated with acute respiratory disease in chickens and turkeys, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly affected by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and/or bacteria such as *Escherichia coli*. In turkeys there is synergism with avian pneumovirus infection. A more chronic form of the disease may occur and can cause reduced egg production in breeders and layers.

Lesions of the respiratory tract take the form initially of excess mucous exudate followed by catarrhal and caseous exudate, which may form amorphous masses in the air sacs. In turkeys and game birds the swollen infraorbital sinuses contain mucoid to caseous exudate.

MG or MS disease in chickens may superficially resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease (Chapter 2.3.14) and avian infectious bronchitis (Chapter 2.3.2). These may be present in mixed infection with MG or MS. Infections with *Haemophilus paragallinarum* (now *Avibacterium paragallinarum*)*,* and *Pasteurella multocida*, should also be ruled out. MG in turkeys may be confused with avian pneumovirus infections and the presence of sinusitis may also suggest infection with *Pasteurella multocida*, *Chlamydia* (Chapter 2.3.1) or MS. Infectious synovitis caused by MS should be differentiated from *Staphylococcus aureus* infection and from infectious tenosynovitis caused by reovirus.

Chickens with infectious synovitis may exhibit pale combs, lameness and retarded growth. Swellings may occur around joints. Greenish droppings containing large amounts of urates are commonly seen. Joints may contain a viscous, creamy to grey exudate in the joint and along tendon sheaths, along with hepatosplenomegaly and mottled, swollen kidneys (17). Respiratory signs and lesions are similar to those observed with MG, except that they are generally milder, and, as with MG, there is a synergistic effect with other respiratory agents (19). MS strains exhibit significant variability with respect to their virulence and tissue tropism (18, 22, 26).

B. DIAGNOSTIC TECHNIQUES

The presence of MG or MS can be confirmed by isolating the organism in a cell-free medium or by detecting its DNA directly in infected tissues or swab samples. Serological tests are also widely used for diagnosis. When

results are equivocal the birds are usually resampled, although chicken embryos or chickens may be inoculated with suspect material.

1. Identification of the agent

- **Culture**

  Samples are taken from live birds, fresh carcasses or the carcasses of birds that have been frozen when fresh. From live birds, swabs may be taken from the choanal cleft, oropharynx, oesophagus, trachea, eyes, cloaca and phallus. In the case of dead birds, samples may be taken from the nasal cavity, infraorbital sinus, trachea, or air sacs. Exudates can be aspirated from the infraorbital sinuses and joint cavities.

  Samples may also be collected from dead-in-shell embryos or chickens or poults that have broken the shell but failed to hatch. Samples can be taken from the inner surface of the vitelline membrane, and from the oropharynx and air sacs of the embryo.

  All samples should be examined as soon as possible after collection. If transportation is necessary, small pieces of tissue should be placed in mycoplasma broth, or swabs should be vigorously agitated in 1–2 ml of mycoplasma broth and then discarded. Alternatively, the swabs can be dipped in mycoplasma broth before the specimens are taken (36) and then replaced in the swab holders for transportation. An ice pack or some other means of chilling should be included as MG and MS die rapidly at room temperature. Serial dilutions of specimens in mycoplasma broth may be of value because the presence of specific antibodies or antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth unless they are diluted out.

  Several suitable culture media have been formulated (10) and those suitable for isolation of avian mycoplasmas can be purchased from Mycoplasma Experience, Reigate, Surrey, United Kingdom. Mycoplasma media generally contain a protein digest and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors. It is important that each new batch of medium be tested with recently isolated MG cultures of low *in-vitro* passage because some components, especially the yeast extract and the serum may vary in their ability to support growth.

  The medium developed by Frey *et al.* is widely used in the United States of America (USA) and other countries for isolation of MG and MS (2, 11). Nicotinamide adenine dinucleotide (NAD) is a growth requirement for the primary isolation of MS, but it may be omitted from the medium for the cultivation of MG.

  The following broth and agar media are also satisfactory:

  - Part A: Pleuropneumonia-like organism (PPLO) broth base without crystal violet (Difco) (14.7 g); distilled or deionised water (700 ml).

  - Part B: Pig serum (heated at 56°C for 1 hour) (150 ml): 25% (w/v) fresh yeast extract (100 ml); 10% (w/v) glucose solution (10 ml); 5% (w/v) thallous acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). Thallous acetate can be toxic to humans and the precautions for its use should be followed. The pH is adjusted to 7.8. Pig serum may be replaced by horse serum, but it is important to ascertain that it supports the growth of MG.

  Part A is autoclaved at 121°C, at 1 atmospheric pressure for 15 minutes and, after cooling, is added to Part B, which has previously been sterilised by filtration.

  For the corresponding solid medium, 10 g of purified agar, known to support the growth of MG, is added to part A above. The mixture is autoclaved as before and kept in a water bath at 56°C. The constituents of part B, omitting the phenol red, are mixed separately and then incubated at 56°C. Parts A and B are mixed carefully to avoid the production of air bubbles, and are dispensed into 50 mm dishes using 7–9 ml/dish. Excess surface moisture can be removed by a short incubation at 37°C. Plates are stored in an airtight container at approximately 4°C for up to 2 weeks.

  Fresh yeast extract is available commercially, although it is preferable to prepare it 'in-house' by taking active dry baker's yeast (250 g) and suspending it in distilled water (1 litre). This is heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 *g*. The supernatant fluid is decanted and adjusted to pH 8.0 with 0.1 M NaOH. This is clarified by centrifugation or by filtration, and then sterilised by filtration. The extract is stored at –20°C. Reagent grade glucose (10 g) is dissolved in distilled or deionised water (100 ml) and adjusted to pH 7.8–8.0 with 0.1 M NaOH. It is sterilised by filtration and stored at 4°C. Reagent grade thallous acetate is dissolved (5 g) in distilled or deionised water (100 ml), filter-sterilised and stored at –20°C. Penicillin solution (10^6 IU benzyl penicillin in 5 ml distilled water) is stored at 4°C, and has a shelf life of 1 week. For isolation from heavily contaminated samples, penicillin concentration can be increased to

2000 units/ml or ampicillin, 0.5–1.0 mg/ml, maybe used instead. Phenol red (0.1 g) is ground in 0.1 M NaOH (2.8 ml), and then made up to 100 ml in sterile distilled water and autoclaved at 115°C at 1 atmosphere for 30 minutes. It is stored at 4°C. (Note: Thallous acetate is highly toxic and care should be taken, especially when preparing the stock solution.)

Specimens are inoculated on to mycoplasma agar and into broth. Solid medium may help detection of slow-growing mycoplasma colonies, which can be overgrown by saprophytes in broth. It may be necessary to make serial dilutions up to 10^{-3} for successful isolation. Inoculated plates are incubated at 37°C in sealed containers. Increased humidity and CO_2 tension in the atmosphere have been reported to enhance growth; these conditions may be obtained by the inclusion of damp paper or cotton wool, and by flushing the container with 5–10% CO_2 in nitrogen, by placing a lighted candle in the container, or by using a CO_2 incubator or suitable gas-generating system.

The caps of liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates are examined daily for colonies with a stereoscopic microscope; after that they are examined less frequently. Cultures from field material should not be discarded as negative for at least 20 days.

Broth medium should be examined daily for acidity, indicated by a change from red to orange or yellow in the indicator. Any observable growth is subcultured on to solid medium immediately. Even if no colour change occurs, subculture on to solid medium should be made after 7–10 days or earlier as the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by MG.

Mycoplasma colonies on solid medium can usually be recognised, although they may not have the typical 'fried egg' appearance. Bacterial colonies may appear on the first passage, but they are often more pigmented and fail to passage on mycoplasma media.

Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG or MS and necessitate purification of the culture by cloning.

Immunological and DNA detection methods can be used to identify mycoplasma isolates. They include the indirect fluorescent antibody (IFA) and immunoperoxidase (IP) tests, both of which are simple, sensitive, specific and rapid to perform; growth inhibition (GI); and metabolism inhibition (MI). Purified (cloned) cultures are required for the GI and MI tests, but not for the IFA or IP test. IFA and IP can detect the presence of more than one species of mycoplasma, as the colonies specific for the antiserum will react while the others will not. However, *M. imitans*, a mycoplasma species that is serologically related to MG and that presents the same biochemical properties has been isolated from ducks, geese and sometimes from other nondomestic bird species in some countries. It may be distinguished from MG by use of a PCR-RFLP (polymerase chain reaction/restriction fragment length polymorphism), as described by Kempf (16). Alternatively, colonies of the isolate can be examined by immunofluorescence using serial dilutions of antisera to MG and *M. imitans* in parallel. The homologous antiserum should have a considerably higher titre.

DNA detection methods for identifying MG or MS directly in tissues or for identifying laboratory isolates are discussed below and are usually based on the PCR.

In certain circumstances where results of the above methods are not conclusive, inoculation of chick embryos or bioassays in live chicks may be appropriate. However these techniques are time-consuming and costly and tend to have been replaced by PCR technology, although they remain a useful research tool. The specimens required for inoculation of chicken embryos are the same as those used for the inoculation of artificial media. They are prepared in broth from which thallous acetate is omitted, incubated for 30–60 minutes at 37°C, and then a 0.05–0.1 ml aliquot is inoculated into the yolk sac of several 6–8-day-old chicken embryos derived from mycoplasma-free flocks. The eggs are candled daily and embryos that die within 24 hours of inoculation are discarded. Any further dead embryos are kept refrigerated until cultured and those surviving after 5 days are placed at 4°C for 4 hours to kill them and to reduce haemorrhages on opening. The yolk is subcultured into broth and on to agar. Yolk lipid tends to obscure colonies so it is essential to streak the yolk thinly or, preferably, to dilute it first in mycoplasma broth.

Bioassays may be performed by the inoculation of a homogenate of suspect material into at least four 8–16 week-old susceptible mycoplasma-free chickens. Diagnosis is confirmed by the recovery of the mycoplasma from these birds, demonstration of its DNA and/or the demonstration of specific antibodies (28).

- **Immunological methods**

  Immunofluorescence and IP procedures for diagnosis are generally applied to suspect laboratory isolates rather than directly to infected exudates or tissues. This is because the organisms are too small to recognise

conclusively under the light microscope and because the corresponding negative and positive control exudate/tissue is unlikely to be readily available.

a) Indirect fluorescent antibody test

The recommended technique for the IFA test (31) requires an agar culture of the unknown isolate, consisting of numerous small discrete colonies, a known MG or MS culture as a positive control, and a culture of a another mycoplasma species, such as *M. gallinaceum* or *M. gallinarum* as a negative control. Also required are polyclonal rabbit anti-MG or MS serum, a normal rabbit serum and an anti-rabbit immunoglobulin fluorochrome-conjugated serum. Sera may be prepared in species other than rabbits, but monoclonal antibodies (MAbs) should not be used because MG or MS demonstrates variable expression of its surface epitopes and an MAB may fail to recognise the organism. Suitable working dilutions in sterile phosphate buffered saline (PBS; 0.01 M, pH 7.2) of the anti-MG or MS serum and the conjugate are first determined by cross-titration, and are selected for use at two-to-four-fold dilutions less than the actual end-points. These are applied to the colonies of mycoplasmas to be identified that have been previously grown on agar plates as indicated below.

- **Test procedure**

i) From colony-bearing agar plates, cut blocks of about 1.0 × 0.5 cm and place them on to labelled microscope slides with the colonies uppermost.

ii) To make subsequent orientation possible, cut off the lower right hand corner of the blocks. One block with the unknown isolate, a block with the known MG culture, a block with the known MS culture and a block with a different but known mycoplasma culture are placed on one slide. A block of the unknown isolate is placed on another slide.

iii) Add a drop of suitably diluted MG (or MS) antiserum to the surface of each block of the first slide and add normal rabbit serum to the single block on the second slide.

iv) Incubate all blocks for 30 minutes at room temperature in a humid atmosphere.

v) Place each block in a labelled tube containing PBS, pH 7.2 and wash for 10 minutes in a rotary mixer, then similarly rewash, and finally return the blocks to the original microscope slides.

vi) Blot excess moisture from the sides of the blocks. Add one drop of the diluted conjugate to each block, and incubate and wash as before.

vii) Return the blocks to their original slides, and examine the colonies by incident light using fluorescence microscopy.

Interpretation of the results is subjective and requires some expertise; comparisons with the controls are essential, and they must give the correct reactions.

Some laboratories use fluorescein-conjugated antiserum in a direct fluorescent antibody test (DFA). A technique that is widely used for DFA is one in which the reagents are applied successively within stainless steel cylinders placed on the original mycoplasma agar plate (32). Although this is quick and easy to perform, the results obtained are less specific than using the indirect method, which is therefore preferred.

b) Indirect immunoperoxidase test

This involves a similar principle to the IFA test except that the binding of specific antibodies to colonies *in situ* is detected by adding an anti-rabbit immunoglobulin that has been conjugated to the enzyme peroxidase. A positive reaction is then developed by adding an appropriate substrate which, on oxidation, produces coloured colonies. An immunobinding procedure can also be used in which the test colonies are blotted on to nitrocellulose (21) and then reacted in a similar manner. As with IFA, polyclonal sera should be used for serotyping isolates by IP. The advantage of the IP test over immunofluorescence is that the IP test does not require an expensive fluorescence microscope.

c) Growth inhibition test

In the GI test, the growth of mycoplasmas is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be high-titred, monospecific and prepared in mammalian hosts as poultry sera do not always inhibit mycoplasma growth efficiently. The organism under test must be in pure culture (cloned) and several dilutions should be tested; a concentration of 10^4 colony-forming units (CFU/ml) is optimal. The rate of growth of the organism may influence growth inhibition, and it is helpful to retard growth initially by incubating at 27°C for 24 hours, followed by incubation at 37°C thereafter. Details of the test and its interpretation are published elsewhere (6).

• **Nucleic acid detection methods**

An alternative to conventional culture and identification is the use of specific DNA detection methods. MG or MS may be detected by hybridisation with DNA probes, but now it is much more common to use the PCR to amplify specific portions of DNA in the test material. At least one commercial MG DNA test kit uses a PCR directly on material extracted from swabs. One commercial company produces a kit to detect MG field strains and one that identifies the vaccine F strain. Several 'in-house' PCR-based tests have also been published for MG including a multiplex PCR, which is designed to detect all four avian mycoplasma pathogens (34), but which has not been validated with clinical samples. Several methods are cited by Kempf (16) and, in addition, a manual published by Lauerman (23) contains a validated PCR assay for MG, MS, and other avian mycoplasmas based on unique sequences contained in the 16S rRNA gene. This method for MG is presented below. In the USA, a PCR based on the *mgc2* gene of MG (12) or the *vlhA* gene of MS (14) is becoming more widely used, because preliminary strain identification can be made by sequencing of the PCR product; it must be remembered that unrelated strains may occasionally share the same sequence.

a) DNA isolation

DNA is extracted from swab samples (three–five may be pooled) suspended in 1 ml of PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. The suspension is centrifuged for 30 minutes at 14,000 g at 4°C. The supernatant is carefully removed with a Pasteur pipette and the pellet is suspended in 25 µl PCR-grade water. The tube and the contents are boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 g for 5 minutes. The DNA is in the supernatant.

b) Primers

The MG primers consist of the following sequences.

MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3'

MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'

For MS, the following primers are used:

MS–F: 5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'

MS–R: 5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'

c) Polymerase chain reaction

The reaction mixture should be prepared in a separate clean area using a set of dedicated pipetters. For one 50 µl PCR reaction the mixture is as follows:

H_2O Ultra-pure	35.75 µl
10 × PCR Buffer	5.00 µl
dNTP(l0 mM)	1.00 µl
F Primer (20 pmole/µl)	0.50 µl
R Primer (20 pmole/µl)	0.50 µl
Taq (5 U/µl)	0.25 µl
$MgCl_2$ (50 mM)	2.00 µl

A 45 µl volume of the reaction mixture is dispensed into each PCR tube. The reaction mixture should be overlaid with a few drops of light weight mineral oil unless the thermocyler is equipped with a heated lid. The tubes are then taken to another clean area where the appropriate DNA sample (5 µl) is added to each tube. Positive and negative controls should be used in each run.

The tubes are then placed in a thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and soak at 4°C.

d) Electrophoresis

PCR products are detected by conventional 2% agarose gel electrophoresis, incorporating appropriate size markers, followed by examination under UV light. The PCR product for MG is 185 bp. Visualisation of the PCR products should be carried out in a separate laboratory area, well separated from all other steps in the PCR procedure.

PCR tests still tend to be carried out by specialist laboratories and should probably be regarded as useful adjuncts to the present diagnostic methods once their validity is firmly established. Great care needs to be taken to avoid contamination of samples with MG or MS DNA from nearby post-mortem rooms, culture

laboratories or from positive amplicates from previous PCR runs (see Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases, for appropriate safeguards). However one commercial kit referred to above is now licensed by the United States Department of Agriculture (USDA) as a diagnostic method and approved for use in the National Poultry Improvement Plan (NPIP). It should be noted that PCR tests are not validated for testing day-old birds for accurate detection of infection.

Molecular methods are also available for differentiation of MG and MS strains (16), but their use tends to be restricted at present to specialist laboratories. A rapid and accurate method for DNA fingerprinting uses arbitrary primed PCR or random amplified polymorphic DNA (RAPD). This technique uses short, arbitrary PCR primers, which generate reproducible patterns in agarose gels (8). This method is rapid and simple, and has proven to be very useful for rapid identification of strains of MG for epidemiological studies. However, there may be problems with reproducibility, so strains to be compared must be run on the same gel. Also, interpretation of banding patterns which appear to be similar can be difficult.

Gene-targeted sequencing (GTS), using PCR primers for the *mgc2, gapA, pvpA*, and MGA_0309 genes of MG can be used to provide an accurate and reproducible method of typing of strains, which will allow rapid global comparisons between laboratories (9). Preliminary strain identification with diagnostic PCR primers for the *mgc2* gene of MG or the *vlhA* gene of MS by sequencing of the PCR product allows for preliminary strain identification without the need for prior isolation of the organism (14, 15). However, unrelated strains sometimes have identical sequences using these primers, and further characterisation may be necessary.

2. Serological tests

The serological tests in common use may lack specificity and/or sensitivity; their use is strongly recommended for monitoring flocks rather than for testing individual birds. Diagnosticians wishing to use such tests are advised to establish the test sensitivity and specificity (Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases) under their own laboratory conditions. It should also be noted that these tests have not been validated for use with sera from day-old birds or from game birds (4).

The most commonly used tests are RSA, ELISA and HI although several others have been described such as radioimmunoassay, microimmunofluorescence and IP assay. The number of sera to be tested within a flock depends on the level of detection and the confidence limits required. Minimal requirements may be laid down for international trade and the frequency of testing may also be stipulated as, for example, in the European Communities Council Directive 90/539/EEC. Minimal requirements and approved tests are also set out for members of the NPIP of the USA.

Poultry companies using ELISA technology for screening large numbers of sera for virus antibodies may find this type of assay convenient also for mycoplasma testing. The ELISA technology will not be described in detail here because several MG kits are available commercially. Instead, the details of the HI test are provided as the reagents needed for this test are not widely available commercially.

a) Rapid serum agglutination test

Sera are collected from a sample of the flock and, if not tested immediately, are stored at 4°C and not frozen. The test should be carried out at room temperature (20–25°C) within 72 hours of serum collection and the reagents should also be at room temperature. Prior centrifugation will reduce nonspecific reactions. The RSA antigens are available commercially, but they may vary in specificity and sensitivity from different manufacturers and from batch to batch. They must be stored according to the manufacturer's instructions. Suitable RSA-stained antigens may also be prepared 'in-house' using culture methods as described in Section B.1.; these are then stained with crystal violet dye. Quality control standards for mycoplasma antigens for serological tests are described below.

- **Test procedure (1)**

i) Drop one volume (approximately 0.02 ml) of serum on to a clean white tile or glass plate followed by one volume of stained MG or MS antigen. Do not allow the serum to dry out before addition of the antigen. It is important to shake the antigen bottle vigorously and frequently during use to keep the correct amount of antigen in suspension.

ii) Use a stirring rod to spread the mixture over a circular area of approximately 1.5 cm diameter. Rock the tile or plate for 2 minutes. Agglutination is indicated by flocculation of the antigen within 2 minutes.

iii) Include known positive and negative controls in the test.

iv) Retest serial dilutions of any sera that agglutinate after heating at 56°C for 30 minutes. If they still react strongly, they are considered to be positive on dilution (1/4 or more).

In the USA, MG and MS positive reference antisera can be obtained from the USDA National Veterinary Services Laboratories (NVSL), and in Europe from AFSSA Ploufragan[1], France. MG and MS and control sera produced in chickens or in turkeys and with a range of titres can be purchased. Sets of antisera can be purchased also from the University of Georgia Department's of Avian Medicine, subject to availability.

There are no international standards for interpreting these tests, but a high proportion of positive sera in a flock (10% or more) indicates MG infection, especially if confirmed by HI test or ELISA. For further confirmation, the flock should be retested within a month. Inconclusive results make it necessary to attempt to isolate the organism or to demonstrate the presence of its DNA. Doubtful results for MG or MS should be investigated by performing tests with MS antigen (and *vice versa*) as infection with these organisms sometimes causes cross-reactions.

Tests can be conducted on yolk as well as sera although the yolk must first be diluted or extracted.

b) Haemagglutination inhibition test

MG and MS are capable of haemagglutinating avian red blood cells (RBCs), and specific antibodies in sera cause inhibition. A strain should be selected that grows well and haemagglutinates reliably. The HI test requires a satisfactory haemagglutinating MG and MS antigen, washed fresh chicken or turkey RBCs, as appropriate, and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of the mycoplasma cells in PBS. It may be difficult to sustain a supply of high-titred broth culture antigen; however, the use of concentrated antigen (usually containing 25–50% glycerol and stored at –70°C), increases the likelihood of nonspecific reactions. In the USA, MG and MS haemagglutination (HA) antigen can be purchased from the NVSL.

The HI test follows well-known procedures (1). The HA titre of the antigen is first determined in doubling dilutions, the HA unit being defined as the least amount of antigen giving complete HA in the test system employed. The HI test should be performed using 4 HA units by the following method or a method having equivalent sensitivity as determined by tests with known positive sera.

All HA titrations and HI tests are best performed in multiwell plastic plates with V-shaped wells and using constant volumes of 50 µl. A positive and a negative control serum are incorporated into each test. One row of eight wells is required for each serum under test.

- **Test procedure**

i)   Add 50 µl of PBS to the first well in each row.

ii) Add 8 HA units of antigen in 50 µl volumes to the second well in each row and add 50 µl of 4 HA units of antigen to each of wells 3 to 8.

iii) Add 50 µl of a previously-prepared 1/5 dilution of the serum under test to the first well, mix, and transfer 50 µl to the second well, and so on, and discard 50 µl from the last well. The first well is the serum control well.

iv) Six wells are required for the antigen control. Add 50 µl of PBS to wells 2 to 6, inclusive, and add 50 µl of the 8 HA unit antigen to wells 1 and 2. Mix the contents of well 2 and transfer 50 µl to well 3, mix and repeat up to well 6, and discard 50 µl.

v)   Two wells are required for the RBC control. Add 50 µl of PBS to each of these.

vi) Add 50 µl of a 0.5% suspension of RBCs (chicken cells for chicken serum and turkey for turkey serum) to all wells.

vii) Shake the plate lightly to ensure thorough mixing of the well contents, and read after standing for approximately 50 minutes at room temperature or when the antigen titration is reading 4 HA units. For reading, the plate should be tilted and only those wells in which the RBCs 'stream' at the same time as those in the RBC control wells should be considered to be inhibited. The serum control should show a clear button of RBCs and the positive and negative controls should react as expected. The HI titre is the highest serum dilution exhibiting complete inhibition of HA.

Sera giving nonspecific HA must be adsorbed to remove all nonspecific haemagglutinins so that a clear button is obtained in the control well without HA antigen. The adsorption is carried out by incubating 1 ml of the serum dilution with 6–8 drops of packed washed chicken or turkey RBCs. The cells are removed after incubation at 37°C for 10 minutes, and the supernatant is tested for haemagglutinating activity.

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1   Agence française de sécurité sanitaire des aliments (AFFSA) Ploufragan, Mycoplasmology Bacteriology Unit, 22440 Ploufragan, France.

There is no official definition of positive and negative results for international trade but the NPIP of the USA states that titres of 1/80 or above are considered to be positive and titres of 1/40 are strongly suspicious.

c) Enzyme-linked immunosorbent assay

Several commercial MG and MS antibody ELISA kits are marketed. The sensitivity is determined to some extent by the manufacturer's recommendations for the cut-off levels for positive and suspicious reactions. Sensitivity may sometimes be 'damped down', to avoid the well-known cross-reaction between MG and MS. One ELISA uses an MAb that recognises an epitope on a 56 kDa polypeptide of MG (7). In this system, ELISA plates are coated with whole cell MG antigen and the sera under test are added as in the conventional indirect ELISA, but the reaction is assessed by the extent of blocking that occurs when the conjugated MAb is added. A similar ELISA has also been marketed for MS. One advantage is that the system can be used for sera from any avian species without adaptation.

•   **Quality control of *Mycoplasma gallisepticum* and *M. synoviae* antigens**

i)   *Mycoplasma gallisepticum* antigens

Antigens are usually prepared from the S6 strain or the A5969 strain of MG. Antigens prepared from other strains may also be used when necessary.

MG antigen for the RSA test: The methods of quality control described below apply solely to suspensions of MG stained with a suitable dye and containing preservative and intended for use in the rapid plate agglutination test with serum. Such antigens are available commercially.

On microscopic examination, the antigen should appear as a homogeneous suspension without floccules or precipitates and the suspending liquid should be free from residual dye. It must be free from contamination with bacteria and fungi. The pH must be between 6.5 and 7. It must be stored at 5±3°C and be warmed to room temperature before use.

The sensitivity and specificity of the antigen is determined with respect to its reaction with known positive sera of high and low titre and known negative sera. A positive reaction is recognised by the formation of coloured floccules and the clearing of the suspending medium. The criteria described above continue to apply until the expiry date declared by the manufacturer.

MG antigen for the HI test: The test is preferably performed with live, actively growing cultures. The antigen must be free from contamination with bacteria and fungi.

MG antigen for the ELISA: It may be difficult to prepare satisfactory antigen for use in the indirect ELISA without considerable prior experimentation and confirmation of sensitivity and specificity. Use of a reliable commercial kit is probably the best approach for most diagnostic laboratories. Some kits are now USDA-licensed and approved for use in the NPIP in the USA.

ii)   *Mycoplasma synoviae* antigens

Antigens prepared from the WVU 1853 strain or other suitable strains should be used.

Mycoplasma synoviae antigen for the RSA test: the specifications apply as for MG antigen for the RSA test.

Mycoplasma synoviae antigen for the HI test: the same specifications apply as for MG antigen for the HI test.

iii)   Additional comments

Sera giving nonspecific reactions to the RSA test do not usually give a positive reaction in the HI test using live HA antigen. Positive RSA reactions can be confirmed by the HI test with sera taken after the first 2–3 weeks of infection (the time taken for HI antibodies to develop). However, the HI test tends to be strain specific (20) and therefore may lack sensitivity. ELISA may be a useful alternative.

Samples of serum should not be frozen before use in RSA tests. They should be free from haemolysis and contamination to avoid nonspecific reactions. The use of inactivated vaccines for other diseases may result in nonspecific reactions. Samples should be tested as soon as possible (within 72 hours) because mycoplasma antibodies may deteriorate on storage. Sera may be inactivated in a water bath at 56°C for 30 minutes.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The preferred method of control is to maintain MG- and MS-free flocks. Vaccination should be considered only in situations where field exposure is inevitable, such as on multi-age sites. Potential exposure of neighbouring poultry flocks should also be carefully considered.

Two types of vaccines are available for the control of MG. These are mild to avirulent MG strains used as live vaccines, or inactivated oil-emulsion bacterins. The subject of MG vaccination has been reviewed by Whithear (35). Although there is antigenic variability among MG strains, it is thought that vaccination with a single strain is sufficient.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

- **Live vaccines: methods of use**

The use of live vaccines is equivalent to 'controlled exposure'. The objective is to infect the flock with a mild, immunogenic MG strain at an age when little or no significant damage occurs. Such exposure results in resistance to challenge later in life, such as on multi-age commercial sites. Successfully vaccinated birds are resistant to respiratory disease, airsacculitis, and egg production drops caused by MG. Vaccination also results in reduced levels of egg transmission in breeders.

The F strain of MG has been the most commonly used vaccine strain (5). It is a naturally occurring strain of mild to moderate virulence for chickens, but it is virulent for turkeys. It ordinarily spreads slowly from bird to bird. When administered to healthy chickens via the upper respiratory tract, little or no respiratory reaction is observed. However, when administered by aerosol or in the presence of other respiratory disease agents, such as Newcastle disease or infectious bronchitis virus, respiratory signs and airsacculitis may result. Vaccinated chickens are permanent carriers, so a single dose is adequate. Use of F strain vaccine in each replacement flock on a multi-age site will eventually result in displacement of the field strain with the vaccine strain. Strains ts-11 and 6/85 are avirulent and spread to unvaccinated birds does not occur or occurs very poorly when birds are in very close contact (25).

Commercial pullets are usually vaccinated between 12 and 16 weeks of age, but vaccination of younger or older birds is permissible. It is essential that vaccination occurs before the flock is naturally infected. Vaccination in cases of probable early field exposure can be carried out in birds as young as 2–4 weeks of age. For the F strain, intranasal or eyedrop administration is preferred. Administration in the drinking water may result in some birds being missed unless the procedure is carried out properly. Aerosol administration should also be done carefully, so that all birds are exposed. A respiratory reaction should be expected at approximately 5–7 days after vaccination if aerosol administration is used. Vaccinated flocks should be tested with the agglutination test approximately 3–4 weeks post-vaccination to be sure that all birds were properly exposed. It is desirable that birds be vaccinated at an age when there is no reaction to other respiratory vaccines. Strain ts-11 should be administered by eyedrop, and 6/85 is given as a fine spray. Vaccination with ts-11 results in a low but distinctive serological response by serum plate agglutination, HI, and ELISA, but vaccination with 6/85 does not ordinarily result in a serological response. No post-vaccination reaction should be observed with 6/85 or ts-11. Flocks vaccinated with F strain or ts-11 are culture positive for the life of the flock, but 6/85 may be difficult to recover later than 4–6 weeks after vaccination.

Commercial live vaccines should be used within 1–2 hours after reconstitution. Lyophilised vaccine should be stored at 4°C. Some manufacturers supply the vaccine frozen. Such vaccine should be stored in liquid nitrogen, dry ice, or at –70°C or colder. Live MG vaccine is not stable for long periods at ordinary freezer temperatures. Storage for more than a few days at –20°C should be avoided.

Strains 6/85 and ts-11 are inherently safer than F strain, although the level of protection may be somewhat less, and may be useful as the primary vaccine strain on a multi-age site or as a 'second generation vaccine' on sites previously using F strain vaccine. They may also be preferred in situations where inadvertent exposure of neighbouring poultry flocks is of concern. F strain displaces wild-type MG more efficiently than either ts-11 or 6/85, but ts-11 has been used to eradicate F strain MG from a multi-age commercial egg-production site (33). Multi-age sites where strain 6/85 is consistently used often test MG-negative, suggesting that it has displaced the wild-type strain.

Live vaccines have also been used in some countries in broiler breeder pullets. In Australia, ts-11 live vaccine is being extensively used in broiler breeder pullets as well as in commercial layers. F strain vaccine has been used in broiler breeder pullets raised under multi-age conditions in some Latin American countries for several years; more recently there has been limited use of strains ts-11 and 6/85. There has been limited use of the 6/85 strain as a vaccine for commercial turkeys in the USA, but no good data on its effectiveness are available. Generally, vaccination of turkeys with live vaccines is not recommended and vaccination of broilers with either live or inactivated vaccines has not been successful. None of the vaccines has been validated for use in game birds.

A live vaccine for MS is available in several countries for use in broiler breeder and layer chickens. It is produced from a temperature-sensitive mutant, MS-H (29). Its characteristics and method of use are similar to those for the MG vaccine, ts-11.

- **Inactivated vaccines: method of use**

MG bacterins are prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant. A high antigen content is essential.

Bacterins are ordinarily used in commercial pullets to provide protection against egg-production drops that occur after MG exposure on multi-age layer sites (13). They may also be used to reduce the level of egg transmission in breeder pullets. Use of bacterins in broilers is limited by the fact that birds vaccinated before 1–2 weeks of age are not protected. Although bacterins may provide protection against respiratory signs, airsacculitis, and egg-production losses, vaccinated flocks are readily infected. The duration of immunity is not known, but most flocks are exposed within 1–2 months after vaccination.

Administration is by the intramuscular or subcutaneous route, usually with a dose of 0.5 ml per bird. There is a risk that a persistent reaction at the site of vaccination will require trimming of carcasses of spent fowl vaccinated by the intramuscular route, so subcutaneous administration in the upper dorsal part of the neck is the most commonly used route. Two doses are preferred, but cost and labour considerations may dictate the use of a single dose, usually between 16 and 18 weeks of age for commercial pullets. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination crews should exercise proper methods of biosecurity when travelling between flocks. Vaccine should be stored at 2–8°C up to the time of use. It should not be frozen or exposed to strong light.

A similar bacterin for MS is also licensed in the USA, but it has received limited use.

1. Seed management

a) Characteristics of the seed

- **Live vaccine**

The vaccine strain should be immunogenic, must readily colonise the upper respiratory tract, and cause minimal damage to the respiratory system. A strong antibody response does not necessarily correlate with immunity.

The seed culture should be free from all extraneous agents. The culture should be cloned to ensure purity. If desired, restriction endonuclease patterns of the mycoplasmal DNA on agarose gels can be run to be sure of the identity and purity of the strain.

The seed culture should be stable with no tendency to revert to virulence. This can be confirmed with ten back passages in susceptible chickens. Contact chickens can be introduced at weekly intervals. If necessary, tracheal swabs can be taken from infected chickens and can then be inserted into the trachea of contact chickens. Transmission of the organism should be proven. The resulting isolate can then be used to challenge susceptible chickens.

- **Killed vaccine**

For killed vaccines the most important characteristics are high yield and good antigenicity. It is assumed, but not proven, that virulent strains are desirable. The seed culture should be free from all extraneous organisms.

b) Method of culture

The seed culture may be propagated in a medium similar to that described above (Section B.1) For live vaccines, the broth culture is lyophilised or frozen at –70°C or colder. For bacterins the culture must be concentrated and resuspended in a small volume of saline or PBS before the emulsion is prepared.

c) Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. Chickens should be vaccinated by the same route that will be used in the field. Vaccinated birds should be challenged, and protection should be determined against respiratory signs, nasal discharge, and/or airsacculitis. Ideally, protection against egg-production losses should be evaluated, but such challenge trials are expensive and cumbersome.

Efficacy test: Groups of 20 specific pathogen free (SPF) chickens or at least mycoplasma-free chickens, 2 weeks of age or older, are vaccinated by eyedrop or other route of administration with one field dose of live vaccine, or subcutaneously or intramuscularly with one dose (usually 0.5 ml) of bacterin. A similar group of unvaccinated chickens is maintained separately as controls. All chickens should be challenged with a 24-hour broth culture of a virulent strain of MG, 2–3 weeks post-vaccination. A simple challenge method is inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac. All birds are necropsied 7–10 days post-challenge, and air sac lesions are scored. Alternative methods are to challenge by inoculating 0.1 ml into the infraorbital sinus and examining the birds for nasal discharge from 7 to 14 days post-challenge or to challenge by aerosol and measure the thickness of the tracheal mucosa on microscopic sections at four to six equidistant predetermined points (35).

2. Method of manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry. Special care must be taken to avoid MG contamination of other products manufactured in the same facility.

Production of vaccine should be on a seed-lot system, using a suitable MG strain of known origin, passage history, and purity. The growth medium is similar to that given above. The serum used in the growth medium should be inactivated at 56°C for 1 hour to prevent contamination with any mycoplasmal organism that may be present, and filter sterilised. A source of SPF serum is desirable.

Broth medium is inoculated, with a rapidly growing inoculum, at a rate of approximately 5% (v/v). Incubation is at 37°C. Production can be in batches using large flasks or in a fermenter. In batch cultures, harvest is approximately 24 hours after inoculation. Live vaccines are preserved by lyophilisation or by freezing at –70°C, in liquid nitrogen, or on dry ice.

For bacterin production, the antigen must be concentrated, usually by centrifugation, ultrafiltration, or other suitable method. Bacterins are made as water-in-oil emulsions, typically 80% mineral oil, 20% aqueous, with suitable emulsifying agents.

3. In-process control

Antigen content: At harvest, the titre should be from 10^8 to 10^9 CFU/ml. The antigen concentration of bacterins is difficult to standardise but may be based on packed cell volume, which is typically 1% (v/v) packed cells in the final product.

Inactivation of killed vaccines: Inactivation is frequently done with either beta-propiolactone or formaldehyde. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine organism and potential contaminants.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation should be carried out by culture in mycoplasma broth on each batch of both the bulk harvest after inactivation and the final product. No evidence of growth of mycoplasma should be observed.

Sterility of killed vaccines: Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the British Pharmacopoeia (Veterinary) 1985.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

- **Live vaccine safety test**

The birds vaccinated in the efficacy test given above can be used to evaluate the safety of the vaccine.

- **Killed vaccine safety test**

Birds vaccinated in the efficacy test described above may be observed for adverse local or systemic effects.

c)   Potency

Potency tests for both live and killed vaccine can be conducted by the procedures given above for the efficacy test. The titre of live vaccines should be sufficient to induce infection by the route recommended; 10^5 CFU/dose is sufficient for eyedrop administration of live F strain vaccine. The recommended dose of ts-11 is $\geq 10^{7.7}$ colour changing units (CCU)/dose and for 6/85 a dose of 10^7–10^8 CFU was effective in challenge trials. For MS-H, doses of $\geq 4.8 \times 10^5$ were shown to be effective.

d)   Duration of immunity (killed vaccine)

Because flocks are generally exposed within 1–2 months after vaccination, duration of immunity is not a primary consideration. After field challenge, resistance is considered to be permanent.

e)   Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

f)   Preservatives

A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistency throughout the shelf life should be checked.

A suitable preservative that has already been established for such purposes should be used. Mycoplasmas are susceptible to many antibacterials except for penicillins; such antibiotics should not be included as preservatives.

g)   Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident, the person should go at once to a hospital, taking the vaccine package with him or her. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injection. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

Personnel vaccinating birds with live virus vaccines by aerosol spray should wear protective clothes and masks.

5.   Tests on the final product

a)   Safety

See Section C.4.b.

b)   Potency

See Section C.4.c.

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NB: There is an OIE Reference Laboratory for Avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

AVIAN TUBERCULOSIS

SUMMARY

Avian tuberculosis is an important disease which affects companion, captive exotic, wild and domestic birds. The disease is most often caused by Mycobacterium avium (*serotypes 1, 2 and 3*) *and* M. genavense. *The most significant cause of poultry disease is* M. avium.

Clinical signs of the disease vary depending on the organs involved. The classical presentation is characterised by chronic and progressive wasting and weakness. Diarrhoea is common. Some birds may show respiratory signs and occasionally sudden death occurs. Some birds may develop granulomatous ocular lesions.

Mycobacterium tuberculosis *is less commonly the cause of infection in birds, often as a result of transmission from pet bird owners, and clinical signs differ from those caused by the more commonly occurring species of mycobacteria.*

Mycobacterium avium complex *and* M. intracellulare *can also infect an extensive range of different animal species such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species.* Mycobacterium genavense *has also been reported in a dog and an immunocmpromised cat. Disease onset in birds is normally more rapid with* M. genavense *than with* M. avium.

In humans, M. avium *and* M. genavense *are capable of inducing a progressive disease that is refractory to treatment, mostly in immunocompromised hosts. All manipulations involving the handling of open live cultures or of material from infected birds must be carried out with adequate biohazard containment.*

Diagnosis of tuberculosis in birds depends on the demonstration of Mycobacterium *spp. in the dead bird, or the detection of an immune response, cellular or humoral, in the live bird.*

Identification of the agent: *Where clinical signs of tuberculosis are seen in the flock, or typical lesions of tuberculosis are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a positive diagnosis. If acid-fast bacilli are not found, but typical signs or lesions are present in the birds, culture of the organism must be attempted. Any acid-fast organism isolated should be identified by biochemical, nucleic-acid-based tests, serological or chromatographical (*e.g. high performance liquid chromatography [*HPLC*]) *criteria.*

Tuberculin test and serological tests: *These tests are normally used to determine the prevalence of disease in a flock, or to detect infected birds. When used to detect the presence of tuberculosis in a flock, they should be supported by the necropsy of any birds that give positive reactions.*

*In domestic fowl, the tuberculin test in the wattle has been the test of choice. This test is less useful in other species of bird. A better test, especially for waterfowl, is the whole blood stained-antigen agglutination test (*Rozanska*). It is more reliable and has the advantage that it will give a result within a few minutes, while the bird is still being held. The tests are not reliable in caged birds.*

Requirements for vaccines and diagnostic biologicals: *No vaccines are available for use in birds. An antigen preparation stained with 1% malachite green is available for the whole blood agglutination test. Avian tuberculin purified protein derivative is the standard preparation for use in the tuberculin test of domestic poultry.*

A. INTRODUCTION

Several mycobacterial species can be involved in the aetiology of avian tuberculosis. The disease is most commonly produced by infection with *Mycobacterium avium* complex (serotypes 1, 2 and 3) and *M. genavense* (25). Other species, such as *M. intracellulare*, *M. scrofulaceum*, *M. fortuitum*, *M. tuberculosis* and *M. bovis* are less common causes of avian tuberculosis (25). *Mycobacterium avium complex* and *M. intracellulare* are capable of infecting an extensive range of different animal species such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species (26, 27).

Mycobacterium avium complex consists of three subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. sylvaticum, and *M. avium* subsp. *paratuberculosis* (28). The latter is the causal agent of Johne's disease, or paratuberculosis, in ruminants and other mammalian species (see Chapter 2.1.11 Paratuberculosis). Although successful experimental infections with *M. a. paratuberculosis* in poultry have been reported (11), there is no evidence that this organism is involved in the aetiology avian tuberculosis.

Most *M. a. avium* isolates from birds have a repetitive sequence IS901 in their genome and produce a characteristic three-band pattern in IS1245 restriction fragment length polymorphism (RFLP) (20). There is convincing evidence that the presence of IS901 correlates with pathogenicity in birds (6, 15). This repetitive sequence is also present in *M. a. sylvaticum* that is capable to produce tuberculosis in birds. IS901 has only been detected in *M. avium* strains with serotypes 1, 2 and 3 (15, 20) that are apparently more pathogenic to birds than other serotypes (25). On the basis of genetic and phenotypic differences it has recently been proposed to divide *M. a. avium* into two subspecies: *M. a. hominissuis* for human and porcine isolates and *M. a. avium* for bird-type isolates (13). The isolates designated as *M. a. hominissuis* had polymorphic multiband IS1245 patterns and were able to grow at 24 and 45°C. In comparison, the avian isolates designated as *M. a. avium* had the conserved three-band pattern in IS1245 RFLP and were unable to grow at 24 and 45°C (13). It is worth noting that the typical features of bird-isolates, the three-band pattern in IS1245 RFLP and presence of IS901, have also been found in cervine and bovine isolates of *M. a. avium* (14).

Tuberculosis in birds is most prevalent in chickens and in wild birds raised in captivity. Turkeys are quite susceptible, but duck and geese are comparatively resistant. The practices of allowing poultry to roam at large on the farm (free range) and of keeping the breeders for several years are conducive to the spread of tuberculosis among them. Infected individuals and contaminated environment (water and soil) are the main source of infection (25). *Mycobacteria* can survive for several months in the environment (25).

In most cases, infected birds show no clinical signs, but they may eventually become lethargic and emaciated. Many affected birds show diarrhoea and comb and wattles may regress and become pale. Affected birds are usually older than one year. Some show respiratory signs and sudden death may occur, dyspnoea is less common, and granulomatous ocular lesions (16) and skin lesions have been reported. Under intensive husbandry conditions, sudden death may occur, often associated with severe lesions in the liver; such lesions are easily observed at post-mortem examination (25).

The primary lesions of tuberculosis in birds are nearly always in the intestinal tract. Such lesions take the form of deep ulcers filled with caseous material containing many organisms, and these are discharged into the lumen and appear in the faeces. Before the intestinal tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall, but when the intestine is opened, the true nature of the mass becomes evident. Typical caseous lesions are nearly always found in the liver and spleen, and these organs usually are greatly enlarged because of the formation of new tuberculous tissue. The lungs and the other tissues are ordinarily free from lesions even in advanced cases.

In most species of affected bird, tuberculous-like lesions are mostly found in the intestinal tract, liver and spleen. Lesions in other organs are less common. Exceptions include pigeons, waterfowl, and some finches, in which the disease begins primarily in the respiratory tract.

It is essential to bear in mind that *M. avium*, *M. intracellulare* and *M. genavense* are capable of giving rise to a progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals (25). All manipulations involving the handling of open live cultures or of material from infected birds must be performed with adequate biohazard containment (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

If there is a characteristic history of tuberculosis in the flock and typical lesions are found in birds at post-mortem, the detection of acid-fast bacilli in smears or sections from affected organs, stained by the Ziehl–Neelsen method,

is normally sufficient to establish the diagnosis. Occasionally a case will occur, presumably as a result of large infecting doses giving rise to acute overwhelming disease, in which affected organs, most obviously the liver, have a 'morocco leather' appearance with fine greyish or yellowish mottling. In such cases acid-fast organisms may not be found, but careful inspection will reveal parallel bundles of brownish refractile bacilli. Prolongation of the hot carbol fuchsin stage of Ziehl–Neelsen staining to 10 minutes will usually reveal that these are indeed acid-fast bacilli, with unusually high resistance to penetration of the stain. Recently, DNA probes and polymerase chain reaction (PCR) techniques have been used to identify the agent. Traditionally, *M. avium* is separated from common nonchromogenic slow-growing organisms by their ability to grow at 42°C (*M. a. avium*) and biochemical tests such as the Tween hydrolysis test, pyrazinamidase, growth on thiophen-2-carboxylic acid hydrazide (TCH)-containing media and tellurite reduction. *Mycobacterium genavense* is particularly fastidious and has special requirements for growth and identification.

- **Culture**

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no acid-fast bacilli are seen in smears or sections, an attempt must be made to isolate the causative organism from the necropsy material. Liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali or acid to eliminate rapidly growing microorganisms before culture (see Chapter 2.4.7 Bovine tuberculosis). *Mycobacterium avium* grows best on media such as Lowenstein–Jensen, Herrold's medium, Middlebrook 7H10 and 7H11 or Coletsos, with 1% sodium pyruvate added. It may occasionally be necessary to incorporate mycobactin, as used for the isolation of *M. paratuberculosis* and *M. silvaticum*. Growth may be confined to the edge of the water of condensation. Cultures should be incubated for at least 8 weeks. Typically *M. avium* produces 'smooth' colonies, within 2–4 weeks; rough variants do occur. Shorter incubation times can be achieved using the liquid culture BACTEC system.

For *M. genavense*, use of the BACTEC system with no additives but with pH 6.0 and a lowered oxygen tension is recommended (18, 19). The optimal solid medium is Middlebrook 7H11 medium acidified to pH 6 and supplemented with blood and charcoal (17).

Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified under the denomination of *M. avium* complex (MAC). Seroagglutination, which is based on sugar residue specificity of surface glycopeptidolipids, allows classification of MAC organisms into 28 serovars. More sophisticated typing methods directed at cell-wall-specific targets are currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to major serovars, and high performance liquid chromatography (HPLC). Serovars 1 to 6, 8 to 11 and 21 are currently ascribed to *M. avium*, and serovars 7, 12 to 20 and 25 to *M. intracellulare*. However, no consensus was achieved on other serovars, and some isolates cannot be typed (9). Tuberculosis in birds is usually caused by *M. avium* types 1, 2, or 3. If one of these is found, it may be assumed to be the cause of the disease. If the isolate is not one of these, further identification tests must be carried out. However, it should be borne in mind that superficial tuberculosis lesions in caged birds, especially psittacines, may be caused by *M. tuberculosis*. Hence, if rough colonies of mycobacteria are isolated from such birds, they should be tested for growth at 42°C. If the isolate will not grow at 42°C, *M. tuberculosis* should be suspected.

- **Nucleic acid recognition methods**

Specific and reliable genetic tests for speciation are currently available (22). Commercial nucleic acid hybridisation probes have become a 'gold standard' for distinction between *M. avium* and *M. intracellulare* cultures. *M. genavense* can also be distinguished with these tests. A further probe that covers the whole MAC was also developed, as genuine MAC strains have been described that fail to react with specific *M. avium* and *M. intracellular* probes (23). These tests use a chemiluminescent-labelled, single-stranded DNA probe that is complementary to the ribosomal RNA of the target organism. The labelled DNA–RNA hybrids are measured in a luminometer. Various in-house molecular methods have been reported for the identification of mycobacterial cultures, including MAC. A multiplex PCR method for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex has some advantages (4). 16S rRNA sequencing (10) or PCR amplification followed by either hybridisation with species-specific probes or restriction enzyme analysis (5, 24, 29) may also be used. Even though some of these methods would theoretically detect the agent directly in tissue samples, none of them has been validated for this use. Therefore, molecular identification of MAC is currently performed on organisms previously isolated by culture. Sequencing of hsp65 to distinguish between subsets of *M. avium* has also been found useful (30).

Regarding intraspecies genotyping, pulsed-field gel electrophoresis of large DNA restriction fragments has proved to be highly sensitive (12). Also, a number of DNA mobile elements have been identified that may be exploited for

this purpose. Insertion sequence IS1245, which is virtually *M. avium* specific, was shown to be the most discriminative for the analysis of strain relatedness (2, 7). A standardised method consisting of IS1245 restriction fragment length polymorphism (RFLP) analysis was recently proposed (31). Bird infection was found to be caused by a particular subset of *M. avium* strains that are characterised by specific, highly conserved RFLP patterns with IS1245 and IS901, in addition to serovars 1, 2 or 3 (20).

Recently O'Grady *et al.* performed RFLP investigation using probes derived from IS901, IS1245 and IS1311 to study the molecular epidemiology of *M. avium* and *M. intracellulare* infection, in particular to gain an understanding of the sources of infection in humans (14).

If specialised typing facilities are not available, the likelihood that the organism isolated is the cause of the disease may be established by pathogenicity tests. It is preferred that these be carried out on the species of bird being investigated, but failing that, domestic fowl or Japanese quail may be used. Young adult birds are best. An inoculum is prepared by putting a small square of aluminium foil and some glass beads in a screw-capped container, which is then sterilised and weighed. A loopful of culture is then placed on the foil and the whole is reweighed. Finally, sufficient sterile normal saline solution is added to suspend the culture at 0.1 mg/ml. Birds are then inoculated intravenously with 1 ml of the suspension. If the organism is virulent, the bird will die in 5–6 weeks or, by that time, the bird will have extensive lesions filled with acid-fast bacilli.

2. Immunological methods

Tests used for export depend on the importing requirement of individual countries. In the main, the tuberculin test or the haemagglutination (stained antigen) test are most frequently used for export testing of poultry.

a) Tuberculin test

The most widely used test in domestic fowl, and the only test for which an international standard for the reagent exists, is the tuberculin test. The tuberculin is the standard avian purified protein derivative (PPD). Birds are tested by intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of tuberculin (containing approximately 2000 International Units [IU]), using a very fine needle of approximately 10 mm × 0.5 mm. The test is read after 48 hours and a positive reaction is any swelling at the site, from a small firm nodule approximately 5 mm in diameter to gross oedema extending into the other wattle and down the neck. With practice, even very small wattles on immature birds can be inoculated successfully. However, in immature birds the comb may be used, although results are not so reliable. Tuberculin testing of the wattle in turkeys is much less reliable than in the domestic fowl. Inoculation in the wing web has been recommended as being more efficient, but this is still not as good as for domestic fowl. Other birds may also be tested in the wing web, but results are not generally satisfactory. The bare ornamental skin areas on Muscovy ducks and some species of pheasant can be used, but reliability is doubtful and interpretation difficult. Testing in the foot web of waterfowl has also been described; the test is not very sensitive and is often complicated by infections of the inoculation site.

In pheasants, the tuberculin test can be performed in either of two ways. In the first, 0.05 ml or 0.1 ml of tuberculin is injected into the skin of the lower eyelid. A positive result is indicated by marked swelling at the site of injection after 48 hours. Alternatively, 0.25 ml of tuberculin is injected into the thoracic muscles and the birds are observed for 6–10 hours. Infected birds will show signs of depression and keep aside from the flock, and there may be cases of sudden death. No clinical signs will be provoked in uninfected birds.

b) Stained antigen test

- **Preparation of the antigen**

An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test (21). The strain used for preparation of the stained antigen must be smooth and not autoagglutinate in saline suspension. It must conform to the characteristics of the *M. avium* species.

A strain that will detect infection with any serotype is recommended to be used instead of the specific serotype that is most likely to be encountered (in Europe serotype 2 for domestic fowl, serotype 1 for waterfowl). It may be preferable to use a strain that is highly specific for the serotype it detects. The specificity of strains can be determined only by testing them as antigens, although in general a serotype 2 antigen will always detect serotype 3 infection and vice versa. Serotype 1 strains appear to detect more often a wide spectrum of infection, and will often also detect infections with mycobactin-dependent mycobacteria or *M. silvaticum*. There is no reason not to use a culture containing more than one strain of *M. avium*, provided that it shows the desired properties of sensitivity and specificity. Consistency of results between batches will be easier with the use of pure cultures.

The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9 containing 1% sodium pyruvate for better growth. Good growth should be obtained in approximately 7 days. The liquid culture is used as seed for bulk antigen preparation.

Antigen for agglutination tests is best grown on solid medium, such as Löwenstein–Jensen or 7H11, containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles. The use of solid medium maximises the chance of detecting any contamination, and antigens grown in some liquid media are not agglutinated by specific antibody. Liquid seed culture should be diluted (on the basis of experience) to give discrete colonies on the solid medium. This will usually give the best yield, and again increases the chance of detecting contamination. About 10 ml of inoculum will usually be enough to allow it to wash over the whole surface, and provide sufficient moisture to keep the air in the bottle near 100% humidity.

The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most strains. The antigen is harvested by the addition of sterile glass beads and twice the volume of sterile normal saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently to wash off all the growth and the washing is collected into a sterile bottle and reincubated at 37°C for 7 days. The killed bacilli are then washed twice in sterile normal saline with 0.2% formalin by centrifugation and resuspension. This sequence is safer than the original method in which the washing was carried out before the incubation that kills the organisms. Finally the organisms are again centrifuged and resuspended in sterile normal saline containing 0.2% formalin and 0.4% sodium citrate, to a concentration of about 1010 bacteria per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland's scale.

Cultures for antigen should be inspected for contamination daily for the first 5 days of incubation. The suspension made from the culture washings is also re-examined microscopically (for likely contaminants such as yeasts), and rechecked by culture to ensure that the formalin has killed the mycobacteria.

- **Validation of the antigen**

Cultures should be checked by Gram staining for the presence of organisms other than mycobacteria.

One or more batches for agglutinating antigen must be tested for efficacy in naturally or artificially infected tuberculous birds by comparison with a standard preparation of known potency. The potency relative to that of the standard preparation must not differ significantly from that declared on the label. Each bottle of antigen must be tested with normal chicken serum (to detect autoagglutination) and *M. avium* positive chicken serum of low and high antibody content. This should be done, where possible, alongside a previous batch of stained antigen. Those bottles that give satisfactory agglutination reactions with the antisera can now be pooled and the antigen stained. This is done by the addition of 3 ml of 1% malachite green solution per 100 ml of suspension. If possible, the stained antigen should now be checked using whole blood just as the unstained antigen was tested with serum. The agglutinating antigen should keep for at least 6 months in the refrigerator at 4°C, and much longer if frozen at –20°C or below. If a batch has not been used for a long time it should be rechecked, especially for autoagglutination.

The only safety test needed is the culture test of the unwashed antigen after 7 days of incubation, to ensure that all the bacilli are dead.

- **Test procedure**

The stained-antigen agglutination test has been used with good results, especially in both domestic and ornamental waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh whole blood, obtained by venipuncture, on a white porcelain or enamel tile. The mixture is rocked for 2 minutes and examined for agglutination. The agglutination may be coarse, in which case it is obvious, or quite fine, in which case it may be most clearly seen as an accumulation of the malachite-green-stained antigen around the edge of the drop, leaving the centre a normal blood-red colour. This test is especially useful for screening large flocks for immediate culling, and therefore has advantages over the tuberculin test for the control of the disease, even in domestic fowl. It has also been claimed that in domestic fowl it is more reliable than the tuberculin test.

Note on limitation of use

Neither the tuberculin test with avian tuberculin nor the stained-antigen agglutination test is likely to be of any value in cases of *M. tuberculosis* infection in caged birds.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available.

Avian tuberculin is a preparation made from the heat-treated products of growth of *M. avium*. It is used by intradermal injection to reveal delayed hypersensitivity as a means of identifying birds infected with or sensitised to the same species of tubercle bacillus.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Strains of *M. avium* used to prepare seed cultures should be identified as to species by appropriate tests. They should be shown to be free from contaminating organisms and to be capable of yielding a product of satisfactory quality. The strains recommended by the European Union (EU), for example, are D4ER and TB56. Reference may also be made to the World Health Organization (32).

b) Method of culture

The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown on solid media, it will be necessary to adapt the organism to grow as a floating culture. This is most easily accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g. Watson Reid's medium). When the culture has been adapted to liquid medium, it can be maintained by passage at 2–4-week intervals (1, 8).

The production culture substrate must be shown to be capable of producing a product that conforms to the standards of the European Pharmacopoeia or other international standards (3). It must be free from ingredients known to cause toxic or allergic reactions.

c) Validation

The strains of *M. avium* used as seed cultures must be shown to be free from contaminating organisms.

Seed lots must be shown to be efficacious in producing tuberculin with sufficient potency. The necessary tests are described in Section C.4 below.

2. Method of manufacture

Avian tuberculin may be made by the following three methods:

a) Old tuberculin

The organism is cultivated in glycerol broth medium, killed by heating in flowing steam, and filtered to remove cells. The filtrate is concentrated by heat and sterilised by filtration.

b) Heat-concentrated synthetic-medium tuberculin

As for old tuberculin but the glycerol broth medium is replaced by a synthetic medium (modified Dorset-Henley's synthetic medium).

c) Purified protein derivative

As for heat-concentrated synthetic-medium (HCSM) tuberculin but, instead of being concentrated by heat, the protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid [TCA] are used), washed and resuspended. PPD tuberculin is recommended as it gives fewer false-positive reactions and can be standardised more precisely. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Mercurial derivatives should not be used. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile neutral glass containers, which are then sealed to prevent contamination. The product may be freeze-dried.

3. In-process control

The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time period. Any flasks showing contamination or grossly abnormal growth should be discarded after autoclaving. As incubation proceeds, the surface growth of many cultures becomes moist and may sink into the medium or to the bottom of the flask. In PPD tuberculins, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be

pH 6.6–6.7. The protein level of the PPD concentrate is determined by the Kjeldahl method. Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.

4. Batch control

a) Sterility

Sterility testing is generally performed according to the European Pharmacopoeia or other guidelines (see also Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

Tuberculin PPD can be examined for freedom from living mycobacteria using the culture method described previously. This culture method, which does not require use of animals, is used in many laboratories and its use is encouraged over the use of animals for this purpose. The following is the previously described method, using experimental animals, to evaluate safety of PPD. Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

c) Residual infectivity

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days, and are examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture.

Each filled container must be inspected before it is labelled, and any showing abnormalities must be discarded.

d) Sensitising effect

To test the sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test are each injected intradermally on each of three occasions with the equivalent of 500 IU of the preparation under test in a 0.1 ml volume. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

e) Potency

The potency of avian tuberculin is determined in guinea-pigs sensitised with M. avium, by comparison with a standard preparation calibrated in IU.

Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by administering to each, by deep intramuscular injection, a suitable dose of inactivated or live M. avium. The test is performed between 4 and 6 weeks later as follows: Shave the guinea-pigs' flanks so as to provide space for three-to-four injections on each side. Prepare at least three dilutions of the tuberculin under test and at least three dilutions of the standard preparation in isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the dilutions to the injection sites randomly according to a Latin square design. The dilutions are injected intradermally in volumes of 0.1 or 0.2 ml.

Between 24 and 28 hours, the diameters of the reactions are measured and the results are calculated using standard statistical methods, taking the diameters to be directly proportional to the logarithms of the concentrations of the tuberculins. The estimated potency must be not less than 75% and not more than 133% of the potency stated on the label. The test is not valid unless the fiducial limits of error ($p = 0.95$) are not less than 50% and not more than 200% of the estimated potency. If the batch fails a potency test, the test may be repeated one or more times provided that the final estimate of potency and of fiducial limits is based on the combined results of all the tests.

It is recommended that avian tuberculin should contain the equivalent of at least 25,000 IU/ml, giving a dose for practical use of 2500 IU/0.1 ml.

f) Specificity

One or more batches of tuberculin may be tested for specificity together with a standard preparation of bovine tuberculin by comparing the reactions produced in guinea-pigs sensitised with *M. bovis* using a procedure similar to that described in Section C.4.d. In guinea-pigs sensitised with *M. bovis*, the potency of the preparation of avian tuberculin must be shown to be not more than 10% of the potency of the standard preparation of bovine tuberculin used in the potency test.

g) Stability

During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C). Freeze-dried preparations may be stored at higher temperatures (but not exceeding 25°C) protected from the light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

Provided the tuberculins are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the end of the following periods subsequent to the last satisfactory potency test: Liquid PPD tuberculins: 2 years; lyophilised PPD tuberculins: 8 years; HCSM tuberculins diluted: 2 years.

h) Preservatives

Antimicrobial preservatives or other substances that may be added to a tuberculin, must have been shown not to impair the safety and effectiveness of the product. The maximum permitted concentrations for phenol is 0.5% (w/v) and for glycerol it is 10% (v/v). The pH should be between 6.5 and 7.5.

i) Precautions (hazards)

Experience both in humans and animals led to the observation that appropriately diluted tuberculin injected intradermally results in a localised reaction at the injection site without generalised manifestations. Even in very sensitive persons, severe, generalised reactions are extremely rare and limited.

5. Tests on the final product

a) Safety

A test for the absence of toxic or irritant properties must be carried out according to the specifications of the European Pharmacopoeia (see also Section C.4.b).

b) Potency

The potency of tuberculins must be estimated by biological methods. These methods must be used for HCSM and PPD tuberculins; they are based on the comparison of the tuberculins to be tested with standard tuberculins (see also Section C.4.d).

<div align="center">

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NB: There is an OIE Reference Laboratory for Avian tuberculosis Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int.

CHAPTER 2.3.7.

DUCK VIRUS ENTERITIS

SUMMARY

*Duck virus enteritis (*DVE*) or duck plague is an acute contagious infection of ducks, geese and swans (*order Anseriformes*) caused by a herpesvirus. Diagnosis is based on a combination of assessing the clinical signs, gross pathology and histopathology supported by identification of the virus by either isolation or polymerase chain reaction.*

Identification of the agent: *The virus may be isolated from the liver, spleen and kidneys of birds dying from this infection. Virus may be recovered by infecting susceptible ducklings, in which the disease can be reproduced; by inoculating embryonated Muscovy duck eggs on the chorioallantoic membrane; or by inoculating cell cultures of duck embryo or Muscovy duck embryo origin. The identity of the virus can be confirmed by neutralisation tests using specific antiserum to inhibit pathological changes in the duck embryos or the cytopathological effects in the cell cultures, or by direct or indirect immunofluorescence tests on infected cell cultures. Alternatively the DVE DNA may be detected by the polymerase chain reaction from the oesophagus, liver and spleen of DVE-infected birds as well as from Muscovy duck embryos or cells used for virus isolation.*

Serological tests: *Immunological tests have little value in the diagnosis of acute DVE infection. Serum neutralisation tests* in ovo *and* in vitro *have been used to monitor exposure to DVE in wildfowl.*

Requirements for biological products: *A live attenuated virus vaccine is available to control DVE in birds over 2 weeks of age. Ducks are vaccinated subcutaneously or intramuscularly for active immunity. Vaccine virus is not thought to spread from vaccinated to unvaccinated stock. An inactivated vaccine has been reported to be efficacious in laboratory tests, but has not been developed or licensed for large-scale use.*

A. INTRODUCTION

Duck virus enteritis (DVE) is an acute, sometimes chronic, contagious virus infection that occurs naturally only in ducks, geese and swans, all members of the family Anatidae of the order Anseriformes. The aetiological agent, a herpesvirus, is a member of the alphaherpesvirinae subfamily of the Herpesviridae. DVE may also be referred to as duck plague, anatid herpes, eendenpest, entenpest and peste du canard. The infection has not been reported in other avian species, mammals or humans.

In domestic ducks and ducklings, DVE has been reported in birds ranging from 7 days of age to mature breeders. In susceptible flocks the first signs are often sudden, high and persistent mortality with a significant drop in egg production. In chronically infected partially immune flocks only occasional deaths occur. Recovered birds may be carriers and may shed the virus in the faeces or on the surface of eggs over a period of years (18, 21). Recently, DVE limited solely to Muscovy ducks has been observed in the USA (2, 5) .

Clinical signs and gross pathology associated with a DVE outbreak vary with the species, age and sex of the affected birds, and the virulence of the virus. In breeder ducks the range of signs include eye watering and pasted eye-lids associated with photophobia, polydypsia, loss of appetite, ataxia, watery diarrhoea and nasal discharge. Birds often have ruffled feathers and soiled vents. Sick birds may maintain an upright stance by using their wings for support, but their overall appearance is one of weakness and depression. In ducklings 2–7 weeks of age, losses may be lower than in older birds and the signs associated with DVE infection include dehydration, loss of weight, a blue colouration of the beaks and blood-stained vents.

At necropsy, there is little evidence of emaciation in adult ducks that have died. In mature males, prolapse of the penis may occur. In mature females, haemorrhages may be observed in ovarian follicles. The gross lesions are characterised by vascular damage, with tissue haemorrhages and free blood in the body cavities, eruptions, or

annular haemorrhages and diphtheroid lesions of the mucosal surfaces of the digestive tract, lesions of the lymphoid organs and retrograde changes of the parenchymatous organs. Collectively, these lesions are pathognomonic for DVE. The pathology and histopathology of DVE in white Pekin ducks has been reviewed (19). Microscopic lesions are characterised by vascular damage and its consequences in visceral organs. Eosinophilic intranuclear inclusions and cytoplasmic inclusions in epithelial cells of the digestive tract are typically present.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Primary isolation of the virus is best achieved from samples of liver, spleen or kidney tissue, which have been homogenised in buffered saline containing antibiotics and clarified by low-speed centrifugation (1800 *g*). Isolation may be attempted by inoculating such homogenates into cell cultures, ducklings or duck embryos.

a) Cell cultures

Cell culture is reported as the method of choice for isolation of DVE virus, but may not always be successful. If attempted, isolations may be made in primary duck embryo fibroblasts (DEF) (23) or, preferably primary Muscovy duck embryo fibroblasts (MDEF) (9, 14,). Muscovy duck embryo liver (MDEL) cells are thought to be even more sensitive (R.E. Gough, pers. comm.). Cell monolayers grown in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin are washed with serum-free MEM and then inoculated with the clarified sample homogenate suspected to contain DVE virus. After incubation for 1 hour at 37°C to allow for virus adsorption, the cultures are maintained on MEM containing 2% FCS, 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin, and incubated in an atmosphere containing 5% CO_2. The cytopathic effect (CPE) is characterised by the appearance of rounded clumped cells that enlarge and become necrotic 2–4 days later. Cultures should be stained with a fluorescent antibody conjugate using a direct or indirect method to identify the virus (see Section B.1.d). Cells can also be fixed and then stained with haematoxylin and eosin to show syncytial formation, intranuclear inclusions and marked cytoplasmic granulation. It has been reported (11) that the isolation of DVE in MDEF cells is favoured by incubation at temperatures between 39.5°C and 41.5°C. However, an elevated temperature does not appear to be essential for isolation, which is often carried out at 37°C. More than one passage in cell culture may be necessary to isolate the virus. This virus isolation method in cell cultures may be modified to a plaque assay by overlaying the cell monolayer with maintenance medium containing 1% agarose. As the virus can be cell associated, sequential passaging should be carried out by trypsinising potentially infected cells and replanting them, as well as inoculating fresh cells with infected culture supernatant from the previous passage.

b) Ducklings

When inoculated intramuscularly, 1-day-old susceptible ducklings die within 3–12 days; uninoculated ducklings, housed separately, should be maintained as controls at the same time. Muscovy ducklings (*Cairina moschata*) are more susceptible than white Pekin ducklings. Both macroscopic and microscopic lesions typical of DVE should be seen on post-mortem examination. The diagnosis may be confirmed either by vaccinating ducklings against DVE and challenging them subsequently with the virus isolate or by immunofluorescence. However, virulent strains of the virus exist, against which the vaccine may be ineffective (13). In the author's experience of natural infections occurring in Muscovy ducks, this method of virus isolation has proved more sensitive than cell culture methods.

c) Duck embryos

Primary virus isolations can be made by inoculation on to the chorioallantoic membrane (CAM) of 9–14 day embryonated Muscovy duck eggs. The embryos may die, showing characteristic extensive haemorrhages 4–10 days after inoculation. Two to four serial blind passages of the homogenised CAMs may be necessary before isolation can be effected. This method is not as sensitive as that using susceptible day-old ducklings.

Embryonated chicken eggs are not very susceptible to infection with field strains of DVE. The virus can nevertheless be adapted to chicken embryos by serial passages. Pekin duck embryos vary in their susceptibility to strains of DVE virus.

d) Immunological methods

Serological tests used to confirm the identity of newly isolated virus include neutralisation assays performed in either embryonated eggs or cell cultures. A plaque assay for DVE in duck embryo cell cultures has been described (4). In the author's laboratory a microtitre assay using primary MDEF or MDEL cells is used. Provided a hyperimmune antiserum of sufficiently high titre is used, a fluorescent antibody test (direct or indirect) for DVE in DEF, MDEF or MDEL cells is the next most sensitive assay after isolation in 1–9-day old

ducklings (8). A reverse passive haemagglutination test for DVE has been described (6), but it is reported to be less sensitive than immunofluorescence and plaque assays. An avidin–biotin–peroxidase method of immunoperoxidase staining to detect DVE antigen in formalin-fixed, paraffin-embedded sections of liver and spleen from experimentally infected birds has been described (12). The identity of the virus may also be confirmed by negative stain electron microscopy, but this alone is not positive confirmation that the herpesvirus observed is DVE virus. Immunoelectron microscopy has been developed recently using DVE hyperimmune serum (15).

e) Nucleic acid recognition methods

Recently, detection of DVE virus by polymerase chain reaction (PCR) has been reported (10, 11, 16, 17). Primers have been identified that are able to amplify DNA from DVE virus present in various tissues, including oesophagus, liver and spleen, from an original outbreak and after passage from Muscovy duck embryos. The following is an example protocol for PCR methods for detection of DVE virus; other protocols exist.

- **PCR method[1]**

This DNA extraction procedure can be used on disrupted cell suspensions from DVE-infected tissue culture, 10% ground tissue suspensions, or cloacal swab material in transport medium. This method is used to prepare duck plague DNA for the known positive PCR controls.

- **Extraction of viral DNA**

Note: All product transfers in steps i to v are performed in a biological safety cabinet.

i) For a 10% ground tissue suspension, add 400 µl to a 1.5 ml microfuge tube and microfuge at 16,000 **g** for 5 minutes. Transfer the supernatant to a new tube and go to step ii.

ii) For tissue culture suspensions and cloacal swab material, add 400 µl of the sample, or supernatant from step i above, to a 1.5 ml tube and microfuge at 16,000–20,000 **g** for 45 minutes to pellet the virus.

iii) Discard the supernatant and resuspend the pellet with 200 µl of Tris/ethylene diamine tetra-acetic acid (EDTA) buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

iv) Add 10 µl of a 5 µg/µl proteinase K solution to give a final concentration of 0.2 µg/µl, mix thoroughly, and incubate at 56°C for 1 hour.

v) Add 25 µl of 10% sodium dodecyl sulfate (SDS) solution to give a final SDS concentration of 1%, mix thoroughly, and incubate at 37°C for 1 hour.

vi) Add 15 µl of 5 M NaCl to give a final concentration of 0.3 M and mix thoroughly.

vii) Add 300 µl of fresh phenol buffered with Tris/HCl, pH 8.0, to the tube, and mix by inverting 50 times.

viii) Microfuge the tube at 16,000 **g** for 5 minutes and transfer the top aqueous phase (sample) to a new tube.

ix) Repeat the phenol extraction steps vii and viii once more.

x) Add 500 µl of ether to the tube, mix thoroughly, and microfuge at 16,000 **g** for 1 minute.

xi) Discard the top aqueous phase (ether) and repeat the ether extraction step (step x) once more.

xii) Heat the tube with the lid open at 56°C for about 15 minutes or until the smell of ether is gone.

xiii) Split the tube contents in two and add 2.25 times the sample volume of 100% ethanol to each tube, mix the tube contents by inverting the tube several times, and leave at room temperature (22°C) for 30 minutes.

xiv) Microfuge the tube at 16,000 **g** for 45 minutes and discard the supernatant.

xv) Add 200 µl of 70% ethanol to gently wash the pellet and then microfuge at 16,000 **g** for 15 minutes.

xvi) Discard the supernatant and dry the pellet at 56°C for 30–45 minutes with the tube lid open.

xvii) Resuspend the DNA in 30 µl distilled water that is RNAase and DNAase free.

xviii) Store the sample tube at 4°C until tested (few days) or at –20°C for long-term storage.

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1 Provided by Dr W.R. Hansen, US Geological Survey, Biological Resources Division, National Wildlife Health Center, 6006, Schroeder Road, Madison, WI 53711, USA. This procedure uses the following commercial items: GeneAmp PCR Reagent Kits containing dNTPs, 10× amplification buffer for hot start PCR, Taq DNA polymerase, Lambda PCR control reagents, and Ampliwax beads (Applied Biosystems), and a 100 base pair molecular size ladder (Invitrogen)

- **Polymerase chain reaction**

Lower reaction mixtures for the DVE PCR and the lambda control are prepared in advance in a biosafety cabinet using the kit manufacturer's recommended methods for a hot start PCR. The lower reaction mixture is dispensed into tubes, sealed with Ampliwax at 80°C, as recommended by the manufacturer, and stored at 4°C for 1–2 months.

PCR primers for DVE DNA-directed DNA polymerase gene

Primer 1 sequence: 5'-GAA-GGC-GGG-TAT-GTA-ATG-TA-3' (forward)

Primer 2 sequence: 5'-CAA-GGC-TCT-ATT-CGG-TAA-TG-3' (reverse)

i)   The upper reaction mixture is prepared according to the kit manufacturer's recommendations the day of the test, and distributed to each sample tube including DVE and lambda control tubes.

ii)   Add 10 µl of DNA suspension from the stored sample tubes to the PCR lower reaction tubes with corresponding labels.

iii)   Place known DVE DNA diluted to 1 pg/10 µl into one control tube and 10 µl of distilled water into the no DNA control tube. Add 10 µl of lambda DNA supplied in the kit and 10 µl of water to the corresponding lambda control tubes.

iv)   Place all the tubes in a thermal cycler that is programmed as follows:

One cycle:	Hold 94°C for 2 minutes
	Hold 37°C for 1 minute
	Hold 72°C for 3 minutes
35 cycles:	Hold 94°C for 1 minute
	Hold 55°C for 1 minute
	Hold 72°C for 2 minutes
One cycle:	Hold 72°C for 7 minutes
	Hold 4°C until stored

PCR tubes are stored at 4°C until the samples are examined for amplification products.

- **Electrophoretic analysis of PCR products**

i)   A fresh 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) is prepared from a 10× stock for agarose preparation and for use in the electrophoresis chamber.

ii)   A 1% agarose solution is prepared in TAE buffer, heated to dissolve the agarose, and, when cool, poured into a gel former with a comb.

iii)   The solidified gel is placed into the electrophoresis chamber and TAE running buffer is added.

iv)   PCR test samples, including the DVE and lambda controls, are mixed 1/10 with 1 µl of loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 0.01 M Tris/HCl, pH 8.0, and 50% [v/v] glycerol) and 10 µl of each is added to individual wells of the gel. The 100 bp molecular size markers are added to each side of the gel.

v)   Run the gel for 1 hour at 120 volts and then stain in a 1% ethidium bromide solution for 20 minutes. Destain the gel for 45 minutes in deionised water and view the gel on a UV-illuminated light box. Photograph the gel to record results.

- **Interpretation of the results**

A 500 bp amplification band in the lambda control sample indicates the PCR ran successfully. A 446 bp band in the DVE known DNA control indicates the DVE primers are working. A 446 bp band in the unknown test sample indicates DVE viral DNA was present. No amplification products will be present in the DVE or lambda no DNA controls. If bands appear in these negative control products, cross-contamination occurred during the test set-up and the test must be repeated.

f)   Strain variation

Although strains of DVE differ considerably in virulence, there is little reported evidence of serological variation.

2.   Serological tests

Serological tests have little value in the diagnosis of acute DVE infections, but assays based on serum neutralisation in embryonated eggs and cell cultures have been used to monitor antibodies following exposure to

DVE in wildfowl. The humoral response to natural infection with DVE virus is often low and antibodies may be short-lived (7); it is assumed that cell-mediated immunity also plays a role in the infection (18). However, detection of neutralising antibodies to DVE virus in serum is possible. Virus neutralisation (VN) (22) assays using a constant-serum/varying-virus method may be performed in chicken or duck embryos by using embryo-adapted virus, or in cell cultures. Neutralisation indices (NI) (28) between 0 and 1.5 were detected in domestic and wild waterfowl that had not been exposed to DVE; a NI of 1.75 or greater was considered to be evidence of prior exposure to DVE virus (3). Alternatively, sera may be screened using a constant-virus/varying-serum method. In the author's laboratory a microtitre neutralisation assay using primary MDEF or DEF is used. Serial twofold dilutions of each serum sample (heat-inactivated at 56°C) are prepared in 50 µl of serum-free MEM in microtitre plates. Approximately $10^{2.0}$ TCID$_{50}$ (50% tissue culture infective dose) of DVE virus in 50 µl of MEM is added to each well and the mixtures are allowed to react at 37°C for 1 hour. A suspension of primary MDEF or DEF in MEM supplemented with 2 mM L-glutamine, 0.17% sodium bicarbonate and 10% FCS, are adjusted to contain 3×10^5 cells per ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO_2 atmosphere. Following incubation, cells are observed daily by light microscopy and finally fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e. there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 3 log$_2$ is usually considered to be negative. A VN titre of 8 or greater is considered to be significant and is evidence of exposure to DVE virus (7). VN antibody may also be detected using cell cultures by mixing sera at a single dilution, e.g. 1/10, with 100–200 TCID$_{50}$ virus and then testing inoculated cell cultures for non-neutralised virus by immunofluorescence. Although this method is not quantitative, it can be useful for screening large numbers of sera. These latter methods, using constant-virus/varying-serum, are much more economical on sera than the NI methods.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A live attenuated virus vaccine can be used to control DVE in birds over 2 weeks of age (18, 19). Fattening or breeding ducks may be vaccinated subcutaneously or intramuscularly to produce an active immunity. The vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducks, as the unvaccinated birds remain susceptible to infection.

An inactivated vaccine has been reported to be as efficacious as modified live vaccine (20). This vaccine has been tested only under laboratory conditions; it has not been tested on a large scale and is not licensed.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

DVE vaccine can be prepared from a strain of the virus that has been attenuated by serial passage in embryonated chicken eggs. In the USA the vaccine strain seed was originally imported from Holland and has been serially passaged 41–46 times.

b) Method of culture

The seed virus should be prepared in 8–11-day-old specific pathogen free (SPF) embryonated chicken eggs by inoculating on to the CAM followed by incubation at 37°C. The seed may be stored at –70°C or lower in the form of a homogenate of the embryo CAM in buffered saline.

c) Validation as a vaccine

The seed virus should be shown to be free from extraneous viruses pathogenic to ducks, chickens and turkeys. It should also be free from bacterial, fungal and mycoplasmal contaminants.

The identity of the virus should be confirmed by a VN test conducted with specific antiserum using the constant-serum/varying-virus method. This test should be performed in embryonated chicken eggs. The antiserum should reduce the virus titre by at least $10^{1.75}$ ELD$_{50}$ (50% embryo lethal dose).

The immunogenicity of the vaccine can be assessed in DVE-susceptible ducks or ducklings by inoculating the recommended vaccine dose intramuscularly and challenging intramuscularly 21 days later with virulent DVE virus. The vaccinated birds should survive challenge while unvaccinated control birds should die. This test should be carried out on the master seed but need not be done routinely on each vaccine batch

produced. For release of subsequent batches, the titre of the virus should be a sufficient indication of vaccine potency.

Once frozen at –70°C or lower the vaccine stores well for at least 1 year with little loss in titre. Once issued, the vaccine should not be refrozen, it should be kept at 4°C and used as soon as possible.

2. Method of manufacture

The vaccine is produced in 8–11-day-old SPF embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. Most embryo deaths occur between 48 and 96 hours after inoculation. The embryos, their CAMs and chorioallantoic fluids are harvested, pooled and homogenised in buffered saline and clarified by low-speed centrifugation (1800 **g**). The preparation is diluted as appropriate, and a stabiliser is incorporated. It is then dispensed into vials and preferably frozen rapidly to –70°C or lower.

3. In-process control

Eggs that have been inoculated should be candled 24 hours later to identify any embryos that have died from nonspecific causes.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

A group of 1-day-old ducklings susceptible to DVE should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

The final product should be free from contamination by bacteria, fungi, and mycoplasma as well as extraneous viruses potentially pathogenic to poultry.

c) Potency

The virus titre of the vaccine should be determined in 9–11-day-old embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. The vaccine should contain a minimum of $10^{3.0}$ ELD_{50} per dose at time of use.

d) Duration of immunity

Immunity in vaccinated ducks should last throughout a breeding season. Annual re-vaccination is recommended (19).

e) Stability

When stored at –70°C or lower the vaccine is stable for at least 1 year. Potency testing should be repeated after this time on an aliquot of vaccine to determine whether virus titre has been maintained. Once thawed the vaccine should not be refrozen, it should be maintained at 4°C in a refrigerator but for no longer than 1 week. Lyophilised vaccine should be stored at 4–8°C and used before the stated expiry date.

f) Preservatives

No preservatives are added to the vaccine.

g) Precautions (hazards)

None.

5. Tests on final product

The vaccine is issued as a vial of frozen concentrated vaccine virus together with a bottle of sterile diluent (phosphate buffered saline), on which standard sterility checks have been made (see Section C.4.a).

a) Safety

No additional testing is performed after the batch testing.

b) Potency

No additional testing is performed after the batch testing.

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DUCK VIRUS HEPATITIS

SUMMARY

Hepatitis in ducks can be caused by at least three different viruses. The more common and internationally widespread is duck hepatitis virus (DHV) type I, now designated as an unclassified picornavirus, which causes a highly lethal, acute, contagious infection in ducklings under 6 weeks of age and, frequently, under 3 weeks of age. It does not occur in older birds. This infection is often referred to simply as duck virus hepatitis.

DHV type II has been reported in the United Kingdom only. It occurred in ducklings from 10 days to 6 weeks of age, and caused pathological changes similar to those of DHV type I. From electron microscopy and molecular studies it is considered to be an astrovirus.

DHV type III has been reported only in the United States of America. It causes similar liver lesions in young ducklings, but is less virulent than DHV type I. It is believed to be a picornavirus, serologically unrelated to type I virus.

Diagnosis of hepatitis in ducklings is based on the characteristic disease pattern in the flock, gross pathological changes, the recovery of virus from dead ducklings, and the reproduction of the disease in susceptible ducklings.

Identification of the agent: *It is not possible to distinguish among DHV types I, II and III on the basis of clinical findings and pathology, but distinctions can be made from the responses of ducklings, embryonated eggs and cell cultures to the isolated viruses. Alternatively DHV type I RNA may be detected by a one-step reverse-transcriptase polymerase chain reaction from duckling liver and also from allantoic fluid and embryo liver from inoculated duck eggs.*

Serological tests: *Serological tests have little value in the diagnosis of the acute infections caused by DHV types I, II and III.*

Serum neutralisation tests in ovo *have been used with all three viruses and* in-vitro *tests have been developed for DHV type 1. These tests have been used for virus identification, assay of immune responses to vaccination and epidemiological surveys.*

Requirements for vaccines and diagnostic biologicals: *DHV type I infections can be controlled by the use of live attenuated virus vaccines and an inactivated virus vaccine. They are administered to breeder ducks to confer passive immunity to ducklings. Live attenuated virus vaccines may also actively immunise DHV type-I-susceptible day-old ducklings.*

Ducklings susceptible to DHV type I may also be passively protected with a chicken egg yolk antibody preparation.

DHV type III infections can be controlled by the use of a live attenuated virus vaccine given to breeder ducks to confer passive immunity to ducklings.

A. INTRODUCTION

Duck hepatitis is caused by at least three different viruses, namely duck hepatitis virus (DHV) types I, II and III. The most common is DHV type I, which is designated as an unclassified picornavirus and may require to be assigned to a new genus within the Picornaviridae (4, 10, 15). DHV type II is an astrovirus, and DHV type III is considered to be a picornavirus serologically unrelated to DHV type I.

A new serotype of DHV named N-DHV that can cause high mortality with characteristic liver lesions has been reported (16). This virus, recovered from both mule ducklings and goslings, is antigenically unrelated to DHV type I, and although phylogenetically distinct, it is still closely related to DHV type I.

Until the present, DHV type I has only been associated with causing disease in mallard and Pekin ducklings but it has now been reported to cause pancreatitis and encephalitis in Muscovy ducks (6).

These viruses, which cause acute infections, should not be confused with duck hepatitis B virus, a hepadnavirus classified in the same group as mammalian hepatitis B virus. The significance of this infection for the duck is not fully understood.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) DHV type I

DHV type I causes a highly contagious infection of ducks. It is of no known public health significance. The disease is an acute, rapidly spreading, often fatal virus infection of young ducklings. It usually affects ducklings under 6 weeks of age and often much younger. The clinical disease is characterised by lethargy and ataxia. Ducklings lose their balance, fall on their sides and kick spasmodically prior to death. At death the head is usually drawn back in the opisthotonos position. The whole disease sequence is rapid and can take as little as 1–2 hours. Practically all mortality in a flock will occur within 3–4 days, with most deaths on the second day. Gross pathological changes appear chiefly in the liver, which is enlarged and displays distinct punctate and ecchymotic haemorrhages. Spleen enlargement and swelling of the kidneys with some congestion of renal blood vessels may also be apparent. Microscopic changes in the liver are characterised by extensive hepatocyte necrosis and bile duct hyperplasia, together with varying degrees of inflammatory cell response and haemorrhage.

The clinical and pathological observations are highly indicative of DHV type I infection. The virus can readily be recovered from liver tissue by homogenisation as a 20% (w/v) suspension in buffered saline. The suspension is clarified, and can then be treated further (if desired) with 5% chloroform (v/v) for 10–15 minutes at ambient temperature. DHV type I is resistant to this treatment.

The presence of DHV type I is usually confirmed by one or more of the following procedures:

i)   By subcutaneous or intramuscular inoculation of the isolate into ducklings between 1 and 7 days of age that are susceptible to DHV type I. The characteristic clinical disease should follow, with deaths occurring within 18–48 hours of inoculation, often in under 24 hours. The ducklings should show the gross pathology attributable to DHV type I. Virus should be re-isolated from the livers.

ii)   By inoculation of serial dilutions of the liver homogenate into the allantoic sac of embryonated duck eggs (10–14 days) or chicken eggs (8–10 days). Duck embryos die between 24 and 72 hours later, whereas chicken embryos are more variable and erratic in their response and usually take 5–8 days to die. Gross pathological changes in the embryos include stunting and subcutaneous haemorrhages over the whole body, with oedema particularly of the abdominal and hind limb regions. The embryo livers may be red and yellowish, swollen and may show some necrotic foci. In embryos that take longer to die, the greenish colour of the allantois is more pronounced, and both the liver lesions and stunting become more evident.

iii) By inoculation of primary cultures of duck embryo liver (DEL) cells, which are particularly sensitive (17). Dilutions of the liver homogenate containing DHV type I cause a cytopathic effect (CPE), which is characterised by cell rounding and necrosis. When overlaid with a maintenance medium containing 1% agarose (w/v), the CPE gives rise to plaques approximately 1 mm in diameter.

• **Immunological tests**

Such tests have not been used extensively for the routine identification of DHV type I infection. Various virus neutralisation (VN) assays have been described, which may assume greater significance if DHV types II and III infections become more widespread. The tests that have been described (2, 17–19) include:

i)   Passive subcutaneous immunisation of 1–7-day-old ducklings susceptible to DHV type I with 1–2 ml specific hyperimmune serum or specific egg yolk antibody. These ducklings are then challenged intramuscularly or subcutaneously, 24 hours later with at least $10^{3.0}$ LD_{50} (50% lethal dose) of the virus

isolate. A control group of uninoculated ducklings is similarly challenged. Identification of infection is based on 80–100% survival in the passively immune ducklings and 80–100% mortality in the controls.

ii) 1–7-day old DHV type-I-susceptible and DHV type I maternally immune ducklings are challenged intramuscularly or subcutaneously with at least $10^{3.0}$ LD_{50} of the virus isolate. Identification is based on 80–100% losses in the susceptible ducklings and 80–100% survival in the maternally immune ducklings.

iii) Serial tenfold dilutions of the virus isolate are mixed with equal volumes of DHV type-I-specific hyperimmune serum diluted between 1/5 and 1/10. The mixtures are allowed to react at room temperature for 1 hour and are then inoculated (0.2 ml) subcutaneously into susceptible ducklings, also via the allantoic cavity (0.2 ml) of embryonated duck eggs and on to primary DEL cell monolayer cultures. Controls in each case consist of the virus isolate mixed with control serum.

There is little evidence for antigenic variation among DHV type I isolates. However, a variant, DHV type Ia, isolated in the United States of America (USA) only partially reacts with the classical type I virus in cross serum neutralisation tests (12, 20). Other variants have been reported from India and Egypt, but nothing further is known about them. Recent reports of disease in Muscovy ducks from France (6) and a N-DHV from Chinese Taipei (16) have raised new questions about duck virus hepatitis.

- **Nucleic acid recognition methods**

Although some recent publications have revealed the molecular structure of DHV type I (4, 10, 15) only one has reported a one-step reverse-transcriptase polymerase chain reaction (RT-PCR) to detect DHV type I (9). This is the method outlined below.

- **Polymerase chain reaction**

This method has been extracted from (9). It is based on primers specific to amplify a region of the 3D gene.

- **Detection of DHV-I from duck and chicken embryo organs**

Supernatants prepared from duckling livers infected with DHV type I are collected and filtered (0.2 µm). The allantoic cavities of each of five 11-day-old duck and 9-day-old chicken embryonated eggs are inoculated with 0.2 ml viral supernatant. The allantoic fluid and liver samples are collected from embryos inoculated with two reference strains and each liver sample is ground in a tissue grinder and phosphate buffered saline is added to make 10% suspensions. Liver sample suspensions and allantoic fluid are centrifuged at 2000 **g** for 30 minutes, the supernatants are treated with the Viral Gene-spin™ viral DNA/RNA extraction kit and the nucleic acids are used for one-step RT-PCR.

- **Nucleic acid extraction**

Extractions of viral RNA are performed using the Viral Gene-spin™ viral DNA/RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea). In brief, a total of 150 µl of the sample for extraction is mixed with 250 µl lysis buffer. For the RT-PCR sensitivity tests, 50 µl of diethylpyrocarbonate (DEPC)-treated distilled water is added to 100 µl of tenfold virus dilution of the samples before mixing with the lysis buffer. A 350-µl aliquot of binding buffer is added to the mixture and triturated; the total 750 µl is placed into a minispin column, which is spun at room temperature for 1 minute at 13,000 rpm in a microcentrifuge. The flow-though is discarded and two cycles of washing–spinning–flow-through-removal are performed using washing buffers A and B (500 µl each), followed by a final spin for 1 minute to dry the membrane. The column is transferred to a new 1.5-ml collection tube and RNA is eluted by addition of 40 µl elution buffer and centrifugation for 1 minute at 13,000 rpm.

After measuring RNA concentrations using the NanoDrop ND-1000 (NanoDrop, Wilmington, DE), the samples are stored at −20°C.

- **One-step RT-PCR**

The one-step RT-PCR is conducted using the Maxime RT-PCR PreMix kit (iNtRON Biotechnology). The 20-µl reaction mixtures contain 1 U of OptiScript reverse transcriptase, 2.5 mM dNTPs, 2.5 U i-Star*Taq* DNA polymerase, and RT-PCR buffer (50 mM Tris/HCl and 75 mM KCl). In addition, the following components are included in the reaction: 4 µl (50 ng) RNA or DNA template, 1 µl (10 pmol/µl) of each specific primer (DHV-1 ComF and DHV-1 ComR), and DEPC-treated dH_2O to a total reaction volume of 20 µl.

A T-gradient thermal cycler (Biometra, Gottingen, Germany) is used for one-step RT-PCR. Reverse transcription is performed at 45°C for 30 minutes, after which the enzyme is inactivated at 94°C for 5 minutes. PCR amplification is conducted using an initial denaturation for 20 seconds at 94°C; followed by

40 cycles of annealing for 30 seconds at 52°C, extension for 30 seconds at 72°C, and denaturation for 20 seconds at 94°C; and a final extension for 5 minutes at 72°C. Reactions are stored at 4°C.

- **Detection of one-step RT-PCR products**

PCR products (10 µl) are separated by electrophoresis (100 V) in horizontal 1.5% agarose gels (iNtRON Biotechnology) and Tris-acetate buffer (40 mM Tris-acetate, 1 mM ethylenediamine tetra-acetic acid). Gels are stained with ethidium bromide (0.5 ug/ml), visualised under ultraviolet light, and photographed.

- **Interpretation of results**

A DNA fragment of 467 bp is amplified by one-step RT-PCR using RNA extracted from the livers of ducklings infected with reference DHV type I strains. Negative control RNA is obtained from an uninfected duckling liver and does not amplify under the same conditions.

b) DHV type II

DHV type II infection of ducks has only been reported from the United Kingdom (1, 5). It is an acute, fatal infection of ducklings producing clinical and pathological signs similar to DHV type I. Affected birds may show signs of polydypsia and usually die within 1–2 hours of appearing sick.

Gross pathological changes include multiple haemorrhages, both punctate and confluent bands in the liver, swollen pale kidneys with congested blood vessels, and enlarged spleens. The alimentary tract is often empty although the small intestine may contain mucus, and haemorrhagic areas are occasionally seen. Petechial haemorrhages are also occasionally seen on the heart. Histologically, changes in the liver are similar to those seen in DHV type I infections; the extent of bile duct hyperplasia may be greater than with DHV type I, but this is relative. DHV type II has astrovirus-like morphology and virions are 28–30 nm in diameter. It is classified in the family Astroviridae as duck astrovirus I (DAstV-I) (5, 11).

The virus may be recovered in 20% (w/v) homogenised liver suspensions in buffered saline. This can be used to inoculate:

i) Susceptible ducklings, in which the response can be variable. A mortality rate of up to 20% may occur within a period of 2–4 days. The gross pathology is similar to that observed in field cases (5). This is in contrast to the findings with DHV type I infection, which is more virulent and rapid in its effect.

ii) Embryonated chicken or duck eggs, either via the amniotic cavity or yolk sac. These may respond, erratically, after four passages, but no deaths may be seen during earlier passages. Embryos take 6–10 days to show evidence of infection; when this occurs there is stunting with green necrotic livers.

- **Immunological tests**

Immunological tests have not been employed routinely as the serological response to infection of both ducklings and duck embryos is poor. However, a neutralisation assay has been applied (5) for virus identification by inoculating chicken embryos via the amniotic cavity with constant-serum/varying-virus mixtures.

Cross protection tests have been performed in 2–4-day old ducklings (5); these are inoculated with antisera to types I or II, then challenged 3 days later with the virus isolate. This technique could distinguish DHV type II from types I and III.

c) DHV type III

DHV type III has been reported in the USA only. Losses of up to 20% occur in ducklings immune to DHV type I (7, 13). DHV type III causes an acute infection of young ducklings with clinical signs similar to those seen in type I infections.

The gross pathology is also similar to type I infection. The liver surface is pale and mottled with many red bands and some petechial haemorrhages. The spleen is paler, but not noticeably enlarged, and the kidneys may show patchy congestion.

The virus can be recovered from homogenised liver suspensions and is resistant to treatment with 5% chloroform. The virus can be isolated by:

i) Inoculating the isolate intramuscularly into susceptible ducklings. The mortality rate may reach 20% with 60% morbidity. No deaths occur in the first 24 hours and all losses ensue between day 2 and day 4 after inoculation. Intravenous inoculation is more effective; type III infection is less virulent than type I.

ii) Inoculating the isolate on to the chorioallantoic membrane (CAM) of 10-day-old embryonated duck eggs. The response is erratic, but some embryo mortality always occurs within 7–10 days. The membranes assume a dry crusty appearance, beneath which they are oedematous. The embryos may be stunted and oedematous with skin haemorrhages. The liver, kidneys and spleen are enlarged.

Attempts to cultivate the virus in hens' eggs have not been successful.

Attempts to induce a CPE with the virus in tissue cultures have not been successful, but the virus has been detected by direct immunofluorescence in experimentally infected DEL and duck embryo kidney (DEK) cell monolayer cultures (7).

2. Serological tests

These do not apply to diagnosis as the clinical disease is too acute.

All three DHV types have been used in virus neutralisation tests *in ovo*, but their success depends on the expression of the virus in the assay system used; with type II and III viruses this can be a problem. *In-vitro* tests have been developed for DHV type I; these include a plaque reduction assay and a microtitre assay (17, 18). The plaque reduction assay may be performed using either primary DEK or DEL cells. Primary cell culture monolayers are prepared in Eagle's minimal essential medium (MEM) containing 5–10% fetal calf serum (FCS), 2 mM glutamine, 0.17% sodium bicarbonate and gentamicin. Trypsinised cells are seeded into 5 cm diameter Petri dishes, then incubated at 37°C in a 5% CO_2 atmosphere. Monolayers should be nearly confluent at 24–48 hours post-seeding. The monolayers are washed twice with serum-free MEM or Hank's balanced salt solution to remove all traces of FCS before infecting with DHV type I. Equal volumes of DHV type I suspended in serum-free MEM, adjusted to 200 plaque-forming units (PFU) per 0.1 ml, are mixed with equal volumes of serially diluted duck sera (twofold dilutions in MEM). The serum samples should be heat inactivated at 56°C for 30 minutes before testing. The virus/serum mixtures are incubated at 37°C for 1 hour, then 0.1-ml aliquots are added to the confluent cell monolayers, three dishes per dilution. The plates are left for 30 minutes at room temperature (20–22°C), then overlaid with agarose maintenance medium (MEM containing 2% chicken serum and 0.1–0.2% FCS to which agarose had been added to a final concentration of 1% [w/w]). The plates are then placed at 37°C in a 5% CO_2 atmosphere. The number of plaques produced is recorded after 48 hours' incubation. Plaques may be observed using an oblique light source, or alternatively monolayers may be fixed with 10% formol-buffered saline and stained with 1% crystal violet. Serum antibody titres are expressed as the reciprocal of the highest serum dilution that reduces the plaque count by 50%.

A microtitre neutralisation assay may be performed using primary DEK cells. Serial twofold dilutions of each serum sample (heat-inactivated) are prepared in 50 µl of serum-free Eagle's basal medium (BME) in microtitre plates. Approximately $10^{2.0}$ $TCID_{50}$ (50% tissue culture infective dose) of DHV type I in 50 µl of BME is added to each well and the mixtures are allowed to react at 37°C for 1 hour. Primary DEK cells are suspended in BME supplemented with 10% tryptose phosphate broth, 2 mM L-glutamine, 0.17% sodium bicarbonate and 2–4% chicken serum, and are adjusted to contain 3×10^5 cells/ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO_2 atmosphere. Following incubation, cells are fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e., there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 4 $\log_2$ is considered to be negative.

These neutralisation tests have been used to assay humoral immune responses to vaccination and for epidemiological surveys, as well as for virus identification.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

DHV type I can be controlled by the use of a live attenuated virus vaccine. This is given to breeder ducks so that immunity is transferred via the yolk to newly hatched birds. Live vaccine virus can also be used to actively immunise newly hatched DHV type-I-susceptible ducklings (3). An inactivated DHV type I vaccine is also effective when administered to breeder ducks that have been primed with live vaccine or previously field exposed to live DHV type I; progeny from these breeders have maternal immunity (18). Ducks may also be passively protected by inoculation of antibodies in chicken egg-yolk.

An attenuated live virus DHV type II vaccine has been used to protect ducklings only under experimental conditions (5).

DHV type III infections have been controlled by the use of attenuated live virus vaccines given to breeder ducks, so that the immunity is transferred via the yolk sac to the hatching ducklings.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

The type I virus vaccine seed used most commonly in Europe is derived from an isolate passaged in embryonated chicken eggs 53–55 times, that in the USA for live and inactivated vaccines has been passaged 84–89 times.

The type II virus vaccine seed originated from an isolate attenuated by 25 serial passages in embryonated chicken eggs (1), and has been employed only experimentally under field conditions (R.E. Gough, personal communication).

The type III vaccine seed has been attenuated by 30 serial passages in embryonated duck eggs inoculated via the CAM.

b) Method of culture

The seed viruses of types I and II are handled similarly. They should be prepared in 9–10-day-old specific pathogen free (SPF) embryonated chicken eggs inoculated via the allantoic route and incubated at 37°C. They can be stored as embryo homogenates in buffered saline at –70°C or below for several years.

The type III seed virus is prepared in 10-day-old SPF duck embryos, inoculated on to the CAM, and incubated for 6–10 days at 37°C. It may be stored as a homogenate of CAM and embryos at –70°C or below.

c) Validation as a vaccine

All seed viruses should be shown to be free from extraneous viruses that are pathogenic for ducks, chickens or turkeys. The seeds should be free from all microbiological and fungal contamination.

In the case of newly hatched ducklings, attenuated live DHV type I replicates rapidly and results in an immunity within 48–72 hours of vaccination. This immunity persists throughout the susceptible period of life (3). However in ducklings protected by vaccination of their parents, the level of maternally derived immunity decreases over the first 2 weeks of life, but such ducklings can be actively re-immunised with attenuated virus given subcutaneously or orally at about 7–10 days of age (8, 14). Alternatively, the immunity can be enhanced by the administration of specific hyperimmune serum or of egg yolk antibody prepared from eggs laid by chickens actively hyperimmunised against DHV type I.

Breeder ducks primed with live DHV type I and then given, intramuscularly, a single dose of inactivated type I vaccine produced maternally immune progeny through a complete laying cycle (18).

2. Method of manufacture

DHV types I and II viruses are treated similarly. The vaccine is produced in 9–10-day-old SPF embryonated chicken eggs inoculated via the allantoic route, and incubated at 37°C. Most embryo deaths occur within 2–3 days in the case of DHV type I, but with type II, the deaths do not occur until 6–10 days after inoculation, although they are harvested at 3–5 days for maximum virus yield. The embryo harvests are homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at –70°C or below. Subsequently, they may be stored satisfactorily between –20°C and –40°C. DHV type I attenuated vaccine is also available as a lyophilised preparation that may be stored at 2–8°C. The reconstituted vaccine may be used with or without the incorporation of aluminium hydroxide in the diluent.

In the case of inactivated DHV type I vaccine, the embryo harvests are homogenised and clarified by low-speed centrifugation and then further purified by treatment with chloroform (final concentration 10% [v/v]). This preparation is then inactivated with freshly prepared binary ethylenimine (BEI). The inactivated virus is then blended with an adjuvant such as LES-STM[1]; 0.2 % (v/v) formalin is added as a preservative (18).

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1 A preparation of *Salmonella typhimurium* (STM), a B cell mitogen, in a lipid emulsion system (LES). Available from Ribi Immunochem Research, Hamilton, Montana 59840, USA.

The type III vaccine is prepared in 10-day-old SPF duck eggs inoculated via the CAM with attenuated DHV type III and incubated at 37°C. Most embryo deaths occur between 6 and 10 days. Eggs containing dying embryos, together with their CAMs, are harvested and homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at –70°C or below.

- **Egg yolk antibody**

    Virulent DHV type I prepared from duckling livers or attenuated virus may be used to hyperimmunise SPF chickens for egg-yolk antibody production. Eggs are collected from the hyperimmunised birds and stored at 4°C until time of production. The yolks are separated, pooled and blended with an antifoaming agent. The mixture is diluted with buffered saline containing no more than 0.2% (v/v) formalin as a preservative. The dispensed product is stored at 4°C and has a shelf life of 1 year. Tests are carried out for sterility in the usual way for the absence of contaminants.

3. In-process control

Any embryo deaths within the first 24 hours of inoculation should be discarded as nonspecific deaths.

The identity of the virus type should be confirmed by a VN test conducted with specific antiserum by a constant-serum/varying-virus method. In the case of types I and II viruses, the tests are performed in embryonated chicken eggs; with type III virus the tests are done in embryonated duck eggs. The antiserum should reduce the titre of the respective virus by at least $10^{2.0}$ ELD_{50} (50% embryo lethal dose).

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the Chapter 1.1.9.

b) Safety

A group of 1–3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of types I and II), or subcutaneously (in the case of type III), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHV type I vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

c) Potency

For DHV types I and II viruses, the virus titre of the vaccine should be determined in 9–10-day-old embryonated chicken eggs inoculated into the allantoic cavity and incubated at 37°C. The immunogenicity, of the vaccine for ducklings susceptible to type I or II virus can be assessed by inoculating subcutaneously a minimum of $10^{3.3}$ ELD_{50} per duckling of the vaccine virus and challenging subcutaneously 72 hours later with $10^{3.0}$ LD_{50} per duckling of virulent DHV virus type I or II (3). At least 80% of the vaccinated birds should survive and, in the case of type I, at least 80% of the controls should die; in the case of type II, a 20% mortality in the controls is more realistic.

The immunogenicity of the inactivated vaccine is considered to be satisfactory if a four-fold or greater increase in neutralising antibody titre can be demonstrated following administration to ducklings that have been previously primed with live attenuated DHV type I.

For type III virus, the titre of the vaccine should be determined in 10-day-old embryonated duck eggs inoculated on to the CAM. Immunogenicity tests in ducklings have proved difficult because of the variable pathogenicity of the challenge virus for ducklings.

Potency tests on yolk antibody are done by determining the neutralising index (NI) for the product in embryonated hens' eggs using the constant-yolk/varying-virus method. A minimum NI of $10^{3.0}$ is considered to be satisfactory. The efficacy of the product is determined by inoculating a group of susceptible ducklings with the recommended dose of egg yolk antibody. A second group is left untreated. After 24 hours each group is challenged with virulent DHV type I virus. The product is adjudged efficacious if at least 80% of the treated ducklings survive and at least 80% of the controls die.

d) Duration of immunity

Breeder ducks given live attenuated DHV type I vaccine two or three times at 12, 8 and 4 weeks before coming in to lay, and breeder ducks given live attenuated DHV type III vaccine twice at 12 and 4 weeks before coming in to lay should produce passively immune progeny throughout a breeding season. However, it is usually recommended to revaccinate every 3 months with DHV type I vaccine and every 6 months with DHV type III vaccine after the onset of lay. DVH type I attenuated vaccine can also be supplied as a lyophilised preparation that is blended with a diluent containing aluminium hydroxide, just before administration. This is given at 7 weeks of age with a second dose 2 weeks before onset of lay. This should provide maternally immune progeny throughout a complete laying cycle. No information on the use of DHV type II vaccine in breeder ducks is available.

Live attenuated DHV type I or type II vaccine given subcutaneously or intramuscularly to 1-day-old ducklings protects against the disease for the duration of their susceptibility. No information is available on the use of DHV type III vaccine to actively immunise 1-day-old ducklings.

Breeder ducks primed with live DHV type I and then given a single dose of inactivated DHV type I vaccine intramuscularly, should produce maternally immune progeny through a complete laying cycle (18).

Egg-yolk antibody offers passive immunisation in the face of an outbreak. The duration of its efficacy is short-lived.

e) Stability

Aqueous preparations of live attenuated DHV type I, II and III vaccines when stored frozen at –70°C or lower should remain stable for at least 1 year. Once thawed these vaccines should be held at 4°C and used within 1 week. Live lyophilised vaccines may be stored at 2–8°C and should retain their potency for at least 1 year.

The inactivated DHV type I vaccine is blended with adjuvant and can be stored at 4°C for at least 20 months without loss of immunogenicity.

Egg-yolk antibody can be stored for up to 1 year at 4°C.

f) Preservatives

No preservatives are added to the live attenuated DHV type I, II and III vaccines.

Formalin (up to 0.2% [v/v]) is added to the DHV type I inactivated vaccine, and to the egg-yolk antibody preparation.

g) Precautions (hazards)

The inactivated DHV type I vaccine should be shaken well to ensure that it is completely blended before use.

5. Tests on the final product

The live attenuated DHV type I and III vaccines are issued as vials of lyophilised or frozen concentrated vaccine virus together with bottles of sterile diluent, on which standard sterility checks have been made (see Section C.4.a). The DHV type II live attenuated vaccine has only been made experimentally.

a) Safety

No additional testing is performed after the batch testing on any of the products.

b) Potency

No additional testing is performed after the batch testing on any of the products.

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FOWL CHOLERA

SUMMARY

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is distributed world-wide. Fowl cholera outbreaks often manifest as acute fatal septicaemia. Diagnosis depends on isolation and identification of the causative bacterium, Pasteurella multocida*. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of myriad bacteria in blood smears, or impression smears of tissues such as liver or spleen. Mild or chronic forms of the disease also occur where the disease is endemic, with localised infection primarily of the respiratory and skeletal systems.*

Identification of the agent: Pasteurella multocida *is readily isolated, often in pure culture, from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, or from the caseous exudate characteristic of chronic fowl cholera lesions. It is a facultative anaerobic bacterium that grows best at 37°C. Primary isolation is usually accomplished using media such as dextrose starch agar, blood agar, and trypticase–soy agar. Isolation may be improved by the addition of 5% heat-inactivated serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation and are discrete, circular, convex, translucent, and butyraceous. The cells are coccobacillary or short rod-shaped, 0.2–0.4 × 0.6–2.5 μm in size, stain Gram negative, and generally occur singly or in pairs. Bipolar staining is evident with Wright or Giemsa stains.*

Identification of P. multocida *is based on the results of biochemical tests, which include carbohydrate fermentation, enzyme production, and selected metabolite production.*

Serological characterisation of strains of P. multocida *includes capsular serogrouping and somatic serotyping. DNA fingerprinting can differentiate among* P. multocida *having the same capsular serogroup and somatic serotype. These characterisations require a specialised laboratory with appropriate diagnostic reagents.*

Serological tests: *Serological tests are rarely used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis through isolation and identification of the causative organism generally precludes the need for serodiagnosis.*

Requirements for vaccines and diagnostic biologicals: *The* P. multocida *vaccines in general use are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple serotypes. Two doses of the killed vaccine are typically required. Live culture vaccines tend to impart greater protective immunity, but are used less frequently because of potential post-vaccinal sequelae such as pneumonitis and arthritis. Multivalent vaccines typically incorporate somatic serotypes 1, 3, and 4 as they among the more commonly isolated avian serotypes. Safety and potency testing of bacterins usually use the host animal. Final containers of live cultures are tested for potency by bacterial counts.*

A. INTRODUCTION

Fowl cholera is a contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida*. It typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality. Chronic infections also occur with clinical signs and lesions related to localised infections. The pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection. Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic

septicaemia. Fowl cholera is not considered to have zoonotic potential as avian isolates are generally nonpathogenic in mammals exposed by the oral or subcutaneous routes. Other bacterial diseases, including salmonellosis, colibacillosis, and listeriosis in chickens, and pseudotuberculosis, erysipelas, and chlamydiosis in turkeys, may present with clinical signs and lesions similar to fowl cholera. Differentiation is based on isolation and identification as *P. multocida* is readily cultured from cases of fowl cholera.

B. DIAGNOSTIC TECHNIQUES

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is often fatal (3, 7). In the peracute form, fowl cholera is one of the most virulent and infectious diseases of poultry. Diagnosis depends on identification of the causative bacterium, *P. multocida*, following isolation from birds with signs and lesions consistent with this disease. Presumptive diagnosis may be based on the observance of typical signs and lesions and/or on the microscopic demonstration of bacteria showing bipolar staining in smears of tissues, such as blood, liver, or spleen. Mild forms of the disease may occur.

All avian species are susceptible to *P. multocida*, although turkeys may be the most severely affected. Often the first sign of disease is dead birds. Other signs include: fever, anorexia, depression, mucus discharge from the mouth, diarrhoea, ruffled feathers, drop in egg production coupled with smaller eggs, increased respiratory rate, and cyanosis at the time of death. Lesions that are often observed include: congested organs with serosal haemorrhages, enlarged liver and spleen, multiple small necrotic areas in the liver and/or spleen, pneumonia, and mild ascites and pericardial oedema. Birds that survive the acute septicaemic stage or those infected with organisms of low virulence may develop chronic fowl cholera, characterised by localised infections. These infections often involve joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by bacterial colonisation with necrosis, fibrinosuppurative exudate, and degrees of fibroplasia.

Diagnosis depends on isolation and identification of the causative organism.

1. Identification of the agent

Pasteurella multocida is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished using media such as blood agar, trypticase–soy agar or dextrose starch agar, and isolation may be improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not require supplemental serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation. They usually are discrete, circular, convex, translucent, and butyraceous. Capsulated organisms usually produce larger colonies than those of noncapsulated organisms. Watery mucoid colonies, often observed with mammalian respiratory tract isolates, are very rare with avian isolates. The cells are coccobacillary or short rod-shaped, usually 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found in tissue smears show bipolar staining with Wright or Giemsa stains or methylene blue, and are usually encapsulated.

Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that succumb to the acute form of the disease, and from exudative lesions of birds with the chronic form of the disease, is generally easily accomplished. Isolation from those chronically affected birds that have no evidence of disease other than emaciation and lethargy is often difficult. In this condition or when host decomposition has occurred, bone marrow is the tissue of choice for isolation attempts. The surface of the tissue to be cultured is seared with a hot spatula and a specimen is obtained by inserting a sterile cotton swab, wire or plastic loop through the heat-sterilised surface. The specimen is inoculated directly on to agar medium or into tryptose or another broth medium, incubated for a few hours, transferred to agar medium, and incubated again.

Identification is based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential. Those carbohydrates that are fermented include: glucose, mannose, galactose, fructose, and sucrose. Those not fermented include: rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, m-inositol, and salicin. Mannitol is usually fermented. Arabinose, maltose, lactose, and dextrin are usually not fermented. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. *Pasteurella multocida* does not cause haemolysis, is not motile and only rarely grows on MacConkey agar. It produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, beta-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; indole and hydrogen sulphide are produced, and methyl red and Voges–Proskauer tests are negative. Detection of hydrogen sulphide production may require lead acetate-laden paper strips suspended above a modified H_2S liquid medium (8). Commercial biochemical test kits are available.

Differentiation of *P. multocida* from other avian *Pasteurella* spp. and *Riemerella* (*Pasteurella*) *anatipestifer* can usually be accomplished using the tests and results indicated in Table 1. Laboratory experience has

shown that *P. multocida* is most easily identified by its colony morphology and appearance in Gram stains. Positive reactions to indole and ornithine decarboxylase are the most useful biochemical indications.

Table 1. *Tests used to differentiate* Pasteurella multocida *from other avian*
Pasteurella *species and* Riemerella anatipestifer

Test*	Pasteurella		Riemerella
	multocida	*gallinarum*	*anatipestifer*
Haemolysis on blood agar	–*	–	v
Growth on MacConkey's agar	–	–	–
Indole production	+	–	–
Gelatin liquefaction	–	–	+u
Catalase production	+	+	+
Urease production	–	–	v
Glucose fermentation	+	+	–
Lactose fermentation	–u	–	–
Sucrose fermentation	+	+	–
Maltose fermentation	–u	+	–
Ornithine decarboxylase	+	–	–

*Test reaction results: – = no reaction; + = reaction; v = variable reactions; –u = usually no reaction; +u usually a reaction.

Antigenic characterisation of *P. multocida* is accomplished by capsular serogrouping and somatic serotyping. Capsular serogroups are determined by a passive haemagglutination test (1, 2). Capsular serogroups, determined by a passive haemaglutination test, are A, B, D, E, and F. All but serogroup E have been isolated from avian hosts. A nonserological disk diffusion test that uses specific mucopolysaccharidases to differentiate serogroups A, D, and F has been developed (6).

Somatic serotypes are usually determined by an agar gel immunodiffusion (AGID) test (4, 5). Serotypes 1 through 16 have been reported; all 16 serotypes have been isolated from avian hosts (8). The most effective characterisation involves determination of both serogroup and serotype. These determinations require a specialised laboratory with appropriate diagnostic reagents. To determine the serotype, the laboratory prepares the unknown bacterial culture as antigen for the AGID test and then must test it against all 16 serotype-specific antisera. Antigens present in a single isolate may react with multiple serotype-specific antisera resulting in bi- or trinomial serotypes, as illustrated by the 3, 4 and 3, 4, 12 strains (8).

- **Somatic typing procedure using the gel diffusion precipitin test**

  i) Inoculate a dextrose starch agar (DSA) plate (20 × 150 mm containing 70 ml of medium or two 15 × 100 mm plates containing 20 ml of medium per plate) with cells from a pure culture of *Pasteurella multocida* by using a sterile cotton swab. Swab the entire surface of the plate(s). Incubate the plate(s) in a 37°C incubator for 18–24 hours. This procedure is used to produce antigen for positive control purposes or to prepare antigen from diagnostic cultures.

  ii) Harvest the cells from the plate(s) using 2.5 ml of 0.85% saline with 0.6% formaldehyde and a sterile hockey stick. Place the cells in a tube using a sterile pipette.

  iii) Autoclave the cells at 100°C for 1 hour.

  iv) Centrifuge the cell suspension mixture at 13,300 ***g*** for 20 minutes.

  v) Remove the supernatant and place in a sterile tube.

  vi) Prepare the agar gel for use in the gel diffusion precipitin test (GDPT) by placing 17.0 g of NaCl, 1.8 g of agar noble, and 200 ml of distilled water into a 500 ml flask. Microwave the contents of the flask with the cap loose for 2.5 minutes. Swirl the contents of the flask and microwave again for 2.5 minutes. Allow the agar to cool slightly for 10–15 minutes. Do not prepare less than 200 ml of agar in a microwave. Dehydration during the microwave process can increase the agar concentration and negatively impact or inhibit diffusion.

vii) Place 5 ml of melted agar onto the surface of a 75 × 25 mm plain glass microscope slide. It is important that the slides are level prior to dispensing the agar. Allow the agar to cool (approximately 30 minutes) completely.

viii) Wells are cut in the agar bed. The wells are 3 mm in diameter and 3 mm apart from edge-to-edge. Frequently an Ouchterlony template is used to create two or three replicates of wells per slide. Each replicate has a centre well and is surrounded by four wells located at 90° angles (from centre).

ix) Reference antiserum is always placed in the centre well (of a replicate). Antigen from a diagnostic or reference culture is placed in one of the surrounding wells within a replicate. Each well is filled to capacity.

x) The slides are incubated within a moist chamber in a 37°C incubator for 48 hours. Precipitin lines of a reaction can be best observed with subdued lighting from underneath the slide. When present, reactions should occur between the centre and surrounding well(s) as an arc of precipitin. Sometimes these reactions are close to the edge of a well. Slides should be carefully examined. Diagnostic cultures can react to more than one reference somatic antiserum.

xi) Positive controls should be used. Reference antiserum should be tested against reference antigen each time the test is performed.

DNA fingerprinting of *P. multocida* by restriction endonuclease analysis (REA) has proved valuable in epidemiological investigations fowl cholera in poultry flocks. Isolates of *P. multocida* having both capsular serogroup and somatic serotype in common may be distinguished by REA. Ethidium-bromide-stained agarose gels are analysed following electrophoresis of DNA digested with either *Hha*I or *Hpa*II endonuclease (10).

2. Serological tests

Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for serodiagnosis. Serological tests, such as agglutination, AGID, and passive haemagglutination, have been used experimentally to demonstrate antibody against *P. multocida* in serum from avian hosts; none were highly sensitive. Determinations of antibody titres using enzyme-linked immunosorbent assays have been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but not for diagnosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Fowl cholera may be caused by any of 16 Heddleston serotypes of *P. multocida*, although certain serotypes appear to be more often associated with disease. The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide or oil adjuvant, prepared from inactivated cells of serotypes selected on the basis of epidemiological information. Commercial bacterins are usually composed of serotypes 1, 3, and 4. Vaccination plays a significant role in the control of this disease. Live vaccines containing modified *P. multocida* are not generally used except in North America.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Bacterin is normally administered by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are typically administered at 2–4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course. Live vaccines are typically administered in the drinking water. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

1. Method of manufacture

The general method for production of *P. multocida* bacterins is presented here. Production cultures of each bacterial isolate to be included in the final product are prepared. The cultures are typically started in small vessels and subpassaged into progressively larger volumes of media until the desired production volume is achieved. Each production culture is inactivated by formalin or other acceptable means. All of the component cultures are mixed, and usually blended, with an adjuvant prior to filling sterile final containers.

The following section is based on the requirements for *P. multocida* bacterins and vaccines as found in Title 9, United States Code of Federal Regulations. Other countries may have slightly different requirements.

2. Master seed management

a) Characteristics of the seed

All strains of *P. multocida* to be incorporated into a bacterin or vaccine must be well characterised, of known serotype, pure, safe and immunogenic. The culture(s) that is evaluated and characterised is designated by lot number and called a master seed. All cultures used in the production of licensed bacterins or vaccines must be derived from an approved master seed(s) and must be within an accepted number of passages from the master seed lot.

b) Validation as a vaccine

i) *Efficacy*

Products prepared from candidate master seeds must be shown to be effective against challenge infection. Efficacy must be demonstrated in each animal species (chickens, turkeys, ducks, psittacines) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. The lot of product used to demonstrate efficacy must be produced from the highest allowable passage of master seed.

For live avian *Pasteurella* vaccines, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 days after vaccination and are observed for 10 days after challenge. A satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive.

The arithmetic mean count of colony-forming units in the lot of product that is used to demonstrate efficacy is used as the minimum standard (immunogenicity standard) for all subsequent production lots of vaccine.

Efficacy of bacterins must be demonstrated similarly prior to licensure. However, no immunogenicity standards are derived from the lot that was used to demonstrate initial efficacy; each production lot is satisfactorily tested in a vaccination-challenge trial prior to release for sale and distribution.

ii) *Safety*

The safety of master seeds used in the production of live vaccines must be evaluated prior to licensing. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. At least 8 of 10 birds must show no unfavourable reactions attributable to the master seed. Additionally, the master seeds must be tested for reversion to virulence and evaluated for excretion from the host and transmission to other target species.

The safety of each production lot is tested by methods described in Section C.4.c.

3. In-process control

The purity of the cultures is determined at each stage of production prior to inactivation. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) and/or by culture. Killed cultures are tested for completeness of inactivation. Analytical assays to determine the levels of formaldehyde or other preservatives are done on bulk vaccine and must be within specified limits. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used for immunogenicity studies.

4. Batch control

a) Sterility

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.26 or 113.27 (9). (See also Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

Safety testing is conducted on each bulk or filled vaccine lot. Live vaccines are tested according to the method described in C.1.c.ii, except that only one representative animal species is required. Bacterins

are administered according to label recommendations, and the birds are observed for 14 days; at least 18 of 20 birds must show no unfavourable reactions attributable to the bacterin.

c) Potency

Each production lot of bacterin or live vaccine must be tested for potency by a test that is related to, and considered predictive of, efficacy. Potency tests are performed on the product in its final form.

Bacterins are tested for potency in a vaccination-challenge trial. Separate groups of birds (20 vaccinates, 10 controls) must be challenged with each of the serotypes of *P. multocida* for which protection is claimed. Bacterins are administered according to the dose and route recommended on the label. Two doses are administered 3 weeks apart, and all birds are challenged 2 weeks after the second dose. The birds are observed for 14 days after challenge. For a satisfactory test, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die.

The potency of live vaccine lots is determined by a bacterial count performed on reconstituted lyophilised product in its final container. The mean bacterial count of any vaccine lot at the time of preparation must be sufficiently high to ensure that at any time prior to product expiration, the count is at least twice the immunogenicity standard. (The European Pharmacopoeia requires a count that is at least equal to the immunogenicity standard.)

d) Stability

The acceptability of the shelf life of a vaccine is confirmed by testing the product for potency at the end of the approved shelf life. At least three lots of vaccine are tested and must pass established potency requirements. Vaccines are stored at 2–7°C and protected from freezing. Partly used packs should be discarded at the end of a day's operations.

e) Preservatives

Any preservatives must be added within specified limits. Preservatives are generally added to vaccines to limit the growth of any contaminants introduced when the rubber cap is pierced with a needle. Ideally, multidose vaccination equipment should be used whereby the vaccine pack is entered only once with a sterile needle.

f) Precautions (hazards)

Vaccines prepared with aluminium-based adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate problems, but medical advice should be sought as there is a risk of infection via a contaminated needle.

Vaccines prepared with oil based adjuvants may cause more severe reactions at the site of injection, which may manifest as large nodules. Care should be taken to administer these vaccines correctly. Operator self injection requires immediate medical attention, involving prompt incision and irrigation of the site.

5. Tests on final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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FOWLPOX

SUMMARY

Fowlpox is a disease of chickens and turkeys caused by a DNA virus of the genus Avipoxvirus *of the family Poxviridae. Its distribution is world-wide. It is slow-spreading and characterised by the formation of proliferative lesions and scabs on the skin, and diphtheritic lesions in the upper parts of the digestive and respiratory tracts. In the case of the cutaneous form, the mortality rate is usually low and affected birds are more likely to recover than those with the diphtheritic form. In the diphtheritic form, proliferative lesions involving the nasal passages, larynx or trachea can result in respiratory distress and death from suffocation.*

Fowlpox causes a transient drop in egg production and a reduced growth rate in young birds.

Identification of the agent: *Fowlpox should be suspected where skin eruptions occur on exposed areas. Histological examination of cutaneous or diphtheritic lesions reveals epithelial hyperplasia with intracytoplasmic inclusions in affected cells. Elementary bodies may be detected in smears from lesions by the use of the Gimenez method. Electron microscopy of lesions will detect virus particles with the characteristic poxvirus morphology by negative staining or in ultrathin sections of the lesion.*

The diphtheritic form of fowlpox involving the trachea must be differentiated from infectious laryngotracheitis, which is caused by a herpesvirus and is characterised by the presence of intranuclear inclusion bodies.

Virus isolation is done by inoculation on to chorioallantoic membranes of 9–12-day-old developing chicken embryos or avian cell cultures.

Serological tests: *Immune responses to fowlpox virus may be demonstrated by the use of virus neutralisation, agar gel immunodiffusion, immunofluorescence, or passive hemagglutination tests, enzyme-linked immunosorbent assay and by immunoblotting.*

Requirements for vaccines and diagnostic biologicals: *Modified live fowlpox or pigeon pox virus vaccines of chicken embryo or avian cell culture origin are available commercially. The use of vaccines is indicated in areas where the disease is endemic, or on premises where infection has been diagnosed.*

A. INTRODUCTION

The morphology of the fowlpox virus is like that of other viruses of the poxviridae family. The mature virus (elementary body) is brick shaped and measures about 330 × 280 × 200 nm. The outer coat is composed of random arrangements of surface tubules. The virion consists of an electron-dense centrally located biconcave core or nucleoid with two lateral bodies in each concavity and surrounded by an envelope. The 288 kbp fowlpox virus genome encodes for over 250 genes.

Fowlpox has a world-wide distribution and is caused by a DNA virus of the genus *Avipoxvirus* of the family *Poxviridae* (17, 21). Its incidence is variable in different areas because of differences in climate, management and hygiene or the practice of regular vaccination. It can cause drops in egg production, or retarded growth in younger birds.

Fowlpox is a slow-spreading virus disease of chickens and turkeys, characterised in the cutaneous form (dry pox) by the development of proliferative lesions, ranging from small nodules to spherical wart-like masses on the skin of the comb, wattle and other unfeathered areas. In the diphtheritic form (wet pox), slightly elevated white opaque

nodules develop on the mucous membranes. They rapidly increase in size to become a yellowish diphtheritic membrane. Lesions occur on the mucous membranes of the mouth, oesophagus, larynx or trachea. The mortality rate is higher in the diphtheritic form than in the cutaneous form, sometimes nearing 50% particularly in young birds. Integration of reticuloendotheliosis virus (REV) sequences has been observed in the genome of fowlpox virus (11, 13). It is interesting that this insertion event occurred over 50 years ago (7). While most field strains contain REV provirus, vaccine strains have only remnants of long terminal repeats (13). Virulence is enhanced by the presence of REV provirus in the genome of field strains of fowlpox virus. Complete sequence of the genome of a vaccine-like strain of fowlpox virus has been determined (1). The functions of the majority of the genes are not known at this time. It is however, interesting that the virus tends to persist in the poultry environment for extended periods of time where other viruses may not survive. In this regard the presence of photolyase gene and A-type inclusion body gene in the virus genome appear to protect the virus from environmental insults (15, 16). Antigenic cross-reactivity is observed among avianpox viruses and it appears that many genes are conserved. Limited studies on antigenic, genetic and biologic comparison of fowlpox virus with other avianpox viruses especially those that infect the wild birds are available. Recently, complete sequence of canarypox virus genome has become available.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Fowlpox virus multiplies in the cytoplasm of epithelial cells with the formation of large intracytoplasmic inclusion bodies (Bollinger bodies) that contain smaller elementary bodies (Borrel bodies). The inclusions can be demonstrated in sections of cutaneous and diphtheritic lesions by the use of haematoxylin and eosin (H&E), acridine orange or Giemsa stains (19). The elementary bodies can be detected in smears from lesions, for example by the Gimenez method (18), which is described below. Electron microscopy can be used to demonstrate viral particles of typical poxvirus morphology by negative staining or in ultrathin sections of infected tissues (3).

a) A smear technique for fowlpox

    i)   Place a drop of distilled water and the lesion (cutaneous or diphtheritic) on a clean slide. Prepare a thin smear by pressing the lesion with another clean slide and rotating the upper slide several times.

    ii)   Air dry and gently fix the smear over a flame.

    iii)   Stain the smear for 5–10 minutes with freshly prepared primary stain (8 ml stock solution[1] of basic fuchsin mixed with 10 ml of phosphate buffer[2], pH 7.5, and filtered through Whatman filter paper No. 1).

    iv)   Wash thoroughly with tap water.

    v)   Counterstain with malachite green (0.8% in distilled water) for 30–60 seconds.

    vi)   Wash the smear with tap water and then dry.

    vii)   Examine the smear under oil immersion. The elementary bodies appear red and are approximately 0.2–0.3 µm in size.

b) Virus isolation

Fowlpox virus can be isolated by the inoculation of suspected material into embryonated chicken eggs. Approximately 0.1 ml of tissue suspension of skin or diphtheritic lesions, with the appropriate concentration of antibiotics, is inoculated on to the chorioallantoic membranes (CAMs) of 9–12-day-old developing chicken embryos. These are incubated at 37°C for 5–7 days, and then examined for focal white pock lesions or generalised thickening of the CAMs. Histopathological examination of the CAM lesions will reveal eosinophilic intracytoplasmic inclusion bodies following staining with H&E (19, 22).

Primary chicken embryo fibroblasts, chicken embryo kidney cells, chicken embryo dermis cells, or the permanent quail cell line QT-35, can also be used to propagate fowlpox virus (6, 10). The adaptation of virus strains to cell cultures is an important requirement for plaque formation, as not all strains will form plaques initially.

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1   Stock solution: A solution of basic fuchsin (5 g) in 95% ethanol (100 ml) is slowly added to a second solution of crystalline phenol (10 g) in distilled water (900 ml). This stock solution, kept in a tightly screw-capped glass bottle, is incubated for 48 hours at 37°C, and then stored at room temperature.

2   Phosphate buffer, pH 7.5: $NaH_2PO_4H_2O$ (2.47 g) and Na_2HPO_4 (11.65 g) are added to distilled water (1000 ml) and stored at 4°C.

c) **Molecular methods**

Restriction fragment length polymorphism (RFLP) analysis can be used for comparison of field isolates and vaccine strains of fowlpox virus (6, 10). However, this procedure is not used in routine diagnosis.

Cloned genomic fragments of fowlpox virus can be used effectively as nucleic acid probes for diagnosis of fowlpox. Viral DNA isolated from lesions can be detected by hybridisation either with radioactively or nonradioactively labelled genomic probes. This method is especially useful for differentiation of fowlpox from infectious laryngotracheitis when tracheal lesions are present (5).

Genomic DNA sequences of various sizes can be amplified by the polymerase chain reaction (PCR) using specific primers (4, 8). This technique is useful when there is only an extremely small amount of viral DNA in the sample.

2. Serological tests

Although both cell-mediated immunity (CMI) and humoral immunity play an important role in poxvirus infections, routine use of the CMI test is not convenient. Therefore, serological tests, such as virus neutralisation (VN), agar gel immunodiffusion (AGID), passive haemagglutination and fluorescent antibody tests as well as the enzyme-linked immunosorbent assay (ELISA), are used to measure specific humoral antibody responses. Evidence of successful immunisation with vaccine can be determined by examining a flock 7–10 days after vaccination for 'takes'. A take consists of a swelling of the skin or a scab at the site where the vaccine was applied and its presence is evidence of successful immunisation.

a) **Virus neutralisation**

After virus/serum interaction, the residual virus activity may be assayed in embryonating chicken eggs or in cell cultures (9). This technically demanding test may not be convenient for routine diagnosis. Only some selected strains of the virus have plaque-forming ability in chicken embryo cells. Neutralising antibodies develop within 1–2 weeks of infection.

b) **Agar gel immunodiffusion**

Precipitating antibodies can be detected by reacting test sera against viral antigens. The antigen can be derived by sonication and homogenisation of infected skin or CAM lesions as well as by treatment of infected cell cultures as described in Section B.2.f below. The lysed suspension is centrifuged and the supernatant is used as antigen. Gel-diffusion medium is prepared with 1% agar, 8% sodium chloride and 0.01% thiomersol. The viral antigen is placed in the central well and the test sera are placed in the peripheral wells. It is important to include a positive and negative control serum. The plates are incubated at room temperature. Precipitation lines develop in 24–48 hours after incubation of the antigen with antibody to homologous or closely related strains. The test is less sensitive than the ELISA (2) or the passive haemagglutination test (23).

c) **Passive haemagglutination**

Tanned sheep or horse red blood cells are sensitised with a partially purified fowlpox viral antigen (20). The antigen is prepared from infected CAMs or cells as described in Section B.2.f below. Passive haemagglutination is more sensitive than AGID. The test will give cross-reactions among avian pox viruses.

d) **Fluorescent antibody tests**

Direct or indirect immunofluorescence tests will reveal specific intracytoplasmic fluorescence in infected cells. The latter test is commonly used and involves two steps: the antibody against fowlpox virus is reacted with the antigen in the infected cells, followed by a secondary fluorescein-isothiocyanate-labelled antibody against chicken gamma globulin (e.g. goat anti-chicken). Such labelled antibodies are available commercially. In this regard, formalin-fixed tissue sections can be used effectively for fluorescent antibody tests.

e) **Immunoperoxidase**

Specific staining of cytoplasmic inclusions is achieved when horseradish-peroxidase-conjugated specific polyclonal antibody against fowlpoxvirus is reacted with the hydrated sections of fowlpox-infected fixed tissues (CAM and skin) or cell culture. Similar results are obtained when either polyclonal or monoclonal antibodies are used in an indirect test. An advantage of the technique is that the sections can be examined with the light microscope and can be stored for an extended period without loss of colour (19).

f)   Enzyme-linked immunosorbent assay

ELISAs have been developed to detect humoral antibodies to fowlpox virus. They are capable of detecting antibody 7–10 days after infection (2), but commercial kits for this test are not available.

Fowlpox virus antigens are prepared either from infected QT-35 cell monolayers or CAM lesions. Infected QT cells are pelleted (700 *g* for 10 minutes at 4°C), washed with isotonic buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM ethylene diamine tetra-acetic acid [EDTA]) followed by lysis in hypotonic buffer (10 mM Tris, pH 8.0, 10 mM KCl, 5 mM EDTA) containing 0.1% Triton X-100 and 0.025% beta-mercaptoethanol. Nuclei and cellular debris are removed by low-speed centrifugation (500 *g* for 5 minutes at 4°C) and the resulting supernatant is used as a source of fowlpox virus antigens for ELISA or immunoblotting. To isolate viral antigen from CAM lesions, initial grinding of the lesions with subsequent detergent treatment as described earlier would be required. Virus propagated in chicken embryo fibroblasts and chicken embryo dermis cells has also been used for antigen. The antigen preparation is as described for QT cells.

Wells of microtitre plates are coated with 1 µg of soluble fowlpox virus antigen in 100 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C (2, 19). Each well is then rinsed once with wash solution (0.29 M NaCl, 0.05% Tween 20) and then blocked with phosphate buffered saline (PBS, pH 7.4) containing 3% bovine serum albumin (BSA) for 1 hour at 37°C. After one wash, serial dilutions of the test sera in PBS containing 1% BSA are added to the wells. After rocking for 2 hours at 37°C, the wells are washed three times prior to the addition of 100 µl/well horseradish-peroxidase-conjugated goat anti-chicken IgG (H+L) antibodies[3] at a recommended dilution in PBS. After 2 hours' incubation at 37°C and three subsequent washes, 100 µl of the peroxidase substrate TMB[3] is added to each well. Reactions are terminated by the addition of 1 M phosphoric acid and absorbance at 450 nm is recorded using an ELISA plate reader[4].

g)   Immunoblotting

Antigenic variations r between strains of fowlpox virus can be evaluated by means of immunoblotting or Western Blotting. In this method, viral antigens separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) are reacted either with polyclonal or monoclonal antibodies against fowlpox virus (6, 12, 14). This method is not convenient for routine diagnosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Early studies indicated the feasibility of protecting chickens from fowlpox by the use of pigeon pox or fowlpox viruses (21, 23). Vaccination is indicated in areas where fowlpox is endemic or on premises where infection has been diagnosed previously. Live fowl and pigeon pox virus vaccines, and also fowlpox vectored vaccines that protect against pox, are available commercially. These vaccines are derived from chicken embryos or avian cell cultures.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Passively acquired immunity should be taken into consideration during vaccination of progeny from flocks that have either had a recent natural infection or been recently vaccinated. As passive immunity (for 2–3 weeks) may interfere with vaccine virus multiplication, such progeny should be vaccinated only after the decline of passively acquired antibody. Fowlpox vaccine is applied by a wing web stab method.

1.   Seed management

a)   Characteristics of the seed

A master seed virus (MSV) must be established and used according to a seed-lot system. A record must be kept of its origin, passage history and characteristics. Viruses used may be either fowlpox or pigeon pox viruses. The MSV must be propagated in suitable premises with materials that meet approved standards, and must be tested for freedom from contamination as well as for identity and purity.

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3   Kirkegaard and Perry Laboratories, Gaithersberg, Maryland, United States of America.
4   Dynatech, Chantilly, Virginia, United States of America.

b) Method of culture

The MSV may be propagated in specific pathogen free (SPF) chicken embryos, using the CAMs, or in avian cell cultures, such as primary chicken embryo fibroblasts, chicken embryo kidney or chicken embryo dermis.

c) Validation as a vaccine

i) *Purity*

The MSV may be neutralised with a specific hyperimmune serum before testing for purity. Because of difficulty in neutralising avian pox virus, it is acceptable to centrifuge the MSV at 1000 **g** for 20 minutes, followed by filtration through a 0.2 µm filter. The neutralised or filtered MSV is then used in tests to demonstrate freedom from extraneous agents. These tests should be done in embryonating eggs or avian cell cultures, to demonstrate absence of extraneous virus replication, and in SPF chickens, to demonstrate freedom from antibodies to extraneous agents.

ii) *Safety*

Vaccines should be prepared only from virus that is a stable attenuated strain or a naturally occurring isolate of low virulence.

The vaccine must be shown to be safe by the recommended route of administration, which is wing web stab, in all ages of susceptible birds. A suitable test is to take ten SPF chickens and inoculate each by piercing the wing web with a needle dipped in the vaccine. The birds are observed for 7–10 days for evidence of 'takes' and for the absence of adverse effects attributable to the vaccine. A 'take' consists of swelling of the skin or a scab at the site where the vaccine was applied and is evidence of successful vaccination. The safety test should be repeated after at least six serial passages of the virus in SPF chickens to show that there has been no reversion to virulence.

iii) *Efficacy*

Data should be obtained using the highest passage level (fifth passage from the master seed) and the lowest titre of virus to be used in the final product: 20 SPF chickens of the minimum age indicated for vaccination should receive one dose of vaccine by the recommended method. The chickens, together with 20 unvaccinated chickens of the same age and source, should be challenged 3 weeks later by scarification with a virulent strain of fowlpox virus. The birds should be observed for 3 weeks. Ninety per cent of the control birds should develop lesions due to the challenge virus and at least 90% of the vaccinated birds should remain free from such lesions.

2. Method of manufacture

Vaccine is manufactured on a seed-lot system from the validated MSV. This must be done in approved premises designed to avoid the risk of contamination. All media and cell cultures must be tested to ensure freedom from contamination.

3. In-process control

During the process of validation as a vaccine, the efficacy data must be compared to the virus content of the vaccine. A suitable potency can thus be established. The vaccine should be filled into final containers to ensure that each container has sufficient virus to achieve the specified potency.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the Chapter 1.1.9.

b) Safety

The safety test described in Section C.1.c.ii above, except the requirement for six passages in SPF chickens, should be used on each batch of vaccine.

c) Potency

Virus content tests should be carried out using each of at least three containers. The dilutions should span 0–100% infection range, using five-fold dilution steps and at least seven replicates per dilution. Tests should

be done in parallel with a standard vaccine, if available. Each lot of vaccine should be titrated in the diluent provided for its use. The virus titre should not normally be higher than 1/10 of the dose at which the vaccine has been shown to be safe and must not be lower than the release titre determined in the test for efficacy. A suitable potency for an attenuated live fowlpox vaccine is likely to be in the region of 10^5 EID_{50} (50% embryo infective dose) per ml.

d) Duration of immunity

The efficacy test given in Section C.1.c.iii may be used to determine the duration of immunity (approximately 6–12 months) by testing at intervals after vaccination, using separate groups of birds for each test.

e) Stability

Evidence of stability must be presented to justify the shelf life. This should be based on virus titrations carried out at intervals until 3 months beyond the requested shelf life on at least six batches of vaccine kept under recommended storage conditions.

f) Preservatives

Preservatives are not used in live vaccines.

g) Precautions (hazards)

It is usually recommended not to vaccinate birds that are in lay. Avoid human contact with the live vaccine. Standard fowlpox vaccine is not to be used in pigeons, though they can be vaccinated with pigeon pox vaccine. In many countries, pigeon pox vaccine has been superseded by attenuated live fowlpox vaccine designed for use in day-old chicks. These products have been safely used in pigeons in the absence of an available pigeon pox vaccine.

5. Tests on the final product

a) Safety

The safety test described in Section C.1.c.ii above is used on each batch.

b) Potency

The potency test described in Section C.4.c above is used on each batch.

ACKNOWLEDGMENT

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FOWL TYPHOID AND PULLORUM DISEASE

SUMMARY

Definition of the disease: *Pullorum disease of chickens is caused by* Salmonella enterica *subspecies* enterica *serovar Gallinarum biovar Pullorum* (Salmonella *Pullorum*)[1]*. At this time the serovar is referred to as Gallinarum in some parts of the world and Pullorum in others; in this chapter the serovar will be referred to as Gallinarum or Pullorum according to the biovar under discussion. In its acute form, Pullorum disease is almost exclusively a septicaemic disease of young chickens. However, the organism may also be associated with disease in turkey poults and may be carried subclinically or lead to reduced egg production and hatchability plus a range of atypical signs in older birds. Ovarian transmission is a major route by which the organism can spread. Game birds and 'backyard' poultry flocks may act as reservoirs of infection, and wild birds may act as vectors for the organism and as such are important in the epidemiology of the disease.*

Fowl typhoid in chickens and turkeys is caused by S. Gallinarum biovar Gallinarum *and is more often observed in the later growing period and in mature stock. Disease is often characterised by rapid spread with high morbidity and acute or subacute mortality. Red mites may be involved in the transmission of disease and persistence in poultry houses*

Salmonellosis caused by Salmonella bongori *or other subspecies of* Salmonella enterica *is covered in chapter 2.9.9 of this* Terrestrial Manual.

Description of disease: *Clinical signs in chicks and poults include anorexia, diarrhoea, dehydration, weakness and death. In mature birds Pullorum disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur. Fowl typhoid is a more acute septicaemic condition which mainly affects mature birds and may be particularly severe in commercial laying flocks.*

Identification of the agent: *Samples should not be taken from birds or eggs that have recently been treated with antimicrobial drugs. Swabs or aseptically collected samples from infected tissues, or intestinal and cloacal contents should be used for diagnostic testing. Other materials that may be sampled include eggs, embryos, faecal droppings and hatcher debris, especially fluff, dust and broken eggshells and chick box linings. Samples of tissues such as caecal tonsils and spleen from infected birds are preferable to faecal and environmental samples. Tissue samples should be inoculated into nonselective and selective enrichment broth and into selective agar medium, such as brilliant green agar, as soon as possible after collection. In case of delay, samples should be stored at 4°C. Typical colonies can be identified by serological and biochemical tests.*

Serological tests: *These are satisfactory for establishing the presence and estimating the prevalence of infection within a flock. The test used in the field is the rapid whole blood plate agglutination test. This test is unreliable in turkeys and ducks as many uninfected birds may give positive reactions. In the laboratory a serum agglutination test is used, either as a rapid plate test or as a tube test. These can be applied as macro- or microagglutination tests though the latter may be more likely to give false-positive results with turkey sera. Any positive reactors should be confirmed as being infected by culture at post-mortem examination. Enzyme-linked immunosorbent assays have been reported but no commercial test is available.*

The use of vaccines to control S. Enteritidis *infections in chickens may cause problems in the interpretation of serological results.*

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1   See the note in Chapter 2.9.9 Salmonellosis for the principles followed concerning the nomenclature of *Salmonella*.

> ***Requirements for vaccines and diagnostic biologicals:*** *Live and inactivated vaccines are available for fowl typhoid in some countries. The most commonly used vaccine is a live vaccine derived from the stable rough strain of S. Gallinarum known as '9R'.*

A. INTRODUCTION

Fowl typhoid and pullorum disease, caused by *Salmonella enterica* subspecies *enterica* serovars Gallinarum biovars Gallinarum and Pullorum, respectively, are widely distributed throughout the world but they have been eradicated from commercial poultry in many developed countries of Western Europe, the United States of America (USA), Canada, Australia and Japan. In the United States and the United Kingdom the serovar is referred to as Pullorum (8); in this chapter the terms serovar Gallinarum or Pullorum will be used. However, *S.* Gallinarum has recently recurred in some European countries (18). *Salmonella* Pullorum remains in wild and game birds. *Salmonella Gallinarum* and *S.* Pullorum are host adapted to avian species and are considered to pose a minimal zoonotic risk (19), although the genome is continually evolving, which could theoretically widen the host range in future (12).

Salmonellosis caused by *Salmonella bongori* or other subspecies of *Salmonella enterica* is covered in chapter 2.9.9 of this *Terrestrial Manual*.

Clinical signs are typical of a septicaemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality occurs in birds of 2–3 weeks of age. In older birds disease may be mild or inapparent. In breeding flocks reduced egg production and hatchability may be the only signs, and trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important transmission routes for the disease.

Post-mortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalised congestion of tissues and an inflamed unabsorbed yolk sac. Longer standing infections commonly lead to typhlitis with development of necrotic caecal casts and small necrotic foci in the liver, lungs and other viscera. Small lesions in the liver and spleen of Pullorum-infected birds may show a 'white spot' appearance that is not seen with Gallinarum; however, this lesion is not pathognomic. These *Salmonella* are very poor at colonisation and survival in the gastrointestinal tract is often indicative of later stages of clinical disease. Adult birds may develop misshapen or shrunken ovaries with follicles attached by pedunculated fibrous stalks. Variant strains of *S.* Pullorum do not normally cause clinical disease or may result in mild, nonspecific signs but may lead to seroconversion.

In fowl typhoid, as well as generalised signs of septicaemia, the liver is usually enlarged, dark and friable with a distinctive coppery bronze sheen that may only develop after exposure to air. The bone marrow is also often dark brown. Although clinical signs and post-mortem findings of pullorum disease and fowl typhoid may be highly suggestive of the conditions, they are not sufficiently distinct from other causes of septicaemia to be pathognomic. It is therefore necessary to confirm disease by isolation of the organisms. Serological tests can be used to establish the presence of the disease in a flock.

B. DIAGNOSTIC TECHNIQUES

In its acute form, pullorum disease is almost exclusively a disease of young chickens, and the agent can be recovered from almost all organs, tissues and faeces. In older birds that have become carriers, *S.* Pullorum is most commonly recovered from the ova and oviduct; and it is recovered only occasionally from other organs and tissues, including the alimentary tract. In the acute phase of fowl typhoid the organism is also widely distributed, but in carrier birds, the organism is found most often in the liver, spleen and reproductive tract, and occasionally in the caeca.

• Culture

Salmonella Pullorum and *S.* Gallinarum belong to the Kauffmann–White scheme serogroup D, along with *S.* Enteritidis, which is thought to be closely related. The organisms are Gram negative nonsporogenic rods 1.0–2.5 µm in length and 0.3–1.5 µm in width. They are considered to be non-motile under normal conditions but inducement of flagellar proteins and motility has been shown in some strains of *S.* Pullorum when grown in special media (9).

For optimal recovery of the organisms, the birds being sampled should not have been treated with antimicrobial drugs for approximately 2–3 weeks previously.

Samples may be obtained from live birds, preferably after identifying highly sero-positive birds. Fresh or freshly chilled carcasses, egg materials, fresh faeces, or any contaminated materials from housing, incubators or transport boxes may also be taken. Swabs may be taken from the cloaca of live birds. Samples from visibly abnormal tissues are preferable to faecal and environmental samples. Aseptic samples can be taken from the spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions. The surface is seared with a hot spatula and a sample is obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilised surface. The demonstration of infection in serological reactor birds that are apparently normal may, in some cases, require the culture of large volumes of homogenised tissues as well as direct swabbing. Tissue pools may be made from tissues collected from a number of birds.

When floor litter or faecal material is sampled, it should be remembered that *S.* Pullorum and *S.* Gallinarum are more difficult to isolate from faecal and environmental samples than other salmonellae and it is always preferable to culture sick or recently deceased birds . Environmental samples may include sock or boot swabs, floor faeces, moist and dry litter and swabs from open drinkers. Red mites associated with poultry which are infected with *S.* Gallinarum often contain the organism after feeding and can be cultured. These samples should be cultured by direct inoculation of a selective enrichment broth such as selenite cysteine, followed by plating on selective media such as brilliant green agar (17).

Both *S.* Pullorum and *S.* Gallinarum grow well on nonselective media, but selective and enrichment media have been described that contain substances to inhibit the growth of extraneous organisms. *Salmonella* Pullorum may grow slowly and produce very small colonies on selective media so incubation of plates for 48 hours is recommended. The efficiency of recovery of *Salmonella* varies according to circumstances, and experience in the use of a medium is an important but unquantifiable factor. Some complex media may have an inhibitory effect on these organisms, so that it is advisable to use both nonselective and selective media for isolation from tissues. Both solid media and broths can be employed. As the toxic properties of selective media may vary, it is preferable to monitor these by comparing growth of control cultures on both types of medium. The inhibitory media should grow at least 75% of the colonies of the corresponding non-inhibitory medium (3, 4, 7, 13).

All the media mentioned below are examples of commonly used media, but there are many others found to be equally satisfactory.

Non-inhibitory media include nutrient agar and blood agar, on which colonies are seen to be smooth, translucent, slightly raised, and about 1–2 mm in diameter. *Salmonella* Gallinarum grows more rapidly than *S.* Pullorum and produces larger colonies with a distinctive smell resembling that of seminal fluid on most media. Broths include buffered peptone water and nutrient and meat infusion broths or universal pre-enrichment broth.

- **Selective media include:**

  MacConkey agar: the agar is inhibitory to non-enteric organisms, it differentiates lactose fermenters (pink colonies) from nonlactose fermenters (colourless colonies). NaCl is omitted to limit the spread of *Proteus* colonies. *Salmonella* colonies are smooth and colourless. *Salmonella* Pullorum produces smaller colonies than other salmonellae. MacConkey is the agar of choice for direct plating from tissues.

  Xylose lysine deoxycholate agar: the agar is inhibitory to non-enteric organisms. *Salmonella* Pullorum grows sparsely as small red translucent colonies. *S.* Gallinarum colonies are small, dome-shaped, and may have a central black spot due to H_2S production, but this reaction may be delayed or variable.

  Brilliant green agar (*BGA*): the agar is inhibitory to coliforms and most *Proteus* strains; useful for distinguishing enteric organism colonies. Salmonellae form low, convex, pale red, translucent colonies of 1– 3 mm in diameter, similar to *Citrobacter*. *Proteus* forms pin-point colonies, *Pseudomonas aeruginosa* appears as small red colonies, and lactose fermenters are green. *Salmonella* Pullorum produces smaller more pale colonies than other salmonellae. BGA is the agar of choice following enrichment.

  Brilliant green sulphapyridine agar: the agar is inhibitory to coliforms and *Proteus* strains. The sulphapyridine is added to stabilise selectivity in the presence of egg materials. *Salmonella* Pullorum produces small colonies.

  Salmonella Pullorum and Gallinarum grow poorly and do not produce typical colonies on newer chromogenic agars such as Rambach agar.

- **Liquid enrichment and selective media include:**

  Selenite F broth: inhibitory to coliforms but not *Proteus*, improved by addition of brilliant green. Loss of activity after 24 hours. Selenite cysteine broth is more stable. Although selenite broths are considered to be preferable for isolation of *S.* Pullorum and *S.* Gallinarum from faeces, if there are difficulties with issues of toxicity or shelf life in particular laboratories the other enrichment broths mentioned below may be used. Most of these other broths are however designed to be used following a nonselective enrichment stage and

S. Gallinarum and S. Pullorum are readily overgrown by competitor organisms in nonselective faecal culture resulting in false-negative tests. Direct selective enrichment is therefore recommended for faeces and intestinal or environmental samples. Nonselective enrichment may give better results for tissues obtained by aseptic post-mortem where there should be no competing organisms (16).

Tetrathionate/brilliant green broth: inhibitory to coliforms and *Proteus*, but may also inhibit some strains of *S.* Pullorum*/*Gallinarum.

Rappaport–Vassiliadis soya (*RVS*) *peptone broth:* for selective enrichment following pre-enrichment, use 1 part inoculum to 100 parts medium. *Salmonella* Pullorum and Gallinarum are more likely to be overgrown by other organisms during pre-enrichment of faeces or intestinal contents than salmonellae that are not host-adapted so direct enrichment with RVS may also be attempted.

- **Recovery of salmonellae**

The methods for recovering *S.* Pullorum and *S.* Gallinarum vary according to the origin of the samples. Although their isolation from cloacal swabs and faeces may be unrewarding, examination of tissues taken at post-mortem is usually more successful. The methods are as follows:

Cloacal swabs and fresh faeces from live birds: swabs dipped in nutrient broth are suitable, small swabs being used for young chickens. The swabs are streaked on selective media, and placed in enrichment broth. The plates and the broth are incubated at 37°C. At this temperature, *Proteus* and *Pseudomonas* organisms tend to be inhibited relative to *Salmonella*. Higher temperatures may be used with some broths, e.g. 41.5°C for Rappaport–Vassiliadis (RV). Subcultures are made on to selective media after 24 and 48 hours.

Gall-bladder contents: swabs of gall-bladder contents are streaked on to nonselective and selective agars and placed in inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture on to selective agar after 24 and 48 hours.

Organs and tissues: swabs or segments of tissues are taken in an aseptic manner from individual tissues and lesions and cultured on to nonselective and selective media, and into similar nonselective and selective broths. These are incubated at 37°C and subcultured on to selective agar after 24 and 48 hours. Intestinal material in selective broths may also be incubated at 40°C; *S.* Gallinarum grows well but there may be some inhibition of *S.* Pullorum at this temperature.

Carrier birds: larger amounts of material may be required to identify the carrier birds. The ovary and oviduct are the tissues of choice for *S.* Pullorum, and the liver and gall-bladder as well as ovary and oviduct should be tested for *S.* Gallinarum. In practice it is usually best to pool samples from a variety of tissues including the spleen. Tissues are homogenised in a small volume of broth and directly plated out. Approximately 10 ml of homogenate is also added to 100 ml of nonselective enrichment broth (e.g. buffered peptone water) and selective enrichment broth (e.g. selenite cysteine broth or brilliant green broth), and incubated at 37°C. These broths are subcultured on to nonselective and selective agar after 24 hours.

Alimentary canal, including the caecal tonsils and intestinal contents: after grinding or homogenisation in a small volume of broth, 10 ml of the homogenate is incubated in 100 ml of selective enrichment broth at 37°C. In general better isolation is achieved with selenite cysteine broth.

Eggshells: the broken eggshells are placed in a tenfold volume of enrichment broth (e.g. selenite cysteine broth). The broth is incubated at 37°C and subcultured on to selective agar after 24 and 48 hours.

Egg contents: Aseptically taken contents of fresh eggs are homogenised and mixed with 200 ml of buffered peptone water or nutrient broth, incubated at 37°C, and subcultured on to nonselective and selective agar after 24 and 48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated.

Embryos: Homogenised viscera and swabs from the yolk sacs of well developed embryos may be streaked on to nonselective and selective agar, one swab being placed in 10 ml of both nonselective and enrichment broth (e.g. selenite cysteine broth or brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to nonselective and selective agars after 24 and 48 hours.

Environmental samples: These include hatcher fluff, debris and macerated egg/chick waste samples and chick box liners or floor faecal or litter samples; 25 g is mixed with 225 ml of enrichment broth (e.g. selenite cysteine broth, brilliant green broth), incubated at 37°C, and subcultured on to selective agar after 24 and 48 hours.

Polymerase chain reaction (PCR) based tests may also be used, but have not been fully validated internationally (15).

1.   Identification of the agent

Typical *S.* Gallinarum colonies on nonselective media are round, translucent, glistening, domed, smooth, and 1–2 mm in diameter after 24–48 hours' incubation. *Salmonella* Pullorum colonies are slightly smaller and translucent. On selective media their appearance varies with the medium, but suspect colonies can be investigated serologically by testing for 'O'9 somatic antigens, observing for motility and testing biochemically.

After incubation for 20–24 hours, the plates should be examined carefully for the presence of typical *S.* Pullorum and *S.* Gallinarum colonies. If growth is slight after 24 hours' incubation, the plates should be reincubated for a further 24 hours and examined again. For biochemical and serological confirmation from each plate, five typical or suspect colonies should be chosen for further examination. If there are fewer than five typical or suspect colonies, all of them should be taken for further examination. Selected colonies should be streaked on to the surface of nutrient agar, in a manner that allows the growth of separate colonies. For biochemical confirmation, only pure cultures taken from nonselective media should be used. The following media should be streaked using an inoculating loop: triple sugar iron (TSI) agar; lysine iron agar (or l-lysine decarboxylation medium); urea agar according to Christensen; tryptone/tryptophan medium for indole reaction; glucose with an inverted Durham tube for acid and gas production; dulcitol, maltose, ornithine decarboxylation medium and semisolid agar, for motility. The reactions shown in Table 1 occur.

Identification kits are commercially available, for example Analytical Profile Index (API) system for Enterobacteriaceae. However, care must be taken when using API because *S.* Pullorum may be misidentified as *Hafnia* spp. Molecular tests using ribotyping techniques and PCR have been developed in research laboratories (10), but are not generally available for confirmation of *S.* Gallinarum and *S.* Pullorum.

For serological confirmation as to serogroup, colonies from nonselective media (nutrient or blood agar) are used. The first stage is elimination of autoagglutinable strains. For this, material taken from a single colony of pure culture is transferred to a glass slide and mixed with a drop of sterile saline. The slide is rocked gently or the drop stirred with a loop for 30–60 seconds and observed for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope. If the bacteria have clumped into more or less distinctive units, the strain is considered to be autoagglutinable and must not be submitted to the following tests. If the bacterial sample is recognised as non-autoagglutinable, it is tested with a polyvalent 'O' (A–G) antiserum. For this purpose, the material from a single colony is dispersed in the drop of polyvalent 'O' antiserum on the glass slide to obtain a homogenous and turbid suspension. After gently rocking for 30–60 seconds, the reaction is observed against a dark background for agglutination. Alternatively the slide agglutination test may be carried out with smaller volumes of suspension under a dissecting microscope. In this case a portion of the colony to be checked is added to a loopful of saline on the microscope slide to produce a light suspension to check for autoagglutination ('rough strains'). If no agglutination takes place, one or two loops of antisera are added, the drop stirred with a loop and observed for agglutination. *Salmonella* Pullorum and *S.* Gallinarum should agglutinate with polyvalent 'O' antisera but not with polyvalent flagella (poly 'H' phase 1 and phase 2) antisera. If the reaction is positive, the single colony is tested further in the same manner using group-specific sera for *S.* Pullorum and *S.* Gallinarum serotypes ('O'9 antiserum). After serogrouping, isolates may be sent to a reference laboratory for serotyping.

Table 1. Biochemical investigation of Salmonella *Pullorum and* S. *Gallinarum*

	Salmonella Pullorum	*Salmonella* Gallinarum
TSI glucose (acid formation)	+	+
TSI glucose (gas formation)	v	–
TSI lactose	–	–
TSI saccharose	–	–
TSI hydrogen sulphide	v	v
Gas from glucose (medium with Durham tube)	+	–
Urea hydrolysis	–	–
Lysine decarboxylation	+	+
Ornithine decarboxylation	+	–
Maltose fermentation	– or late +	+
Dulcitol	–	+
Motility	–	–

+ = 90% or more positive reaction within 1 or 2 days; – = No reaction (90% or more); v = Variable reactions.

It is also possible to confirm and differentiate *S.*Gallinarum by specific PCR (18).

- **Test procedure for culture of visceral, faecal, intestinal and environmental samples for *S.* Pullorum and *S.* Gallinarum**

    i)   Where possible, begin laboratory procedures on the same day as samples are collected.

    ii) Homogenise the material as much as possible by manual mixing, gentle macerating or stomaching with a small volume of sterile saline if the material is dry.

    iii) Stir the mixture with a small rectal swab or loop and streak thickly on to one-quarter of a brilliant green agar plate. (Swabs from uncontaminated tissues sampled in an aseptic manner can also be streaked on to blood agar.)

    iv) From this deposit of material on the plate, streak the rest of the plate to obtain individual colonies.

    v)   Add 5–25 g of the homogenised sample to freshly made selenite cysteine broth (see note on liquid enrichment and selective media above) to make a 1:10 sample to broth ratio. Shake or stir to disperse the sample in the broth.

    vi) Incubate the brilliant green agar plates and selenite cysteine broth at 37°C for 24 hours.

    vii) Examine the plate after 24 hours' culture. Carry out agglutination tests on up to five suspect colonies with polyvalent 'O' (A–G) antisera and polyvalent H (phase 1 and phase 2) antisera. If agglutination is unclear subculture suspect colonies on to nutrient agar or blood agar and repeat tests after 24 hours' incubation of those media.

    viii) If poly 'O' is positive then check with 'O'9 antiserum. If 'O'9 is positive and poly 'H' is negative, this is indicative of the possible presence of *S.* Pullorum or *S.* Gallinarum.

    ix) If there are no positive colonies on the brilliant green agar plate, streak out a 10 µl loop of incubated selenite cysteine broth onto brilliant green agar as in step iv above.

    xi) Incubate the brilliant green agar plates at 37°C for 24 hours and re-incubate the previous (negative) brilliant green agar plates and the selenite cysteine broths for a further 24 hours.

    xi) Repeat examination of plates as in step vii above.

    xii) If plates are still negative, replate from selenite cysteine broth and incubate brilliant green agar plate, that was inoculated in step ix, for a further 24 hours and examine as in step vii above.

    xiii) Confirm *S.* Pullorum and *S.* Gallinarum using biochemical tests as shown in Table 1. Isolates can be sent to a *Salmonella* reference laboratory for confirmation of serotype and for phage typing of *S.* Pullorum.

- **Molecular epidemiology**

    Standard molecular 'fingerprinting' techniques used for *Salmonella*, such as plasmid profile analysis, pulsed field gel electrophoresis or ribotyping can be used for investigating outbreaks of *S.* Pullorum or *S.* Gallinarum. It is often necessary to use combinations of such methods to obtain maximum discrimination.

2. Serological tests

Serological tests are best applied as a flock test as results for individual birds will vary according to the stage of infection. It is therefore necessary to take sufficient individual samples to determine infection in the flock. The number of samples will depend on the expected prevalence and level of confidence desired (see Chapter 1.1.1 Collection and shipment of diagnostic specimens). If the test is to be used for detecting individual infected birds for culling, it should be repeated at least twice and preferably until the whole flock has given at least two negative tests.

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination (RST), tube agglutination and micro-agglutination (21). Other invasive *Salmonella* such as *S.* Enteritidis and *S.* Typhimurium may give false-positive results in serological tests for *S.* Pullorum.

Both *S.* Pullorum and *S.* Gallinarum possess 'O' antigens 9 and 12 and may also posess O antigen 1. However, in the case of *S.* Pullorum, there is a variation in the ratio of 12_1, 12_2 and 12_3; the standard strain contains more 12_3 than 12_2, while the reverse is true of the variant form. Intermediate forms also exist. (There appears to be no such form variation in the case of *S.* Gallinarum.) As this variation occurs, it is necessary to use a polyvalent antigen in immunodiagnostic tests. The same antigen is used to detect both *S.* Pullorum and *S.* Gallinarum, but detection of the latter may be relatively poor (17).

a)   Rapid whole blood agglutination test

The rapid whole blood agglutination test can be used under field conditions for detecting both *S.* Pullorum and *S.* Gallinarum, and the reactors can be identified immediately. However, it is not reliable in turkeys as the test results in a significant proportion of false-positive results. Chickens can be tested at any age,

although some authorities specify a minimum age of 4 months (21, 22) and positive results from chicks less than 4 weeks of age may be due to maternal antibodies.

- **Preparation of stained antigen for the rapid whole blood or rapid serum agglutination test**

Incubate one standard form strain of *S.* Pullorum (antigenic structure 9, 12_1, 12_3) and one variant form (antigenic structure 9, 12_1, 12_2) at 37°C and harvest separately until final mixing for the complete antigen.

Sow strains on to separate agar slopes, incubate at 37°C for 24 hours, emulsify growth with sterile normal saline and spread an inoculum over an agar plate to produce easily selected discrete colonies. For this the plates are incubated for 48 hours, a number of colonies are marked out and each is tested for agglutination on a slide with 1/500 acriflavine in saline. Smooth-phase colonies do not produce agglutination. Pick off typical colonies that do not produce any agglutination, seed on to agar slopes, and incubate for 24 hours. Emulsify the growth in saline and evenly distribute 2 ml over the surface of the medium (200 ml) in a Roux or similar flask. Incubate the flasks for 60 hours.

For harvesting the bacterial growth, flood the surface of each flask with enough sterile buffered formol saline, pH 6.5 (8.5 g/litre sodium chloride, 10 ml/litre neutral formalin, 4 ml/litre 0.5 M sodium phosphate: made up to 1 litre with distilled water, pH adjusted to 6.5 using 1 M orthophosphoric acid or 1 M sodium hydroxide), to give dense cell suspensions (about 10 ml per flask). Add 12–15 sterile glass beads of 3–5 mm diameter and rock the flasks until all the growth is in even suspension; leave in a vertical position for at least 15 minutes. Check the morphology and purity of the suspensions by preparing and examining Gram-stained films. Bulk the suspension from each flask containing the same strains. To each 100 ml of suspension, add 200 ml of absolute alcohol. Shake the mixture and allow to stand for 36 hours or until precipitation is complete. Check the agglutinability of the standard and variant precipitate by first centrifuging a sample to separate the alcohol, which is removed, dilute with normal saline and test with a known positive and negative serum. If satisfactory, remove the clear supernatant alcohol (centrifugation at 2000 *g* for 10 minutes may be helpful in precipitation), and add sufficient phosphate buffered saline (PBS) containing 10% (v/v) glycerol to standardise the density to 75 × No. 1 Wellcome opacity tube (or 50 × tube No. 1.0 on the McFarland scale). Add equal volumes of standard and variant strains, and add 1% (v/v) of 3% (w/v) alcoholic crystal violet solution to the final mixture, and allow to stand for 48 hours at room temperature. Store in a tightly closed container at 0–4°C for up to 6 months. To assess safety, carry out a culture test on blood agar for nonviability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for *S.* Pullorum and *S.* Gallinarum, and against a negative serum. If possible, also test with known positive and negative serum and blood from positive and negative chickens.

Stained antigen products for the whole blood plate agglutination test are available commercially, and although there seems to be some slight differences in their sensitivity (5), it is unlikely that poultry flocks infected with the different variants of *S.* Pullorum would be missed.

- **Test procedure**

i)   Use a clean white tile marked into squares of about 3 × 3 cm. If a tile with 3 × 4 squares is used, up to 12 blood samples can be tested at the same time.

ii)   Place 1 drop (about 0.02 ml) of crystal-violet-stained antigen in the centre of each square.

iii)   Obtain a sample of fresh whole blood. This is conveniently done by making a stab of a wing vein using a needle with a triangular point.

iv)   Place an equal size drop of fresh whole blood next to a drop of antigen.

v)   Mix the drops of antigen and blood using a fine glass rod, which is wiped clean between samples.

vi)   Use a gentle rocking motion to keep the drops agitated for up to 2 minutes. Several tests may be carried out simultaneously on the same tile, but the drops should not be allowed to dry out during this time. In very warm conditions, larger drops may be required to avoid drying out.

vii)   A positive reaction is indicated by easily visible clumping of the antigen within 2 minutes.

viii)   A negative reaction is indicated by absence of clumping of the antigen within 2 minutes.

ix)   Include known positive and negative control sera on each testing occasion, using them in the same way as the blood.

x)   On completion of a set of tests, the tile is washed and dried, ready for further use.

In the absence of positive reactions, any doubtful reactions can only be interpreted in the light of the previous *Salmonella* testing history of the flock. Where there are positive reactors, any doubtful reactor should be regarded as positive. Also, recently infected birds may not show a typical positive reaction until they are retested after 3–4 weeks.

b) Rapid serum agglutination test

The RST is performed in the same manner, except that serum is substituted for whole blood. For export test purposes an initial screening of sera by RST followed by confirmation of positives by the tube agglutination test is the optimal approach. Ideally serum samples tested by any method should be tested within 72 hours of collection as nonspecific reactions may increase in older samples. Fresh samples can be frozen for later testing if a delay is unavoidable.

c) Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at an initial dilution of 1/25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen[2]. Positive and negative control sera are included in each test. The antigen is prepared from unstained *S.* Pullorum or *S.* Gallinarum cultures diluted to a concentration of No. 1 on the McFarland scale (as described above). The mixture is incubated at 37 or 50°C for 18–24 hours before reading. A positive reaction consists of a granular white deposit with a clear supernatant fluid; a negative reaction shows uniform turbidity. Samples positive at a dilution of 1/25 are retested at a higher range of dilutions and a titre of 1/50 is usually considered to be positive, although this figure seems to vary in the literature. In many cases a single dilution of 1/50 is used but this may fail to detect some flock infections if only small numbers of samples are taken.

d) Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller volumes of reagents. The test is performed in microtest plates. Sera are first diluted by adding 10 µl of serum to 90 µl of normal saline, and then adding 100 µl of previously standardised stained microtest antigen to give a final dilution of 1/20. By titrating the serum in doubling dilutions and adding an equal volume of standardised stained antigen, an end-point (titre) can be obtained. The plates are sealed and incubated at 37°C for 18–24 or 48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate. Titres of 1/40 are usually considered to be positive but this test is more liable to produce false-positive results with turkey sera.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay (ELISA).

ELISA techniques have been described for detecting antibodies to *S.* Pullorum and *S.* Gallinarum (14). The indirect ELISA using lipopolysaccharide antigen is likely to be the most sensitive and specific serological flock test for *Salmonella*, including *S.* Gallinarum and *S.* Pullorum. It is relatively easy to perform with serum or yolk, and can be used for quantifying the titre of antibody (1, 2, 22). No commercial ELISA kits for *S.* Pullorum and *S.* Gallinarum are currently available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Although both live and inactivated vaccines have been prepared for use against *S.* Gallinarum, the vaccine most widely used is made from the rough 9R strain (6). It has only been employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (20), and may stimulate the production of transient antibodies. It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination.

Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection. Autogenous vaccines can also be used to control clinical disease. Control can best be achieved by biosecurity, hygiene, good management, monitoring and removal of infected flocks. Commercially available 9R vaccines are commonly used for reduction of *S.* Enteritidis in laying flocks (11).

1. Seed management

a) Characteristics of the seed

Live fowl typhoid vaccine is a suspension of suitably attenuated living organisms of a rough strain of *S.* Gallinarum, e.g. 9R. The organisms in the vaccine give the biochemical reactions characteristic of *S.* Gallinarum. Colonies of a 24-hour culture prepared from the vaccine on nutrient agar plates are rough

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2   For preparation of small volumes of somatic antigens see Chapter 2.9.9 Salmonellosis.

when examined by the acriflavine slide test. The culture should not contain the somatic antigens characteristic of the smooth forms of *S.* Gallinarum.

b) Method of culture

Salmonella Gallinarum is grown on or in a suitable medium, such as nutrient agar or broth, for 24 hours at 37°C.

c) Validation as a vaccine

There is no satisfactory method of assessing the protection afforded by the vaccine in the field. However, experience has shown that the vaccine can provide some benefit in some situations where control cannot be achieved by hygiene and management alone. The potency test described below may be used to provide evidence of efficacy.

2. Method of manufacture

The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture of *S.* Gallinarum (9R) and incubation at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation.

Alternatively the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between 5×10^6 and 5×10^7 organisms.

3. In-process control

The culture used for inoculation of the production cultures and the harvested cells are examined microscopically using Gram staining to check for purity.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Six healthy, susceptible (preferably specific pathogen free [SPF]) chickens, 8–16 weeks of age, are each injected subcutaneously with ten doses of vaccine, and are observed for at least 7 days; no local or systemic reaction should develop.

c) Potency

Fifteen healthy chickens, 8–16 weeks of age, of the Light Sussex or Rhode Island Red breeds, or crosses of these, and taken from a stock that is free from *S.* Pullorum infection, are each injected subcutaneously with a quantity of vaccine corresponding to one field dose, i.e. 5×10^7 viable organisms. After an interval of 21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for approximately 18 hours. The chickens are then challenged by oral administration of 1 ml of a broth suspension containing 5×10^7 organisms of a virulent strain of *S.* Gallinarum mixed with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%). All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

d) Duration of immunity

The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at stages during lay. A booster dose during lay may be required, but should not be used during lay in flocks providing eggs for human consumption.

e) Stability

The shelf life of the vaccine can be measured by conducting potency tests at periods after manufacture. These should be done on at least six samples. Potency should remain satisfactory for at least 1 year.

f) **Preservatives**

No preservatives are used.

g) **Precautions (hazards)**

The organism is not known to be pathogenic to humans, and there are no special risks associated with the manufacture of either the vaccine or the antigen. However, the vaccine may establish a persistent infection in carrier birds and can precipitate disease in already infected chickens.

5. Tests on the final product

a) **Safety**

The safety test described in Section C.4.b should be used on a representative sample from each batch of final product.

b) **Potency**

The potency test described in Section C.4.c should be used on a representative sample from each batch of final product.

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INFECTIOUS BURSAL DISEASE
(Gumboro disease)

SUMMARY

Infectious bursal disease (IBD) is caused by a virus that is a member of the genus Avibirnavirus of the family Birnaviridae. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two serotypes of IBDV are recognised. These are designated serotypes 1 and 2. Both serotypes can be differentiated by cross-neutralisation assays. Clinical disease has been associated with only serotype 1 and all commercial vaccines are prepared against this serotype. Antigenic variants of IBD serotype 1 have been described and these may require special vaccines for maximum protection. Very virulent strains of classical serotype 1 are now common and are causing serious disease in many countries.

Clinical disease due to infection with the IBDV, also known as Gumboro disease, can usually be diagnosed by a combination of characteristic signs and post-mortem lesions. Laboratory confirmation of disease, or detection of subclinical infection, can be carried out by demonstration of a humoral immune response in unvaccinated chickens or by detecting the presence of viral antigen or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful.

Identification of the agent: *Isolation of IBDV is not usually carried out as a routine diagnostic procedure. Specific antibody-negative chickens may be used for this purpose, as may cell cultures or embryonating eggs from specific antibody-negative sources. However, some difficulty may be experienced if using the latter two systems as the virus does not readily adapt to them. If successful, the identity of the virus can be confirmed by the virus neutralisation (VN) test.*

The agar gel immunodiffusion (AGID) test can be used to detect viral antigen in the bursa of Fabricius. A portion of the bursa is removed, homogenised, and used as antigen in a test against known positive antiserum. This is particularly useful in the early stages of the infection, before the development of an antibody response. An immunofluorescence test using IBDV-specific chicken antiserum can also be used to detect antigen in bursal tissue. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) based on plates coated with IBDV-specific antibodies have also been described for the demonstration of IBDV antigens in bursal homogenates. The reverse-transcription polymerase chain reaction (RT-PCR) with specific primers may be used to detect viral genomic RNA in the bursa of Fabricius.

Strain characterisation: *IBDV strains can be further identified by testing their pathogenicity in specific antibody-negative chickens, by investigating their antigenic reactivity in cross VN assays or in tests based on monoclonal antibodies, by determining the nucleotide sequence of RT-PCR amplification products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these different approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.*

Serological tests: An AGID, VN or ELISA may be carried out on serum samples. The infection usually spreads rapidly within a flock of birds. Because of this, only a small percentage of the flock needs to be tested to detect the presence of antibodies. If positive reactions are found in unvaccinated birds then the whole flock must be regarded as infected.

Requirements for vaccines and diagnostic biologicals: Both live attenuated and inactivated (killed) vaccines are available to control the disease. A live recombinant vaccine expressing the VP2 antigen of IBDV has also been licensed recently. It is important that live vaccines be stable, with no tendency to revert to virulence on passage. To be effective, the inactivated vaccines need to have a high antigen content.

Live vaccines are used to produce an active immunity in young chickens. A complementary approach to this is to provide chickens with passive protection by vaccinating the parents using a combination of live and killed vaccines. Effective vaccination of breeding stock is therefore of great importance.

Live vaccines: Attenuated strains of IBD viruses are used. These are referred to as either mild, intermediate, or 'intermediate plus' ('hot') vaccines. The mild vaccines cause limited bursal damage, while the intermediate and intermediate plus vaccines cause some lymphocytic depletion in the bursa of Fabricius. Usually none of the vaccine types causes immunosuppression when used in birds over 14 days old that have been hatched from IBD immune parents.

Mild vaccines are rarely used in broilers, but are used widely to prime broiler parents prior to inoculation with inactivated vaccine. Intermediate and 'hot' vaccines are more capable of overcoming low levels of maternally derived antibodies (MDA). Live vaccines are usually administered by spray or in drinking water. In the absence of MDA, mild vaccines are given at 1-day old. When MDA are present at 1 day of age, vaccination should be delayed until MDA in most of the flock has waned. The best schedule can be determined by serological testing of the birds to detect the time at which MDA has fallen to a low level. More recently, vaccines have been developed that can be administered in ovo at 18 days of incubation.

Killed vaccines: These are usually used to stimulate high and uniform levels of antibody in parent chickens so that the progeny will have high and uniform levels of MDA. The killed vaccines may occasionally be used in young valuable birds with MDA. The killed vaccines are manufactured in oil emulsion adjuvant and given by injection. They must be used in birds already sensitised by primary exposure, either to live vaccine or to field virus. This can be checked serologically. High levels of MDA can be obtained in breeder birds by giving, for example, live vaccine at approximately 8 weeks of age, followed by inactivated vaccine at approximately 18 weeks of age.

A. INTRODUCTION

Infectious bursal disease (IBD) is caused by a virus that is a member of the genus *Avibirnavirus* of the family *Birnaviridae*. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two distinct serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 virus causes clinical disease in chickens younger than 10 weeks. Older chickens usually show no clinical signs. Antibodies are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with Serotype 2 virus (20).

B. DIAGNOSTIC TECHNIQUES

Isolation and identification of the agent provide the most certain diagnosis of IBD, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate (25). In practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, or on detection of the virus in tissues, using immunological or molecular methods.

1. Identification of the agent

Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity with severe depression in most birds lasting for 5–7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2–3 days. Usually between 5% and 10% of birds die, but mortality can reach 30–40%. The main clinical signs are watery diarrhoea, ruffled feathers, reluctance to move, anorexia, trembling and prostration. Post-mortem lesions include dehydration of the muscles with numerous ecchymotic haemorrhages, enlargement and discoloration of the kidneys, with urates in the tubules. The bursa of Fabricius shows the main diagnostic lesions. In birds that die at the peak of the disease outbreak, the bursa is enlarged and turgid with a pale yellow discoloration. Intrafollicular haemorrhages may be present and, in some cases, the bursa may be completely haemorrhagic giving the appearance of a black cherry. Peribursal straw-coloured oedema will be present in many bursae. Confirmation of clinical disease or detection of subclinical disease is best done by using immunological methods as the IBDV is difficult to isolate. For virus isolation, the methods described below should be followed. Differentiation between serotypes 1 and 2 or between serotype 1 subtypes or pathotypes should be undertaken by a specialised laboratory (e.g. the OIE Reference Laboratories for infectious bursal disease [see Table given in Part 3 of this *Terrestrial Manual*]).

a) Sample preparation

Remove the bursae of Fabricius aseptically from approximately five affected chickens in the early stages of the disease. Chop the bursae using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each), and homogenise in a tissue blender. Centrifuge the homogenate at 3000 ***g*** for 10 minutes. Harvest the supernatant fluid for use in the investigations described below. Filtration through a 0.22 µ filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre.

b) Identification by the agar gel immunodiffusion test

A protocol for the AGID test is described in Section B.2.a. For detection of antigen in the bursa of Fabricius by AGID, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue, and the freeze–thaw exudate may be used to fill the wells.

c) Identification by immunofluorescence

Sections of bursa are prepared using a microtome cryostat, dried at room temperature and then fixed in cold acetone. Fluorescent-labelled IBDV-specific antisera are applied to the sections, which are then incubated at 37°C for 1 hour in a humid atmosphere. At the end of the incubation period, they are washed for 30 minutes using phosphate buffered saline (PBS), pH 7.2, then rinsed in distilled water. The sections are mounted using buffered glycerol, pH 7.6, and examined by UV microscopy for IBDV-specific fluorescence (27).

d) Identification by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA)

Different protocols have been described for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) (12, 19, 36). Briefly, ELISA plates are coated with IBDV-specific antibodies. Depending on the chosen AC-ELISA protocol, the capture antibody may be a mouse anti-IBDV monoclonal antibody (MAb), or a mix of such MAbs, or a chicken post-infectious anti-IBDV polyclonal serum. It has been suggested that AC-ELISAs using polyclonal antibodies may have a higher sensitivity. Samples of bursal homogenates (see above) diluted 1/10 to 1/25 (w/v) in a suitable dilution buffer are incubated in the coated wells. Unbound antigens are discarded at the end of the incubation period by washing with a suitable washing buffer (e.g. PBS, pH 7.2 + 0.2% Tween 20). The captured antigens are then revealed, as in an indirect ELISA, with a detection antibody (which must have been developed from a different animal species than the capture antibody), followed by an enzyme conjugate that binds to the detection antibody only (in some protocols the detection antibody may be directly conjugated to the enzyme), followed by the enzyme substrate. Finally, optical densities, which parallel the amount of captured IBDV antigens, are read with an ELISA reader.

The AC-ELISA is based on the use of samples possibly containing live virus and should be performed only in suitable containment facilities such as a class II safety cabinet. All liquid (washing buffers) and solid wastes should be considered to be contaminated by IBDV and decontaminated accordingly before disposal.

Critical steps in the implementation or assessment of AC-ELISA are i) the need to perform extensive washings between each step of the reaction to keep background reactions low, ii) the requirement for known positive and negative samples to be included in each assay as controls, and iii) the need for both the

capture and detection antibodies to positively react with all serotype 1 IBDV strains (i.e. neither capture nor detection should critically depend on IBDV antigenic variation that occurs among serotype 1 strains).

e) Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation (8, 16, 43). The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (24, 43). This method can detect the genome of IBDV, which is unable to grow in cell culture, because it is not necessary to grow the virus before amplification.

RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotidic sequence. Different areas of the genome will be amplified depending on the location from which the primers have been selected. The example below allows the amplification of the middle third of the gene encoding the outer capsid protein VP2 (9, 10).

- **Extraction of nucleic acids**

Single-stranded RNA is extremely susceptible to degradation by RNases. IBDV double-stranded RNA (dsRNA) genome resists degradation by RNases. However, infected cells also contain IBDV-derived positive-sense single-stranded RNA species that can be used as a template at the RT step and may contribute to improving the sensitivity of the assay. It is thus important that RNA extraction be performed using gloves and RNase-free reagents and labware.

IBDV RNA can be extracted from infected tissues using some kits available from commercial suppliers of molecular biology reagents. Alternatively, IBDV RNA can be extracted by adding 1% (weight/volume final concentration) sodium dodecyl sulphate and 1 mg/ml proteinase K to 700 μl of virus suspension (e.g. bursal homogenate). Incubate for 60 minutes at 37°C. Nucleic acids are obtained using a standard protocol for phenol/chloroform extraction (caution: phenol is toxic and should be handled and disposed accordingly). Nucleic acids are harvested from the final aqueous phase by ethanol precipitation and are resuspended in RNase-free distilled water or a suitable buffer. Water-diluted RNA should be kept frozen at a temperature below –20°C until use.

- **Reverse transcription**

A variety of reverse transcriptases are commercially available. Follow the supplier's instructions to prepare the RT reaction mix. Use the 'lower' PCR primer (complementary to the positive strand of IBDV genome, see below) for reverse transcription, as this allows the synthesis of cDNA both from the positive strand of IBDV dsRNA genome and from IBDV-derived positive-sense single-stranded RNAs previously contained in infected cells. Alternatively, random primers (hexanucleotides) can be used to prime cDNA synthesis.

The IBDV RNA matrix must be denatured before transfer to the RT reaction mix. Add one part (by volume) molecular biology grade dimethylsulphoxide to four parts the unfrozen solution of IBDV RNA. Heat for 3 minutes at 92°C and chill on ice; an alternative method is to heat for 5 minutes and immediately incubate the mixture in liquid nitrogen. Transfer the relevant volume of denatured matrix to the reaction mix. Incubate according to the instructions of the enzyme supplier.

The cDNA solution obtained after the RT step should be kept frozen at a temperature below –20°C. Delaying the PCR step for several weeks after the cDNA synthesis may cause false-negative PCR results.

- **Polymerase chain reaction**

A variety of DNA polymerases suitable for PCR are commercially available. Follow the manufacturer's instructions to prepare the PCR reaction mix. Protocols for the amplification and molecular typing of IBDV have been reviewed recently (44). As an example, the U3/L3 and +290/–861 pairs of PCR primers shown below can be suggested and have been found useful for amplifying the middle third of the VP2 gene in segment A of serotype 1 IBDV strains (9, 10), and a region at the 5' extremity of IBDV segment B (21), respectively. Both regions have been shown to be suitable for molecular epidemiology studies (22). Although a significant number of IBDV strains have two nucleotide changes at position 35 (G–A) and 38 (T–C) of the U3 primer (including isolates from Japan [OKYM], Hong Kong [HK46], UK [UK661], Nigeria [N4]), it has been shown that the U3-L3 primer pair successfully amplifies some of these viruses that exhibit both mutations. This is probably because the 3' extremity of U3 is highly conserved. However, as with most PCR assays, IBDV strains may exist with nucleotide changes at the annealing positions of the primers, thus requiring the use of other primers for optimised RT-PCR detection.

The combination of segment A- and segment B-targeted RT-PCR protocols enhances the probability that, if present, serotype 1 IBDV will indeed be detected; it also allows a thorough genetic characterisation of the IBDV strains detected.

Nucleotide sequence of the U3 and L3 IBDV-specific PCR primers (specific for Segment A, VP2 gene):

Upper U3: 5'-**TGT-AAA-ACG-ACG-GCC-AGT**-<u>GCA-TGC</u>-*GGT-ATG-TGA-GGC-TTG-GTG-AC*-3'

Lower L3: 5'-**CAG-GAA-ACA-GCT-ATG-ACC**-<u>GAA-TTC</u>-*GAT-CCT-GTT-GCC-ACT-CTT-TC*-3'

Nucleotide sequence of the +226 and –793 IBDV-specific PCR primers (specific for Segment B, VP1 gene):

Upper +290: 5'-**TGT-AAA-ACG-ACG-GCC-AGT**-<u>GAA-TTC</u>-*AGA-TTC-TGC-AGC-CAC-GGT-CTC-T*-3'

Lower -861: 5'-**CAG-GAA-ACA-GCT-ATG-ACC**-<u>CTG-CAG</u>-*TTG-ATG-ACT-TGA-GGT-TGA-TTT-TG*-3'

The U3 and L3 primers are both 44 nucleotides long, whereas primers +290 and –861 are 46 and 47 nucleotides long, respectively. The four primers include an IBDV-specific 3' extremity (in italics in the sequence shown above) corresponding to nucleotide positions 657–676 and 1193–1212 of IBDV segment A in primers U3 and L3, respectively (numbering as in segment A of strain P2, Acc No X84034), and to nucleotide positions 290–311 and 861–883 of IBDV segment B in primers +290 and –861, respectively (numbering as in segment B of strain D6948, Acc No AF240687). The IBDV-specific extremity is coupled to a non-IBDV 5' extremity (bold type in the sequence above) corresponding to the M13 and RM13 universal primers in the upper and lower primers, respectively. The M13 and RM13 universal primers are commonly used as primers in DNA sequencing reactions, so that purified PCR products resulting from amplification with the U3/L3 and +290/–861 primer pairs can be easily sequenced in both directions. Finally, restriction sites (underlined in the above sequence) are included for the following restriction endonucleases: *Sph*I (in primer U3), *Eco*RI (in primers L3 and +290), and *Pst* I (in primer –861). These restriction sites are positioned so that the PCR products resulting from amplification with the U3/L3 or +290/–861 pairs can be cloned if required. The U3/L3 pair generates a 604 base pair (bp) product, 516 bp of which are specific of the amplified IBDV sequence and encompass the region encoding the hyper-variable region of the VP2 protein. The +290/–861 pair generates a 642 bp product, 549 bp of which are specific of the amplified IBDV sequence. Both products are derived from genomic regions that are suitable for phylogenetic analysis (9, 10, 21, 22).

Perform an initial denaturation step as recommended by the DNA polymerase supplier, followed by 35 cycles, each including one denaturation, one annealing and one elongation step. In such cycles, denaturation at 95°C for 30 seconds and annealing at 64°C for 45 seconds may be used with both the U3/L3 and +290/–861 primer pairs (the annealing temperature should be adapted if other primers are used). The parameters for the elongation step should be set according to the supplier's recommendations.

Revelation may be performed by electrophoresis with the PCR products and DNA molecular weight markers in a 1% agarose gel stained with ethidium bromide (caution: ethidium bromide is toxic and carcinogenic. It should be handled and disposed accordingly).

Three PCR reactions should be performed for each cDNA sample (pure, 10- and 100-fold diluted cDNA) to avoid false-negative results due to PCR inhibition in mixes containing high amounts of the cDNA preparation.

Each PCR should include negative and positive control reactions. Protocols that include an internal control to test for the presence of PCR inhibitors have been developed (35).

Delaying the PCR for several weeks after the RT step may cause false negative PCR results.

f)   Isolation of virus in cell culture

Inoculate 0.5 ml of sample into each of four freshly confluent chicken embryo fibroblast (CEF) cultures (from a specific pathogen free [SPF] source) in 25 cm^2 flasks. Adsorb at 37°C for 30–60 minutes, wash twice with Earle's balanced salt solution and add maintenance medium to each flask. Incubate the cultures at 37°C, observing daily for evidence of cytopathic effect (CPE). This is characterised by small round refractive cells. If no CPE is observed after 6 days, discard the medium, then freeze and thaw the cultures and inoculate the resulting lysate into fresh cultures. This procedure may need to be repeated at least three times. If CPE is observed, the virus should be tested against IBDV antiserum in a tissue culture virus neutralisation (VN) test (see below). The more pathogenic IBDV strains usually cannot be adapted to grow in CEF unless the virus has first been submitted to extensive serial passage in embryos (see below).

g)   Isolation of virus in embryos

Inoculate 0.2 ml of sample into the yolk sac of five 6–8-day-old specific antibody negative (SAN) chicken embryos and on to the chorioallantoic membrane (1) of five 9–11-day-old SAN chicken embryos. SAN embryos are derived from flocks shown to be serologically negative to IBDV. Candle daily and discard dead embryos up to 48 hours post-inoculation. Embryos that die after this time are examined for lesions. Serotype 1 IBD produces dwarfing of the embryo, subcutaneous oedema, congestion and subcutaneous or intracranial haemorrhages. The liver is usually swollen, with patchy congestion producing a mottled effect.

In later deaths, the liver may be swollen and greenish, with areas of necrosis. The spleen is enlarged and the kidneys are swollen and congested, with a mottled effect. If lesions are observed, the virus should then be tested against a monospecific anti-IBDV serum in an embryo-revealed virus neutralisation assay.

Serotype 1 IBDV usually causes death in at least some of the embryos on primary isolation.

Serotype 2 IBDV does not induce subcutaneous oedema or haemorrhages in the infected embryos, but embryos are of a smaller size with a pale yellowish discolouration.

For the preparation of embryo-propagated stock virus or for subsequent passaging, embryos with lesions or embryos suspected to be infected, respectively, are harvested aseptically. Their head and limbs are discarded and the main body is minced as described in Section B.1.a for the preparation a virus suspension.

h)   Isolation of virus in chickens

This method has been used in the past but is no longer recommended due to animal welfare concerns. Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eye-drop route with 0.05 ml of sample. Kill the chickens 72–80 hours after inoculation, and examine their bursae of Fabricius. The bursae of chickens infected with virulent serotype 1 IBDV appear yellowish (sometimes haemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plugs of caseous material are occasionally found. The plicae are petechiated.

The presence of lesions in the bursae of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursae from both groups may be used as antigen in an agar gel immunodiffusion (AGID) test against known positive IBD antiserum (see Section B.1.b).

The extent of bursal damage may vary considerably with the pathogenicity of the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives a limited indication on strain pathogenicity.

The bursae of chickens infected with serotype 2 IBDV do not exhibit any gross lesions.

i)   Strain differentiation

IBDV strains can be further identified by testing their pathogenicity in SAN chickens, by investigating their antigenic reactivity in cross VN tests or using MAbs, by determining the nucleotide sequence of RT-PCR amplification products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.

- **Pathogenicity testing**

Studies to compare the pathogenicity of IBDV strains must be carried out in secure biocontainment facilities to avoid the dissemination of the studied virus (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities). SAN birds with a known microbial status (ideally SPF chickens) must be used to avoid interference by contaminating agents.

The main variables when comparing the results of pathogenicity trials are the breed, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus, and the possible presence of contaminating agents in the inoculum. Light layer breeds have been reported to be more susceptible than heavy broilers (42). Differences in susceptibility may also occur between different SPF chicken lines. The highest susceptibility to acute IBD occurs in chickens between 3 and 6 weeks of age (25). (The influence of the immune status is described in Section C.) A high dose of challenge virus such as that recommended in Section C.1.c is necessary so that all inoculated chickens become infected at once without requiring bird-to-bird transmission of the inoculated virus. Finally, the presence in the inoculum of contaminating agents, such as adenovirus or chicken infectious anaemia virus, may modify the severity of IBD and signs observed after challenge (32).

The terms 'variant', 'classical' and 'very virulent' have been used to describe IBDV strains that exhibit a different pathogenicity. Based on the signs and lesions observed in two lines of White Leghorn SPF chickens during acute experimental IBD following a 10^5 50% embryo infective dose (EID_{50}) challenge, North American 'Variant' IBDVs induce little if any clinical signs and no mortality but marked bursal lesions, 'Classical' IBDVs induce approximately 10–50% mortality with typical signs and lesions whereas 'very virulent' IBDVs induce approximately 50–100% mortality with typical signs and lesions (Eterradossi *et al.,* personal observation).

- **Antigenicity testing**

Antigenic relatedness among IBDV strains may be assayed in cross VN tests, which correlate best with cross protection. Such tests have to be performed in SAN embryonated eggs when the studied viruses do not grow in CEF (e.g. very virulent IBDV [vvIBDV]). Differences in cross VN results among serotype 1 IBDV strains have led to the definition of serotype 1 'subtypes', some of which include the antigenically 'variant' North American IBDV isolates (15).

Another approach to the study of genetic relatedness is the use of mouse MAbs that bind to IBDV neutralising epitopes. Several panels of MAbs exist world-wide (12, 13, 37). Some of the MAbs have been included in commercially available kits, but no unified MAb panel as yet been proposed. All neutralising epitopes of IBDV characterised to date have been mapped into a major immunogenic domain in the middle third (amino acid positions 200 to 340) of the VP2 outer capsid protein (10, 33, 40). This region is termed 'VP2 variable domain' because most amino acid changes observed among IBDV strains are clustered in it. Within vVP2, four amino acid stretches are of critical importance to antigenicity and are referred to as vVP2 hydrophilic peaks. These are amino acid positions 210 to 225 (major peak A), 249 to 252 (minor peak 1), 281 to 292 (minor peak 2) and 313 to 324 (major peak B) (2, 41). Both North American 'variants' and 'very virulent' IBDV exhibit in these areas amino acid changes that correlate with epitope variation (9, 40). To date, no antigenic marker has been shown to correlate strictly with IBDV pathogenicity.

- **Molecular identification**

Most efforts at molecular identification have focused on the characterisation of the larger segment of IBDV (segment A) and especially of the vVP2 encoding region. Several protocols have been published on characterisation using restriction endonucleases of RT-PCR products. These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism) (17, 24, 46). The usefulness of the information they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Some sites involved in antigenicity have already been identified (see above), however, restriction sites reliably related to virulence still need to be defined and validated. Nucleotide sequencing of RT-PCR products, although more expensive than restriction analysis, provides an approach to assessing more precisely the genetic relatedness among IBDV strains. Markers have been demonstrated experimentally, using a reverse genetics approach, for cell culture-adapted strains, which exhibit amino acid pairs 279 N–284 T (23) or 253 H–284 T (28). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 I and 299 S) (3, 9, 24). However, it is not yet known whether these amino acids play a role in virulence or if they are merely an indication of the clonal origin of most vvIBDV isolates. Several recent studies indicate that although VP2 is an important virulence determinant, it may not be the only one (4). It has been reported that segment A and B of IBDV mostly co-evolve (i.e. most significant IBDV clusters, such as vvIBDV-related strains, may be identified by analysis of both genome segments), however some potentially reassortant viruses have been identified (21).

2. Serological tests

a) Agar gel immunodiffusion test

The AGID test is the most useful of the serological tests for the detection of specific antibodies in serum, or for detecting viral antigen or antibodies in bursal tissue.

Blood samples should be taken early in the course of the disease, and repeat samples should be taken 3 weeks later. Because the virus spreads rapidly, only a small proportion of the flock needs to be sampled. Usually 20 blood samples are enough. For detection of antigen in the bursa of Fabricius, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in a scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue.

- **Preparation of positive control antigen**

Inoculate 3–5-week-old susceptible chickens, by eye-drop, with a clarified 10% (w/v) bursal homogenate known to contain viable IBDV[1]. Kill the birds 3 days post-inoculation, and harvest the bursae aseptically. Discard haemorrhagic bursae and pool the remainder, weigh and add an equivalent volume of cold distilled water (or of a suitable buffer such as PBS or tryptose phosphate broth) and an equivalent volume of undiluted methylene chloride. (Caution: methylene chloride is toxic and possibly carcinogenic. It should be handled and disposed accordingly. A possible alternative to avoid health hazards caused by methylene chloride is to use trichlorotrifluoroethane). Thoroughly homogenise the mixture in a tissue blender and centrifuge at 2000 *g* for 30 minutes. Harvest the supernatant fluid and dispense into aliquots for storage at –40°C. The antigen contains live virus and should be handled only in suitable containment facilities such as

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1   A suitable classical strain of IBDV (serotype 1, classical pathotype) is strain 52/70, otainable from one of the OIE Reference Laboratories (see Table given in Part 3 of this *Terrestrial Manual*).

a class II safety cabinet. If required, the antigen can be inactivated prior to dispensing: add 0.3% (v/v) ß-propiolactone to the harvested supernatant, then further incubate at 37°C for 2 hours. It is important that incubation takes place on an orbital shaker or a mechanical rocker, so that any inner part of the vial that has been in contact with live virus indeed gets into contact with ß-propiolactone. Dispense and store as above. Check the efficacy of the inactivation process by attempting IBDV isolation from the inactivated antigen, with three serial passages on SAN embryonated eggs (see Section B.1.g).

- **Preparation of positive control antiserum**

Inoculate 4–5-week-old susceptible chickens, by eye-drop, with 0.05 ml of a clarified 10% (w/v) bursal homogenate known to contain viable IBDV[1]. Exsanguinate 28 days post-inoculation. Pool and store serum in aliquots at –20°C.

- **Preparation of agar**

Dissolve sodium chloride (80 g) and phenol (5 g) in distilled water (1 litre) (caution: phenol is toxic and should be handled and disposed of accordingly). Add agar (12.5 g) and steam until the agar has dissolved. To avoid the health and environmental hazards caused by the use of phenol, another suitable recipe for the preparation of agar is as follows: sodium chloride (80 g), kalium dihydrogenophosphate (0.45 g), sodium hydrogenophosphate dihydrate (1.19 g), agar (10 g) and distilled water to a final volume of 1 litre (final pH 7.1 at 20–25°C). This second recipe can be homogenised by heating up to 90°C under agitation. While the mixture is still very hot, filter it through a pad of cellulose wadding covered with a few layers of muslin and dispense the medium in 20 ml volumes into glass bottles. The medium without phenol can further be sterilised by autoclaving at (at most) 115°C for 15 minutes. Store the bottles at 4°C until required for use.

- **Test procedure**

i) Prepare plates from 24 hours to 7 days before use. Dissolve the agar by placing in a steamer or boiling water bath. Take care to prevent water entering the bottles.

ii) Pour the contents of one bottle into each of the required number of 9 cm plastic Petri dishes laid on a level surface. (Some laboratories prefer to pour the gel on 25 × 75 mm glass slides, 3 mm deep.)

iii) Cover the plates and allow the agar to set, and then store the plates at 4°C. Poured plates may be stored for up to 7 days at 4°C. (If the plates are to be used the same day that they are poured, dry them by placing them opened but inverted at 37°C for from 30 minutes to 1 hour.)
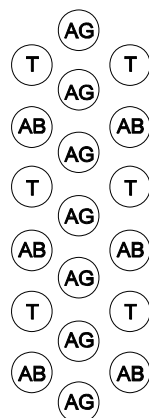

AG = Positive antigen

AB = Positive antiserum

Fig. 1. Protocol for tests for antibody.

T = test sera
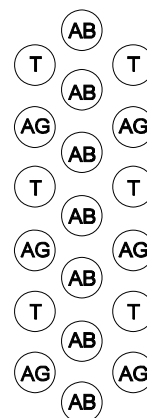
Fig. 2. Protocol for tests for antigen.

T = test tissues

Notes:
1. The linear pattern of wells is preferred although a hexagonal pattern may be used. Each test serum or bursa should be placed adjacent to a positive control antibody (AB) or antigen (AG), respectively.
2. Wells, 3 mm deep, 6 mm in diameter, and 3 mm apart (or wells of any other size previously shown to be effective), are used.

iv) Cut three vertical rows of wells 6 mm in diameter and 3 mm apart, using a template and tubular cutter.

v) Remove the agar from the wells by aspiration or remove using a pen and nib, taking care not to damage the walls of the wells.

vi) Using a pipette, dispense 50 µl of the test sera into the wells as shown in Figure 1.

Or, for the detection of IBDV antigens in bursae:

Dispense small pieces of finely minced test bursae by means of curved fine-pointed forceps into the wells, as shown in Figure 2, to just fill the wells. Alternatively, the freeze–thaw exudate of minced tissues can be used to fill the wells.

vii) Dispense 50 µl of the positive and negative control reagents into the relevant wells.

viii) Incubate the plates at between 22°C and 37°C for up to 48 hours in a humid chamber to avoid drying the agar.

ix) Examine the plates against a dark background with an oblique light source after 24 and 48 hours.

- **Quantitative agar gel immunodiffusion tests**

The AGID test can also be used to measure antibody levels by using dilutions of serum in the test wells and taking the titre as the highest dilution to produce a precipitin line (5). This can be very useful for measuring maternal or vaccinal antibodies and for deciding on the best time for vaccination; however, this AGID quantitative determination has now been largely replaced by the ELISA.

b) Virus neutralisation tests

VN tests are carried out in cell culture. The test is more laborious and expensive than the AGID test, but is more sensitive for detecting antibody. This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV 1 and 2 serotypes.

First, 0.05 ml of virus diluted in tissue culture medium to contain 100 $TCID_{50}$ (50% tissue culture infective doses) per 0.05 ml is placed in each well of a tissue-culture grade microtitre plate (Spearman–Kärber [1] or the Reed & Muench [30]). The test sera are heat-inactivated at 56°C for 30 minutes. Serial doubling dilutions of the sera are made in the diluted virus. After 30 minutes at room temperature, 0.2 ml of SPF chicken embryo fibroblast cell suspension, with a cell density allowing confluent layers to be obtained after 24 hours of incubation, is dispensed into each well. Plates are sealed and incubated at 37°C for 4–5 days, after which the monolayers are observed microscopically for typical CPE. The end-point (serum titre) is expressed as the reciprocal of the highest serum dilution that did not show CPE. To reduce test-to-test and operator-to-operator variation, a standard reference antiserum may be included with each batch of tests[2] and the titre of the virus suspension must be reassessed in each new experiment using a sufficient number of repeats (wells) per virus dilution.

c) Enzyme-linked immunosorbent assay

ELISAs are in use for the detection of antibodies to IBD. Coating the plates requires a purified, or at least semipurified, preparation of virus, necessitating special skills and techniques. Methods for preparation of reagents and application of the assay were described by Marquardt *et al.* in 1980 (26). Commercial kits are available.

The test sera are diluted according to the established protocol or kit instructions and each is dispensed into the requisite number of wells. After incubation under the appropriate conditions, the sera are discarded from the plates, and the wells are washed thoroughly. Anti-chicken immunoglobulins conjugated to an enzyme are dispensed into the wells, and the plates are again incubated as appropriate. The plates are emptied and rewashed before substrate containing a chromogen that gives a colour change in the presence of the enzyme used is added to the plate. After a final incubation step, the substrate/chromogen reaction is stopped by addition of a suitable stopping solution and the colour reactions are quantified by measuring the optical density of each well. The Sample to Positive (S/P) ratio for each test sample is calculated.

d) Interpretation of results

The AGID test is surprisingly sensitive, though not as sensitive as the VN test; the latter will often give a titre when the AGID test is negative. Positive reactions indicate infection in unvaccinated birds without maternal antibodies. As a guide, a positive AGID reaction in a vaccinated bird or young bird with maternal antibody indicates a protective level of antibody. ELISA gives more rapid results than VN or AGID and is less costly in terms of labour, although the reagents are more expensive. VN and AGID titres correlate well, but as VN is more sensitive, AGID titres are proportionally lower. Correlation between ELISA and VN and between ELISA and AGID is more variable depending on the source of the ELISA reagents. When testing for the decay of maternally derived antibodies (MDA), it is not uncommon to find residual VN antibodies at an age when ELISA results are already negative. A formula has been devised that allows ELISA titres to be used to calculate the optimal age for vaccination (18), which will vary depending on the vaccine used. Nonspecific positive reactions may occur with most ELISAs because they are usually designed for monitoring vaccine

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2 A suitable reference antiserum may be obtained from the OIE Reference Laboratories (see Table given in Part 3 of this *Terrestrial Manual*).

responses, in which case sensitivity is regarded as more important than specificity. This should be taken into account when the ELISA is used for diagnosis. In commercial chicken flocks, the possibility that a serotype 1 ELISA antigen also detects antibodies induced by a natural infection with serotype 2 IBDV cannot be ruled out, however this possible cross reactivity has not yet been demonstrated to interfere with serological monitoring programmes of IBD based on the ELISA.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two types of vaccine are mostly available for the control of IBD. These are live attenuated vaccines, or inactivated oil-emulsion adjuvanted vaccines (39). A live recombinant vaccine expressing IBDV antigens has also been licensed (7).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

To date, IBD vaccines have been made with serotype 1 IBDV only, although a serotype 2 virus has been detected in poultry. The serotype 2 virus has not been associated with disease, but its presence will stimulate antibodies. Serotype 2 antibodies do not confer protection against serotype 1 infection, neither do they interfere with the response to type 1 vaccine. There have been numerous descriptions of antigenic variants of serotype 1 virus (31). Cross-protection studies have shown that inactivated vaccines prepared from 'classical' serotype 1 virus require a high antigenic content to provide good protection against some of these variants. IBD vaccines that contain both classical and variant IBD serotype 1 viruses are now licensed. vvIBDV strains with limited antigenic changes as compared with 'classical' serotype 1 viruses have emerged since 1986. Active immunisation with a 'classical' serotype 1 virus or vaccine provides a good protection against the vvIBDVs (16), however the latter viruses are less susceptible to neutralisation by maternally derived antibodies than 'classical' pathogenic viruses (42).

•   **Live vaccines: methods of use**

Live IBD vaccines are produced from fully or partially attenuated strains of virus, known as 'mild', 'intermediate', or 'intermediate plus' ('hot'), respectively.

Mild or intermediate vaccines are used in parent chickens to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine. They are susceptible to the effect of MDA so should be administered only after all MDA has waned. Application is by means of intramuscular injection, spray or in the drinking water, usually at 8 weeks of age (34).

Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only minimal levels of MDA. This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decay. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels. The timing of additional applications will depend on the antibody titres of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD, and the third dose 7–10 days later. The route of administration is by means of spray or in the drinking water. Intramuscular injection or eye-drop is used rarely. If the vaccine is given in the drinking water, clean water with a neutral pH must be used that is free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. It is possible to divide the medicated water into two parts, giving the second part 30 minutes after the first.

Recently, technology has been developed to deliver live vaccine into eggs during the incubation period. Live vaccine virus is blended with IBD antibody and the complex is injected *in ovo* at 18 days of incubation. The eggs go on to hatch and the vaccine virus is released when the chicks are about 7 days of age. In this way, the problem of maternally derived IBD antibody is overcome and the chicks are effectively immunised (14).

A live recombinant vaccine that uses a viral vector (herpes virus of turkeys) to express the VP2 antigen of IBDV in chickens has been licensed recently in Europe. There is limited information available on the use of this vaccine.

Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, it is possible that IBD vaccines that cause bursal damage could interfere with the response to other vaccines. Only healthy birds

should be vaccinated. The vials of lyophilised vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

• Inactivated vaccines: method of use

Inactivated IBD vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field virus during rearing (6). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. Occasionally, inactivated vaccines may be used in programmes combining inactivated and live vaccines, in young valuable birds with high MDA levels reared in areas with high risk of exposure to virulent IBDV. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular into the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. Vaccine should be stored at between 4°C and 8°C. It should not be frozen or exposed to bright light or high temperature.

Only healthy birds, known to be sensitised by previous exposure to IBDV, should be vaccinated. Used in this way the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (45). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression. It has been shown that bursal damage occurring after about 15 days of age has little effect on immunocompetence as by that time the immunocompetent cells have migrated into the peripheral lymphoid tissues. However, if there is a threat of exposure to infection with very virulent IBDV, live vaccines should be applied as described above. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

1.   Seed management

a)   Characteristics of the seed

• Live vaccine

The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens. This includes freedom from contamination with other strains of IBDV. For vaccine strains that claim to be attenuated and nonimmunosuppressive, the seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out at least five consecutive chicken-to-chicken passages at 3–4-day intervals using bursal suspension as inoculum in SPF chickens of the minimum age recommended for vaccination. It must be shown that the virus was transmitted. A histological comparison is then made to show that there is no difference between bursae from birds inoculated with the initial and the final passage material. Bursal scoring (29) and imaging techniques have been developed.

Test for immunosuppression: An important characteristic is that the virus should not produce such damage to the bursa of Fabricius that it causes immunosuppression in susceptible birds. Live vaccines of the 'intermediate' or 'intermediate plus' type may be licensed even though they may be capable of causing immunosuppression. A possible protocol for the experimental assessment of immunosuppression is the following: The vaccine is administered by injection or eye-drop, one field dose per bird, to each of 20 SPF chickens, at 1-day old. A further group of birds of the same age and source are housed separately as controls. At 2 weeks of age, each bird in both groups is given one field dose of live Newcastle disease vaccine by eye-drop. Alternatively, the IBDV vaccine may be administered at the minimum age recommended for vaccination, and the Newcastle disease vaccine at the time when bursal lesions induced by the IBDV vaccine are maximal. The haemagglutination inhibition (HI) response of each bird to Newcastle disease vaccine is measured 2 weeks after the administration of the Newcastle Disease vaccine, and the protection is measured against challenge with $10^{5.0}$ to $10^{6.5}$ ELD_{50} (50% embryo lethal doses) Herts 33/56 strain (or similar) of Newcastle disease virus. The IBD vaccine fails the test if the HI response and protection afforded by Newcastle disease vaccine is significantly less (<0.01) in the group given IBD vaccine than in the control group. In countries where Newcastle disease virus is exotic, an alternative is to use sheep erythrocytes or *Brucella abortus*-killed antigen as the test antigen, measuring the response using the haemagglutination or serum agglutination test, respectively. However, another live vaccine is a preferable test system because it also evaluates cell-mediated immunity.

• Killed vaccine

For killed vaccines, the most important characteristics are high yield and good antigenicity. Both virulent and attenuated strains have been used. The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens (38).

b) Method of culture

Seed virus may be propagated in various culture systems, such as SPF chicken embryo fibroblasts, or chicken embryos. In some cases, propagation in the bursa may be used. The bulk is distributed in aliquots and freeze-dried in sealed containers. There have been claims that bursal origin vaccines are better immunogens than tissue culture vaccines. In controlled studies, it was concluded that both vaccines, when containing similar antigenic mass, elicited similar immune responses.

c) Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge. In the case of killed vaccines, a test must be carried out in older birds that go on to lay, using the recommended vaccination schedule, so that their progeny can be challenged to determine resistance due to MDA at the beginning and end of lay.

- **Live vaccine**

Efficacy test: Administer one vaccine dose of the minimum recommended titre to each of 20 SPF chickens of the minimum age of vaccination. Inoculate separate groups for each of the recommended routes of application. Leave 20 chickens from the same hatch as uninoculated controls. After 14 days, challenge each of the chickens by eye-drop with approximately 100 CID_{50} (50% chicken infective dose) of a virulent strain of IBDV as recommended by one of the OIE Reference Laboratories for IBD (see Table given in Part 3 of this *Terrestrial Manual*). Observe the chickens daily for 10 days. Register the number of birds that die or exhibit IBD signs. Perform a histological examination of the bursa in chickens that survive at day 10. The vaccine fails the test unless at least 90% of the vaccinated chickens survive without showing either clinical signs or severe lesions in the bursae of Fabricius at the end of the observation period. If more than half the controls do not show IBD signs, or one or more control chicken does not exhibit severe lesions of the bursa of Fabricius, or control or inoculated birds die from causes not attributable to the test, the test is invalid. Lesions are considered to be severe if at least 90% of follicles show greater than 75% depletion of lymphocytes. Providing results are satisfactory, this test need be carried out on only one batch of all those batches prepared from the same seed lot.

- **Killed vaccine**

Efficacy test: At least 20 unprimed SPF birds are given one dose of vaccine at the recommended age (near to point-of-lay) at least one of the recommended routes; an alternative recommended procedure is to test one dose of vaccine in the recommended routes listed on the label, using 20 unprimed SPF birds for each route. The antibody response is measured between 4 and 6 weeks after vaccination by serum neutralisation with reference to a standard antiserum[3].

Eggs are collected for hatching 5–7 weeks after vaccination, and 25 progeny chickens are then challenged at 3 weeks of age by eye-drop with approximately 100 CID_{50} of a recognised virulent strain of IBDV. Ten control chickens of the same breed but from unvaccinated parents are also challenged. Protection is assessed 3–4 days after challenge by removing the bursa of Fabricius from each bird; each bursa is then subjected to histological examination or tested for the presence of IBD antigen by the agar gel precipitin test. Not more than three of the chickens from vaccinated parents should show evidence of IBD infection, whereas all those from unvaccinated parents should be affected.

These procedures should be repeated towards the end of the period of lay when the vaccinated birds are at least 60 weeks of age, but, on this occasion challenge of the progeny should be undertaken when they are 15 days old.

The efficacy test should be repeated on primed birds vaccinated by the recommended schedule. The final dose of killed vaccine is given at the earliest recommended age. Chickens hatched from fertile eggs collected at the beginning and the end of lay are tested for protection against challenge as described above.

These tests need to be performed once only using a typical batch of vaccine.

2. Method of manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

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3   See footnote 2

Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. SPF eggs must be used for all materials employed in propagation and testing of the vaccine. Live vaccines are made by growth in eggs or cell cultures. Inactivated IBD vaccines may be made using virulent virus grown in the bursae of young birds, or using attenuated, laboratory-adapted strains of IBDV grown in cell culture or embryonated eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% suspension of bursal material in water, with suitable emulsifying agents.

3.   In-process control

Antigen content: Having grown the virus to high concentration, its titre should be assayed by use of cell cultures, embryos or chickens as appropriate to the strain of virus being used. The antigen content required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be effective in laboratory and field trials.

Inactivation of killed vaccines: This is often done with either ß-propiolactone or formalin. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria, that may arise from the starting materials.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch of both the bulk harvest after inactivation and the final product. An alternative approach is to test inactivation of the final or bulk harvest, but not both. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or chickens, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

Sterility of killed vaccines: Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia.

4.   Batch control

a)   Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b)   Safety

- **Live vaccine safety test**

Ten field doses of vaccine are administered by eye-drop to each of 15 SPF chickens of the minimum age recommended for vaccination and not older than 2 weeks. The chickens are observed for 21 days. If more than two chickens die due to causes not related to the vaccine, the test must be repeated. The vaccine fails the test if any chickens die or show signs of disease attributable to the vaccine. This test is performed on each batch of final vaccine.

- **Killed vaccine safety test**

Ten SPF birds, 14–28 days of age, are inoculated by the recommended routes with the recommended dose or twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. The test is performed on each batch of final vaccine.

c)   Potency

- **Live vaccine potency test**

A potency test (virus titration) in eggs or cell cultures must be carried out on each serial (batch) of vaccine produced. In addition, the method described in Section C.1.c 'Live vaccine (efficacy test)' must be used on one batch representative of all the batches prepared from the same seed lot.

- **Killed vaccine potency test**

Ten SPF chickens, approximately 4 weeks of age, are each vaccinated with one dose of vaccine given by the recommended route. An additional ten control birds of the same source and age are housed together with the vaccinates. The antibody response of each bird is determined 4–6 weeks after vaccination in a VN test with reference to a standard antiserum. The mean antibody level of the vaccinated birds should not be

significantly less than the level recorded in the test for protection. No antibody should be detected in the control birds. This test must be carried out on each batch of final vaccine.

d) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at the requested shelf life or as an alternative at 3 months beyond

e) Preservatives

A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistence throughout shelf life should be checked. A suitable preservative already established for such purposes should be used.

f) Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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NB: There are OIE Reference Laboratories for Infectious bursal disease (Gumboro disease) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

MAREK'S DISEASE

SUMMARY

Marek's disease (MD) is a lymphomatous and neuropathic disease of domestic fowl caused by an alphaherpesvirus.

Diagnosis is made on clinical signs and gross or microscopic lesions. Chickens may become persistently infected with MD virus (MDV) without developing clinical disease. Infection by MDV is detected by virus isolation and the demonstration of viral antigen or antibodies.

MD is prevented by vaccination with monovalent or multivalent live virus vaccines of various types. The vaccine is injected in ovo *or at hatch.*

In chickens, MD occurs at 3–4 weeks of age or older and is most common between 12 and 30 weeks of age. Clinical signs observed are paralysis of the legs and wings, with enlargement of peripheral nerves, but nerve involvement is sometimes not seen, especially in adult birds. Depending on the strain of MDV, lymphomatosis can occur, especially in the ovary, liver, spleen, kidneys, lungs, heart, proventriculus and skin. As opposed to the uniform cell population that comprises the tumours caused by lymphoid leukosis, the nerve infiltration and lymphomas caused by MDV consist of lymphoid cells of various types. Tumours that resemble those produced by MDV are induced by the avian retrovirus, reticulo-endothelial virus (REV). Differentiation of MD from lymphoid leukosis is important. Lesions caused by reticuloendotheliosis virus can also be confused with those of MD.

Identification of the agent: *Under field conditions, most chickens become infected with MDV during the first few weeks of life and then carry the infection throughout their lives, often without developing overt disease. The infection is usually detected by inoculating live buffy coat cells on to monolayer cultures of chicken kidney cells or duck embryo fibroblasts, in which characteristic viral plaques develop within a few days. Two serotypes of MDV are recognised – 1 and 2 – and a third serotype is represented by the related herpesvirus of turkeys (HVT). Serotype 1 includes the virulent strains and serotype 2 the naturally avirulent strains. MD viral antigen can be detected in the feather tips of infected birds using a radial precipitin test.*

Serological tests: *Antibodies to MDV develop within 1–2 weeks of infection and are commonly recognised by the agar gel immunodiffusion test, the indirect fluorescent antibody test, and sometimes by other serological tests such as enzyme-linked immunosorbent assay.*

Requirements for vaccines and diagnostic biologicals: *MD is prevented by vaccinating chickens* in ovo *or at 1 day of age. Live viral vaccines are used. HVT, in either a cell-free (lyophilised) form, or a cell-associated ('wet') form, is most commonly used. Attenuated variants of serotype 1 strains of MDV are the most commonly used vaccine type; also serotype 2 strains may also be used, particularly in bivalent vaccines, together with HVT (serotype 3). Serotype 1 and 2 vaccines are only available in the cell-associated form. Bivalent vaccines consisting of serotypes 1 and 3 or trivalent vaccines consisting of serotypes 1, 2, and 3 are also used. The bivalent and trivalent vaccines have been introduced to combat the very virulent strains of MDV that are not well controlled by the usual monovalent vaccines.*

Vaccination greatly reduces clinical disease, but not persistent infection by MDV. The vaccine viruses are also carried throughout the life of the fowl and are continued to be shed, which results in the ubiquitous presence of MDV.

A. INTRODUCTION

Marek's disease (MD) (14, 25, 33) is a disease of domestic fowl (chickens) caused by a herpesvirus. Birds get infected by inhalation of infected dust from the poultry houses, and following a complex life cycle, the virus is shed from the feather follicle of infected birds (4). MD occurs at 3–4 weeks of age or older and is most common between 12 and 30 weeks of age. MD is associated with several distinct pathological syndromes, of which the lymphoproliferative syndromes are the most frequent and are of most practical significance. In the classical form of the disease, characterised mainly by the involvement of nerves, mortality rarely exceeds 10–15% and can occur over a few weeks or many months. In the acute form, in which there is usually lymphoma formation in the viscera, a disease incidence of 10–30% in the flock is not uncommon and outbreaks involving up to 70% can occur. Mortality may increase rapidly over a few weeks and then cease, or can continue at a steady or slowly falling rate for several months. Currently, the acute form of the disease with extensive visceral lymphomas is most prevalent. In its classical form, the most common clinical sign of MD is partial or complete paralysis of the legs and wings. In the acute form, birds are often severely depressed and some may die without showing signs of clinical disease. Non-neoplastic disease involving brain pathology with vasogenic oedema resulting in transient paralysis is increasingly recognised with MD induced by the more virulent strains.

In the classical form, the characteristic finding is enlargement of one or more peripheral nerves. Those most commonly affected and easily seen at post-mortem are the brachial and sciatic plexuses, celiac plexus, abdominal vagus and intercostal nerves. Affected nerves are often two or three times their normal thickness, the normal cross-striated and glistening appearance is absent, and the nerve may appear greyish or yellowish, and sometimes oedematous. Lymphomas are sometimes present in the classical form of MD, most frequently as small, soft, grey tumours in the ovary, and sometimes also in the lungs, kidneys, heart, liver and other tissues. 'Grey eye' caused by an iridocyclitis that renders the bird unable to accommodate the iris in response to light and causes a distorted pupil is common in older (16–18 week) birds, and may be the only presenting sign.

In the acute form, the typical finding is widespread, diffuse lymphomatous involvement of the liver, gonads, spleen, kidneys, lungs, proventriculus and heart. Sometimes lymphomas also arise in the skin around the feather follicles and in the skeletal muscles. Affected birds usually have enlarged peripheral nerves, as in the classical form. In younger birds, liver enlargement is usually moderate in extent, but in adult birds the liver may be greatly enlarged and the gross appearance identical to that seen in lymphoid leukosis, from which the disease must be differentiated. Nerve lesions are often absent in adult birds with MD.

In both the classical and acute forms of MD, the disease starts as a proliferation of lymphoid cells, which is progressive in some cases and regressive in others. The peripheral nerves may be affected by proliferative, inflammatory or minor infiltrative changes, which are termed type A, B, and C lesions, respectively. The A-type lesions consist of infiltration by proliferating lymphoblasts, large, medium and small lymphocytes, and macrophages, and appear to be neoplastic in nature. The B-type lesion is characterised by interneuritic oedema, infiltration by mainly small lymphocytes and plasma cells, and Schwann cell proliferation, and appears to be inflammatory. The C-type lesion consists of a light scattering of mainly small lymphocytes, and is often seen in birds that show no gross lesions or clinical signs. It is thought to be a regressive, inflammatory lesion. Demyelination frequently occurs in nerves affected by the A- and B-type lesions, and is responsible for the clinical paralysis.

Lymphomas in the visceral organs and other tissues are similar cytologically to the lymphoproliferations in the A-type lesions in nerves. Usually the lymphoid cells are of mixed types, often with a preponderance of small and medium lymphocytes, but sometimes, particularly in acute MD in adult birds, large lymphocytes and lymphoblasts may predominate.

The heterogeneous population of lymphoid cells in MD lymphomas, as seen in haematoxylin-and-eosin-stained sections, or in impression smears of lymphomas stained by May–Grünwald–Giemsa, is an important feature in differentiating the disease from lymphoid leukosis, in which the lymphomatous infiltrations are composed of uniform lymphoblasts. Another important difference is that, in lymphoid leukosis, gross lymphomas occur in the bursa of Fabricius, and the tumour has an intrafollicular origin and pattern of proliferation. In MD, although the bursa is sometimes involved in the lymphoproliferation, the tumour is less apparent, diffuse and interfollicular in location. Peripheral nerve lesions are not a feature of lymphoid leukosis as they are in MD. The greatest difficulty comes in distinguishing between lymphoid leukosis and forms of MD sometimes seen in adult birds in which the tumour is lymphoblastic with marked liver enlargement and absence of nerve lesions. If post-mortems are conducted on several affected birds, a diagnosis can usually be made based on gross lesions and histopathology. However there are other specialised techniques described. The expression of a Meq biochemical marker has been used to differentiate between MD tumours, latent MDV infections and retrovirus-induced tumours (3). The procedure may require specialised reagents and equipment and it may not be possible to carry out these tests in laboratories without these facilities. Other techniques, such as detection by immunofluorescence of activated T cell antigens present on the surface of MD tumour cells (MD tumour-associated surface antigen or MATSA), or of B-cell antigens or IgM on the tumour cells of lymphoid leukosis can give a presumptive diagnosis, but these are not specific to MD tumour cells.

Nerve lesions and lymphomatous proliferations induced by certain strains of reticuloendotheliosis virus are similar, both grossly and microscopically, to those present in MD. Although reticuloendotheliosis virus is not

common in chicken flocks, it should be borne in mind as a possible cause of lymphoid tumours; its recognition depends on virological and serological tests on the flock. Reticuloendotheliosis virus can also cause neoplastic disease in turkeys, ducks, quail and other species. Another retrovirus also causes lymphoproliferative disease in turkeys. Although chicken flocks may be seropositive for reticuloendotheliosis virus, neoplastic disease is rare. The main features in the differential diagnosis of MD, lymphoid leukosis and reticuloendotheliosis are shown in Table 1. Peripheral neuropathy is a syndrome that can easily be confused with the neurological lesions caused by MD virus (MDV). This is not very common but its incidence may be increasing in some European flocks (3).

There are no recognised health risks to humans working with MDV or the related herpesvirus of turkeys (HVT).

Table 1. *Features useful in differentiating Marek's disease, lymphoid leukosis and reticuloendotheliosis*

Feature	Marek's disease	Lymphoid leukosis	Reticuloendotheliosis[*]
Age	Any age. Usually 6 weeks or older	Not under 16 weeks	Not under 16 weeks
Signs	Frequently paralysis	Non-specific	Non-specific
Incidence	Frequently above 5% in unvaccinated flocks. Rare in vaccinated flocks	Rarely above 5%	Rare
Macroscopic lesions			
Neural involvement	Frequent	Absent	Infrequent
Bursa of Fabricius	Diffuse enlargement or atrophy	Nodular tumours	Nodular tumours
Tumours in skin, muscle and proventriculus, 'grey eye'	May be present	Usually absent	Absent
Microscopic lesions			
Neural involvement	Yes	No	Infrequent
Liver tumours	Often perivascular	Focal or diffuse	Focal
Spleen	Diffuse	Often focal	Focal or diffuse
Bursa of Fabricius	Interfollicular tumour and/or atrophy of follicles	Intrafollicular tumour	Intrafollicular tumour
Central nervous system	Yes	No	No
Lymphoid proliferation in skin and feather follicles	Yes	No	No
Cytology of tumours	Pleomorphic lymphoid cells, including lymphoblasts, small, medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts	Lymphoblasts	Lymphoblasts
Category of neoplastic lymphoid cell	T cell	B cell	B cell

[*]Reticuloendotheliosis virus may cause several different syndromes. The bursal lymphoma syndrome is most likely to occur in the field and is described here.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Infection by MDV in a flock may be detected by isolating the virus from the tissues of infected chickens. The ubiquitous nature of MDV must be remembered and the diagnosis of MD should be based on a combination of

MDV isolation or detection of the genome by PCR and clinical disease. Commonly used sources are buffy coat cells from heparinised blood samples, or suspensions of lymphoma cells or spleen cells. When these samples are collected in the field, it is suggested that they be transported to the laboratory under chilled conditions. As MDV is highly cell associated, it is essential that these cell suspensions contain viable cells. The cell suspensions are inoculated into monolayer cultures of chicken kidney cells or duck embryo fibroblasts (chicken embryo fibroblasts are less sensitive for primary virus isolation). Serotype 2 and 3 viruses (see Section C.1.a) are more easily isolated in chicken embryo fibroblasts than in chicken kidney cells. Usually a 0.2 ml suspension containing from 10^6 to 10^7 live cells is inoculated into duplicate monolayers grown in plastic cell culture dishes (60 mm in diameter). Inoculated and uninoculated control cultures are incubated at 38.5°C in a humid incubator containing 5% CO_2. Alternatively, closed culture vessels may be used. Culture medium is replaced at 2-day intervals. Areas of cytopathic effects, termed plaques, appear within 3–5 days and can be enumerated at about 7–10 days.

Another, less commonly used source of MDV for diagnostic purposes is feather tips, from which cell-free MDV can be extracted. Tips about 5 mm long, or minced tracts of skin containing feather tips, are suspended in an SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid) buffer for extraction and titration of cell-free MDV (9). The buffer is made as follows: 0.2180 M sucrose (7.462 g); 0.0038 M monopotassium phosphate (0.052 g); 0.0072 M dipotassium phosphate (0.125 g); 0.0049 M L-monosodium glutamate (0.083 g); 1.0% bovine albumin powder (1.000 g); 0.2% EDTA (0.200 g); and distilled water (100 ml). The buffer is sterilised by filtration and should be at approximately pH 6.5.

This suspension is sonicated and then filtered through a 0.45 µm membrane filter for inoculation on to 24-hour-old drained chicken kidney cell monolayers. After absorption for 40 minutes, the medium is added, and cultures are incubated as above for 7–10 days.

Using these methods, MDV of serotypes 1 and 2 may be isolated, together with the HVT (serotype 3), if it is present as a result of vaccination. With experience, plaques caused by the different virus serotypes can be differentiated fairly accurately on the basis of time of appearance, rate of development, and plaque morphology. HVT plaques appear earlier and are larger than serotype 1 plaques, whereas serotype 2 plaques appear later and are smaller than serotype 1 plaques.

MDV and HVT plaques may be identified as such using specific fluorescent antibodies raised in chickens. Monoclonal antibodies may be used to differentiate serotypes (17).

- **Polymerase chain reaction**

    Genomes of all three serotypes have been sequenced (2, 18). Polymerase chain reaction (PCR) tests have been developed for the diagnosis MD; and a real time RT-PCR has been described (1, 5, 16). In addition differentiation of some oncogenic and non-oncogenic strains of serotype 1 MDV, and of MDV vaccine strains of serotypes 2 and 3 (6, 7, 15, 27, 34) have been described. PCR may also be used to quantitate virus load in tissues (5, 7, 8, 24) or differentially detect MDV and HVT in the blood or feather tips (5, 13).

2. Serological tests

The presence of antibodies to MDV in non-vaccinated chickens from about 4 weeks of age is an indication of infection. Before that age, such antibodies may represent maternal transmission of antibody via the yolk and are not evidence of active infection.

Viruses, antigens and antisera are usually available from OIE Reference Laboratories for Marek's Disease (see Table in Part 3 of this *Terrestrial Manual*), but international standard reagents have not yet been produced.

a) Agar gel immunodiffusion

There is no prescribed test for trade, but the agar gel immunodiffusion (AGID) test is employed most commonly to detect antibody. The test is conducted using glass slides coated with 1% agar in phosphate buffered saline containing 8% sodium chloride. Adjacent wells are filled with antigen or serum and these are incubated in a humid atmosphere at 37°C for 24 hours for diffusion to take place; positive sera show reactions of identity with known positive serum and antigen. The antigen used in this test is either disrupted MDV-infected tissue culture cells or an extract of feather tips, or skin containing feather tracts obtained from MDV-infected chickens. The cell culture antigen is prepared by propagating MDV in chicken kidney cells or chicken embryo fibroblast cells. When cytopathic effect is confluent, the cells are detached from the culture vessel and suspended in culture medium or phosphate buffered saline without tryptose phosphate broth (presence of tryptose phosphate broth may produce non-specific precipitin lines) at a concentration of about 1×10^7 cells/ml. This suspension is then freeze–thawed three times and used as antigen.

- **Test procedure**

i) Make a 1% solution of Difco Bactoagar in 8% sodium chloride by standing the mixture in a boiling water bath.

ii) Either a microscope slide or a Petri dish can be used and the agar is poured to a thickness of 2–3 mm.

iii) Cut holes in the agar using a template with a centre well and 6 wells spaced at equal distance around the centre well. The diameter of wells should be approximately 5.3 mm, and the wells should be about 2.4 mm apart. A template with cutters is commercially available.

iv) The antigen is placed in the centre well and the standard antiserum is placed in alternate exterior wells. Serum samples to be tested are placed in the remaining three wells so that .a continuous line of identity is formed between an unknown sample that is positive and the known positive control sera.

v) Incubate the slide for 24 hours at 37°C in a humid container and read the results over a lamp in a darkened room.

A variation of the AGID test may be used to detect MDV antigen in feather tips as an indication of infection by MDV. Glass slides are prepared with a coating of 0.7% agarose (e.g. A37) in 8% sodium chloride, containing MDV antiserum. Tips of small feathers are taken from the birds to be examined and are inserted vertically into the agar, and the slides are maintained as described above. The development of radial zones of precipitation around the feather tips denotes the presence in the feather of MDV antigen and hence of infection in the bird.

b) Other tests

Other tests for MDV antibody include the direct and indirect fluorescent antibody test. These demonstrate the ability of a test serum to stain MDV plaques in cell cultures (28, 29). These tests are group specific and more sensitive than the AGID test. A virus neutralisation test for the ability of a serum to neutralise the plaque-forming property of cell-free MDV can also be employed. However, this test is more suitable for research purposes than for routine diagnostic use. Enzyme-linked immunosorbent assays (ELISA) for detecting MDV antibodies are available (10, 25, 35). To prepare antigen for the ELISA, wells of a 96-well microtitre plate are coated with MDV-infected cells.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Control of MD is essentially achieved by the widespread use of live attenuated vaccines (21). Commercial biological products used in the control of MD are the cell-associated or cell-free (lyophilised) live virus or HVT, respectively (see below). Although genetically engineered recombinant vaccines have been developed (23), they are currently not in commercial use. Marek's disease vaccines are injected *in ovo* at the 17th or 18th day of embryonation (26) or subcutaneously at hatch. The requirements for producing vaccines are outlined below, and in Chapter 1.1.8 Principles of veterinary vaccine production, but other sources should be consulted for further information on the procedures (11, 12, 19, 20, 22, 30). Protocols are given in the British Pharmacopoeia Monograph 589, and the US Code of Federal Regulations, Volume 9, part 113 (31). The guidelines in this *Terrestrial Manual* are intended to be general in nature and maybe supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Viruses of the MDV group are classified under three serotypes – 1, 2, and 3 – on the basis of their antigenic relatedness.

Serotype 1: This includes all the pathogenic strains of the virus, ranging from strains that are very virulent plus (e.g. 648A), very virulent (e.g. Md/5, Md/11, Ala-8, RB-1B), virulent (e.g. HPRS-16, JM GA), mildly virulent (e.g. HPRS-B14, Conn A) and finally to weakly virulent (e.g. CU-2, CVI-988). These strains may be attenuated by passage in tissue culture, with loss of pathogenic properties but retention of immunogenicity, to provide strains that have been used as vaccines. Those that have been used commercially include attenuated HPRS-16 and CVI-988 (Rispens) strains. Attenuated variants of the very virulent stains have been used in experimental vaccines to protect against the variant form of acute MD caused by the very virulent stains. Md11/75C/R2/23 is one such strain (32) licensed for use in the United States of America. Serotype 1 vaccines are prepared in a cell-associated ('wet') form that must be stored in liquid nitrogen.

Serotype 2: This includes naturally avirulent strains of MDV (e.g. SB-1, HPRS-24, 301B/1, HN-1), and several of these have been shown to provide protection against virulent strains. The SB-1 and 301B/1 strains have been developed commercially and used, particularly with HVT, in bivalent vaccines for protection against the very virulent strains. Serotype 2 vaccines exist only in the cell-associated form.

Serotype 3: This contains the strains of naturally avirulent HVT (e.g. FC126, PB1), which are widely used as a monovalent vaccine, and also in combination with serotype 1 and 2 strains in bivalent or trivalent vaccines against the very virulent strains of MDV. HVT may be prepared in a cell-free form as a freeze-dried (lyophilised) vaccine or in a cell-associated ('wet') form.

b)   Method of culture

The substrates used for commercial vaccine production are primary chicken embryo fibroblasts (CEF) derived from specific pathogen free (SPF) flocks or duck embryo fibroblasts. CEF from SPF flocks are preferred to duck cells because more is known about chicken-embryo-transmitted pathogens and methods for their detection.

c)   Validation as a vaccine

Methods for testing SPF flocks for freedom from infection are available (20, 30). SPF chicken flocks should be free from avian adenoviruses, including egg-drop syndrome 76 virus, avian encephalomyelitis virus, avian leukosis virus (subgroups A, B and J), avian nephritis virus, avian reoviruses, avian rotaviruses, chicken anaemia virus, fowl pox virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza type A virus, MDV, *Mycoplasma gallisepticum, Mycoplasma synoviae,* Newcastle disease virus, reticuloendotheliosis virus, *Salmonella* spp., and turkey rhinotracheitis virus.

SPF duck flocks should be free from avian adenoviruses, avian reoviruses, *Chlamydia*, duck virus enteritis, duck virus hepatitis types I and II, influenza type A virus, Newcastle disease virus, *Pasteurella* (now *Riemerella*) *anatipestifer,* reticuloendotheliosis virus, and *Salmonella* infections.

Freedom from other infections may also be required as they become recognised.

The master seed virus should be shown to be non-pathogenic for chickens by inoculating ten times the field dose into 1-day-old SPF chickens of a strain susceptible to MD, to ensure that it does not cause gross lesions or significant microscopic lesions of MD by 120 days of age. It should be noted that some vaccine strains of MDV and HVT may produce minor and transient microscopic nerve lesions.

No increase in virulence should occur during six serial passages of the vaccine strain in 1-day-old SPF MD-susceptible chickens. Ten times the field dose of vaccine is inoculated initially and then passaged by inoculation of heparinised blood at 5–7-day intervals, and tests for viraemia are run to check that virus is transferred at each passage. The birds receiving the final passage are kept for 120 days and should be free from MD lesions. However, some strains such as Rispens, may cause some mild MD lesions. The important observation is that the virulence should not change. This is a difficult test because the genetic resistance of the chickens fundamentally affects the apparent virulence of the virus, so does the type of inoculum. After successful completion of laboratory safety tests, the safety of the strain should be confirmed in extensive field trials.

Seed virus must be free from the agents listed for SPF flocks and from other contaminants that may be acquired in the laboratory. A vaccine strain derived from turkeys must also be free from lymphoproliferative disease virus and haemorrhagic enteritis virus.

The ability of the master seed virus – and derived virus at the limit of the passage range used to produce vaccinal virus (usually not more than five tissue culture passages) – to protect against MD must be determined. Standardised protection tests are published. They involve vaccination of MD-susceptible SPF chickens at 1 day of age and challenge with sufficient virulent MDV 8 days later to cause at least a 70% incidence of MD in unvaccinated chickens. Two types of tests are used. In the protection index test, a single field dose (1000 PFU) (plaque-forming units) of vaccine is given and the incidence of MD in vaccinated birds is compared with that in unvaccinated birds. Protective indices should be greater than 80, i.e. vaccinated birds should show at least 80% reduction in the incidence of gross MD, compared with unvaccinated controls.

A PD_{50} (50% protective dose) test is also used, involving the inoculation of five four-fold serial dilutions of vaccine virus selected to provide protection above and below the 50% level, followed by challenge 8 days later to determine the PD_{50} value. The assays are conducted using a standard reference vaccine for comparison. The PD_{50} may be as low as 4 PFU, but higher values can be obtained depending on the vaccine strain, whether cell-free or cell-associated and the presence or absence of maternal antibodies in the test chickens. On the basis of the PD_{50} test, it has been suggested that the minimum vaccine field dose should be the greater of two values: 10^3 PFU or 100 PD_{50}.

Extensive field trials of a new vaccine strain in the presence of field challenge should be conducted, using different breeds of birds of varying MDV maternal antibody status, to ensure efficacy and persistence of immunity. Experience suggests that vaccinal immunity, once acquired, is lifelong.

2. Method of manufacture

Substrate cells are seeded into flat-bottomed vessels for stationary incubation, or into cylindrical vessels for rolled incubation. Media commonly used are Eagle's minimal essential medium, or 199 medium, buffered with sodium bicarbonate and supplemented with 5% calf serum. Incubation is at 38–39°C for 48 hours.

For cell-associated vaccine, cultures are infected with production HVT or MDV seed-virus stock, in cell-associated form, which is usually two passages beyond the master seed stock. Cultures are incubated for 48 hours then the infected cells are harvested by treating the washed cell sheet with an EDTA/trypsin solution to allow the cells to begin to detach. The flasks are then returned to the incubator (38.5°C) to allow complete detachment. The cells are subjected to low-speed centrifugation, and then resuspended in the freezing mixture consisting of cell growth medium containing 7.5–15% dimethylsulphoxide (DMSO), and held at 4°C or dispensed immediately into the final vaccine containers, usually glass ampoules, which are flame sealed and frozen in liquid nitrogen.

Cell-free lyophilised vaccine may be prepared from HVT, but not from MDV strains. For the production of this form of vaccine, HVT-infected cultures are incubated for 72 hours, infected cells are detached from the vessel as described above, or scraped from the walls of the vessel. The cells are suspended in a small volume of growth medium, centrifuged, and resuspended in a buffered stabiliser solution containing 8% sucrose, but free from protein to prevent frothing. The cell suspension is sonicated to release virus, the cell debris is removed, the suspension is diluted with a complete stabiliser – such as SPGA – filled into the final containers, and lyophilised.

The dilution rate for both cell-associated and cell-free vaccines is based on previous experience, as is the number of doses required per container, because the virus content of the harvested material cannot be assayed prior to filling the final containers. The virus content of the finished product can subsequently be added to the label.

3. In-process control

For optimal results in preparing cell-associated vaccine, a slow rate of freezing (1–5°C per minute) and rapid thawing are essential. The infectivity titre of the infected cells, and hence the number of doses per ampoule, are determined after filling the ampoules. Similarly for cell-free vaccine, the virus content of the final suspension, and hence the number of doses per container, is determined after filling.

4. Batch control

a) Identity

Using immunofluorescence assay (IFA) with monospecific serum, checks should be carried out to show that the product is of the same specificity as the seed virus. This is best done using monoclonal antibodies.

b) Safety and sterility

Extensive testing is required of the materials used to produce the vaccine, and of the final product. Substrate cells should come from an SPF flock, in particular, free from vertically transmitted agents. Substances of animal origin used in the preparation of vaccines such as serum, trypsin, and bovine serum albumin, must be free from extraneous agents.

Batches of the final vaccine produced should be tested for freedom from contaminating bacteria, fungi, mycoplasma and the viruses listed for SPF flocks; tests for purity of the diluent should also be conducted. Suitable tests for the detection of extraneous agents at all stages of vaccine production are recommended by several official bodies (20, 22, 30) and in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials.

Ten doses of vaccine or a quantity of diluent equivalent to two doses of vaccine should be inoculated into separate groups of ten 1-day-old SPF chickens. No adverse reactions should occur during a 21-day observation period.

c) Potency

The standard dose of each type of vaccine is 1000 PFU per chicken or egg. Virus content assays are conducted on batches of vaccine to ensure that the correct dose per bird will be achieved.

d) Duration of immunity

A test for duration of immunity is carried out on the seed virus only. Such immunity is apparently lifelong.

e) Stability

Tests for stability are carried out on six representative batches of vaccine to show that titre is maintained during the stated shelf life of the vaccine. These tests should be conducted under the conditions of storage of the vaccine. The lyophilised product should have a shelf life of 12 months when stored at 2–8°C. Manufacturers may double the virus content of the vaccine to compensate for some loss of titre during storage. Appropriate diluting fluids are provided for use with cell-associated and freeze-dried vaccines. The stability of reconstituted vaccine over a 2-hour period should be tested.

f) Preservatives

Preservatives are not included in the vaccine or diluent.

g) Precautions (hazards)

With cell-associated vaccine, care is necessary to avoid injury from ampoules that may explode when they are removed from liquid nitrogen. Eye protection must be worn. During use, reconstituted vaccine must be kept cool and cell-associated vaccine should be agitated to keep cells in suspension.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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NB: There are OIE Reference Laboratories for Marek's disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

NEWCASTLE DISEASE

SUMMARY

Newcastle disease (ND) is caused by specified viruses of the avian paramyxovirus type I (APMV-1) serotype of the genus Avulavirus belonging to the family Paramyxoviridae. There are nine serotypes of avian paramyxoviruses designated APMV-I to APMV-9.

NDV has been shown to be able to infect over 200 species of birds, but the severity of disease produced varies with both host and strain of virus. The less pathogenic strains may induce severe disease when exacerbated by the presence of other organisms or by adverse environmental conditions. The preferred method of diagnosis is virus isolation and subsequent characterisation.

Identification of the agent: *Suspensions in an antibiotic solution prepared from tracheal and cloacal swabs (or faeces) obtained from live birds, or of faeces and pooled organ samples taken from dead birds, are inoculated into the allantoic cavity of 9–11-day-old embryonating fowl eggs. The eggs are incubated at 37°C for 4–7 days. The allantoic fluid of any egg containing dead or dying embryos, as they arise, and all eggs at the end of the incubation period are tested for haemagglutinating activity.*

Any haemagglutinating agents should be tested for specific inhibition with a monospecific antiserum to NDV. NDV (APMV-1) may show some antigenic cross-relationship with some of the other avian paramyxovirus serotypes, particularly APMV-3 and APMV-7.

The pathogenicity of any newly isolated virus can be assessed by determining the intracerebral pathogenicity index. The pathogenicity of isolates can also be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. NDV is subject to official control in most countries and the virus has a high risk of spread from the laboratory; consequently, appropriate laboratory biosafety and biosecurity must be maintained; a risk assessment should be carried out to determine the level needed.

Serological tests: *The haemagglutination inhibition test is used most widely in NDV serology, its usefulness in diagnosis depends on the vaccinal immune status of the birds to be tested and on prevailing disease conditions.*

Requirements for vaccines and diagnostic biologicals: *Live viruses of low virulence (lentogenic) or of moderate virulence (mesogenic) are used for the vaccination of poultry depending on the disease situation. Inactivated vaccines are also used.*

Live vaccines may be administered to poultry by various routes. They are usually produced by harvesting the infective allantoic/amniotic fluids from inoculated embryonating fowl eggs; some are prepared from infective cell cultures. The final product should be derived from the expansion of master and working seeds.

Inactivated vaccines are given intramuscularly or subcutaneously. They are usually produced by the addition of formaldehyde to infective virus preparations, or by treatment with beta-propiolactone. Most inactivated vaccines are prepared for use by emulsification with a mineral or vegetable oil.

If pathogenic forms of NDV are used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

A. INTRODUCTION

Newcastle disease (ND) is caused by specified viruses of the avian paramyxovirus type I (APMV-I) serotype of the genus *Avulavirus* belonging to the subfamily Paramyxovirinae, family Paramyxoviridae. The paramyxoviruses isolated from avian species have been classified by serological testing into nine serotypes designated APMV-1 to APMV-9; ND virus (NDV) has been designated APMV-1 (6).

Since its recognition in 1926, ND is regarded as being endemic in many countries. Prophylactic vaccination is practised in all but a few of the countries that produce poultry on a commercial scale.

One of the most characteristic properties of different strains of NDV has been their great variation in pathogenicity for chickens. Strains of NDV have been grouped into five pathotypes on the basis of the clinical signs seen in infected chickens (15). These are:

1. Viscerotropic velogenic: a highly pathogenic form in which haemorrhagic intestinal lesions are frequently seen;

2. Neurotropic velogenic: a form that presents with high mortality, usually following respiratory and nervous signs;

3. Mesogenic: a form that presents with respiratory signs, occasional nervous signs, but low mortality;

4. Lentogenic or respiratory: a form that presents with mild or subclinical respiratory infection;

5. Asymptomatic enteric: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut (7) and even in infections of specific pathogen free (SPF) birds, considerable overlapping may be seen. In addition, exacerbation of the clinical signs induced by the milder strains may occur when infections by other organisms are superimposed or when adverse environmental conditions are present.

As signs of clinical disease in chickens vary widely and diagnosis may be complicated further by the different responses to infection by different hosts, clinical signs alone do not present a reliable basis for diagnosis of ND. However, the characteristic signs and lesions associated with the virulent pathotypes will give rise to strong suspicion of the disease.

NDV is a human pathogen. Reported infections have been non-life threatening and usually not debilitating for more than a day or two (18). The most frequently reported and best substantiated clinical signs in human infections have been eye infections, usually consisting of unilateral or bilateral reddening, excessive lachrymation, oedema of the eyelids, conjunctivitis and sub-conjunctival haemorrhage. Although the effect on the eye may be quite severe, infections are usually transient and the cornea is not affected. Reports of other clinical symptoms in humans infected with NDV are less well substantiated, but suggest a more generalised infection may sometimes occur resulting in chills, headaches and fever, with or without conjunctivitis. There is evidence that both vaccinal and virulent (for poultry) strains of NDV may infect and cause clinical signs in humans. There is no evidence of human-to-human spread.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

ND, as defined in Section B.1.f of this chapter, is subject to official control in most countries and the virus has a high risk of spread from the laboratory; consequently, a risk assessment should be carried out to determine the level of biosafety and biosecurity needed for the diagnosis and characterisation of the virus. The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

a) Samples for virus isolation

When investigations of ND are the result of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples from dead birds should consist of oro-nasal swabs, as well as samples collected from lung, kidneys, intestine (including contents), spleen, brain, liver and heart tissues. These may be collected separately or as a pool, although intestinal samples are usually processed separately from other samples.

Samples from live birds should include both tracheal and cloacal swabs, the latter should be visibly coated with faecal material. Small delicate birds may be harmed by swabbing, but the collection of fresh faeces may serve as an adequate alternative.

Where opportunities for obtaining samples are limited, it is important that cloacal swabs (or faeces) and tracheal swabs (or tracheal tissue) be examined as well as organs or tissues that are grossly affected or associated with the clinical disease. Samples should be taken in the early stages of the disease.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4, containing antibiotics. Protein-based media, e.g. brain–heart infusion (BHI) or tris-buffered tryptose broth (TBTB), have also been used and may give added stability to the virus, especially during shipping. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml); streptomycin (2 mg/ml); gentamycin (50 µg/ml); and mycostatin (1000 units/ml) for tissues and tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the solution to pH 7.0–7.4 following the addition of the antibiotics. If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days.

b) Virus culture

The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1000 g for about 10 minutes at a temperature not exceeding 25°C are inoculated in 0.2 ml volumes into the allantoic cavity of each of at least five embryonated SPF fowl eggs of 9–11 days' incubation. After inoculation, these are incubated at 35–37°C for 4–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

c) Virus identification

HA activity detected in bacteriologically sterile fluids harvested from inoculated eggs may be due to the presence of any of the 16 haemagglutinin subtypes of influenza A viruses or of the eight other paramyxovirus serotypes. (Nonsterile fluid could contain bacterial HA.) NDV can be confirmed by the use of specific antiserum in a haemagglutination inhibition (HI) test. Usually chicken antiserum that has been prepared against one of the strains of NDV is used.

Cross-reactions in HI tests between NDV and some of the other APMVs, especially APMV-3 and APMV-7 serotype viruses may cause some problems that can be resolved by the use of suitable antigen and antiserum controls.

d) Pathogenicity index

The extreme variation in virulence of different NDV isolates and the widespread use of live vaccines means that the identification of an isolate as NDV from birds showing clinical signs does not confirm a diagnosis of ND, so that an assessment of the virulence of the isolate is also required (see Section B.1.f below 'Definition of Newcastle disease'). In the past such tests as the mean death time in eggs, the intravenous pathogenicity test and variations of these tests have been used (27), but by international agreement, a definitive assessment of virus virulence is based on the intracerebral pathogenicity test. The current OIE definition (Section B.1.f below) also recognises the advances made in understanding the molecular basis of pathogenicity and allows confirmation of virus virulence, but not lack of virulence, by *in-vitro* tests that determine the amino acid sequence at the F0 protein cleavage site.

- **Intracerebral pathogenicity index**

i) Fresh infective allantoic fluid with a HA titre >2^4 (>1/16) is diluted 1/10 in sterile isotonic saline with no additives, such as antibiotics.

ii) 0.05 ml of the diluted virus is injected intracerebrally into each of ten chicks hatched from eggs from an SPF flock. These chicks must be over 24-hours and under 40-hours old at the time of inoculation.

iii) The birds are examined every 24 hours for 8 days.

iv) At each observation, the birds are scored: 0 if normal, 1 if sick, and 2 if dead. (Birds that are alive but unable to eat or drink should be killed humanely and scored as dead at the next observation. Dead individuals must be scored as 2 at each of the remaining daily observations after death.)

v) The intracerebral pathogenicity index (ICPI) is the mean score per bird per observation over the 8-day period.

The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic and asymptomatic enteric strains will give values close to 0.0.

e)   Molecular basis for pathogenicity

During replication, NDV particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious. This post-translation cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all NDV strains.

It would appear that the F0 molecules of viruses virulent for chickens can be cleaved by a host protease or proteases found in a wide range of cells and tissues, and thus spread throughout the host damaging vital organs, but F0 molecules in viruses of low virulence are restricted in their cleavability to certain host proteases resulting in restriction of these viruses to growth only in certain host-cell types.

Most ND viruses that are pathogenic for chickens have the sequence ^{112}R/K-R-Q-K/R-R^{116} at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, the N-terminus of the F1 protein, whereas the viruses of low virulence have sequences in the same region of ^{112}G/E-K/R-Q-G/E-R^{116} and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence ^{112}G-R-Q-K-R-F^{117}, but give high ICPI values. Thus there appears to be the requirement of at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 if the virus is to show virulence for chickens.

Several studies have been done using molecular techniques to determine the F0 cleavage site sequence by reverse-transcription polymerase chain reaction (RT-PCR), either on the isolated virus or on tissues and faeces from infected birds, followed by analysis of the product by restriction enzyme analysis, probe hybridisation or nucleotide sequencing with a view to establishing a routine *in vitro* test for virulence (for a review see ref. 2). Determination of the F0 cleavage sequence may give a clear indication of the virulence of the virus, and this has been incorporated into the definition of ND (see Section B.1.f).

In the diagnosis of ND it is important to understand that the demonstration of the presence of virus with multiple basic amino acids at the F0 cleavage site confirms the presence of virulent or potentially virulent virus, but that failure to detect virus or detection of NDV without multiple basic amino acids at the F0 cleavage site using molecular techniques does not confirm the absence of virulent virus. Primer mismatch, or the possibility of a mixed population of virulent and avirulent viruses means that virus isolation and an *in-vivo* assessment of virulence will still be required.

Analyses of viruses isolated in Ireland in 1990 and during the outbreaks of ND in Australia since 1998 have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence (5, 45). Virulent NDV has also been generated experimentally from low virulence virus by passage in chickens (39).

f)   Definition of Newcastle disease

It seems likely that the vast majority of birds are susceptible to infection with ND viruses of both high and low virulence for chickens, although the clinical signs seen in birds infected with NDV vary widely and are dependent on factors such as: the virus, host species, age of host, infection with other organisms, environmental stress and immune status. In some circumstances infection with the extremely virulent viruses may result in sudden high mortality with comparatively few clinical signs. Thus the clinical signs are variable and influenced by other factors so that none can be regarded as pathognomonic.

Even for susceptible hosts, such as chickens, ND viruses show a considerable range of virulence. Generally, variation consists of clusters around the two extremes in the ICPI test, but, for a variety of reasons, some viruses may show intermediate virulence.

The enormous variation in virulence and clinical signs means it is necessary to define carefully what constitutes ND for the purposes of trade, control measures and policies. The definition of ND currently in use in all member states of the European Union is defined in Directive 92/66/EEC (20).

The OIE definition for reporting an outbreak of ND is:

'Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

   a)   The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater.

   or

b) *Multiple basic amino acids have been demonstrated in the virus* (*either directly or by deduction*) *at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.'*

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues –4 to –1 from the cleavage site.'

g) Monoclonal antibodies

Mouse monoclonal antibodies (MAbs) directed against strains of NDV have been used in HI tests to allow rapid identification of NDV without the possible cross-reactions with other APMV serotypes that may occur with polyclonal sera. MAbs have been produced that give reactions in HI tests that are specific for particular strains or variant NDV isolates (6, 10).

Panels of MAbs have been used to establish antigenic profiles of NDV isolates based on whether or not they react with the viruses. This has proven to be a valuable method for grouping and differentiating isolates of NDV, and has been particularly valuable to the understanding of the epidemiology of outbreaks (10).

h) Phylogenetic studies

Development of improved techniques for nucleotide sequencing, the availability of sequence data of more ND viruses in computer databases and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses have led to a considerable increase is such studies in recent years. Considerable genetic diversity has been detected, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific lineages or clades and this has proven valuable in assessing both the global epidemiology and local spread of ND (4, 8, 19, 28, 32, 33, 38, 41, 43, 44).

Although in the past phylogenetic studies have been impracticable as a routine tool, the greater availability and increased speed of production of results obtained using sophisticated, commercially available kits for RT-PCR and automatic sequencers now means such studies are within the capabilities of many more diagnostic laboratories and can give meaningful results that are contemporaneous rather that retrospective (2). Aldous *et al.* (4) proposed that genotyping of NDV isolates should become part of diagnostic virus characterisation for reference laboratories by producing a 375-nucleotide sequence of the F gene, which includes the F0 cleavage site, routinely for all viruses and comparing the sequences obtained with other recent isolates and 18 viruses representative of the recognised lineages and sub-lineages. Such analysis should allow rapid epidemiological assessment of the origins and spread of the viruses responsible for ND outbreaks.

i) Molecular techniques in diagnosis

In addition to the use of RT-PCR and other similar techniques for the determination of the virulence of ND viruses (see Section B.1.e) or for phylogenetic studies (see Section B.1.h), there has been increasing use of such molecular techniques to detect NDV in clinical specimens, the advantage being the extremely rapid demonstration of the presence of virus.

Care should be taken in the selection of clinical samples as some studies have demonstrated lack of sensitivity in detecting virus in some organs and particularly in faeces (23, 25, 30). Tracheal or oropharyngeal swabs are often used as the specimens of choice because they are easy to process and usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. However, tissue and organ samples and even faeces have been used with some success. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens and even with commercial kits care should be taken in selecting the most appropriate or validated for the samples to be analysed.

Usually RT-PCR systems have been used to amplify a specific portion of the genome that will give added value; for example by amplifying part of the F gene that contains the F0 cleavage site so that the product can be used for assessing virulence (3, 14, 23, 25, 29, 37, 38). Perhaps the most serious problem with the use of RT-PCR in diagnosis is the necessity for post-amplification processing because of the high potential for contamination of the laboratory and cross contamination of samples. Extreme precautions and strict regimens for handling samples are necessary to prevent this (see Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases).

One of the strategies used to avoid post-amplification processing is to employ real-time RT-PCR (rRT-PCR) techniques. The advantages of such assays are that rRT-PCR assays based on the fluorogenic hydrolysis probes or fluorescent dyes eliminate the post-amplification processing step and that results can be obtained in less than 3 hours. The most successful application of an rRT-PCR assay was in the USA during the ND outbreaks of 2002–2003, when the assay described by Wise *et al.* (46) was employed and showed a sensitivity of 95% when compared with virus isolation for more than 1400 specimens. The assay has three sets of primers and probes that are used in separate reactions: a matrix primer/probe set that is designed to detect most strains of NDV, a fusion primer/probe set that can identify virulent strains of NDV (including many PPMV-1 viruses) and a primer/probe set designed to detect low virulent strains of the virus. Samples are first screened with the matrix primers/probe then positive specimens are tested with the low virulent and fusion and primers/probe sets to confirm presence of low or highly virulent virus, respectively. The primers and probes in this report were validated on lentogenic, mesogenic and velogenic strains circulating in the United States of America. At the peak of the outbreak, between 1000 and 1500 samples were tested daily by rRT-PCR. A disadvantage of rRT-PCR is that, at present, the special thermocyclers required are extremely expensive and this would deter many laboratories from employing this system.

One further important problem is that while the vast majority of NDV isolates are genetically quite close, some have been shown to be genetically distinct. For example, one group of viruses, which were placed in genogroup 6 by Aldous *et al.* (4) and subsequently Class I by Czegledi *et al.* (24), are so different from all the other NDV isolates, i.e. Class II viruses (24) that different primers would be necessary for their detection in RT-PCR tests.

As with virulence determination, it is important that PCR techniques alone are not used to record a negative result in investigations of suspected ND.

2. Serological tests

NDV may be employed as an antigen in a wide range of serological tests, enabling neutralisation or enzyme-linked immunosorbent assays (ELISA) and HI to be used for assessing antibody levels in birds. At present, the HI test is most widely used for detecting antibodies to NDV in birds, although many poultry producers are using commercial ELISA kits to assess post-vaccination antibody levels.

a) Haemagglutination and haemagglutination inhibition tests

Chicken sera rarely give nonspecific positive reactions in the HI test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken red blood cells (RBCs), so this property should first be determined and then removed by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, shaking gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 ***g*** for 2–5 minutes and the adsorbed sera are decanted.

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply in the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.2, and RBC taken from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution. (If SPF chickens are not available, blood may be taken from unvaccinated birds monitored regularly and shown to be free from antibodies to NDV.) Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

- **Haemagglutination test**

i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.

ii) 0.025 ml of the virus suspension (i.e. infective or inactivated allantoic fluid) is placed in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/5, 1/7, etc.

iii) Twofold dilutions of 0.025 ml volumes of the virus suspension are made across the plate.

iv) A further 0.025 ml of PBS is dispensed to each well.

v) 0.025 ml of 1% (v/v) chicken RBCs is dispensed to each well.

vi) The solution is mixed by tapping the plate gently. The RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination inhibition test**

i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.

ii) 0.025 ml of serum is placed into the first well of the plate.

iii) Twofold dilutions of 0.025 ml volumes of the serum are made across the plate.

iv) 4 HAU virus/antigen in 0.025 ml is added to each well and the plate is left for a minimum of 30 minutes at room temperature, i.e. about 20°C, or 60 minutes at 4°C.

v) 0.025 ml of 1% (v/v) chicken RBCs is added to each well and, after gentle mixing, the RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for about 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre >1/4 (>2^2 or >$\log_2 2$ when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

The value of serology in diagnosis is clearly related to the expected immune status of the affected birds. HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2 4$ when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or $\log_2 3$) or more. Back titration of antigen should be included in all tests to verify the number of HAU used.

HI titres may be used to assess the immune status of a flock. In vaccinated flocks that are being monitored serologically, it may be possible to identify anamnestic responses as the result of a challenge infection with field virus (13), but great care should be exercised as variations may occur from other causes. For example, it has been demonstrated that APMV-3 virus infections of ND-virus-vaccinated turkeys will result in substantially increased titres to NDV (11).

b) **Enzyme-linked immunosorbent assay**

There are a variety of commercial ELISA kits available and these are based on several different strategies for the detection of NDV antibodies, including indirect, sandwich and blocking or competitive ELISAs using MAbs. At least one kit uses a subunit antigen. Usually such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The HI test and ELISA may measure antibodies to different antigens; depending on the system used ELISAs may detect antibodies to more than one antigen while the HI test is probably restricted to those directed against the HN protein. However, comparative studies have demonstrated that the ELISAs are reproducible and have high sensitivity and specificity; they have been found to correlate well with the HI test (1). Conventional ELISAs have the disadvantage that it is necessary to validate the test for each species of bird for which they are used. Competitive ELISAs usually employ MAbs which, because of their specificity for single epitopes, may not recognise all strains of APMV-1.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A detailed account of all aspects of NDV vaccines, including their production and use, has been published (13) and should be referred to for details of the procedures outlined here. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

In this section, conventional live and inactivated vaccines will be considered, as these are still used universally. However, it should be remembered that there has been much recent work on the application of molecular biology techniques to the production of new vaccines, and success has been reported in obtaining protective immunity with recombinant fowlpox virus, vaccinia virus, pigeonpox virus, turkey herpesvirus and avian cells in which the HN gene, the F gene, or both, of NDV are expressed. Several of these recombinant viruses have been licensed for use in certain countries.

NDV strains used in conventional commercial live virus vaccines fall into two groups: lentogenic vaccines, such as Hitchner-B$_1$, La Sota, V4, NDW, I2 and F, and mesogenic vaccines, such as Roakin, Mukteswar and Komarov. Strains from both these groups have been subjected to selection and cloning to fulfil different criteria in their production and application. The mesogenic vaccine viruses all have two pairs of basic amino acids at the F0 cleavage site and ICPI values of around 1.4. This means that infections of birds with these viruses would fall within the intended definition of ND (Section B.1.f), but as these vaccines are used primarily in countries where ND is endemic this may not necessarily preclude their use. Some countries have specified that only lentogenic NDV strains can be used as vaccines (42).

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If ND, as defined in Section B.1.f of this chapter, is used for vaccine production or for vaccine–challenge studies, that part of the facility where this work is done should meet the requirements for Containment Group 4 pathogens as outlined in chapter 1.1.2 of this *Terrestrial Manual.*

Most live virus vaccines are grown in the allantoic cavity of embryonated fowl eggs but some, notably some mesogenic strains, have been adapted to a variety of tissue culture systems.

Live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray, or by intranasal or conjunctival instillation. A lentogenic vaccine for use *in ovo* has been licensed for use in the United States of America. Some mesogenic strains are given by wing-web intradermal inoculation. Vaccines have been constructed to give optimum results through application by specific routes. In general, the more immunogenic live vaccines are more virulent, and are therefore more likely to cause adverse side-effects. For example, vaccination with the La Sota strain will cause considerably greater problems in young susceptible birds than the Hitchner-B$_1$ strain, although La Sota induces a stronger immune response.

Inactivated vaccines are considerably more expensive than live vaccines, and their use entails handling and injecting individual birds. They are prepared from allantoic fluid that has had its infectivity inactivated by the addition of formaldehyde or beta-propiolactone. This is incorporated into an emulsion with mineral oil, and is administered intramuscularly or subcutaneously. Individual birds thus receive a standard dose. There is no subsequent spread of virus or adverse respiratory reactions. Both virulent and avirulent strains are used as seed virus although, from the aspect of safety control, the use of the latter appears more suitable. As no virus multiplication takes place after administration, a much larger amount of antigen is required for immunisation than for live virus vaccination. A high yield of virus to produce a potent vaccine is important, and the Ulster 2C strain is very suitable for this purpose.

The duration of immunity depends on the vaccination programme chosen. One of the most important considerations affecting vaccination programmes is the level of maternal immunity in young chickens, which may vary considerably from farm to farm, batch to batch, and among individual chickens. For this reason, one of several strategies is employed. Either the birds are not vaccinated until 2–4 weeks of age when most of them will be susceptible, or 1-day-old birds are vaccinated by conjunctival instillation or by the application of a coarse spray. This will establish active infection in some birds that will persist until maternal immunity has waned. Revaccination is then carried out 3–4 weeks later. It has been demonstrated that inactivated vaccines may also be usefully employed to vaccinate 1-day-old chicks that have a degree of maternal immunity (17), and the best results of all were obtained when 1-day-old maternally immune chicks were given a combination of live and inactivated vaccines, compared with live or inactivated vaccines given alone (16). Vaccination of fully susceptible 1-day-old birds, even with the most mild of live vaccines, may result in respiratory disease, especially if common pathogenic bacteria are present in significant numbers.

Vaccination after 3 weeks of age is normally practised only in breeding hens and hens laying table eggs. This should be done at sufficiently frequent intervals to maintain an adequate immunity. Vaccination programmes often employ slightly more pathogenic live virus vaccines to boost immunity than those used initially. These more pathogenic live vaccines may also be used following initial vaccination with oil emulsion inactivated vaccines.

When devising a vaccination programme, consideration should be given to the type of vaccine used, the immune and disease status of the birds to be vaccinated, and the level of protection required in relation to any possibility of infection with field virus under local conditions (13). Two examples of vaccination programmes that may be used in different disease circumstances are listed here. For the first example, when the disease is mild and sporadic, it is suggested that the following order of vaccination be adopted: live Hitchner-B$_1$ by conjunctival or spray administration at 1 day of age; live Hitchner-B$_1$ or La Sota at 18–21 days of age in the drinking water; live La Sota in the drinking water at 10 weeks of age, and an inactivated oil emulsion vaccine at point of lay. For the second example, when the disease is severe and more widespread, the same protocol as above is adopted up to 21 days of age, and this is followed by revaccination at 35–42 days of age with live La Sota in the drinking water or as an aerosol; this revaccination is repeated at 10 weeks of age with an inactivated vaccine (or a mesogenic live vaccine) and again repeated at point of lay (13).

1. Seed management

a) Characteristics of the seed

The first principle to consider when selecting a strain for a live NDV vaccine is whether it is to be used as a primary or a secondary vaccine, the main consideration being its pathogenicity. The methods of application and frequency of use are valid considerations. The use of MAbs has demonstrated considerable variation in the antigenicity of different strains (10). This may indicate a need to tailor vaccines more carefully to relate antigenically to any prevalent field virus.

A live vaccine based on NDV strain V4, selected for heat stability, has been introduced to combat the specific problems associated with village chicken rearing in developing countries. The intention is that this vaccine could be coated on food fed to scavenging chickens. To date, trials in different countries have produced mixed results; it may well be that local factors are extremely important in affecting the success of this strategy (40). More recently the thermostable I2 vaccine has been developed specifically for vaccinating village chickens; it is currently recommended that this vaccine be given by eye drop (9).

Use of live vaccines may be restricted by legislation. For example, Commission Decision 93/152/EEC (21) restricts the use of vaccines in member states of the European Union from 1 January 1995 to those for which the master seed has been tested and shown to have an ICPI of <0.4 if no fewer than 10^7 mean egg infectious doses (EID_{50}) are administered to each bird, or <0.5 if no fewer than 10^8 EID_{50} are administered to each bird. The OIE Standards Commission has similarly recommended that while in principle vaccines should have an ICPI < 0.7, in order to account for interassay and interlaboratory variability a safety margin should be allowed so that vaccine master seed virus strains should not have an ICPI exceeding 0.4 (35).

The most important consideration in selecting a seed for the preparation of inactivated vaccine is the amount of antigen produced when grown in embryonated eggs; it is rarely cost-effective to concentrate virus. Both virulent and lentogenic strains have been used as inactivated vaccines, but the former offer an unnecessary risk because the manipulation of large quantities of virulent virus is involved, as well as the dangers of inadequate inactivation and possible subsequent contamination. This risk is reflected in Commission Decision 93/152/EEC (21), which restricts the use of viruses used for inactivated vaccine in member states of the European Union from 1 January 1995 to those for which the master seed has been tested and shown to have an ICPI of <0.7 if no fewer than 10^8 EID_{50} are administered to each bird. Some lentogenic strains grow to very high titres in eggs. Exceptionally high titres can be obtained by the Ulster 2C strain, which has been recommended as a seed for inactivated vaccine (26). However, successful commercial inactivated vaccines are produced when the Hitchner B_1, La Sota or F strains are used as seeds.

In view of the finding that virulent NDV can emerge by mutation from virus of low virulence (5, 39, 45), the introduction of wholly new strains of ND in live vaccines should be considered carefully and the vaccines subjected to evaluation before use.

b) Method of culture

A master seed is established, and from this a working seed. If the strain has been cloned by limiting dilution or plaque selection, the establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml).

c) Validation as a vaccine

Seed viruses of unknown pedigree should be passaged through SPF eggs and cloned before producing the master seed. Some passage through SPF chickens may also be desirable (13). In either case, the master seed should be checked after preparation for sterility, safety, potency and extraneous agents. Some countries also require back passage studies for live NDV vaccine to ensure that the pathogenicity is not increased by cycling through birds (42).

2. Method of manufacture

For vaccine production, a working seed, from which batches of vaccine are produced, is first established by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at –60°C or lower as lyophilised virus does not always multiply to high titre on subsequent first passage (13).

Most ND vaccines are produced in embryonated fowl eggs, and live virus vaccines should be produced in SPF eggs. The method of production is large-scale aseptic propagation of the virus; all procedures are performed under sterile conditions.

It is usual to dilute the working seed in sterile PBS, pH 7.2, so that about 10^3–10^4 EID_{50}/0.1 ml is inoculated into the allantoic cavity of 9- or 10-day-old embryonated SPF fowl eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids aspirated after depression of the embryo. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination before large pools are made for lyophilisation or inactivation. Live vaccines are usually lyophilised. The methodology depends on the machinery used and the expertise of the manufacturers, but this is a very important step as inadequate lyophilisation results in both loss of titre and a reduced shelf life.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000) or beta-propiolactone (a typical final concentration is 1/2000–1/4000). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are not concentrated; the inactivated allantoic fluid is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

Generally oil-based inactivated vaccines are prepared as primary emulsions of water-in-oil. The oil phase usually consists of nine volumes of highly refined mineral oil, such as Marcol 52, Drakeol 6VR and BayolF, plus one volume of emulsifying agent, such as Arlacel A, Montanide 80 and Montanide 888 (36). The aqueous phase is the inactivated virus to which a non-ionic emulsifier such as Tween 80 has been added. The oil phase to aqueous phase ratio is usually 1:1 to 1:4. Manufacturers strive to reach a balance between adjuvant effect, viscosity and stability. Too high viscosity and the vaccine is difficult to inject; too low viscosity and the vaccine is unstable.

3. In-process control

Each batch of live virus vaccine should be tested for viability and potency. For those produced in eggs, the most important process control is testing for bacterial and fungal contamination. This is necessary because of the occasional occurrence of putrefying eggs, which may remain undetected at the time of harvest.

For inactivated vaccines, the efficacy of the process of inactivation should be tested in embryonated eggs, taking 25 aliquots (0.2 ml) from each batch and passaging each three times through SPF embryos (13).

4. Batch control

Most countries have published specifications for the control of production and testing of NDV vaccines (e.g. ref. 34), which include the definition of the obligatory tests on vaccines during and after manufacture.

It is necessary to test the infectivity of live virus vaccines to enable adequate levels of virus to be administered. The virus is usually titrated in embryonated fowl eggs to give the EID_{50}. This involves making tenfold dilutions of virus; 0.1 ml of each dilution is inoculated into between five and seven 9–10-day-old embryonated fowl eggs. After 5–7 days' incubation at 37°C, the eggs are chilled and tested for the presence of haemagglutinin activity, which is an indication of the presence of live virus. The EID_{50} end-point is calculated using a standard formula such as Spearman–Kärber (12).

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

The use of chickens for the testing of vaccines involves the inoculation of ten or more birds of stated age that originate from an SPF flock. Ten doses of live vaccine are administered supraconjunctivally to each bird and the birds are then observed for 21 days. No chicken should show serious clinical signs and none should die from causes attributable to the vaccine (22). An alternative is to use the prechallenge part of the potency test below as a safety test and if unfavourable reactions that are attributable to the product occur, the test is declared inconclusive and the safety test is repeated. If not repeated satisfactorily, the batch is declared unsatisfactory (42).

For inactivated vaccines, a double dose is administered by the recommended route to ten 3-week-old birds, and these are observed for 2 weeks for the absence of clinical signs of disease or local lesions.

c) Potency

Various methods for the testing of NDV vaccines for potency have been proposed. The importance of using a suitable challenge strain for assessment has been stressed (13). Suitable challenge strains are Herts 33 or GB Texas. For live vaccines, the method recommended involves the vaccination of 10 or more SPF or other fully susceptible birds, some countries specify 20 birds (22), at the minimum recommended age by the suggested route using the minimum recommended dose. After 14–21 days, each vaccinated bird and ten control birds are challenged intramuscularly with 10^5 LD_{50} (50% lethal dose) of ND challenge virus The vaccine passes the test if at the end of 10 days, 90% of the vaccinated chickens survive with no signs of disease, but all controls die within 6 days.

For inactivated vaccines, 21–28-day-old SPF or susceptible chickens are used. Three groups of 20 birds are injected intramuscularly with volumes of vaccine equivalent to 1/25, 1/50 and 1/100 of a dose. A group of ten chickens is kept as controls. All the birds are challenged by intramuscular injection of 10^6 LD_{50} of ND challenge virus, 17–21 days later. Chickens are observed for 21 days. The PD_{50} (50% protective dose) is calculated by standard statistical methods. The test is only valid if challenged control birds all die within 6 days. The vaccine complies with the test if the PD_{50} is not less than 50 per dose and if the lower confidence limit is not less than 35 PD_{50} per dose. Some control authorities accept a test at 1/50 only, for animal welfare reasons.

It is not necessary to repeat the potency test on each batch if it has been shown that a representative batch of the final product from the master seed has passed the test.

d) Duration of immunity

The level of immunity reached with any single dose or regimen of ND vaccination will vary enormously with both vaccine and host species. The level of immunity required in a given host (i.e. to protect against death, disease, meat or egg production losses) is extremely complex and difficult to evaluate. Generally some assessment of the longevity of serum antibodies should be made and vaccine regimens adopted to maintain these above an acceptable level (13).

e) Stability

When stored under the recommended conditions the final vaccine product should maintain its potency for at least 1 year. Accelerated stability tests such as reduction of infectivity following incubation at 37°C for 7 days (31) may be used as a guide to the storage capabilities of a batch of live vaccine. Oil emulsion vaccines should also be subjected to accelerated ageing by storing at 37°C, for a minimum of 1 month, without separation of the aqueous and oil phases. The US requires real-time stability to be checked on the first few batches of NDV vaccine. Usually three samples are checked for killed vaccine and 10 for live vaccine. Live virus vaccines must be used immediately after reconstitution. Inactivated vaccines must not be frozen.

f) Preservatives

In most countries, preservatives must not be included in the freeze-dried live product, but antimicrobial preservatives may be incorporated in the diluent used to reconstitute the vaccine. An alternative used in the US is to allow the use of certain preservatives, but they must be indicated on the labelling.

g) Precautions (hazards)

Live NDV vaccines may represent a hazard to humans. ND viruses, both virulent and of low virulence for chickens have been reported to have infected humans, usually causing acute conjunctivitis following direct introduction to the eye. Infections are usually transient and the cornea is not involved.

Mineral oil emulsion vaccines represent a serious hazard to the vaccinator. Accidental injection of humans should be treated promptly by incision and washing of the site, as for a 'grease-gun' injury.

5. Tests on the final product

a) Safety

See Section C.4.b above.

b) Potency

See Section C.4.c above.

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NB: There are OIE Reference Laboratories for Newcastle disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

TURKEY RHINOTRACHEITIS
(avian metapneumovirus)

SUMMARY

Avian metapneumovirus (aMPV) causes an acute highly contagious upper respiratory tract infection primarily of turkeys and chickens. The disease produced by aMPV was originally referred to as avian pneumovirus infection and avian rhinotracheitis; it also has been referred to as turkey rhinotracheitis in turkeys and swollen head syndrome in chickens. Avian metapneumoviruses are single-stranded non-segmented negative-sense RNA viruses belonging to the family Paramyxoviridae, genus Metapneumovirus. *The disease can cause significant economic losses in turkey and chicken flocks, particularly when exacerbated by secondary pathogens. The only avian species known to support the replication of aMPVs, other than turkeys and chickens, are pheasants, Muscovy ducks and guinea fowl. The disease has global distribution in poultry-producing regions with only Oceania and Canada reported to be free of aMPV infection. Four antigentically distinct subtypes, A, B, C and D, of aMPV have been identified by neutralisation with monoclonal antibodies and sequence analysis of the attachment protein, G.*

In susceptible turkeys, infection of the respiratory tract can occur at any age although young poults appear to be more severely affected. The severity of disease and mortality rate is largely influenced by secondary bacterial infection. Infection with aMPV can result in serious egg production problems in turkey breeding flocks. Clinical signs include sneezing, tracheal rales, nasal and ocular discharge and swollen infraorbital sinuses and conjunctivitis. The onset of clinical signs and spread of infection through a flock can be rapid occurring as quickly as 2–4 hours. In chickens, the disease is characterised by apathy and swelling of the face and infraorbital sinuses, leading to mild respiratory signs, egg production problems and swollen head syndrome (SHS). Cerebral disorientation, torticollis and opisthotonus frequently follow. In broilers, aMPV is not a primary pathogen, but is involved with other agents in SHS or other respiratory disease complexes.

Identification of the agent: *Virus isolation in cell cultures, embryonated eggs, and tracheal organ cultures as well as molecular methods for identification of the nucleic acid have all been used successfully to detect aMPV, but the degree of success depends on the strain of virus, type and timeliness of sample collection, as well as storage and handling of specimens. Electron microscopy, virus neutralisation and molecular techniques are widely used to identify the virus. Virus detection and identification can be difficult unless samples are taken early in the course of the disease. Both attenuated and virulent strains of aMPV replicate to the highest titre in the upper respiratory tract tissues of young turkeys, but only for approximately 10 days.*

Monoclonal antibodies to the G glycoprotein have been used in virus neutralisation tests to differentiate subtypes A and B, while neutralisation tests using polyclonal antiserum have shown that subtypes A and B belong to a single serotype. Subtype C is neutralised poorly by subtype A or B monospecific antiserum, and not by monoclonal antibodies that differentiate subtype A and B. These data suggest that subtype C represents a second serotype of aMPV. Monospecific antiserum and monoclonal antibodies can be used for agent identification by virus neutralisation and immunofluorescence staining of infected cell cultures; however antigenic characteristics need to be considered. The immunodiffusion test has also been used to confirm aMPV isolates.

Molecular procedures based on the F, G, M and N genes of aMPV have been used for the detection and or genomic subtyping of aMPV. Proteins coded by the F and G genes are major immunogens and, as such, present nucleotide variability. Nucleotide sequence analysis of the G gene has been used to confirm previous virus neutralisation studies differentiating subtypes A and

B. Conventional reverse-transcriptase polymerase chain reaction (RT-PCR) procedures can also be used for aMPV A and B genomic subtyping. Moreover, nucleotide sequence analysis of the gene encoding the matrix protein (M), further supports the subtype A and B division. Sequence analysis of the M gene of subtype C aMPV shows the United States of America isolate to be distinct from subtype A and B viruses. RT-PCR assays directed to the F and M genes are subtype specific and useful for detection of aMPV when subtype identity of the virus is known. However, a single set of RT-PCR primers directed to the N gene have been shown to detect subtypes A, B and could possibly be used as universal primers for the detection of aMPV.

Serologic tests: Due to difficulties in isolating and identifying aMPV, confirmation of infection is often achieved by serological methods, particularly in unvaccinated flocks. The most commonly employed method is the enzyme-linked immunosorbent assay (ELISA). Other methods that have been used are virus neutralisation (VN), immunofluorescence and immunodiffusion tests. The VN test can be performed in tracheal organ cultures, chicken embryo fibroblast (CEF), chicken embryo liver (CEL) or Vero cell cultures. However, the VN and ELISA show similar sensitivity and the ELISA is the most commonly used assay. Numerous commercial ELISA kits as well as in-house assays have been developed. Differences in sensitivity and specificity between tests have been reported to occur between commercial kits. A homologous strain of aMPV should be used as antigen due to variations in antigenicity. In many countries where the disease is endemic, vaccination is practised complicating interpretation of the results. Ideally, serum samples from animals in the acute phase of disease and from convalescent animals should be obtained for testing. In chickens the serological response to aMPV infection is weak when compared with the response in turkeys.

Requirements for vaccines and diagnostic biologicals: This section is under study, it is anticipated that information on vaccines will be added to the online version of the Terrestrial Manual *from June 2008, subject to approval by the OIE International Committee.*

A. INTRODUCTION

Avian metapneumovirus (aMPV) previously referred to as avian pneumovirus (APV) and avian rhinotracheitis (ART) virus causes an acute, highly contagious upper respiratory tract infection of turkeys and chickens. In turkeys, the virus causes a disease known as turkey rhinotracheitis (TRT). The aetiological agent is an unsegmented single-stranded negative-sense RNA virus of approximately 15 kilo bases with helical symmetry (17). The virus is well characterised as a pneumovirus, but differs from mammalian pneumoviruses at the molecular level and has recently been classified as the type strain of a new genus, *Metapneumovirus,* in the family Paramyxoviridae (35). Recent reports have indicated that similar viruses have been detected in humans associated with respiratory tract infection in children (30, 48). Avian metapneumovirus has no non-structural NS1 and NS2 proteins and the gene order (3'-N-P-M-F-M2-SH-G-L-5') is different from that of mammalian pneumoviruses (3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5') (46). Avian metapneumovirus has been classified into four subtypes; A, B, C and D based on nucleotide sequence analysis and neutralisation with monoclonal antibodies (8, 14). Other subtypes may exist but have not yet been detected and identified.

Infection with aMPV can occur from a very young age in turkeys and is characterised by snicking, rales, sneezing, nasal discharge, foaming conjunctivitis, swelling of the infraorbital sinuses and submandibular edema (37). Secondary adventitious agents can dramatically exacerbate the clinical signs. In an uncomplicated infection, recovery is rapid and the birds appear normal in approximately 14 days. When husbandry is poor or secondary bacterial infection occurs, airsacculities, pericarditis, pneumonia, and perihepatitis may also cause increasing morbidity and mortality rates (12, 28). Secondary agents that have been shown to exacerbate and prolong clinical disease are *Bordetella avium*, *Pasteurella*-like organisms, *Mycoplasma gallisepticum*, *Ornithobacterium rhinotracheale* and *Escherichia coli (*1, 12, 21, 41). Morbidity can be as high as 100%, with mortality ranging from 0.5% in adult turkeys to 80% in young poults (5, 17, 51). Clinical signs of infection in chickens are less characteristic than those in turkeys. Severe respiratory distress may occur in broiler chickens particularly when exacerbated by secondary pathogens such as infectious bronchitis virus, mycoplasmas, and *Escherichia coli* (33, 34). Unlike subtype A and B, the United States of America (USA) strain (subtype C) has not been shown to naturally induce disease in chickens although experimentally infected chickens were shown to be susceptible to a subtype C turkey isolate of aMPV (43). Different strains of aMPV have been shown to have a specific tropism for chickens or turkeys (15). Evidence shows other species of birds can be infected with aMPV, however clinical signs have rarely been reported (18). Viruses similar to subtype C aMPV have been reported to occur in ducks in France associated with respiratory signs and egg production problems (47). Retrospective molecular analysis of viruses isolated in the 1980s from turkeys in France indicates the presence of a fourth subtype of aMPV designated subtype D (3). The results of experimental studies suggest that direct contact is

necessary for bird-to-bird spread of the virus (1, 12). In commercial conditions transmission is also likely to be airborne as the disease is restricted to the respiratory tract. Following experimental infection of 2-week-old turkeys the virus was detected in the respiratory tract for only a few days (2) in birds inoculated with aMPV alone. However, in birds inoculated with aMPV and *B. avium* virus was detected for up to 7 days post-inoculation (dpi) (7). There is no evidence that aMPV can result in a latent infection and no carrier state is known to exist. Although neonatal turkeys are occasionally infected (42) there are no reports of vertical transmission of aMPV.

In growing turkeys virus replication is limited to the upper respiratory tract and is short in duration. Replication of both attenuated and virulent strains of aMPV persist for approximately 10 dpi (12, 51). Limited replication occurs in the trachea and lung, but virus has not been shown to replicate in other tissues following natural infection (9, 10). Sequential histopathological and immunocytochemical studies have shown viral replication in the turbinates causing a serous rhinitis with increased glandular activity, epithelial exfoliation, focal loss of cilia, hyperaemia and mild mononuclear infiltration in the submucosa at 2 dpi. A catarrhal rhinitis with mucopurulent exudate, damage to the epithelial layer and a copious mononuclear inflammatory infiltration in the submucosa was seen 3–4 dpi. Transient lesions were seen in the trachea, with little or no lesions present in the conjunctiva and other tissues (24). Respiratory infection is less severe in laying turkeys; however, there may be a drop in egg production of up to 70% (45) and the quality of eggs during the recovery period, up to 3 weeks, may be poor.

In chickens there is strong evidence to suggest aMPV is one of the aetiological agents of swollen head syndrome (SHS). The syndrome is characterised by respiratory disease, apathy, swelling of infra-orbital sinuses and unilateral or bilateral facial swelling, extending over the head. These signs are frequently followed by cerebral disorientation, torticollis and opisthotonos. Although mortality does not usually exceed 1–2%, morbidity may reach 10%, and egg production is frequently affected (19, 29, 33, 34, 46).

Serological evidence suggests aMPV is widespread throughout the world and of considerable economic importance, particularly in turkeys. Oceania and Canada are the only regions that have not reported aMPV (9, 10, 17). There is serological and molecular evidence that aMPV occurs in a variety of other avian species, including pheasants, guinea fowl, ostriches, passerines and various waterfowl (4, 17, 44), but there is no evidence of serious disease.

B. DIAGNOSTIC TECHNIQUES

1.   Identification of the agent

To maximise the chances of successfully isolating the virus, a multiple approach to diagnosis is recommended. This is particularly relevant when dealing with different subtypes or genotypes that may require varied *in-vitro* methods to isolate the virus. This was illustrated in the USA with the failure to culture subtype C aMPV, which is not associated with ciliostasis, in tracheal organ cultures (13, 41). The agent was cultured following multiple embryo and cell culture passages. This was in contrast to the European experience and elsewhere in which tracheal organ cultures were shown to be the most reliable method for the primary isolation of subtype A and B aMPV (11).

- **Collection and selection of diagnostic specimens**

    It is very important to take samples for attempted virus isolation in the early stages of infection as the virus may be present only in the sinuses and turbinates for a short period. Ideally, the upper respiratory tract of live birds in the acute phase of the disease should be sampled using sterile swabs (17, 45). The most successful samples have been nasal exudates, choanal cleft swabs and scrapings of sinus and turbinate tissue. The virus has also been isolated from trachea and lungs, and occasionally viscera of affected turkey poults (5, 13). Isolation of virus is rarely successful from birds showing severe chronic signs as the extreme clinical signs are usually due to secondary adventitious agents. This certainly applies to SHS of chickens in which the characteristic signs appear to be due to secondary *Escherichia coli* infection. Furthermore, for reasons that are unclear, virus isolation from chickens appears to be more difficult than from turkeys.

    It is essential that samples requiring dispatch to a diagnostic laboratory be sent immediately on ice. When delays of more than 3 days are expected, the samples should be frozen prior to dispatch. Swabs for attempted virus isolation should be sent on ice fully immersed in viral transport medium, but those for polymerase chain reaction (PCR) analysis can be sent dry.

    For virus isolation, a 20% (v/v) suspension of the nasal exudate or homogenised tissue is made in phosphate-buffered saline (PBS) or brain–heart infusion (BHI) broth containing antibiotics at pH 7.0–7.4. This is then clarified by centrifugation at 1000 ***g*** for 10 minutes and the supernatant is passed through a 450 nm membrane filter.

- **Culture and Identification of avian metapneumovirus (aMPV)**

  The best method for primary virus isolation from infected birds is in tracheal organ cultures or embryonating turkey or chicken eggs and subsequent cultivation in cell cultures (5, 13). The original isolation of aMPV in South Africa in the late 1970s and the more recent Colorado aMPV were isolated in embryonating eggs, however subtype A and B aMPV isolations have routinely been conducted in tracheal organ cultures (11). Subtype C aMPV, and perhaps other non-identified APV, do not cause ciliostasis in organ cultures and for this reason embryonated eggs and subsequent cultivation in cell culture is the preferred method of primary virus isolation (17, 41).

  Tracheal organ cultures are prepared from turkey embryos or very young turkeys obtained from flocks free of specific antibodies to aMPV. Tracheas from chicken embryo or 1-to-2-day-old chicks may also be used. Transverse sections of trachea are rinsed in PBS (pH 7.2), placed one section per tube in Eagles medium with antibiotics, and held at 37°C. For inoculation with infective material, the tubes are drained, and 0.1 ml of bacteria-free inoculum is added. After incubation for 1 hour at 37°C growth medium is added and the cultures are incubated at 37°C on a roller apparatus rotating at 30 revolutions per hour. Cultures are examined daily after agitation on a laboratory mixer to remove debris from the lumen. Ciliostasis may occur within 7 days of inoculation on primary passage but usually is produced rapidly and consistently only after several blind passages (17).

  Six-to-8-day-old embryonating chicken or turkey eggs from flocks known to be free of aMPV antibodies are inoculated by the yolk-sac route with 0.2–0.3 ml of bacteria-free material from infected birds and incubated at 37°C. Yolk sac material should be processed for a second blind embryo passage if there is no evidence of infection (embryo stunting or mortality) with the first passage. Within 7–10 days, there is usually evidence of stunting of the embryos with few deaths. Serial passage is often required before the agent causes consistent embryo mortality. Isolation in embryonated eggs is slow, expensive, labour intensive, and requires multiple subsequent cell culture passages for identification (17).

  Various cell cultures have been used for the primary isolation of aMPV, including chicken embryo cells, Vero cells and more recently the QT-35 cells, with varying degrees of success. Primary isolation of subtype C has been conducted with multiple (5–6 serial passages) in Vero cell cultures (4). However, once the virus has been adapted to growth in embryonating eggs or tracheal organ cultures, in which it grows only to low titres, the virus will readily replicate to moderate titres following multiple passages in a variety of primary chicken or turkey embryo cells, Vero cells, and QT-35 cells (5, 9, 10, 20, 38). The virus produces a characteristic cytopathic effect (CPE) with syncytial formation in QT-35 and chicken embryo fibroblast cells within 7 days. Identification of virus-infected cell culture is conducted by immunofluorescence staining of infected cells or by plaque reduction neutralisation assay (PRNA) in QT-35 cells (39, 40).

  By negative-contrast electron microscopy, the virus has a paramyxovirus-like morphology. Pleomorphic fringed particles, roughly spherical and 80–200 nm in diameter are commonly seen. Occasionally much larger filamentous forms are present, which may be up to 1000 nm in length. The surface projections are 13–14 nm in length and the helical nucleocapsid that can sometimes be seen emerging from disrupted particles is 14 nm in diameter with an estimated pitch of 7 nm per turn (7).

- **Molecular Identification**

  Reverse-transcriptase PCR (RT-PCR) is a significantly more sensitive and rapid method for the detection of aMPV than standard virus isolation methods because of the fastidious nature of aMPV (11, 17). RT-PCR procedures targeted to the F, M, N and G genes are used for the detection of aMPV. However, because of molecular heterogeneity between aMPV strains, most RT-PCR procedures are subtype specific or do not detect all subtypes (2, 11, 35, 36). Subtype specific assays are successfully used for the detection and diagnosis of endemic strains (3, 11, 26, 31, 36). However, limitations of subtype-specific assays need to be recognised when conducting diagnostic testing for respiratory disease. Primers directed to conserved regions of the N gene have been shown to have broader specificity, detecting representative isolates from A, B, C, and D subtypes (3). RT-PCR assays directed to the G gene have also been successfully used for genotype or subtype identification (22, 23, 25). A variety of RT-PCR techniques have been developed and evaluated and these have been extensively reviewed elsewhere (11, 32).

  Nasal exudates, choanal cleft swabs, and turbinate specimens collected 2–7 days post-exposure are the preferred specimen (15, 17, 36, 45). It is imperative to collect specimens when clinical signs are first exhibited as recent studies have shown that the maximum amount of virus is present in the trachea and nasal turbinates at 3 days post-inoculation and viral RNA persists for 9 days in the trachea and up to 14 days in the nasal turbinates (49). It has been shown that aMPV can be detected from specimens collected 7–10 days post-exposure, however the viral concentration is considerably less thus reducing success of detection (1, 36). Swabs from a single flock can be pooled in groups of five to increase recovery rate.

Template RNA for RT-PCR can be extracted from homogenised tissue, dry swabs or wet swab pools with silica column or magnetic bead commercial RNA extraction reagents according to manufacturer's protocol. Tracheal swab supernatant and sinus fluid (140 µl/600 µl lysis buffer) specimens can also be processed with the RNeasy® (Qiagen, Valencia, CA) procedure.

Target	Primer ID	Sequence 5'–3'	Position	Product size
N Gene	Nc	5'–TTC-TTT-GAA-TTG-TTT-GAG-AAG-A–3'	632–653	RT primer
	Nx	5'–CAT-GGC-CCA-ACA-TTA-TGT-T–3'	830–812	115
	Nd	5'–AGC-AGG-ATG-GAG-AGC-CTC-TTT-G–3'	716–737	115

i)   Synthesis of the cDNA can be carried out in 20 µl volume with the Nc RT primer and SuperScript II® Rnase H-RT (Invitrogen, Carlsbad, CA) enzyme. Heat 1 µl RT primer (2 pmol), 1 µl dNTP mix (10 mM each), with extracted RNA and sterile distilled water (QS to 20 µl) to 65°C for 5 minutes.

ii)   Chill quickly and pulse centrifuge.

iii) Add 4 µl 5× First-Strand buffer, 2 µl 0.1 M DTT, and 1 µl RNaseOUT® (Invitrogen, Carlsbad, CA).

iv)   Heat contents to 42°C for 2 minutes and add 1 µl (200 units) of SuperScript II®, mix gently.

v)   RT is conducted at 42°C for 50 minutes followed by 70°C for 15 minutes for inactivation of RT enzyme.

vi)   PCR amplification can be conducted with AmliTaq Gold® polymerase (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Amplification conditions are as follows: 94°C for 15 minutes and 30 cycles of 94°C for 20 seconds, 51.0°C for 45 seconds, 72°C for 45 seconds with a final extension of 72°C for 10 minutes.

Several RT-PCR assays directed to the F, G and M genes have been successfully used for subtype identification and detection or diagnosis of endemic aMPV (20, 21, 24). Nucleotide sequence and phylogenetic analysis of the G gene has been used to genotype subtype A, B, and C aMPV and is the recommended procedure for subtype identification of an unidentified virus. Recommended RT-PCR procedures for sequence analysis of the G gene have been described (22, 23). Procedures for the identification of subtype A and B RNA in diagnostic specimens have also been described (31), as have procedures for the detection of subtype A and C viruses (36). Isolation of aMPV from chickens is difficult and has succeeded only in a limited number of cases; for this reason, molecular tests have been used primarily for the detection of aMPV in chickens (11, 25). It is important to remember that PCR detects viral RNA, not live virus, so the significance of a positive PCR result in terms of detecting an active infection remains to be established, particularly if live vaccination is employed routinely.

2.   Serologic tests

Due to difficulties in isolating and identifying aMPV, serology is the most common method of diagnosis of aMPV infections, particularly in unvaccinated flocks. The most commonly employed method is the ELISA; however, virus neutralisation, microimmunofluorescence and immunodiffusion tests have been used (39, 40). A number of commercial and in-house ELISA kits are available that are suitable for testing both turkey and chicken serum; however, differences in sensitivity and specificity between commercial kits have been reported (27, 28). Competitive or blocking ELISA kits incorporating an aMPV-specific monoclonal antibody have been developed. These kits claim to have a broad spectrum of sensitivity and specificity for all subtypes of aMPV and can be used for testing sera from a variety of avian species. In-house ELISA antigens, as described below, have been prepared in a variety of substrates including various cell cultures and tracheal organ cultures (6, 11). Generally, aMPV antibodies are less well detected when a heterologous strain of aMPV is used as antigen, even though the strains appear closely related by virus neutralisation test (11). The situation is further complicated by discrepancies in the ability of different ELISAs to detect vaccinal antibody when different aMPV strains are used as coating antigens (16, 40). In-house assays using a homologous antigen have been used extensively for the surveillance of endemic aMPV strains. Ideally, both acute and convalescent serum samples should be obtained for testing. In chickens, the serological response to aMPV infection is weak when compared to the response in turkeys (12).

•   **Enzyme-linked immunosorbent assay**

Virus is propagated in chicken embryo fibroblast (CEF) or Vero cell cultures until 70–100% of the monolayer is simultaneously infected (3–4 days). The cell culture fluid is decanted and the monolayer washed with PBS (pH 7.2). The monolayer is lysed with 0.5 ml (per 75 cm^2 flask) of a 0.5% non-ionic detergent solution (IGEPAL CA-630 or Nonidet P-40) on a rocking platform for 1 hour at 4°C. Following physical disruption of lysed cells, the whole virus antigen lysate is clarified at 3000 *g* for 15 minutes. Uninfected cell cultures are

treated in the same manner for a negative control antigen. Serial dilutions of antigen are tested against serial dilutions of anti-species IgG horseradish peroxidise conjugate in a checker-board fashion to determine the optimal antigen/conjugate dilution. A working dilution of the aMPV antigen and normal antigen (100 µl) are coated onto flat-bottom microtitre plates with a carbonate/bicarbonate coating buffer (6). Each serum is tested against aMPV and normal antigen for determination of the S/P ratio. Coated plates are incubated at 4°C overnight and washed a total of five times with a Tween 20 wash solution (6) prior to use or three times prior to long-term storage at –70°C. Residual wash solution remains on the plate when the plates are frozen. Following storage and equilibration to room temperature, the plates are washed twice and blotted dry prior to use.

i) Dilute test sera 1/40 in dilution/blocking buffer (6).

ii) Apply 50 µl test sera and working dilutions of positive and negative sera to aMPV antigen and normal antigen-coated wells.

iii) Incubate at room temperature for 1 hour.

iv) Wash plates five times with Tween 20 wash solution

v) Apply 50 µl of the working dilution of anti-species IgG horseradish peroxidise conjugate to each well and incubated for 1 hour at room temperature.

vi) Wash plates five times with Tween 20 wash solution

vii) Apply 100 µl of the prepared ortho-phenylenediame (OPD) substrate solution to each well and incubated for 10 minutes in the dark. Combine the following reagents for preparation of OPD substrate: 243 ml 0.1 M citric acid, 257 ml 0.2 M disodium hydrogen phosphate, adjust pH to 5.0 and QS to 1 liter with distilled water.

viii) Stop the reaction with 25 µl/well of 2.5 M sulphuric acid.

ix) Read the OD at 490/450 nm.

The results are expressed as the OD difference between the virus antigen-coated and negative control antigen-coated wells. Determine the mean OD_{490} reading for each duplicate set of wells with the positive and negative antigen for each serum. A sample with an OD_{490} difference between the antigen-coated and negative control antigen-coated wells of more than 0.2 is considered positive. Sporadic non-specific positive reactions are inherent with the ELISA test, especially with chicken serums, and immunofluorescence may be used for confirmation testing.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

This section is under study, it is anticipated that information on vaccines will be added to the online version of the *Terrestrial Manual* from June 2009, subject to approval by the OIE International Committee.

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NB: There is an OIE Reference Laboratory for Turkey rhinotracheitis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).