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(72) **Inventeurs/Inventors:**
FROST, MELINDA JANE, AU;
KIRKLAND, PETER DANIEL, AU;
FINLAISON, DEBORAH SUSAN, AU

(73) **Propriétaire/Owner:**
INTERVET INTERNATIONAL B.V., NL

(74) **Agent:** GOWLING WLG (CANADA) LLP

(54) **Titre : ESPECES DE PESTIVIRUS**
(54) **Title: PESTIVIRUS SPECIES**

(57) **Abrégé/Abstract:**
The application relates to a pestivirus, designated PMC virus, that is associated with porcine myocarditis syndrome, and the gene and protein sequences derived therefrom. The application further relates to detection methods, vaccine therapeutics, and diagnostic methods using the PMC virus or gene/protein sequences derived therefrom.

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(71) Applicant *(for all designated States except US)*: **MINISTER FOR PRIMARY INDUSTRIES FOR AND ON BEHALF OF THE STATE OF NEW SOUTH WALES** [AU/AU]; Level 17 Parkview, 157 Liverpool Street, Sydney, New South Wales 2000 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FROST, Melinda, Jane** [AU/AU]; 12 Elisabeth Henrietta Circuit, Macquarie Links, NSW 2565 (AU). **KIRKLAND, Peter, Daniel** [AU/AU]; 34 Griffith Avenue, Camden, New South Wales 2570 (AU). **FINLAISON, Deborah, Susan** [AU/AU]; 3/133 Menangle Street, Picton, New South Wales 2571 (AU).

(74) Agent: **WRAY & ASSOCIATES**; Level 4, The Quadrant, 1 William Street, Perth, Western Australia 6000 (AU).

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(54) Title: PESTIVIRUS SPECIES

(57) Abstract: The application relates to a pestivirus, designated PMC virus, that is associated with porcine myocarditis syndrome, and the gene and protein sequences derived therefrom. The application further relates to detection methods, vaccine therapeutics, and diagnostic methods using the PMC virus or gene/protein sequences derived therefrom.

Pestivirus Species

Field of the Invention

The present invention relates to a novel pestivirus, and gene sequences derived from the same. The invention further relates to detection methods, vaccines, therapeutics, and diagnostic methods using the sequences of the present invention.

Background Art

Pestiviruses cause highly contagious and often fatal diseases of pigs, cattle and sheep, which are characterised by damage to the respiratory and gastrointestinal tracts and immune system and can run an acute or chronic course. Infection of the reproductive system may cause embryonic and foetal death, congenital defects and the birth of persistently infected animals. Outbreaks of the diseases associated with pestivirus infections occur in many countries and can cause large economic losses.

The Pestivirus genus of the Flaviviridae comprises three structurally, antigenically and genetically closely related member species: Classical swine fever (CSF) or hog cholera (Francki et al. 1991. Flaviviridae, In the Fifth report of the International Committee on Taxonomy of Viruses, Archiv. Virol. Suppl. 2, Springer Verlag, Vienna p. 223-233.); Bovine viral diarrhoea virus (BVDV) which mainly affects cattle, and Border disease virus (BDV) which mainly affects sheep (Moennig and Plagemann (1992) Adv. Virus Res. 41: 53-98; Moormann et al., (1990) Virology 177: 184-198; Becher et al. (1994) Virology 198: 542-551). Recent studies indicate that there may be several less well recognised viruses that warrant separate taxonomic classification, perhaps as separate species (Avalos-Ramirez et al (2001) Virology 286: 456-465)

The genomes of pestiviruses consist of a positive strand RNA molecule of about 12.5 kb (Renard et al. (1985) DNA 4: 429-438; Moormann and Hulst (1988) Virus Res. 11: 281-291; Becher et al. (1994) Virology 198: 542-551). However, the

positive strand RNA genomes of several cytopathogenic BVDV strains may be considerably larger (Meyers et al. (1991) Virology 180: 602-616; Meyers et al. (1992) Virology 191: 368-386; Qi et al. (1992) Virology 189: 285-292).

An inherent property of viruses with a positive strand RNA genome is that their genomic RNA is infectious, i.e. after transfection of this RNA in cells that support viral replication, infectious virus is produced. As expected, the genomic (viral) RNA of pestiviruses is also infectious (Moennig and Plagemann, (1992) Adv. Virus Res. 41: 53-98).

In 2003 an outbreak of stillbirths and pre-weaning deaths of piglets occurred on two farms in New South Wales, Australia (McOrist et al, (2004) Aust Vet J. 82: 509-511). Key features of the clinical presentation and pathology findings suggested that this disease outbreak was novel and probably due to a virus. Extensive testing for known viruses and some bacteria failed to identify an aetiological agent. To avoid confusion with other important diseases in pigs, the term "porcine myocarditis syndrome" (abbreviated as "PMC") was ascribed to the disease, and the term "PMC virus" given to presumptive agent. Subsequently, the causative agent was identified as a novel pestivirus. The name Bungowannah is proposed for this new virus.

The present invention addresses a need in the art for methods of detecting and/or treating infections caused by the novel PMC virus.

Summary of the Invention

The invention provides an isolated RNA nucleotide sequence corresponding to the PMC virus nucleotide sequence depicted in SEQ ID NO:1, or sequences substantially homologous to SEQ ID NO:1, or fragments thereof.

The invention also provides the isolated DNA nucleotide sequence of the PMC virus of SEQ ID NO:1, or sequences substantially homologous to SEQ ID NO:1, or fragments thereof.

The invention further provides polypeptides encoded by the above RNA and DNA nucleotide sequences and fragments thereof, and/or an isolated PMC virus amino acid sequence as shown in SEQ ID NO: 2 and fragments thereof.

In another aspect, the invention provides methods for detecting the presence of a PMC virus amino acid sequence in a sample, comprising the steps of:

a) contacting a sample suspected of containing a PMC virus amino acid sequence with an antibody that specifically binds to the PMC virus amino acid sequence under conditions which allow for the formation of reaction complexes comprising the antibody and the PMC virus amino acid sequence; and

b) detecting the formation of reaction complexes comprising the antibody and PMC virus amino acid sequence in the sample, wherein detection of the formation of reaction complexes indicates the presence of PMC virus amino acid sequence in the sample.

The invention also provides methods for detecting the presence of a PMC virus antibody in a sample, comprising the steps of:

a) contacting a sample suspected of containing a PMC virus antibody with an amino acid sequence under conditions which allow for the formation of reaction complexes comprising the PMC virus antibody and the amino acid sequence; and

b) detecting the formation of reaction complexes comprising the antibody and amino acid sequence in the sample, wherein detection of the formation of reaction complexes indicates the presence of PMC virus antibody in the sample.

Additionally, the invention provides an in vitro method for evaluating the level of PMC virus antibodies in a biological sample comprising the steps of:

a) detecting the formation of reaction complexes in a biological sample according to the method noted above; and

b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of PMC virus antibodies in the biological sample.

The invention also provides an in vitro method for evaluating the level of PMC virus polypeptides in a biological sample comprising the steps of:

a) detecting the formation of reaction complexes in a biological sample according to the method noted above; and

b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of PMC virus polypeptide in the biological sample.

The present invention further provides methods for detecting the presence or absence of PMC virus in a biological sample, which comprise the steps of:

a) bringing the biological sample into contact with a polynucleotide probe or primer comprising a PMC virus polynucleotide of the invention under suitable hybridising conditions; and

b) detecting any duplex formed between the probe or primer and nucleic acid in the sample.

The present invention also relates to a method for the detection of PMC virus nucleic acids present in a biological sample, comprising:

a) amplifying the nucleic acid with at least one primer as defined above,

b) detecting the amplified nucleic acids.

The present invention also relates to a method for the detection of PMC virus nucleic acids present in a biological sample, comprising:

a) hybridizing the nucleic acids of the biological sample at appropriate conditions with one or more probes as defined above,

b) washing under appropriate conditions, and

c) detecting the hybrids formed.

In a further aspect, the present invention provides a method for the generation of antibodies comprising the steps of:

a) providing a PMC virus polypeptide sequence to a subject; and

b) collecting the antibodies generated in the subject against the polypeptide.

In another aspect of the invention, there is provided a vaccine composition comprising a PMC virus polypeptide or fragment thereof. The invention also provides a vaccine composition comprising a PMC virus nucleotide or fragment thereof that encodes for a PMC virus polypeptide.

Pharmaceutical compositions comprising a PMC virus polypeptide that enhances the immunocompetence of the host individual and elicits specific immunity against the PMC virus are further provided by the invention.

The present invention also provides therapeutic compositions comprising polynucleotide sequences and/or antibodies prepared against the polypeptides of the invention. The present invention further provides therapeutic compositions comprising PMC virus nucleic acid sequences as well as antisense and ribozyme polynucleotide sequences hybridisable to a polynucleotide sequence encoding a PMC virus amino acid sequence according to the invention.

The present invention provides for the use of PMC virus amino acid sequences and/or antibodies according to the invention, for manufacture of a medicament for

modulation of a disease associated with PMC virus. The present invention additionally provides for the use of polynucleotide sequences of the invention, as well as antisense and ribozyme polynucleotide sequences hybridisable to a polynucleotide sequence encoding a PMC virus amino acid sequence according to the invention, for manufacture of a medicament for modulation of a disease associated with PMC virus.

The present invention further provides a method of inducing a protective immune response in an animal or human against PMC virus comprising the steps of:

a) administering to said animal or human an effective amount of a composition of the invention.

The present invention also provides methods for enhancing an animal's immunocompetence and the activity of its immune effector cells against a PMC virus comprising the step of:

a) administering a composition comprising a therapeutically effective amount of a PMC virus peptide or polypeptide.

In addition, the present invention provides a live vector comprising the PMC virus and a heterologous polynucleotide.

In another aspect of the invention, there is provided a method of screening for drugs comprising the steps of:

a) contacting an agent with a PMC virus amino acid sequence or fragment thereof and

b) assaying for the presence of a complex between the agent and the PMC virus amino acid sequence or fragment.

The present invention also provides a method of screening for ligands of the proteins of the PMC virus comprising the steps of:

a) contacting a ligand with a PMC virus amino acid sequence or fragment thereof and

b) assaying for the presence of a complex between the PMC virus amino acid sequence or fragment and a ligand.

In a further aspect of the invention, a test kit may be prepared for the demonstration of the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled immunochemically reactive component obtained by the direct or indirect attachment of the present PMC virus amino acid sequence or a specific binding partner thereto, to a detectable label;

(b) other reagents; and

(c) directions for use of said kit.

Additionally, the invention provides a test kit for the demonstration of the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled antibody to the PMC virus;

(b) other reagents; and

(c) directions for use of said kit.

The invention also provides a test kit for the demonstration of the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled polypeptide derived from the PMC virus;

(b) other reagents; and

(c) directions for use of said kit.

Additionally the present invention provides a test kit prepared for the demonstration of the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled nucleic acid sequence derived from the PMC virus;

(b) other reagents; and

(c) directions for use of said kit.

The present invention also provides a recombinant expression vector comprising a PMC virus nucleic acid sequence or a part thereof as defined above, operably linked to prokaryotic, eukaryotic or viral transcription and translation control elements.

The invention further relates to the hosts (prokaryotic or eukaryotic cells) which are transformed by the above mentioned vectors and recombinants and which are capable of expressing said RNA and/or DNA fragments.

The present invention also relates to a method for the production of a recombinant PMC virus polypeptide, comprising the steps of:

a) transforming an appropriate cellular host with a recombinant vector, in which a PMC virus polynucleotide sequence or a part thereof has been inserted under the control of appropriate regulatory elements,

b) culturing said transformed cellular host under conditions enabling the expression of said insert, and,

c) harvesting said polypeptide.

According to another embodiment the present invention provides methods for preparing a PMC virus amino acid sequence, comprising the steps of:

(a) culturing a cell containing a vector as described above under conditions that provide for expression of the PMC virus amino acid sequence; and

(b) recovering the expressed PMC virus sequence.

Brief Description of the Drawings

Figure 1 shows the DNA sequence of the PMC virus of the present invention;

Figure 2 shows the protein sequence of the PMC virus of the present invention;

Figure 3 shows a map of the location of primers used to sequence the whole virus, the dotted lines underneath are the length of the PCR products produced and sequenced;

Figure 4 shows an ethidium bromide stained 0.8% gel of SISPA applied to DNA and RNA of adaptor PCR (run on Corbett and Eppendorf cycler machines). Arrows indicate where gel was cut to collect bands for purification and cloning (e.g. ER1 = Eppendorf PCR machine, RNA preparation, gel position 1). Lane 1 Eppendorf machine RNA SISPA 10ul of PCR product; Lane 2 Eppendorf machine DNA SISPA 10ul of PCR product; Lane 3 Eppendorf machine RNA SISPA 40ul of PCR product; Lane 4 Eppendorf machine DNA SISPA 40ul of PCR product; Lane 5 Eppendorf machine Blank 40ul of PCR control; Lane 6 Corbett machine RNA SISPA 40ul of PCR product; Lane 7 Corbett machine DNA SISPA 40ul of PCR product; Lane 8 Corbett machine blank 40ul of PCR product; Lane 9 100bp marker.

Figure 5 shows an ethidium bromide stained 1% gel of SISPA applied to DNA and RNA simultaneously to screen colonies for inserts (e.g. ER3 1 = Eppendorf PCR machine, RNA sample position 3 colony 1). Lane 1 ER3 1; Lane 2 ER3 2; Lane 3 ER3 3; Lane 4 ER3 4; Lane 5 ER3 5; Lane 6 ER3 6; Lane 7 ER3 7; Lane 8 ER3 8; Lane 9 ER3 9; Lane 10 ER3 10; Lane 11 ER3 11; Lane 12 ER3 12; Lane 13 Marker 100bp; Lane 14 ER4 1; Lane 15 ER4 2; Lane 16 ER4 3; Lane 17 ER4 4; Lane 18 ER4 5; Lane 19 ER4 6; Lane 20 ER4 7; Lane 21 ER4 8; Lane 22

ER4 9; Lane 23 ER4 10; Lane 24 ER4 11; Lane 25 ER4 12; Lane 26 ER5 1; Lane 27 ER5 2; Lane 28 ER5 3; Lane 29 ER5 4; Lane 30 ER5 5; Lane 31 ER5 6; Lane 32 ER5 7; Lane 33 Marker 100bp; Lane 34 ER5 8; Lane 35 ER5 9; Lane 36 ER5 10; Lane 37 ER5 11; Lane 38 ER5 12; Lane 39 ER6 1; Lane 40 ER6 2; Lane 41 ER6 3; Lane 42 ER6 4; Lane 43 ER6 5; Lane 44 ER6 6; Lane 45 ER6 7; Lane 46 ER6 8; Lane 47 ER6 9; Lane 48 ER6 10; Lane 49 ER6 11; Lane 50 ER6 12; Lane 51 ER7 1; Lane 52 ER7 2; Lane 53 Marker 100bp; Lane 54 ER7 3; Lane 55 ER7 4; Lane 56 ER7 5; Lane 57 ER7 6; Lane 58 ER7 7; Lane 59 ER7 8; Lane 60 ER7 10; Lane 61 ER7 11; Lane 62 ER7 12; Lane 63 ER8 1; Lane 64 ER8 2; Lane 65 ER8 3; Lane 66 ER8 4; Lane 67 ER8 5; Lane 68 ER8 6; Lane 69 ER8 7; Lane 70 ER8 8; Lane 71 ER8 9; Lane 72 ER8 10; Lane 73 Marker 100bp; Lane 74 ER8 11; Lane 75 ER8 12; Lane 76 ER9 1; Lane 77 ER9 2; Lane 78 ER9 3; Lane 79 ER9 4; Lane 80 ER9 5; Lane 81 Marker 100bp; Lane 82 ER9 6; Lane 83 ER9 7; Lane 84 ER9 8; Lane 85 ER9 9; Lane 86 ER9 10; Lane 87 ER9 11; Lane 88; Lane 89 ER10 2; Lane 90 ER10 3; Lane 91 ER10 4; Lane 92 ER10 5; Lane 93 ER10 6; Lane 94 ER10 7; Lane 95 ER10 8; Lane 96 ER10 9; Lane 97 ER10 10; Lane 98 ER10 11; Lane 99 ER10 12.

Figure 6 shows an ethidium bromide stained 1% gel of PCR carried out to screen of colonies for DNA (Eppendorf cycler). Lane 1 ED2 1 = Eppendorf machine, DNA gel cut out 2, colony 1; Lane 2 ED2 2; Lane 3 ED2 3; Lane 4 ED2 4; Lane 5 ED2 5; Lane 6 ED2 6; Lane 7 ED2 7; Lane 8 ED2 8; Lane 9 ED2 9; Lane 10 ED2 10; Lane 11 ED2 11; Lane 12 ED2 12; Lane 13 Marker 100bp; Lane 14 ED3 1; Lane 15 ED3 2; Lane 16 ED3 3; Lane 17 ED3 4; Lane 18 ED3 5; Lane 19 ED3 6; Lane 20 ED3 7; Lane 21 ED3 8; Lane 22 ED3 9; Lane 23 ED3 10; Lane 24 ED3 11; Lane 25 ED3 12; Lane 26 ED4 1; Lane 27 ED4 2; Lane 28 ED4 3; Lane 29 ED4 4; Lane 30 ED4 5; Lane 31 ED4 6; Lane 32 ED4 7; Lane 33 Marker 100bp; Lane 34 ED4 8; Lane 35 ED4 9; Lane 36 ED4 10; Lane 37 ED4 11; Lane 38 ED4 12; Lane 39 ED5 1; Lane 40 ED5 2; Lane 41 ED5 3; Lane 42 ED5 4; Lane 43 ED5 5; Lane 44 ED5 6; Lane 45 ED5 7; Lane 46 ED5 8; Lane 47 ED5 9; Lane 48 ED5 10; Lane 49 ED5 11; Lane 50 ED5 12; Lane 51 ED6 1; Lane 52 ED6 2; Lane 53 ED6 3; Lane 54 ED6 4; Lane 55 ED6 5; Lane 56 ED6 6; Lane 57 ED6 7; Lane 58 ED6 8; Lane 59 ED6 9; Lane 60 Marker 100bp ; Lane 61 ED6 10; Lane 62 ED6

11; Lane 63 ED6 12; Lane 64 ED7 1; Lane 65 ED7 2; Lane 66 ED7 3; Lane 67 ED7 4; Lane 68 ED7 5; Lane 69 ED7 6; Lane 70 ED7 7; Lane 71 ED7 8; Lane 72 ED7 9; Lane 73 ED7 10; Lane 74 ED7 11; Lane 75 ED7 12; Lane 76 ED8 1; Lane 77 ED8 2; Lane 78 ED8 3; Lane 79 ED8 4; Lane 80 ED8 5; Lane 81 ED8 6; Lane 82 ED8 7; Lane 83 ED8 8; Lane 84 ED8 9; Lane 85 ED8 10; Lane 86 ED8 11; Lane 87 ED8 12.

Figure 7 shows an ethidium bromide stained 1% gel of PCR carried out to screen of colonies for RNA inserts (Corbett cycler). Lane 1 CR2 1 = Corbett machine, RNA gel position 2, colony 1; Lane 2 CR2 2; Lane 3 CR2 3; Lane 4 CR2 4; Lane 5 CR2 5; Lane 6 CR2 6; Lane 7 Marker 100bp; Lane 8 Marker 100bp; Lane 9 CR2 7; Lane 10 CR2 8; Lane 11 CR2 9; Lane 12 CR2 10; Lane 13 CR2 11; Lane 14 CR2 12; Lane 15 CR3 1; Lane 16 CR3 2; Lane 17 CR3 3; Lane 18 CR3 4; Lane 19 CR3 5; Lane 20 CR3 6; Lane 21 CR3 7; Lane 22 CR3 8; Lane 23 CR3 9; Lane 24 CR3 10; Lane 25 CR3 11; Lane 26 CR3 12; Lane 27 Marker 100bp; Lane 28 Marker 100bp; Lane 29 CR4 1; Lane 30 CR4 2; Lane 31 CR4 3; Lane 32 CR4 4; Lane 33 CR4 5; Lane 34 CR4 6; Lane 35 CR4 7; Lane 36 CR4 8; Lane 37 ; CR4 9; Lane 38 CR4 10; Lane 39 CR4 11; Lane 40 CR4 12; Lane 41 marker 100bp; Lane 42 marker 100bp; Lane 43 CR5 1; Lane 44 CR5 2; Lane 45 CR5 3; Lane 46 CR5 4; Lane 47 CR5 5; Lane 48 CR5 6; Lane 49 CR5 7; Lane 50 CR5 8; Lane 51 CR5 9; Lane 52 CR5 10; Lane 53 CR5 11; Lane 54 PCR Blank control; Lane 55 marker 100bp.

Figure 8 shows an ethidium bromide stained 1% gel of PCR carried out to screen colonies for DNA (Corbett cycler). Lane 1 marker 100bp; Lane 2 CD3 1 = Corbett machine, DNA gel cut out 3, colony 1; Lane 3 CD3 2; Lane 4 CD3 3; Lane 5 CD3 4; Lane 6 CD3 5; Lane 7 CD3 6; Lane 8 CD3 7; Lane 9 CD3 8; Lane 10 CD3 9; Lane 11 CD3 10; Lane 12 CD3 11; Lane 13 CD3 12; Lane 14 CD4 1; Lane 15 CD4 2; Lane 16 CD4 3; Lane 17 CD4 4; Lane 18 CD4 5; Lane 19 CD4 6; Lane 20 marker 100bp; Lane 21 marker 100bp; Lane 22 CD4 7; Lane 23 CD4 8; Lane 24 CD4 9; Lane 25 CD4 10; Lane 26 CD4 11; Lane 27 CD4 12; Lane 28 CD5 1; Lane 29 CD5 2; Lane 30 CD5 3; Lane 31 CD5 4; Lane 32 CD5 5; Lane 33 CD5 6; Lane 34 CD5 7; Lane 35 CD5 8; Lane 36 CD5 9; Lane 37 CD5 10; Lane 38 CD5 11; Lane 39 CD5 12; Lane 40 marker 100bp; Lane 41 marker 100bp; Lane 42

CD6 1; Lane 43 CD6 2; Lane 44 CD6 3; Lane 45 CD6 4; Lane 46 CD6 5; Lane 47 CD6 6; Lane 48 CD6 7; Lane 49 CD6 8; Lane 50 CD6 9; Lane 51 CD6 10; Lane 52 CD6 11; Lane 53 CD6 12.

Figure 9 shows an ethidium bromide stained 1.5% gel of PCR carried out to confirm authenticity of viral sequence for virus confirmation by nRT-PCR. PCR results confirmed the presence of Pestivirus in clinical specimens (lanes 3, 8 and 23) while EMCV was not present (lane 28) (lanes marked + are PCR positive). Lane 1 Marker 100bp; Lane 2 Blank CR39 primers; Lane 3 SISPA sera CR39 primers; Lane 4 NADL +ve control CR39 primers; Lane 5 EMCV –ve control CR39 primers; Lane 6; Lane 7 Blank ER510 primers; Lane 8 SISPA sera ER510 primers; Lane 9 NADL +ve control ER510 primers; Lane 10 EMCV –ve control ER510 primers; Lane 11; Lane 12 Blank ER55 primers; Lane 13 SISPA sera ER55 primers; Lane 14 NADL +ve control ER55 primers; Lane 15 EMCV –ve control ER55 primers; Lane 16; Lane 17; Lane 18; Lane 19; Lane 20 Marker 100bp; Lane 21 Marker 100bp; Lane 22 Blank ER62 primers; Lane 23 SISPA sera ER62 primers; Lane 24 NADL +ve control ER62 primers; Lane 25 EMCV –ve control ER62 primers; Lane 26; Lane 27 Blank ER41 primers; Lane 28 SISPA sera ER41 primers; Lane 29 NADL +ve control ER41 primers; Lane 30 EMCV –ve control ER41 primers; Lane 31; Lane 32; Lane 33; Lane 34; Lane 35; Lane 36; Lane 37; Lane 38; Lane 39; Lane 40 marker 100bp.

Figure 10 shows a hydrophobicity plot of the PMC virus protein sequence.

Detailed Description of the Invention

New Pestivirus

In accordance with this invention, a new pestivirus has been discovered that differs genetically from known pestiviruses. The new virus is characterised by the RNA sequence corresponding to that shown in SEQ ID NO: 1. The sequence has been deposited as Genbank reference EF100713.

The new virus is hereinafter generally referred to as PMC virus and the condition caused by infection with the PMC virus is PMC.

The PMC virus genome comprises a single open reading frame (ORF), encoding a number of genes. The genes encoded by the ORF of PMC correspond to those of other pestiviruses, being the Npro, capsid, E0, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B genes.

The PMC virus is approximately 40% similar to other pestiviruses on a nucleic acid sequence level. At the protein level, PMC virus has 46-71% identity and 63-83% similarity with other pestiviruses. A comparative analysis of both the nucleic acid and deduced amino acid sequences would suggest that PMC virus is sufficiently unique to warrant consideration for classification as a new species within the pestivirus genus.

Open Reading Frames, Encoded Genes, Features of RNA Genome

The nucleotide sequence of SEQ ID NO:1 encodes a single ORF encoding a number of different genes. The genes encoded by SEQ ID NO:1 correspond to the Npro, capsid, E0, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B genes of other pestiviruses.

The approximate location of the genes of PMC, based on sequence comparison with gi12657941, is indicated in Table 1.

Table 1: Location of proteins within PMC nucleic acid open-reading frame.

PROTEIN	APPROXIMATE DNA POSITION
NPro	419-922
Capsid	923-1219
E0	1220-1885
E1	1886-2473
E2	2474-3604
P7	3605-3820
NS2	3821-5224
NS3	5225-7252
NS4A	7253-7441
NS4B	7442-8482
NS5A	8483-9997
NS5B	9998-12077

Table 2: Location of proteins within PMC protein open-reading frame.

PROTEIN	APPROXIMATE AMINO ACID POSITION
NPro	1-167
Capsid	168-267
E0	268-489
E1	490-685
E2	686-1062
P7	1063-1134
NS2	1135-1602
NS3	1603-2278
NS4A	2279-2341
NS4B	2342-2688
NS5A	2689-3193
NS5B	3194-3886

NUCLEIC ACID SEQUENCES

RNA

The invention provides an isolated RNA nucleotide sequence corresponding to the PMC virus nucleotide sequence depicted in SEQ ID NO:1, or sequences substantially homologous to SEQ ID NO:1, or fragments thereof. The invention further provides an RNA sequence comprising the complement of the PMC virus RNA genome, or fragments thereof.

The RNA sequence may also correspond to a fragment of SEQ ID NO:1. Preferably, the fragment is selected from the following locations of SEQ ID NO:1: position 419-922, 923-1219, 1220-1885, 1886-2473, 2474-3604, 3605-3820, 3821-5224, 5225-7252, 7253-7441, 7442-8482, 8483-9997, 9998-12077. Alternatively, the fragment may be selected from any one of SEQ ID NOs:3 – 15.

Substantial homology or identity exists when a PMC virus polynucleotide sequence or fragment thereof will hybridise to another PMC virus polynucleotide (or a complementary strand thereof) under selective hybridisation conditions.

Selective hybridisation may be under low, moderate or high stringency conditions, but is preferably under high stringency.

Typically, selective hybridisation will occur when there is at least about 55% identity over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75% and most preferably at least about 90%. The length of homology comparison, as described, may be over longer stretches and in certain embodiments will often be over a stretch of at least about nine nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 32 nucleotides and preferably at least about 36 or more nucleotides.

Thus, the polynucleotide sequences of the invention preferably have at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listings herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described below for polypeptides. A preferred sequence comparison program is the GCG Wisconsin Bestfit program.

In the context of the present invention, a homologous sequence is taken to include a nucleotide sequence which is at least 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the nucleic acid level over at least 20, 50, 100, 200, 300, 500 or 819 nucleotides with the corresponding nucleotide sequences set out in SEQ ID NO:1. In particular, homology should typically be considered with respect to those regions of the sequence that encode contiguous amino acid sequences known to be essential for the function of one or more of PMC virus proteins, rather than non-essential neighbouring sequences.

PMC virus polynucleotide sequence fragments of the invention will preferably be at least 15 nucleotides in length, more preferably at least 20, 30, 40, 50, 100 or 200 nucleotides in length. Generally, the shorter the length of the polynucleotide sequence, the greater the homology required to obtain selective hybridisation. Consequently, where a polynucleotide sequence of the invention consists of less than about 30 nucleotides, it is preferred that the percentage identity is greater

than 75%, preferably greater than 90% or 95% compared with the polynucleotide sequences set out in the sequence listings herein. Conversely, where a polynucleotide sequence of the invention consists of, for example, greater than 50 or 100 nucleotides, the percentage identity compared with the polynucleotide sequences set out in the sequence listings herein may be lower, for example greater than 50%, preferably greater than 60 or 75%.

Nucleic acid sequences according to the present invention which are homologous to the sequences as represented by a SEQ ID NO: 1 can be characterized and isolated according to any of the techniques known in the art, such as amplification by means of sequence-specific primers, hybridization with sequence-specific probes under more or less stringent conditions, serological screening methods or via the LiPA typing system.

DNA

The DNA of the new PMC virus also is provided. The DNA sequence is preferably derived from the RNA sequences described above. Most preferably, the DNA sequence is that shown in SEQ ID NO: 1 or fragments thereof.

The invention also provides DNA fragments hybridisable with the genomic RNA of PMC. The DNA or DNA fragment sequence may be derived from the cDNA sequence of the PMC virus or fragments thereof. The DNA, cDNA or fragments thereof may be in the form of recombinant DNAs.

The DNA sequence may also be a fragment of SEQ ID NO:1. Preferably, the fragment is selected from the following locations of SEQ ID NO:1: position 419-922, 923-1219, 1220-1885, 1886-2473, 2474-3604, 3605-3820, 3821-5224, 5225-7252, 7253-7441, 7442-8482, 8483-9997, 9998-12077.

Variant Nucleic Acids

Nucleic acid sequences and fragments, which would include some deletions or mutations which would not substantially alter their ability to hybridizing with the

genome of PMC virus, are also provided by the present invention. Such variants are to be considered as forming obvious equivalents of the RNA, DNA or fragments referred to above.

Other preferred variant nucleic acid sequences of the present invention include sequences which are redundant as a result of the degeneracy of the genetic code compared to any of the above-given nucleic acid sequences of the present invention. These variant nucleic acid sequences will thus encode the same amino acid sequences as the nucleic acid sequences they are derived from. Preferably, the RNAs of these variants, and the related cDNAs derived from said RNAs, are hybridisable to corresponding parts of the RNA and cDNA of PMC virus.

Also included within the present invention are sequence variants of the DNA sequence of SEQ ID NO: 1 or corresponding RNA sequence or fragments thereof, containing either deletions and/or insertions of one or more nucleotides, especially insertions or deletions of 1 or more codons.

Also included are substitutions of some non-essential nucleotides by others (including modified nucleotides and/or inosine).

Particularly preferred variant polynucleotides of the present invention also include sequences which hybridise under stringent conditions with any of the nucleic acid sequences of the present invention. Thus, sequences which show a high degree of homology (similarity) to any of the nucleic acid sequences of the invention as described above are preferred. Particularly preferred are sequences which are at least 80%, 85%, 90%, 95% or more homologous to said nucleic acid sequences of the invention. Preferably, said sequences will have less than 20%, 15%, 10%, or 5% variation of the original nucleotides of said nucleic acid sequences.

Probes and Primers

Primer and probes are further provided, which can be made starting from any RNA or DNA sequence or sequence fragment according to the invention. Preferably, such probes or primers are between about 5 to 50 nucleotides long,

more preferably from about 10 to 25 nucleotides. Probes and primers of the present invention may be used in PCR, sequencing reactions, hybridisation reactions and other applications known to the skilled person.

The present invention also relates to an oligonucleotide primer comprising part of SEQ ID NO: 1, said primer being able to act as a primer for specifically amplifying the nucleic acid of the PMC virus. Preferably, the primer is a single stranded DNA oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to the nucleic acid strand to be copied. The specific length and sequence of the primer used will depend on the complexity of the required DNA or RNA targets, as well as on the conditions of primer use, such as temperature and ionic strength. The fact that amplification primers do not have to match exactly with corresponding template sequence to warrant proper amplification is amply documented in the literature (Kwok et al., 1990).

The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS; Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990; Walker et al., 1992) or amplification by means of Qβ replicase (Lizardi et al., 1988; Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules using primer extension. During amplification, the amplified products can be conveniently labelled either using labelled primers or by incorporating labelled nucleotides. Labels may be isotopic (^{32}P, ^{35}S, etc.) or non-isotopic (biotin, digoxigenin, etc.). The amplification reaction is repeated between 20 and 70 times, advantageously between 25 and 45 times.

The present invention also relates to an oligonucleotide probe comprising part of SEQ ID NO:1, with said probe being able to act as a hybridisation probe for the PMC virus. Preferably, the probe can be used for specific detection and/or classification into types and/or subtypes of PMC virus. Preferably, the probe is a single stranded sequence-specific oligonucleotide sequence which has a

sequence that is complementary to the target sequence of the PMC virus to be detected.

Those skilled in the art will recognise that the stringency of hybridisation will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands and the number of nucleotide base mismatches between the hybridising nucleic acids. Stringent temperature conditions will generally include temperatures in excess of 30°C, typically in excess of 37°C, and preferably in excess of 45°C. Stringent salt conditions will ordinarily be less than 1000 mM, typically less than 500 mM, and preferably less than 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. An example of stringent hybridisation conditions is 65°C and 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0).

Optionally, the probe of the invention is labelled and/or attached to a solid substrate. The solid substrate can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the background level of hybridization remains low. Usually the solid substrate will be a microtiter plate, a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead). Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH_2 groups, SH groups, carboxylic groups, or coupling with biotin or haptens.

The probes of the invention may include also an isolated polynucleotide attached to a label or reporter molecule and may be used to isolate other polynucleotide sequences, having sequence similarity by standard methods. For techniques for preparing and labelling probes see, e.g. Sambrook et al., (1989) or Ausubel et al., (2001).

Oligonucleotides according to the present invention and used as primers or probes may also contain or consist of nucleotide analogues such as phosphorothioates (Matsukura et al., 1987), alkylphosphoriates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may contain interculating agents (Asseline et al., 1984). The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics, reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules, etc.

Recombinant DNAs containing fragments of the DNA sequence of PMC virus are also provided by the present invention, and may be used as, for example, probes. Preferably, the plasmid used to generate the recombinant DNA is a plasmid amplifiable in prokaryotic or eukaryotic cells and carrying said fragments. For example, using cloned DNA containing a DNA fragment of PMC virus as a molecular hybridization probe, either by marking with radionucleotides or with fluorescent reagents, PMC virus RNA may be detected directly, for example, in blood, body fluids and blood products.

Nucleic acid arrays

PMC virus polynucleotide sequences (preferably in the form of probes) may also be immobilised to a solid phase support for the detection of PMC virus. Alternatively the PMC virus polynucleotide sequences will form part of a library of DNA molecules that may be used to detect simultaneously a number of different genes from PMC virus. In a further alternate form of the invention, PMC virus polynucleotide sequences together with other polynucleotide sequences (such as from other bacteria or viruses) may be immobilised on a solid support in such a manner permitting identification of the presence of PMC virus and/or any of the other polynucleotide sequences bound onto the solid support.

Techniques for producing immobilised libraries of DNA molecules have been described in the art. Generally, most prior art methods describe the synthesis of single-stranded nucleic acid molecule libraries, using for example masking techniques to build up various permutations of sequences at the various discrete

positions on the solid substrate. U.S. Patent No. 5,837,832 describes an improved method for producing DNA arrays immobilised to silicon substrates based on very large scale integration technology. In particular, U.S. Patent No. 5,837,832 describes a strategy called "tiling" to synthesize specific sets of probes at spatially defined locations on a substrate that may be used to produce the immobilised DNA libraries of the present invention. U.S. Patent No. 5,837,832 also provides references for earlier techniques that may also be used. Thus polynucleotide sequence probes may be synthesised *in situ* on the surface of the substrate.

Alternatively, single-stranded molecules may be synthesised off the solid substrate and each pre-formed sequence applied to a discrete position on the solid substrate. For example, polynucleotide sequences may be printed directly onto the substrate using robotic devices equipped with either pins or pizo electric devices.

The library sequences are typically immobilised onto or in discrete regions of a solid substrate. The substrate may be porous to allow immobilisation within the substrate or substantially non-porous, in which case the library sequences are typically immobilised on the surface of the substrate. The solid substrate may be made of any material to which polypeptides can bind, either directly or indirectly. Examples of suitable solid substrates include flat glass, silicon wafers, mica, ceramics and organic polymers such as plastics, including polystyrene and polymethacrylate. It may also be possible to use semi-permeable membranes such as nitrocellulose or nylon membranes, which are widely available. The semi-permeable membranes may be mounted on a more robust solid surface such as glass. The surfaces may optionally be coated with a layer of metal, such as gold, platinum or other transition metal. A particular example of a suitable solid substrate is the commercially available BiaCore^{TM} chip (Pharmacia Biosensors).

Preferably, the solid substrate is generally a material having a rigid or semi-rigid surface. In preferred embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate regions for different polymers with, for example, raised regions or etched trenches. It is also preferred that the solid substrate is suitable for the high

density application of DNA sequences in discrete areas of typically from 50 to 100 μm, giving a density of 10000 to 40000 dots/cm^{-2}.

The solid substrate is conveniently divided up into sections. This may be achieved by techniques such as photoetching, or by the application of hydrophobic inks, for example teflon-based inks (Cel-line, USA).

Discrete positions, in which each different member of the library is located may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc.

Attachment of the polynucleotide sequences to the substrate may be by covalent or non-covalent means. The polynucleotide sequences may be attached to the substrate via a layer of molecules to which the library sequences bind. For example, the polynucleotide sequences may be labelled with biotin and the substrate coated with avidin and/or streptavidin. A convenient feature of using biotinylated polynucleotide sequences is that the efficiency of coupling to the solid substrate can be determined easily. Since the polynucleotide sequences may bind only poorly to some solid substrates, it is often necessary to provide a chemical interface between the solid substrate (such as in the case of glass) and the nucleic acid sequences. Examples of suitable chemical interfaces include hexaethylene glycol. Another example is the use of polylysine coated glass, the polylysine then being chemically modified using standard procedures to introduce an affinity ligand. Other methods for attaching molecules to the surfaces of solid substrate by the use of coupling agents are known in the art, see for example WO98/49557.

Binding of complementary polynucleotide sequences to the immobilised nucleic acid library may be determined by a variety of means such as changes in the optical characteristics of the bound polynucleotide sequence (i.e. by the use of ethidium bromide) or by the use of labelled nucleic acids, such as polypeptides labelled with fluorophores. Other detection techniques that do not require the use of labels include optical techniques such as optoacoustics, reflectometry, ellipsometry and surface plasmon resonance (see WO97/49989).

Thus, the present invention provides a solid substrate having immobilized thereon at least one polynucleotide of the present invention, preferably two or more different polynucleotide sequences of the present invention. In a preferred embodiment the solid substrate further comprises polynucleotide sequences derived from genes other than the PMC virus polynucleotide sequence.

Antisense Nucleic Acids and Ribozymes

The present invention also extends to the preparation of antisense nucleotides and ribozymes that may be used to interfere with the expression of PMC virus amino acid sequences at the translational level. This approach utilises antisense nucleic acid and ribozymes to block translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule [See: Weintraub, (1990) *Sci. Am.*, 262:40-46; Marcus-Sekura, (1988) *Anal. Biochem.*, 172:289-295]. In the cell, they hybridise to that mRNA, forming a double-stranded molecule. The cell does not translate an mRNA complexed in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridise to the AUG initiation codon will be particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules when introducing them into infected cells. Antisense methods have been used to inhibit the expression of many genes *in vitro* [Hambor *et al.*, (1988) *J. Exp. Med.*, 168:1237-1245].

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns. By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognise specific nucleotide sequences in an RNA molecule and cleave it [Cech, (1988) *J. Am. Med. Assoc.*, 260:3030-3034]. Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

Investigators have identified two types of ribozymes, *Tetrahymena*-type and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize four-base sequences, while "hammerhead"-type recognize eleven- to eighteen-base sequences. The longer the recognition sequence, the more likely it is to occur exclusively in the target mRNA species. Therefore, hammerhead-type ribozymes are preferable to *Tetrahymena*-type ribozymes for inactivating a specific mRNA species and eighteen base recognition sequences are preferable to shorter recognition sequences.

The PMC polynucleotide sequences described herein may thus be used to prepare antisense molecules against, and ribozymes that cleave, mRNAs for PMC virus amino acid sequences, thus inhibiting expression of the PMC virus polynucleotide sequences.

POLYPEPTIDE SEQUENCES

Polypeptides

The invention also covers polypeptides encoded by the above RNA and DNA nucleotide sequences and fragments thereof. The invention further provides an isolated PMC virus amino acid sequence as shown in SEQ ID NO: 2 and fragments thereof. More desirably, the PMC virus amino acid sequence is provided in substantially purified form. Further provided are polypeptide fragments having lower molecular weights and having peptide sequences or fragments in common with those shown in SEQ ID NO:2.

The term "isolated" is used to describe a PMC virus amino acid sequence that has been separated from components that accompany it in its natural state. Further, a PMC virus amino acid sequence is "substantially purified" when at least about 60 to 75% of a sample exhibits a single PMC virus amino acid sequence. A substantially purified PMC virus amino acid sequence will typically comprise about 60 to 90% W/W of a PMC virus amino acid sequence sample, more usually about 95%, and preferably will be over about 99% pure. Protein purity or homogeneity may be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a

single PMC virus amino acid sequence band upon staining the gel. For certain purposes, higher resolution may be provided by using HPLC or other means well known in the art which are utilised for application.

The invention further contemplates fragments of the PMC virus amino acid sequence. A PMC virus amino acid sequence fragment is a stretch of amino acid residues of at least about five to seven contiguous amino acids, often at least about seven to nine contiguous amino acids, typically at least about nine to 13 contiguous amino acids and, most preferably, at least about 20 to 30 or more contiguous amino acids.

In a highly preferred form of the invention the fragments exhibit ligand-binding, immunological activity and/or other biological activities characteristic of PMC virus amino acid sequences. More preferably, the fragments possess immunological epitopes consistent with those present on native PMC virus amino acid sequences.

As used herein, "epitope" refers to an antigenic determinant of a polypeptide. An epitope could comprise three amino acids in a spatial conformation that is unique to the epitope. Generally, an epitope consists of at least five amino acids, and more usually consists of at least 8-10 amino acids. Methods of determining the spatial conformation of such amino acids are known in the art.

Preferred PMC virus amino acid sequences of the invention will have one or more biological properties (eg in vivo, in vitro or immunological properties) of the native full-length PMC virus amino acid sequence. Alternatively, fragments of the full-length PMC virus amino acid sequence may have one or more biological properties of one or more of the genes which the full length amino acid sequence encodes.

Preferably, the fragments of the full length PMC virus amino acid sequence SEQ ID NO:2 are chosen from the following locations in SEQ ID NO:2: 1-167, 168-267, 268-489, 490-685, 686-1062, 1063-1134, 1135-1602, 1603-2278, 2279-2341,

2342-2688, 2689-3193, 3194-3886. Alternatively, the fragment may be selected from any one of SEQ ID NOs:16 - 27.

Non-functional PMC virus amino acid sequences are also included within the scope of the invention since they may be useful, for example, as antagonists of PMC virus genes. The biological properties of analogues, fragments, or derivatives relative to wild type may be determined, for example, by means of biological assays.

PMC virus amino acid sequences, including analogues, fragments and derivatives, can be prepared synthetically (e.g., using the well known techniques of solid phase or solution phase peptide synthesis). Preferably, solid phase synthetic techniques are employed. Alternatively, PMC virus amino acid sequences of the invention can be prepared using well known genetic engineering techniques, as described *infra*. In yet another embodiment, PMC virus amino acid sequences can be purified (e.g., by immunoaffinity purification) from a biological fluid, such as but not limited to whole blood, plasma, faeces, serum, or urine from animals, including pigs, cattle, sheep, chickens, human beings, dogs, horses, and fish.

Variant Polypeptides

PMC virus amino acid sequence analogues preferably include those having an amino acid sequence wherein one or more of the amino acids is substituted with another amino acid, which substitutions do not substantially alter the biological activity of the molecule.

In the context of the invention, an analogous sequence is taken to include a PMC virus amino acid sequence which is at least 60, 70, 80 or 90% homologous, preferably at least 95 or 98% homologous at the amino acid level over at least 20, 50, 100 or 200 amino acids, with the amino acid sequence set out in SEQ ID NO:1. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for the function of the protein or proteins encoded by the PMC virus RNA, rather than non-essential neighbouring sequences.

Although homology can be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity. The terms "substantial homology" or "substantial identity", when referring to PMC virus amino acid sequences, indicate that the PMC virus amino acid sequence in question exhibits at least about 70% identity with an entire naturally-occurring PMC amino acid sequence or portion thereof, usually at least about 80% identity and preferably at least about 90 or 95% identity.

In a highly preferred form of the invention, a PMC virus amino acid sequence analogue will have 80% or greater amino acid sequence identity to the PMC virus amino acid sequence set out in SEQ ID NO:2. Examples of PMC virus amino acid sequence analogues within the scope of the invention include the amino acid sequence of SEQ ID NO:2 wherein: (a) one or more aspartic acid residues is substituted with glutamic acid; (b) one or more isoleucine residues is substituted with leucine; (c) one or more glycine or valine residues is substituted with alanine; (d) one or more arginine residues is substituted with histidine; or (e) one or more tyrosine or phenylalanine residues is substituted with tryptophan.

PMC virus amino acid sequence derivatives are also provided by the invention and include PMC virus amino acid sequences, analogues or fragments thereof which are substantially homologous in primary structure but which include chemical and/or biochemical modifications or unusual amino acids. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, ubiquitination, labelling, (e.g., with radionucleotides), and various enzymatic modifications, as will be readily appreciated by those well skilled in the art.

In one form of the invention the chemical moieties suitable for derivatisation are selected from among water soluble polymers. The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the

desired polymer based on considerations such as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis and other considerations. For the present proteins and peptides, these may be ascertained using the assays provided herein.

The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol. Polyethylene glycol propionaldehyde may provide advantages in manufacturing due to its stability in water.

In another form of the invention the amino acid sequences may be modified to produce a longer half life in an animal host, for example, by fusing one or more antibody fragments (such as an Fc fragment) to the amino or carboxyl end of a PMC virus amino acid sequence.

Where the PMC virus amino acid sequence is to be provided in a labelled form, a variety of methods for labelling amino acid sequences are well known in the art and include radioactive isotopes such as ^{32}P, ligands which bind to labelled antiligands (eg, antibodies), fluorophores, chemiluminescent agents, enzymes and antiligands which can serve as specific binding pair members for a labelled ligand. The choice of label depends on the sensitivity required, stability requirements, and available instrumentation. Methods of labelling amino acid sequences are well known in the art [See, e.g., Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); and Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. *Current protocols in molecular biology.* Greene Publishing Associates/Wiley Intersciences, New York (2001)].

The PMC virus amino acid sequences of the invention, if soluble, may be coupled to a solid-phase support, e.g., nitrocellulose, nylon, column packing materials (e.g., Sepharose beads), magnetic beads, glass wool, plastic, metal, polymer gels, cells, or other substrates. Such supports may take the form, for example, of beads, wells, dipsticks, or membranes.

The invention also provides for fusion polypeptides, comprising PMC virus amino acid sequences and fragments. Thus PMC virus amino acid sequences may be fusions between two or more PMC virus amino acid sequences or between a PMC virus amino acid sequence and a related protein. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. For example, ligand-binding or other domains may be "swapped" between different fusion polypeptides or fragments. Such homologous or heterologous fusion polypeptides may display, for example, altered strength or specificity of binding. Fusion partners include immunoglobulins, bacterial beta-galactosidase, trpE, protein A, beta-lactamase, alpha amylase, alcohol dehydrogenase and yeast alpha mating factor.

Modified PMC virus amino acid sequences may be synthesised using conventional techniques, or may be encoded by a modified polynucleotide sequence and produced using recombinant nucleic acid methods. The modified polynucleotide sequence may also be prepared by conventional techniques. Fusion proteins will typically be made by either recombinant nucleic acid methods or may be chemically synthesised.

DIAGNOSTICS

In accordance with another embodiment the invention provides diagnostic and prognostic methods to detect the presence of PMC virus using PMC virus glycoproteins, proteins and other peptides and polypeptides (whether obtained in a purified state from PMC virus preparations, or by chemical synthesis) and/or antibodies derived there from and/or PMC virus polynucleotide sequences.

Diagnostic and prognostic methods will generally be conducted using a biological sample obtained from an animal, such as a pig. A "sample" refers to a sample of tissue or fluid suspected of containing a PMC polynucleotide or polypeptide from an animal, but not limited to, e.g., whole blood, blood cells, plasma, serum, milk, faecal samples, tissue and samples of *in vitro* cell culture constituents.

Polypeptide/Antibody-based Diagnostics

Means are provided for the detection of proteins of PMC virus, particularly for the diagnosis of PMC or for the detection of antibodies against PMC virus or its proteins, particularly in subjects afflicted with PMC or more generally in asymtomatic carriers and in animal derived products such as meat. Such methods are also referred to as immunoassays.

The invention thus provides a method for detecting the presence of a PMC virus amino acid sequence in a sample, comprising the steps of:

a) contacting a sample suspected of containing a PMC virus amino acid sequence with an antibody that specifically binds to the PMC virus amino acid sequence under conditions which allow for the formation of reaction complexes comprising the antibody and the PMC virus amino acid sequence; and

b) detecting the formation of reaction complexes comprising the antibody and PMC virus amino acid sequence in the sample, wherein detection of the formation of reaction complexes indicates the presence of PMC virus amino acid sequence in the sample.

Particularly the invention relates to an in vitro process of diagnosis making use of an amino acid sequence encoding an envelope glycoprotein or of a polypeptide bearing an epitope of a glycoprotein from PMC virus or any other viral protein (structural or non-structural) for the detection of anti-PMC virus antibodies in serum, milk or body fluids. Preferably, the antibody used in the above methods

binds to the E0, E1, E2, NS2, NS3, NS4A, NS4B and/or NS5A, NS5B proteins of PMC virus.

The invention also provides a method for detecting the presence of a PMC virus antibody in a sample, comprising the steps of:

a) contacting a sample suspected of containing a PMC virus antibody with an amino acid sequence under conditions which allow for the formation of reaction complexes comprising the PMC virus antibody and the amino acid sequence; and

b) detecting the formation of reaction complexes comprising the antibody and amino acid sequence in the sample, wherein detection of the formation of reaction complexes indicates the presence of PMC virus antibody in the sample.

A method is also provided for the detection of anti-PMC virus antibodies, comprising the steps of:

a) depositing a predetermined amount of one or several PMC virus antigens onto a solid support such as a microplate;

b) introducing increasing dilutions of a biological fluid (e.g., blood serum or plasma, milk, cerebrospinal fluid, lymphatic fluid or other body fluids) onto the antigens and incubating;

c) washing the solid support with an appropriate buffer;

d) adding specific labelled antibodies directed against the antibodies of the subject; and

e) detecting the antigen-antibody-antibody complex formed, which is then indicative of the presence of PMC virus antibodies in the biological fluid.

Preferably, the antibody used in these methods is derived from an affinity-purified polyclonal antibody, and more preferably a mAb. In addition, it is preferable for the antibody molecules used herein be in the form of Fab, Fab', $F(ab')_2$ or F(v) portions or whole antibody molecules.

Particularly preferred methods for detecting PMC virus based on the above methods include enzyme linked immunosorbent assays, radioimmunoassays, immunoradiometric assays and immunoenzymatic assays, including sandwich assays using monoclonal and/or polyclonal antibodies.

Three such procedures that are especially useful utilise either PMC virus amino acid sequences (or fragments thereof) labelled with a detectable label, antibody Ab_1 labelled with a detectable label, or antibody Ab_2 labelled with a detectable label. The procedures may be summarized by the following equations wherein the asterisk indicates that the particle is labelled and "AA" stands for the PMC virus amino acid sequence:

A. $AA^* + Ab_1 = AA^*Ab_1$

B. $AA + Ab^*_1 = AA\ Ab_1^*$

C. $AA + Ab_1 + Ab_2^* = Ab_1\ AA\ Ab_2^*$

The procedures and their application are all familiar to those skilled in the art and accordingly may be utilised within the scope of the present invention. The "competitive" or "blocking" procedure, Procedure A, is described in U.S. Patent Nos. 3,654,090 and 3,850,752. Procedure B is representative of well-known competitive assay techniques. Procedure C, the "sandwich" procedure, is described in U.S. Patent Nos. RE 31,006 and 4,016,043. Still other procedures are known, such as the "double antibody" or "DASP" procedure.

In each instance, the PMC virus amino acid sequences form complexes with one or more antibody(ies) or binding partners and one member of the complex is labelled with a detectable label. The fact that a complex has formed and, if desired, the

amount thereof, can be determined by known methods applicable to the detection of labels.

It will be seen from the above that a characteristic property of Ab_2 is that it will react with Ab_1. This is because Ab_1, raised in one mammalian species, has been used in another species as an antigen to raise the antibody, Ab_2. For example, Ab_2 may be raised in goats using rabbit antibodies as antigens. Ab_2 therefore would be anti-rabbit antibody raised in goats. For purposes of this description and claims, Ab_1 will be referred to as a primary antibody, and Ab_2 will be referred to as a secondary or anti-Ab_1 antibody.

The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals that fluoresce when exposed to ultraviolet light, and others.

A number of fluorescent materials are known and can be utilised as labels. These include, for example, fluorescein, rhodamine and auramine. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

The PMC virus amino acid sequences or their binding partners can also be labelled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re.

Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes, which can be used in these procedures, are known and can be utilized. The preferred enzymes are peroxidase, ß-glucuronidase, ß-D-glucosidase, ß-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Patent Nos. 3,654,090, 3,850,752 and 4,016,043 are referred to by way of example for their disclosure of alternate labelling material and methods.

In another embodiment of the invention there are provided in vitro methods for evaluating the level of PMC virus antibodies in a biological sample comprising the steps of:

a) detecting the formation of reaction complexes in a biological sample according to the method noted above; and

b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of PMC virus antibodies in the biological sample.

Preferably, the antibody used in the above methods binds to the E0, E1, E2, NS2, NS3, NS4A, NS4B and/or NS5A, NS5B proteins of PMC virus.

In another embodiment of the invention there are provided in vitro methods for evaluating the level of PMC virus polypeptides in a biological sample comprising the steps of:

a) detecting the formation of reaction complexes in a biological sample according to the method noted above; and

b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of PMC virus polypeptide in the biological sample.

Preferably, the polypeptide used in the above methods encodes the E0, E1, E2, NS2, NS3, NS4A, NS4B and/or NS5A, NS5B proteins of PMC virus.

Further there are provided *in vitro* methods for monitoring therapeutic treatment of a disease associated with PMC virus in an animal host comprising evaluating, as describe above, the levels of PMC virus antibodies in a series of biological samples obtained at different time points from an animal host undergoing such therapeutic treatment.

The methods for detecting polypeptides using antibodies, or immunoassays, according to the present invention may utilize antigens from the different domains of the new and unique polypeptide sequences of the present invention that maintain linear (in case of peptides) and conformational epitopes (in case of polypeptides) recognized by antibodies in the sera from subjects infected with PMC virus.

It is within the scope of the invention to use, for instance, single or specific oligomeric antigens, dimeric antigens, as well as combinations of single or specific oligomeric antigens.

The PMC virus antigens of the present invention may be employed in virtually any assay format that employs a known antigen to detect antibodies. Of course, a format that denatures the PMC virus conformational epitope should be avoided or adapted.

A common feature of all of these detection methods is that the antigen is contacted with the test specimen suspected of containing PMC virus antibodies under conditions that permit the antigen to bind to any such antibody present in the component. Such conditions will typically be physiologic temperature, pH and ionic strength, using an appropriate predetermined quantity of antigen. The incubation of the antigen with the specimen is followed by detection of immune complexes comprised of the antigen and antibodies derived from the specimen typically by using a labelled second antibody that is directed against the immunoglobulins of the test animal species.

Design of the immunoassays is subject to a great deal of variation, and many formats are known in the art. Protocols may, for example, use solid supports, or immunoprecipitation. Assays which amplify the signals from the immune complex are also known; examples of which are assays which utilize biotin and avidin or streptavidin, and enzyme-labelled and mediated immunoassays, such as ELISA assays. Furthermore, the immunoassay may be, without limitation, in a heterogeneous or in a homogeneous format, and of a standard or competitive type.

In a heterogeneous format, the polypeptide is typically bound to a solid matrix or support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidine fluoride (known as Immunolon™), diazotized paper, nylon membranes, activated beads, and Protein A beads. For example, Dynatech Immunolon™ 1 or Immunlon™ 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic polypeptides is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are know in the art.

In a homogeneous format, the test sample is incubated with the combination of antigens in solution. For example, it may be under conditions that will precipitate any antigen-antibody complexes which are formed. Both standard and competitive formats for these assays are known in the art.

In a standard format, the amount of PMC virus antibodies in the antibody-antigen complexes is directly monitored. This may be accomplished by determining whether labelled anti-xenogeneic (e.g. anti-swine) antibodies which recognize an epitope on anti-PMC virus antibodies will bind due to complex formation. In a competitive format, the amount of PMC virus antibodies in the sample is deduced by monitoring the competitive effect on the binding of a known amount of labelled antibody (or other competing ligand) in the complex.

Complexes formed comprising anti-PMC virus antibody (or in the case of competitive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For example, unlabelled PMC virus antibodies in the complex may be detected using a conjugate of anti-xenogeneic Ig complexed with a label (e.g. an enzyme label).

In an immunoprecipitation or agglutination assay format, the reaction between the PMC virus antigens and the antibody forms a network that precipitates from the

solution or suspension and forms a visible layer or film of precipitate. If no anti-PMC antibody is present in the test specimen, no visible precipitate is formed.

There currently exist three specific types of particle agglutination (PA) assays. These assays are used for the detection of antibodies to various antigens when coated to a support. One type of this assay is the haemagglutination assay using red blood cells (RBCs) that are sensitized by passively adsorbing antigen (or antibody) to the RBC. The addition of specific antigen antibodies present in the body component, if any, causes the RBCs coated with the purified antigen to agglutinate.

To eliminate potential non-specific reactions in the haemagglutination assay, two artificial carriers may be used instead of RBC in the PA. The most common of these are latex particles. However, gelatin particles may also be used. The assays utilizing either of these carriers are based on passive agglutination of the particles coated with purified antigens.

Nucleic Acid-based Diagnostics

The present invention further provides methods for detecting the presence or absence of PMC virus in a biological sample, which comprise the steps of:

c) bringing the biological sample into contact with a polynucleotide probe or primer comprising a PMC virus polynucleotide of the invention under suitable hybridising conditions; and

d) detecting any duplex formed between the probe or primer and nucleic acid sequences in the sample.

According to one embodiment of the invention, detection of PMC virus may be accomplished by directly amplifying PMC virus polynucleotide sequences from biological sample, using known techniques and then detecting the presence of PMC virus polynucleotide sequences.

The present invention thus also relates to a method for the detection of PMC virus nucleic acids present in a biological sample, comprising:

c) amplifying the nucleic acid with at least one primer as defined above,

d) detecting the amplified nucleic acids.

Preferably, the nucleic acid is extracted and/or purified (eg from a from a tissue sample) prior to amplification.

The present invention also relates to a method for the detection of PMC virus nucleic acids present in a biological sample, comprising:

d) hybridizing the nucleic acids of the biological sample at appropriate conditions with one or more probes as defined above,

e) washing under appropriate conditions, and

f) detecting the hybrids formed.

Preferably, the hybridizing conditions are denatured conditions.

Preferably, the nucleic acid is extracted and/or purified (eg from a from a tissue sample) prior to hybridisation. More preferably, the nucleic acid sample is amplified with at least one primer as defined above, after extraction or at least prior to hybridisation. Preferably, said probes are attached to a solid substrate or detected in a liquid phase by photometric or fluorogenic detection or by other methods of visualisation such as by agarose gel electrophoresis.

The present invention also relates to a method as defined above, wherein said nucleic acids are labelled during or after amplification.

Suitable assay methods for purposes of the present invention to detect hybrids formed between the oligonucleotide probes and the nucleic acid sequences in a sample may comprise any of the assay formats known in the art, such as the

conventional dot-blot format, sandwich hybridization or reverse hybridization. For example, the detection can be accomplished using a dot blot format, the unlabelled amplified sample being bound to a membrane, the membrane being incorporated with at least one labelled probe under suitable hybridization and wash conditions, and the presence of bound probe being monitored.

An alternative and preferred method is a "reverse" dot-blot format, in which the amplified sequence contains a label. In this format, the unlabelled oligonucleotide probes are bound to a solid support and exposed to the labelled sample under appropriate stringent hybridization and subsequent washing conditions. It is to be understood that also any other assay method which relies on the formation of a hybrid between the nucleic acids of the sample and the oligonucleotide probes according to the present invention may be used.

In one form of the invention, the target nucleic acid sequence is amplified by PCR and then detected using any of the specific methods mentioned above. Other useful diagnostic techniques for detecting the presence of PMC virus polynucleotide sequences include, but are not limited to: 1) allele-specific PCR; 2) single stranded conformation analysis; 3) denaturing gradient gel electrophoresis; 4) RNase protection assays; 5) the use of proteins which recognize nucleotide mismatches, such as the *E. coli* mutS protein; 6) allele-specific oligonucleotides; and 7) fluorescent *in situ* hybridisation.

In addition to the above methods, PMC virus polynucleotide sequences may be detected using conventional probe technology. When probes are used to detect the presence of the PMC virus polynucleotide sequences, the biological sample to be analysed, such as blood or serum, may be treated, if desired, to extract the nucleic acids. The sample polynucleotide sequences may be prepared in various ways to facilitate detection of the target sequence; e.g. denaturation, restriction digestion, electrophoresis or dot blotting. The targeted region of the sample polynucleotide sequence usually must be at least partially single-stranded to form hybrids with the targeting sequence of the probe. If the sequence is naturally single-stranded, denaturation will not be required. However, if the sequence is

double-stranded, the sequence will probably need to be denatured. Denaturation can be carried out by various techniques known in the art.

Sample polynucleotide sequences and probes are incubated under conditions that promote stable hybrid formation of the target sequence in the probe with the putative PMC virus polynucleotide sequence in the sample. Preferably, high stringency conditions are used in order to prevent false positives.

Detection, if any, of the resulting hybrid is usually accomplished by the use of labelled probes. Alternatively, the probe may be unlabelled, but may be detectable by specific binding with a ligand that is labelled, either directly or indirectly. Suitable labels and methods for labelling probes and ligands are known in the art, and include, for example, radioactive labels which may be incorporated by known methods (e.g., nick translation, random priming or kinasing), biotin, fluorescent groups, chemiluminescent groups (e.g., dioxetanes, particularly triggered dioxetanes), enzymes, antibodies and the like. Variations of this basic scheme are known in the art, and include those variations that facilitate separation of the hybrids to be detected from extraneous materials and/or that amplify the signal from the labelled moiety.

It is also contemplated within the scope of this invention that the nucleic acid probe assays of this invention may employ a cocktail of nucleic acid probes and/or primers capable of detecting PMC virus polynucleotide sequences. Thus, in one example to detect the presence of PMC virus polynucleotide sequences in a cell sample, more than one probe complementary to PMC virus polynucleotide sequences is employed and in particular the number of different probes is alternatively 2, 3, or 5 different nucleic acid probe sequences.

Additionally, the present invention provides a method for detecting viral RNA or DNA comprising the steps of:

a) immobilizing PMC virus on a support (e.g., a nitrocellulose filter);

b) disrupting the virion; and

c) hybridizing with a probe.

Preferably, the probe is labelled. More preferably, the probe is radiolabelled or fluorescent- or enzyme-labelled. Such an approach to detection of virus has already been developed for Hepatitis B virus in peripheral blood (Scotto J. et al. Hepatology (1983), 3, 379-384).

The present invention also provides a method for rapid screening of genomic DNA derived from the tissue of subjects with PMC virus related symptoms to detect proviral PMC virus related DNA or RNA present in the tissues. Thus, the present invention also provides a method for screening the tissue of subjects comprising the steps of:

a) extracting DNA from tissue;

b) restriction enzyme cleavage of said DNA;

c) electrophoresis of the fragments; and

d) Southern blotting of genomic DNA from tissues and subsequent hybridization with labelled cloned PMC virus DNA.

Hybridization *in situ* can also be used.

ANTIGENIC POLYPEPTIDE PRODUCTION

Viral RNA and DNA according to the invention can be used for expressing PMC viral antigens for diagnostic purposes, as well as for the production of a vaccine against PMC virus. The methods which can be used to achieve expression of antigenic polypeptides are multifold:

a) DNA can be transfected into mammalian cells with appropriate selection markers by a variety of techniques, such as calcium phosphate precipitation, polyethylene glycol, protoplast-fusion, etc and the resultant proteins purified.

b) DNA fragments corresponding to genes can be cloned into expression vectors for *E. coli*, yeast or mammalian cells and the resultant proteins purified.

c) The provival RNA or DNA can be "shot-gunned" (fragmented) into prokaryotic expression vectors to generate fusion polypeptides. Recombinants, producing antigenically competent fusion proteins, can be identified by simply screening the recombinants with antibodies against PMC virus antigens.

Particular reference in this respect is made to those portions of the genome of PMC virus which, in the figures, are shown to belong to open reading frames and which encode the products having the polypeptide sequences shown. Preferably, the nucleic acid sequences used in the above methods encode the E0, E1, E2, NS2, NS3, NS4A, NS4B and/or NS5A, NS5B proteins of PMC. Preferably, polypeptides are provided containing sequences in common with polypeptides comprising antigenic determinants included in the proteins encoded and expressed by the PMC virus genome.

ANTIBODIES

Antibodies to PMC proteins

The different peptides according to this invention can also be used themselves for the production of antibodies, preferably monoclonal antibodies specific for the respective different peptides. Thus, according to the invention, PMC virus amino acid sequences produced recombinantly or by chemical synthesis and fragments or other derivatives or analogues thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize the PMC virus amino acid sequence. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and a Fab expression library.

Thus, the present invention provides a method for the generation of antibodies comprising the steps of:

a) providing a PMC virus polypeptide sequence to a subject; and

b) collecting the antibodies generated in the subject against the polypeptide.

Preferably, the ppolypeptide used to generate the antibody is antigenic. More preferably, the polypeptide is chosen from the list comprising the E0, E1, E2, NS2, NS3, NS4A, NS4B and/or NS5A or NS5B proteins of PMC virus. More preferably, the protein used to generate the antibody is the E0, E2, NS2 and/or NS3 proteins or a fragment or derivative thereof For example, in a highly preferred embodiment, a composition of the invention comprises both a PMC virus E0/E2 complex and an PMC virus NS2/NS3 complex.

A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic amino acid sequence contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567, as well as antigen binding portions of antibodies, including Fab, $F(ab')_2$ and F(v) (including single chain antibodies). Accordingly, the phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule containing the antibody combining site. An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin

molecule that contain the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), which portions are preferred for use in the therapeutic methods described herein.

Fab and $F(ab')_2$ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous *et al.* Fab' antibody molecule portions are also well-known and are produced from $F(ab')_2$ portions followed by reduction with mercaptoethanol of the disulfide bonds linking the two heavy chain portions, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts.

For the production of hybridomas secreting said monoclonal antibodies, conventional production and screening methods can be used. These monoclonal antibodies, which themselves are part of the invention, provide very useful tools for the identification and even determination of relative proportions of the different polypeptides or proteins in biological samples, particularly animals samples containing PMC virus or related viruses.

Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Preferably, the adjuvant is pharmaceutically acceptable.

Further examples of adjuvants which may be effective include but are not limited to: N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

Additional examples of adjuvants and other agents include aluminium hydroxide, aluminium phosphate, aluminium potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, Corynebacterium parvum (Propionobacterium acnes), Bordetella pertussis, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, immuno stimulating complexes (ISCOMs), liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminium hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminium hydroxide is approved for human use.

The proportion of immunogenic polypeptide and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminium hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). Conveniently, the vaccines are formulated to contain a final concentration of immunogen in the range of from 0.2 to 200 μg/ml, preferably 5 to 50 μg/ml, most preferably 15 μg/ml.

After formulation, the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or it may be freeze-dried. Lyophilisation permits long-term storage in a stabilised form.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

The PMC virus polypeptides of the invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

Compositions of the present invention may further comprise antigenic polypeptides that are not coupled to PMC virus polypeptides and/or biologically active molecules whose primary purpose is not to serve as an antigen but to modulate the immune response in some other aspect. Examples of biologically molecules that modulate the immune system of an animal or human subject include cytokines.

The term "cytokine" refers to any secreted polypeptide that influences the function of other cells mediating an immune response. Some examples of cytokines include, but are not limited to, interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon-α (IFN-α), interferon-β (IFN-β), interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), tumour necrosis factor-β (TNF-β), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor-β (TGF-β).

Various procedures known in the art may be used for the production of polyclonal antibodies to PMC virus amino acid sequences, or fragment, derivative or analogues thereof.

For the production of antibody, various host animals can be immunised by injection with the PMC virus amino acid sequence, or a derivative (e.g., fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc.

In one embodiment, the PMC virus amino acid sequences or fragment thereof can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH).

Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active

substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward the PMC virus amino acid sequences, or fragments, analogues, or derivatives thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler *et al.*, (1975) *Nature*, 256:495-497, the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, (1983) *Immunology Today*, 4:72], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, (1985) in *Monoclonal Antibodies and Cancer Therapy*, pp. 77-96, Alan R. Liss, Inc.]. Immortal, antibody-producing cell lines can be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. *See, e.g.*, U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890.

In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals. According to the invention, swine antibodies may be used and can be obtained by using swine hybridomas or by transforming B cells with PMC virus *in vitro*. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison *et al.*, (1984) *J. Bacteriol.*, 159-870; Neuberger *et al.*, (1984) *Nature*, 312:604-608; Takeda *et al.*, (1985) *Nature*, 314:452-454] by splicing the genes from a mouse antibody molecule specific for a PMC amino acid sequence together with genes from an antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such chimeric antibodies are preferred for use in therapy of intestinal diseases or disorders, since the antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce PMC virus amino acid sequence-specific single chain antibodies. An additional embodiment of the invention utilises the techniques described for the construction of Fab expression libraries [Huse *et al.*, (1989) *Science*, 246:1275-1281] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a PMC virus amino acid sequence, or its derivatives, or analogues.

Antibody fragments, which contain the idiotype of the antibody molecule, can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Screening for Antibodies

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA, "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc.

In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies that recognise a specific epitope of a PMC virus amino acid sequence, one may assay generated hybridomas for a product that binds to a PMC virus amino acid sequence

fragment containing such epitope. For selection of an antibody specific to a PMC virus amino acid sequence from a particular species of animal, one can select on the basis of positive binding with PMC virus amino acid sequence expressed by or isolated from cells of that species of animal.

Labelling Antibodies

Advantageously, the labelling of the anti-immunoglobulin antibodies is achieved by an enzyme selected from among those which are capable of hydrolysing a substrate, which substrate undergoes a modification of its radiation-absorption, at least within a predetermined band of wavelengths. The detection of the substrate, preferably comparatively with respect to a control, then provides a measurement of the likelihood of exposure of an animal to the virus, or of the effective presence, of the disease.

Thus, preferred methods of immunoenzymatic and also immunofluorescent detections, in particular according to the ELISA technique, are provided. Titrations may be determinations by immunofluorescence or direct or indirect immunoenzymatic determinations. Quantitative titrations of antibodies on the serums studied can be made.

Epitope Bearing Fragments

Antibodies according to the present invention may be generated using polypeptide fragments (or molecules, particularly glycoproteins having the same polypeptidic backbone as the polypeptides mentioned hereinabove) bearing an epitope characteristic of a protein or glycoprotein of PMC virus. The polypeptide or molecule may further have N-terminal and C-terminal extremities respectively either free or, independently from each other, covalently bonded to amino acids other than those which are normally associated with them in the larger polypeptides or glycoproteins of the PMC virus, which last mentioned amino acids are then free or belong to another polypeptidic sequence.

Conjugation to Increase Immunogenicity

Peptide sequences of small size bearing an epitope or immunogenic determinant, (eg those which are readily generated by chemical synthesis), may require coupling or covalent conjugation to a physiologically acceptable and non-toxic carrier molecule in order to increase their in vivo immunogenic character and thus enhance the production of antibodies.

Particularly, the invention relates to antibodies generated using hybrid polypeptides containing any of the epitope bearing-polypeptides which have been defined more specifically hereinabove, recombined with other polypeptides fragments normally foreign to the PMC virus proteins, having sizes sufficient to provide increased immunogenicity to the epitope-bearing-polypeptide. The foreign polypeptide fragments are preferably immunogenically inert and/or do not interfere with the immunogenic properties of the epitope-bearing-polypeptide.

Such hybrid polypeptides, which may contain from 5 up to 150, even 250 amino acids, usually consist of the expression products of a vector which contains a nucleic acid sequence encoding said epitope-bearing-polypeptide expressible under the control of a suitable promoter or replicon in a suitable host.

Said epitope-bearing-polypeptides, particularly those whose N-terminal and C-terminal amino acids are free, may also be generated by chemical synthesis according to techniques well known in the chemistry of proteins.

Examples of carrier molecules or macromolecular supports which can be used for making the conjugates according to the invention are natural proteins, such as tetanic toxoid, ovalbumin, serum-albumins, hemocyanins, etc. Synthetic macromolecular carriers, for example polysines or poly(D-L-alanine)-poly(L-lysine), can also be used. Other types of macromolecular carriers that can be used, which generally have molecular weights higher than 20,000, are known from the literature.

The conjugates can be synthesized by known processes such as are described by Frantz and Robertson [Infection & Immunity, 33, 193-198 (1981)] and by P. E. Kauffman [pplied and Environmental Microbiology", Oct. 1981 Vol. 42, No. 4, pp. 611-614]. For instance, the following coupling agents can be used: glutaric aldehyde, ethyl chloroformate, water-soluble carbodiimides such as(N-ethyl-N'(3-dimethylamino-propyl) carbodiimide, HCl), diisocyanates, bis-diazobenzidine, di- and trichloro-s-triazines, cyanogen bromides and benzaquinone, as well as the coupling agents mentioned in Scand. J. Immunol., 1978, vol. 8, pp. 7-23 (Avrameas, Ternynck, Guesdon).

Any coupling process can be used for bonding one or several reactive groups of the peptide, on the one hand, and one or several reactive groups of the carrier, on the other hand. Coupling is advantageously achieved between the carboxyl and amine groups carried by the peptide and the carrier in the presence of a coupling agent of the type used in protein synthesis, e.g., 1-ethyl-3-(3-dimethylaminoproyl)-carbodiimide, N-hydroxybenzotriazole, etc. Coupling between amine groups respectively borne by the peptide and the carrier can also be made with glutaraldehyde, for instance, according to the method described by Boquet et al. (1982) Molec. Immunol., 19, 1441-1549, when the carrier is haemocyanin.

The immunogenicity of epitope-bearing-peptides can also be increased by oligomerisation thereof, for example in the presence of glutaraldehyde or any other suitable coupling agent. In particular, the invention relates to the water soluble immunogenic oligomers thus obtained, comprising particularly from 2 to 10 monomer units.

VACCINES

The invention also relates to vaccine compositions whose active principle is a polypeptide or fragment thereof of the present invention i.e. the hereinabove disclosed polypeptides of PMC virus, fusion polypeptides or oligopeptides, in association with a suitable pharmaceutically or physiologically acceptable carrier. The present invention further provides immunogenic polypeptides, and more

particularly protective polypeptides, for use in the preparation of vaccine compositions against PMC or related syndromes.

Thus, the present invention provides a vaccine composition comprising a PMC virus polypeptide or fragment thereof.

Preferably, the polypeptide is an antigenic polypeptide. More preferably, the vaccine further comprises a pharmaceutically acceptable carrier or diluent.

The invention also provides a vaccine composition comprising a PMC virus nucleotide or fragment thereof that encodes for a PMC virus polypeptide.

The term "vaccine" as used herein, refers to mean any composition of the invention containing PMC virus peptide or polypeptide or nucleotide sequnces coding for PMC virus polypeptides having at least one antigenic determinant which, when administered to a animal, is capable of stimulating an immune response against the antigenic determinant. It will be understood that the term vaccine does not necessarily imply that the composition will provide a complete protective response. Rather a therapeutic effect will be sufficient.

The phrase "immune response" refers to any cellular process that is produced in the animal following stimulation with an antigen and is directed toward the elimination of the antigen from the animal. The immune response typically is mediated by one or more populations of cells characterized as being lymphocytic and/or phagocytic in nature.

A vaccine may generate an immune response that blocks the infectivity, either partially or fully, of an infectious agent. The administration of the vaccine of the present invention may be for either a prophylactic or therapeutic purpose. When provided prophylactically, the vaccine is provided in advance of any exposure to PMC virus or in advance of any symptom of any symptoms due to PMC virus infection. The prophylactic administration of the immunogen serves to prevent or attenuate any subsequent infection by PMC virus in a mammal or reduce the severity of infection and/or symptoms. When provided therapeutically, the vaccine

is provided at (or shortly after) the onset of the infection or at the onset of any symptom of infection or disease caused by PMC virus. The therapeutic administration of the vaccine serves to attenuate the infection or disease.

The immune response generated against an introduced PMC virus peptide or polypeptide will be dictated by the amino acid constitution of the antigenic peptide or polypeptide. Such determinants may define either humoral or cell mediated antigenic regions. Without being limited to any particular mode of action, it is contemplated that the immune response generated by the PMC virus peptide or polypeptide will preferably include both humoral and cell mediated immune responses. Where a cell mediated immune response is effected it preferably leads to a T cell cascade, and more specifically by means of a cytotoxic T cell cascade.

The term "cytotoxic T cell", as used herein, refers to any T lymphocyte expressing the cell surface glycoprotein marker CD8+ that is capable of targeting and lysing a target cell which bears a major histocompatibility class I (MHC Class I) complex on its cell surface and is infected with an intracellular pathogen.

Preferably, the vaccine composition is developed to generate antibodies against the E0 and E2 envelope glycoproteins and the NS2 and NS3 non-structural proteins.

The vaccine compositions of the present invention may be used to vaccinate animals and humans against infectious diseases, preferably against PMC. The term “animal” includes: mammals such as farm animals including sheep, goats, pigs, cows, horses, llamas, household pets such as dogs and cats, and primates; birds, such as chickens, geese and ducks; fish; and reptiles such as crocodiles and alligators.

The vaccine composition according to the invention preferably contains a nucleotide sequence as described above, either as such or as a vaccine strain or in a vector or host organism, or a polypeptide as described above, in an amount effective for producing protection against a pestivirus infection. The vaccine can

also be a multipurpose vaccine comprising other immunogens or nucleotides encoding these. The vaccines can furthermore contain conventional carriers, adjuvants, solubilizers, emulsifiers, preservatives etc. The vaccines according to the invention can be prepared by conventional methods.

Preferably, the active principle is a peptide containing less than 250 amino acid units, preferably less than 150, particularly from 5 to 150 amino acid residues, as deducible from the complete genome of PMC virus.

The term 'effective amount' refers to an amount of epitope-bearing polypeptide sufficient to induce an immunogenic response in the subject to which it is administered either in a single dose or as part of a series of doses. Preferably, the effective amount is sufficient to effect prophylaxis or treatment, as defined above. The exact amount necessary will vary according to the application. For vaccine applications or for the generation of polyclonal antiserum/antibodies, for example, the effective amount may vary depending on the taxonomic group or species of subject to be treated (e.g. nonhuman primate, primate, etc.), the age and general health and physical condition of the subject, the severity of the condition being treated, the capacity of the subject's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the strain of infecting PMC virus, the particular polypeptide selected and its mode of administration, and other relevant factors. It is also believed that effective amounts will be found within a relatively large, non-critical range. An appropriate effective amount can be readily determined using only routine experimentation.

By way of example, suitable dosages of the vaccine compositions are those which are effective to elicit antibodies in vivo, in the host, particularly a porcine host. Suitable doses range from 10 to 500 μg of polypeptide, protein or glycoprotein, for instance 50 to 100 μg. Other preferred ranges of proteins for prophylaxis of PMC are 0.01 to 1000 μg/dose, preferably 0.1 to 100 μg/dose. Several doses may be needed per subject in order to achieve a sufficient immune response and subsequent protection against PMC.

The immunogenic compositions are conventionally administered using standard procedures, for example, intravenously, subcutaneously, intramuscularly, intraorbitally, ophthalmically, intraventricularly, intracranially, intracapsularly, intraspinally, intracisternally, intraperitoneally, buccal, rectally, vaginally, intranasally, orally or by aerosol administration.

Preferably, the immunogenic composition is administered parenterally, typically by injection, for example, subcutaneously or intramuscularly. However, additional formulations suitable for other methods of administration include oral formulations and suppositories or prepared for pulmonary, nasal or other forms of administration. Dosage treatment may be a single dose schedule or a multiple dose schedule.

The mode of administration of the immunogenic vaccine compositions prepared in accordance with the invention will necessarily depend upon such factors as the stability of the immunogenic compositions under physiological conditions, the intensity of the immune response required etc.

The vaccine compositions of the invention may be co-administered with additional immune response enhancers or biological response modifiers including, but not limited to, the cytokines IFN-α, IFN-γ, IL-2, IL-4, IL-6, TNF, or other cytokine-affecting immune cells. In accordance with this aspect of the invention, the PMC virus peptide or polypeptide is administered in combination therapy with a therapeutically active amount of one or more of these cytokines. In addition, conventional antibiotics may be coadministered with the PMC virus peptide or polypeptide. The choice of suitable antibiotics will however be dependent upon the disease in question.

Parenteral Delivery

The compounds provided herein can be administered by any parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections. Typically, such vaccines are prepared either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein

encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients and carriers, which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Oral Delivery

Contemplated for use herein are oral solid dosage forms, which are described generally in Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatised with various polymers (E.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in Modern Pharmaceutics, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference. In general, the formulation will include a PMC virus polypeptide or polynucleotide, and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of PMC virus polypeptides or polynucleotides. In this respect the PMC virus polypeptides or polynucleotides may be chemically modified so that oral delivery is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the protein (or peptide) molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the protein and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene

glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. Abuchowski et al., 1981, supra; Newmark et al., J. Appl. Biochem., 4:185-189 (1982). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For PMC virus polypeptides or polynucleotides the location of release may be the stomach, the small intestine (the duodenum, the jejunem, or the ileum), or the large intestine. One skilled in the art has available formulations that will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the complex or by release of the biologically active material beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance, a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic i.e. powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, PMC virus polypeptides or polynucleotides may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, alpha-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall and these can include but are not limited to: stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be

used such as sodium lauryl sulphate, magnesium lauryl sulphate, polyethylene glycol of various molecular weights, and Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the complex during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulphate, dioctyl sodium sulphosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethomium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the complex either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the complex are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The complex could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms i.e., gums. Slowly degenerating matrices may also be incorporated into the formulation. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e. the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film-coated tablet; the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose,

hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Pulmonary Delivery

Also contemplated herein is pulmonary delivery of vaccine composition. The PMC virus polypeptides or polynucleotides may be delivered to the lungs of an animal while inhaling and traverses across the lung epithelial lining to the blood-stream.

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered-dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of the complex. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified proteins may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the complex suspended in water at a concentration of about 0.1 to 25 mg of biologically active protein per ml of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the complex suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing the complex and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation. The protein (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 microns, most preferably 0.5 to 5 microns, for most effective delivery to the distal lung.

Nasal Delivery

Nasal delivery of the vaccine comprising PMC virus polypeptides or polynucleotides is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

THERAPEUTIC COMPOSITIONS

Polypeptide Based Therapies

The PMC virus polypeptides according to present invention also can be used as a prophylactic or therapeutic, which may be utilised for the purpose of stimulating humoral and cell mediated responses in animals, such as swine, thereby providing protection against infection with PMC virus. Natural infection with PMC virus induces circulating antibody titres against PMC virus. Therefore, PMC virus amino acid sequence or parts thereof, have the potential to form the basis of a systemically or orally administered prophylactic or therapeutic to provide protection against PMC.

Thus, the invention provides pharmaceutical compositions comprising a PMC virus polypeptide that enhances the immunocompetence of the host individual and elicits specific immunity against pathogens, preferably PMC virus.

The therapeutic regimens and pharmaceutical compositions of the invention are described elsewhere in the specification. These compositions are believed to have the capacity to prevent the onset and progression of infectious disease such as PMC.

Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Compositions of the invention comprising PMC virus polypeptides may also be combined with suitable components to obtain vaccine compositions. Accordingly, in one embodiment the present invention provides a PMC virus amino acid sequence or fragments thereof described herein in a therapeutically effective amount admixed with a pharmaceutically acceptable carrier, diluent, or excipient.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15%, preferably by at least 50%, more preferably by at least 90%, and most preferably prevent, a clinically significant deficit

in the activity, function and response of the animal host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the animal host or to stimulate by at least about 15%, preferably by at least 50%, more preferably by at least 90%, and most preferably completely, a animal's immune system, causing it to generate an immunological memory against the antigenic determinant.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to an animal. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

In a more specific form of the invention there are provided pharmaceutical compositions comprising therapeutically effective amounts of PMC virus amino acid sequence or an analogue, fragment or derivative product thereof together with pharmaceutically acceptable diluents, preservatives, solubilizes, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength and additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol). The material may be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. See, e.g., Martin, Remington's Pharmaceutical Sciences, 18th Ed. 1990, Mack Publishing Co., Easton, PA, pp 1435-1712 that are herein incorporated by reference. The

compositions may be prepared in liquid form, or may be in dried powder, such as lyophilised form.

The present invention also provides for the use of PMC virus amino acid sequences according to the invention, for manufacture of a medicament for modulation of a disease associated with PMC virus.

Antibody Based Therapeutics

The present invention also provides therapeutic compositions comprising antibodies prepared against the polypeptides of the invention.

The antibodies can be used directly as antiviral agents. To prepare antibodies, a host animal is immunized using one or more PMC virus proteins bound to a carrier as described above for vaccines. The host serum or plasma is collected following an appropriate time interval to provide a composition comprising antibodies reactive with the protein(s) of the virus particle. The gamma globulin fraction or the IgG antibodies can be obtained, for example, by use of saturated ammonium sulfate or DEAE Sephadex, or other techniques known to those skilled in the art. The antibodies are substantially free of many of the adverse side effects which may be associated with other anti-viral agents such as drugs.

Such therapeutic antibody compositions may additionally contain one or more of the additional agents described above in relation to polypeptide therapeutics.

The present invention provides for the use of antibodies against the PMC virus according to the invention, for manufacture of a medicament for modulation of a disease associated with PMC virus.

Polynucleotide base therapy

The present invention further provides therapeutic compositions comprising PMC virus nucleic acid sequences as well as antisense and ribozyme polynucleotide

sequences hybridisable to a polynucleotide sequence encoding a PMC virus amino acid sequence according to the invention.

Polynucleotide sequences encoding antisense constructs or ribozymes for use in therapeutic methods are desirably administered directly as a naked nucleic acid construct. Uptake of naked nucleic acid constructs is enhanced by several known transfection techniques, for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

Alternatively the antisense construct or ribozymes may be combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

Also addressed by the present invention is the use of polynucleotide sequences of the invention, as well as antisense and ribozyme polynucleotide sequences hybridisable to a polynucleotide sequence encoding a PMC virus amino acid sequence according to the invention, for manufacture of a medicament for modulation of a disease associated with PMC virus.

Administration of Therapeutic Compositions

It will be appreciated that therapeutic compositions provided accordingly to the invention may be administered by any means known in the art. Therapeutic compositions may be for administration by injection, or prepared for oral, pulmonary, nasal or other forms of administration. The mode of administration of the therapeutic compositions prepared in accordance with the invention will necessarily depend upon such factors as the stability of the complex under physiological conditions, the intensity of the immune response required etc.

Preferably, the pharmaceutical compositions for administration are administered by injection, orally, or by the pulmonary, or nasal route.

Preferably, the therapeutic compositions are administered using standard procedures, for example, intravenously, subcutaneously, intramuscularly, intraorbitally, ophthalmically, intraventricularly, intracranially, intracapsularly, intraspinally, intracisternally, intraperitoneally, buccal, rectally, vaginally, intranasally, orally or by aerosol administration.

The PMC virus amino acid sequence or antibodies derived there from, or polynucleotide sequences are more preferably delivered by intravenous, intra-arterial, intraperitoneal, intramuscular, or subcutaneous routes of administration. Alternatively, the PMC virus amino acid sequence or antibodies derived there from, properly formulated, can be administered by nasal or oral administration. The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition.

The present invention further provides a method of inducing a protective immune response in an animal or human against a PMC virus comprising the steps of:

a) administering to said animal or human an effective amount of a composition of the invention.

The present invention also provides methods for enhancing an animal's immunocompetence and the activity of its immune effector cells against a PMC virus comprising the step of:

a) administering a composition comprising a therapeutically effective amount of a PMC virus peptide or polypeptide.

Live Vector Delivery Agent

In another aspect of the invention, the PMC virus may be used as a live vector for delivery of recombinant antigens.

Thus, the present invention provides a live vector comprising the PMC virus and a heterologous polynucleotide.

Preferably, the heterlolgous polynucleotide is operably linked to the polyneucletide sequence of the PMC virus, such that expression of the polynucleotide sequence of the PMC virus also leads to expression of the heterologous polynucleotide sequence.

Furthermore, the PMC virus may have one or more sections of autologous polynucleotide sequence removed. Removal of such sequence may preferably render the live virus attentauted in pathogenicity in a host subject.

For example, the PMC virus may be used as a delivery vector to deliver gene sequences that encode a protein from a second infective agent into a subject to be vaccinated against the second infective agent. The second infective agent may be a virus (such as classical swine fever virus), a bacteria, a parasite etc.

Alternatively, the PMC virus may be used as a delivery vector to deliver antigens from some other source. For example, a PMC virus vector may be used to deliver antigenic proteins to a subject to stimulate the subject to make antibodies against the antigenic proteins that may be collected for purposes such as use in diagnostic kits etc.

DRUG SCREENING ASSAYS

The present invention also provides assays that are suitable for identifying substances such as drugs, agents or ligands that bind to PMC virus amino acid sequences. In addition, assays are provided that are suitable for identifying substances that interfere with PMC virus amino acid sequences. Assays are also provided that test the effects of candidate substances identified in preliminary *in vitro* assays on intact cells in whole cell assays.

Thus, the present invention provides a method of screening for drugs comprising the steps of:

a) contacting an agent with a PMC virus amino acid sequence or fragment thereof and

b) assaying for the presence of a complex between the agent and the PMC virus amino acid sequence or fragment.

The present invention also provides a method of screening for ligands of the proteins of the PMC virus comprising the steps of:

a) contacting a ligand with a PMC virus amino acid sequence or fragment thereof and

b) assaying for the presence of a complex between the PMC virus amino acid sequence or fragment and a ligand.

One type of assay for identifying substances such as drugs, agents or ligands that bind to PMC virus amino acid sequences involves contacting a PMC virus amino acid sequence, which is immobilised on a solid support, with a non-immobilised candidate substance and determining whether and/or to what extent the PMC virus amino acid sequences and candidate substance bind to each other. Alternatively, the candidate substance may be immobilised and the PMC virus amino acid sequence non-immobilised.

In a preferred assay method, the PMC virus amino acid sequence is immobilised on beads such as agarose beads. Typically this is achieved by expressing the component as a GST-fusion protein in bacteria, yeast or higher eukaryotic cell lines and purifying the GST-fusion protein from crude cell extracts using glutathione-agarose beads. The binding of the candidate substance to the immobilised PMC virus amino acid sequence is then determined. This type of assay is known in the art as a GST pulldown assay. Again, the candidate

substance may be immobilised and the PMC virus amino acid sequence non-immobilised.

It is also possible to perform this type of assay using different affinity purification systems for immobilising one of the components, for example Ni-NTA agarose and hexahistidine-tagged components.

Binding of the PMC virus amino acid sequence to the candidate substance may be determined by a variety of methods well known in the art. For example, the non-immobilised component may be labelled (with for example, a radioactive label, an epitope tag or an enzyme-antibody conjugate). Alternatively, binding may be determined by immunological detection techniques. For example, the reaction mixture can be Western blotted and the blot probed with an antibody that detects the non-immobilised component. ELISA techniques may also be used.

Candidate substances are typically added to a final concentration of from 1 to 1000 nmol/ml, more preferably from 1 to 100 nmol/ml. In the case of antibodies, the final concentration used is typically from 100 to 500 μg/ml, more preferably from 200 to 300 μg/ml.

In a competitive binding assay the PMC virus amino acid sequence or fragment is typically labelled. Free PMC virus amino acid sequence or fragment is separated from that present in a protein:protein complex, and the amount of free (i.e., uncomplexed) label is a measure of the binding of the agent being tested to the PMC virus amino acid sequence or its interference with PMC virus amino acid sequence:ligand binding, respectively.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the PMC virus amino acid sequence and is described in detail in PCT Application WO 84/03564, published on Sep. 13, 1984. Briefly stated, large numbers of different small peptide test compounds are synthesised on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with PMC virus amino acid sequence and

washed. Bound PMC virus amino acid sequence is then detected by methods well known in the art.

This invention also contemplates the use of competitive drug screening assays in which antibodies capable of specifically binding the PMC virus amino acid sequence compete with a test compound for binding to the PMC virus amino acid sequence or fragments thereof. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants of the PMC virus amino acid sequence.

KITS

In a further embodiment of this invention, kits may be prepared to determine the presence or absence of PMC virus in suspected infected animals and/or to quantitatively measure PMC infection. In accordance with the testing techniques discussed above, one class of such kits will contain at least the labelled PMC virus amino acid sequence or its binding partner, for instance an antibody specific thereto, and directions depending upon the method selected, e.g., "competitive," "sandwich," "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Thus, kits for PMC virus serum immunoassay may be either (a) a sandwich type immunoassay, employing a first anti-PMC virus antibody as capture or detector antibody and a second anti-PMC virus antibody as a detector or capture antibody to complement the first anti-PMC virus antibody, or (b) a competitive type immunoassay, employing a anti-PMC virus antibody with a labelled PMC virus antigen or a PMC virus antigen attached to a solid phase.

Accordingly, a test kit may be prepared for the demonstration of the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled immunochemically reactive component obtained by the direct or indirect attachment of the present PMC

virus amino acid sequence or a specific binding partner thereto, to a detectable label;

(b) other reagents; and

(c) directions for use of said kit.

More specifically, the diagnostic test kit may comprise:

(a) a known amount of the PMC virus amino acid sequence as described above (or a binding partner) generally bound to a solid phase to form an immunosorbent, or in the alternative, bound to a suitable tag, or there are a plural of such end products, etc;

(b) if necessary, other reagents; and

(c) directions for use of said test kit.

In a further variation, the test kit may be prepared and used for the purposes stated above, which operates according to a predetermined protocol (e.g. "competitive," "sandwich," "double antibody," etc.), and comprises:

(a) a labelled component which has been obtained by coupling the PMC virus amino acid sequence to a detectable label;

(b) one or more additional immunochemical reagents of which at least one reagent is a ligand or an immobilized ligand, which ligand is selected from the group consisting of:

(i) a ligand capable of binding with the labelled component (a);

(ii) a ligand capable of binding with a binding partner of the labelled component (a);

(iii) a ligand capable of binding with at least one of the component(s) to be determined; or

(iv) a ligand capable of binding with at least one of the binding partners of at least one of the component(s) to be determined; and

(c) directions for the performance of a protocol for the detection and/or determination of one or more components of an immunochemical reaction between the PMC virus amino acid sequence and a specific binding partner thereto.

Kits to detect Antibodies

The invention also provides diagnostic kits for the in vitro detection of antibodies against the PMC virus, which kits comprise any of the polypeptides identified herein and all the biological and chemical reagents, as well as equipment, necessary for performing diagnostic assays.

Accordingly, the invention provides a kit for demonstrating the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled antibody to the PMC virus;

(b) other reagents; and

(c) directions for use of said kit.

Preferably, the polypeptide used in the kit is an antigenic or epitope bearing polypeptide. Most preferably, the polypeptide is a polypeptide encoding, but not exclusively limited to, the E0, E2, NS2 or NS3 protein.

Preferred kits comprise all reagents required for carrying out ELISA assays. Thus preferred kits will include, in addition to any of said polypeptides, suitable buffers and anti-species immunoglobulins, which anti-species immunoglobulins are labelled either by an immunofluorescent molecule or by an enzyme. In the last

instance, preferred kits also comprise a substrate hydrolysable by the enzyme and providing a signal, particularly modified absorption of a radiation, at least in a determined wavelength, which signal is then indicative of the presence of antibody in the biological fluid to be assayed with said kit. Kits may also include labelled monoclonal or polyclonal antibodies that are directed against PMC virus epitopes and these labelled antibodies may be used to block or compete with antibodies from the test specimen. If the activity of the labelled antibody is blocked, no or a reduced reaction will occur and it can be deduced that the test specimen contains antibodies to PMC virus.

The present invention also relates to a diagnostic kit for use in detecting the presence of PMC virus antibodies, said kit comprising at least one peptide as defined above, with said peptide being preferably bound to a solid support.

The peptide, for example, can be attached to a variety of different solid supports to enable the washing away of unreacted reagents during the course of using the kit. These include: microwells, coated test tubes, coated magnetic particles, wands or sticks, and membranes (nitrocellulose and others).

Preferably, the peptides are attached to specific locations on the solid support. More preferably, the solid support is a membrane strip and said peptides are coupled to the membrane in the form of parallel lines. Preferably, the peptide used in the kit is an antigenic or epitope bearing peptide.

The PMC virus antigens of the present invention will typically be packaged in the form of a kit for use in these immunoassays. The kit will normally contain, in separate containers, the PMC virus antigen, control antibody formulations (positive and/or negative), labelled antibody when the assay format requires the same and signal generating reagents (e.g. enzyme substrate) if the label does not generate a signal directly. The PMC virus antigen may be already bound to a solid support or may be provided separately, with reagents for binding it to the solid support. Instructions (e.g. written, tape, CD-ROM, etc.) for carrying out the assay usually will be included in the kit.

Immunoassays that utilize PMC virus antigens are useful in screening samples (such as blood, serum, plasma, milk, body fluids) to detect if the subject from which the tissue was derived has been exposed to or infected with PMC virus.

The solid support used in the kits of the present invention can include polymeric or glass beads, nitrocellulose, microparticles, microwells of a reaction tray, test tubes and magnetic beads.

The signal generating compound can include an enzyme, a luminescent compound, a fluorophore such as fluorescein, a time-resolved fluorescent probe such as a europium chelate, a chromogen, a radioactive element, a chemiluminescent compound such as an acridinium ester or particles such as colloidal gold, plain latex, or dyed latex. Examples of enzymes include alkaline phosphatase, horseradish peroxidase and beta-galactosidase.

Kits to Detect Polypeptides and Antigens

The present invention further provides a diagnostic kit for use in detecting the presence of PMC virus proteins.

Accordingly, the invention provides a kit for demonstrating the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled polypeptide derived from the PMC virus;

(b) other reagents; and

(c) directions for use of said kit.

Preferably, said antibody is bound to a solid support. The antibody can be attached to a variety of different solid supports to enable the washing away of unreacted reagents during the course of using the kit. These include: microwells, coated test tubes, coated magnetic particles, wands or sticks, and membranes

(nitrocellulose and others). Preferably, the antibodies are attached to specific locations on a solid substrate.

The anti-PMC virus antibody can be attached to the solid support by a variety of means such as passive adsorption, covalent coupling, or by using a solid phase pre-coated with a secondary binder such as protein A, protein G, a secondary antibody specific for the primary antibody, avidin, or an antibody specific for a particular ligand (i.e.: biotin, dinitrophenol, fluorescein, and others). In the case of avidin or any of the ligand specific antibodies, it is necessary to covalently attach the ligand to the anti-PMC virus antibody.

For example, ELISA kits may be used to detect the presence of antigens to PMC virus in a sample to demonstrate that an animal is suffering from PMC or is, for example, a non-symptomatic carrier of the virus.

Preferably, the protein to be detected using the present kit is an antigen or an epitope bearing region of a PMC virus protein. Most preferably, the antibody binds to the E0, E2, NS2 or NS3 protein of PMC.

Kits to Detect Nucleic Acid Sequences

The invention also provides kits for screening animals suspected of being infected with PMC virus, or to confirm that an animal is infected with PMC virus, by detecting PMC virus nucleic acid sequences.

Accordingly, the invention provides a kit for demonstrating the presence of PMC virus comprising:

(a) a predetermined amount of at least one labelled nucleic acid sequence derived from the PMC virus;

(b) other reagents; and

(c) directions for use of said kit.

For example, the polynucleotide sequence may be one or more primers, such as those exemplified above, and the instructions for use may be instructions to perform PCR on RNA or DNA extracted from a tissue sample from a subject.

VECTORS, HOST CELLS ETC

Vectors

The present invention also provides a recombinant expression vector comprising a PMC virus nucleic acid sequence or a part thereof as defined above, operably linked to prokaryotic, eukaryotic or viral transcription and translation control elements.

The invention further relates to the hosts (prokaryotic or eukaryotic cells) which are transformed by the above mentioned vectors and recombinants and which are capable of expressing said RNA and/or DNA fragments.

According to another embodiment the present invention provides methods for preparing a PMC virus amino acid sequence, comprising the steps of:

(a) culturing a host cell containing a vector as described above under conditions that provide for expression of the PMC virus amino acid sequence; and

(b) recovering the expressed PMC virus sequence.

This procedure can also be accompanied by the step of:

(c) subjecting the amino acid sequence to protein purification.

The present invention also relates to a method for the production of a recombinant PMC virus polypeptide, comprising the steps of:

a) transforming an appropriate cellular host with a recombinant vector, in which a PMC virus polynucleotide sequence or a part thereof has been inserted under the control of appropriate regulatory elements,

b) culturing said transformed cellular host under conditions enabling the expression of said insert, and,

c) harvesting said polypeptide.

Vectors provided by the present invention will typically comprise a PMC virus polynucleotide sequence encoding the desired amino acid sequence and preferably transcription and translational regulatory sequences operably linked to the amino acid encoding sequence so as to allow for the expression of the antigenic polypeptide in the cell. Preferably, the vector will include appropriate prokaryotic, eukaryotic or viral promoter sequence followed by the PMC virus nucleotide sequences as defined above. The recombinant vector of the present invention may preferably allow the expression of any one of the PMC virus polypeptides as defined above in a prokaryotic, or eukaryotic host or in living mammals when injected as naked RNA or DNA.

The vector may comprise a plasmid, a cosmid, a phage, or a virus or a transgenic animal. Particularly useful for vaccine development may be BCG or adenoviral vectors, as well as avipox recombinant viruses. Examples of such expression vectors are described in Sambrook *et al.*, (1989) supra or Ausubel *et al.*, (2001) supra. Many useful vectors are known in the art and may be obtained from such vendors as Stratagene, New England Biolabs, Promega Biotech, and others.

It may be desirable to use regulatory control sequences that allow for inducible expression of the antigenic polypeptide, for example in response to the administration of an exogenous molecule. Alternatively, temporal control of expression of the antigenic polypeptide may occur by only introducing the polynucleotide into the cell when it is desired to express the polypeptide.

It may also be convenient to include an N-terminal secretion signal so that the antigenic polypeptide is secreted into the cell medium.

Expression vectors may also include, for example, an origin of replication or autonomously replicating sequence and expression control sequences, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, and mRNA stabilising sequences. Secretion signals may also be included where appropriate, from secreted polypeptides of the same or related species, which allow the protein to cross and/or lodge in cell membranes, and thus attain its functional topology, or to be secreted from the cell. Such vectors may be prepared by means of standard recombinant techniques well known in the art and discussed, for example, in Sambrook *et al.*, (1989) or Ausubel *et al.*, (2001).

An appropriate promoter and other necessary vector sequences will be selected so as to be functional in the host, and may include, when appropriate, those naturally associated with outer membrane lipoprotein genes.

Promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used in prokaryotic hosts. Useful yeast promoters include promoter regions for metallothionein, 3-phosphoglycerate kinase or other glycolytic enzymes such as enolase or glyceraldehyde-3-phosphate dehydrogenase, enzymes responsible for maltose and galactose utilization, and others. Vectors and promoters suitable for use in yeast expression are further described in Hitzeman *et al.*, EP 73,675A. Appropriate non-native mammalian promoters might include the early and late promoters from SV40 or promoters derived from murine Moloney leukaemia virus, avian sarcoma viruses, adenovirus II, bovine papilloma virus or polyoma. In addition, the construct may be joined to an amplifiable gene (e.g., DHFR) so that multiple copies of the gene may be made.

While such expression vectors may replicate autonomously, they may also replicate by being inserted into the genome of the host cell, by methods well known in the art.

Expression and cloning vectors will likely contain a selectable marker, a gene encoding a protein necessary for survival or growth of a host cell transformed with the vector. The presence of this gene ensures growth of only those host cells that express the inserts. Typical selection genes encode proteins that a) confer resistance to antibiotics or other toxic substances, e.g. ampicillin, neomycin, methotrexate, etc.; b) complement auxotrophic deficiencies, or c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*. The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art.

Vectors containing PMC virus polynucleotide sequences can be transcribed *in vitro* and the resulting RNA introduced into the host cell by well-known methods, e.g., by injection, or the vectors can be introduced directly into host cells by methods well known in the art, which vary depending on the type of cellular host, including electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent, such as a retroviral genome); and other methods. The introduction of PMC virus polynucleotide sequences into the host cell may be achieved by any method known in the art, including, *inter alia*, those described above.

In a preferred embodiment, the PMC virus polynucleotide is part of a viral vector, such as a baculovirus vector, or infectious virus, such as a baculovirus. This provides a convenient system since not only can recombinant viral stocks can be maintained and stored until ready for use. Desirably, the nucleotide sequence encoding the antigenic peptide or polypeptides is inserted into a recombinant baculovirus that has been genetically engineered to produce antigenic peptide or polypeptides, for instance, by following the methods of Smith et al (1983) Mol Cell Biol 12: 2156-2165. A number of viral transfer vectors allow more than one

polynucleotide sequence encoding a polypeptide to be inserted into the same vector so that they can be co-expressed by the same recombinant virus.

Host Cells

To produce a cell capable of expressing PMC virus amino acid sequences, preferably polynucleotide sequences of the invention are incorporated into a recombinant vector, which is then introduced into a host prokaryotic or eukaryotic cell.

The invention also provides host cells transformed or transfected with a PMC virus polynucleotide sequence. Preferred host cells include yeast, filamentous fungi, plant cells, insect, amphibian, avian species, bacteria, mammalian cells, and human cells in tissue culture. Illustratively, such host cells are selected from the group consisting of *E. coli, Pseudomonas, Bacillus, Streptomyces,* yeast, CHO, R1.1, B-W, L-M, COS 1. COS 7, BSC1, BSC40, BMT10, and Sf9 cells.

Large quantities of PMC virus polynucleotide sequence of the invention may be prepared by expressing PMC virus polynucleotide sequences or portions thereof in vectors or other expression vehicles in compatible prokaryotic or eukaryotic host cells. The most commonly used prokaryotic hosts are strains of *Escherichia coli*, although other prokaryotes, such as *Bacillus subtilis* or *Pseudomonas* may also be used. Examples of commonly used mammalian host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cells, and WI38, BHK, and COS cell lines, although it will be appreciated by the skilled practitioner that other cell lines may be appropriate.

Also provided are mammalian cells containing a PMC virus polynucleotide sequences modified *in vitro* to permit higher expression of PMC virus amino acid sequence by means of a homologous recombinational event consisting of inserting an expression regulatory sequence in functional proximity to the PMC virus amino acid sequence encoding sequence.

The invention is not limited to the production of one antigenic polypeptide at a time in the host cell. Multiple polynucleotides encoding different antigenic polypeptides of interest may be introduced into the same host cell. The polynucleotides may be part of the same nucleic acid molecule or separate nucleic acid molecules.

GENERAL

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

The entire disclosures of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference. No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

As used herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Examples

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these methods in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

Example 1

Sample preparation

Tissue samples were extracted and prepared using a method whose main basis was derived from Allander et al (2001) "A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species." Proc Natl Acad Sci U S A. 98(20): 11609-14, with some modifications to improve the efficiency from Baugh et al (2001) "Quantitative analysis of mRNA amplification by in vitro transcription." Nucleic Acids Res. 29(5): E29. However, the methods were modified to improve efficiency.

1. Preparation of serum samples:

a) Obtain at least 240 μL of supernatant from a tissue homogenate or serum and divide into 2x 120ul lots

b) To each 120ul of sample add 240ul of PBS or H_2O (or take 50ul sera + 100ul PBS)

c) Filter diluted sample through two separate 0.2um filters by centrifuging at 2000xg (wash top of filter and keep at -20°C)

d) Add 25ul DNASE I (250U) to each tube of filtered sample and incubate at 37°C for 2 hr

e) Add 1ul of RNase Cocktail (500U Rnase A, 20000U Rnase T1) to each tube and incubate at RT for 1 hr.

f) Take 1 tube of treated sample (360ul) for RNA extraction and one tube for DNA extraction (add 500ul DNAeasy AL +50ul proteinase K etc and elute in 50ul water).

2. RNA extraction:

a) Divide sample into 90ul lots and add 600ul RLT, ie 4 x 690ul

b) Homogenize by passing through 21G syringe at least 5X

c) Add 690ul of 70% ethanol to each tube of sample and mix by pipetting

d) Apply 700ul of sample to column at a time and centrifuge for 15sec at 10,000 rpm. Place flow through waste in a 5ml container and keep at -80 °C.

e) Add 700ul of buffer RW1 to the column and centrifuge for 15sec at 10,000 rpm. Discard flow through material and collection tube.

f) Transfer column to a new tube and add 500ul of RPE centrifuge for 15sec at 10,000rpm, discard flow through material

g) Repeat step (f) using same tube but centrifuge for 2min at 10,000 rpm.

h) Transfer column to a new tube and centrifuge for 1min at 10,000 rpm.

i) Elute the RNA in 20ul of RNAse free water, let the water sit on the column for 1 minute before centrifuging. Reuse the eluate and centrifuge for 1min at 10,000rpm to collect any left over RNA on column.

j) Store RNA at -80 °C until needed.

3. DNA extraction:

a) To 360ul of sample add 36ul of proteinase K and 360ul of buffer AL, mix by vortex, incubate at 70°C for 10 minutes.

b) Add 360ul of 100% ethanol, mix by vortexing

c) Pipette mixture from step (b) into DNAeasy column and centrifuge at 8,000rpm for 1 minute. Place flow-through into a tube and store at -80°C.

d) Place column in a new tube and add 500ul of AW1 spin at 8,000rpm for 1 minute. Discard flow through and tube.

e) Place column in a new tube and add 500ul of AW2 and spin at 13,000rpm for 3 minutes. Discard flow through and spin for another 1 minute and discard flow through and tube.

f) Place column in a new tube, add 50ul of water and let sit for 1 minute. Spin at 8,000rpm for 1min and collect eluate. Reapply the 50ul eluate and spin again.

g) Store DNA at -80°C until needed.

RNA Sequence-independent Single Primer Amplification (SISPA) for double stranded RNA viruses

The SISPA method employed was developed from that of Baugh et al and Allander et al, to maximise yield and product length while minimising template-independent side reactions. However, the present method is applied to low yield viral RNA, not total mRNA and a melting step has been added.

4. First strand cDNA synthesis

a) Mix together the following:

1ul random hexamers (10pmol)
8ul – 9ul RNA (in H_2O)

b) Mix, heat 90°C 3minutes, spin and put on ice

c) On ice add:

1st strand buffer	4ul
0.1M dTT	2ul
5mM dNTP	2ul
SSIII (400U)	1ul
T4gene32	1ul

1St strand buffer: 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$

d) Mix, spin and heat at 50 °C for 30 minutes

e) Add another 1ul of SSIII and leave for another 30 min at 50 °C

f) Heat inactivate at 70°C for 10 minutes and then place on ice

5. Second Strand cDNA synthesis

a) On ice mix :

H2O	87ul	
5X 2nd strand buffer	30ul	
5mM dNTPs	6ul	
DNA polymerase (40U)	4ul	
E.coli DNA ligase (10U)	1ul	
RNase H (2U)		2ul
1st strand DNA mix (step 1)		20ul

2nd Strand Buffer: 20mM Tris-HCl (pH 6.9), 4.6mM $MgCl_2$, 90mM KCl, 0.15mM b-NAD+, 10mM $(NH_4)_2SO_4$

b) Mix, spin and incubate at 16°C for 2 hrs. *NOTE: can start DNA SISPA whilst this incubation is underway.*

c) Add 10ul (10U) T4 DNA polymerase (1u/ul) and incubate at 16°C for 15 min.

d) Heat 2nd strand synthesis at 72°C 10 minutes, let cool to 37°C.

6. Clean up DNA

a) Spin phase lock at 13,000 rpm for 30 sec at 4°C

b) Add 150ul of step 2 reaction

c) Add equal volume phenol/chloroform 160ul

d) Shake lightly

e) Spin at 13000rpm 5 minutes, 4°C

f) Transfer upper phase to new tube ~160ul

g) Precipitate DNA add 100% ethanol 2.5V i.e 375ul and 1 ul glycogen (20mg/ml) Leave at -20°C for 2 hrs or O/N

h) Spin at 13000rpm 20 minutes, remove S/N off pellet

i) Wash pellet 1 x 70% ethanol 13000rpm 5 min at 4 °C

j) Take pellet up in 35ul of water *NOTE: can stop here and freeze at -80°C until the DNA SISPA sample is also ready.*

DNA SISPA

7. Second DNA strand synthesis

a) Mix together the following:

DNA	50ul
10pmol random hexamers (10pmol/ul)	1ul
5U 3'-5' exo Klenow fragment DNA polymerase	1ul
Buffer (supplied with Klenow fragment DNA polymerase)	1ul
5mM dNTP	1ul
T4gene32	1ul

b) Leave at 37°C for 1hr

8. Clean up DNA

a) Spin phase lock at 13,000 rpm for 30 sec at 4°C

b) Add 60ul of step 1 reaction

c) Add equal volume phenol/chloroform 60ul

d) Shake lightly

e) Spin at 13,000 rpm 5 minutes, 4°C

f) Transfer upper phase to new tube ~60ul

g) Precipitate DNA add 100% ethanol 2.5V i.e 150ul and 1ul glycogen (20mg/ml) Leave at -20°C for 2 hrs or overnight

h) Spin at 13,000 rpm for 20 minutes, remove supernatant off pellet

i) Wash pellet 1 x 70% ethanol 13,000 rpm 5 min at 4°C

j) Take pellet up in 44ul of water *NOTE: can stop here and freeze at -80°C until the RNA SISPA sample is also ready.*

Generation of Recombinant Nucleic Acid Sequences

9. Restriction Digest

a) Add 10U Csp 6.1 (i.e 1ul of 10U/ul stock) to 35ul of sample, add 4ul of Buffer B and 5ul of Csp6I

b) Incubate at 37°C for 2 hr

c) Inactivate at 65°C for 20 minutes

10. Dephosphorylate digested DNA

a) To inactivated restriction digest (50ul) add:

6ul of 10X CIP dephosphorylation buffer
0.3ul of CIP 18U/ul
3.7ul water

CIP Dephosphorylase buffer1X: 0.05M Tris-HCl, 0.1mM EDTA, pH8.5

b) Incubate at 37°C for 30 minutes

c) Add another 0.3ul of CIP 18U/ul and incubate at 37°C for 30 minutes

11. Clean up DNA

a) Spin phase lock at 13,000 rpm for 30 sec at 4°C

b) Add 60ul dephosphorylated DNA

c) Add equal volume (60ul) phenol/chloroform

d) Shake lightly

e) Spin at 13,000 rpm 5 minutes, 4°C

f) Transfer upper phase to new tube ~50ul

g) Precipitate DNA add 2,5 volumes 100% ethanol (150ul) and 1 ul glycogen (20mg/ml) Leave at -20°C for 2 hrs or overnight

h) Spin at 13,000 rpm 20 minutes, remove supernatant off pellet

i) Wash pellet 1 x 70% ethanol, spin 13,000 rpm 5 min at 4°C

j) Dessicate for 2-3 minutes or air dry for 15 minutes

k) Reconstitute in 5.8ul H_2O.

12. Adaptor Ligation

a) Mix together :

T4 DNA ligase (5U/ul)	1.2ul
5x Ligase Buffer	2ul
50 pmol adaptor (phosphorylated ends)	1ul
DNA from Step 3.	5.8ul

Ligase buffer 5X: 330mM Tris-HCl, 25mM $MgCl_2$, 25mM DTT, 5mM ATP, pH 7.5

b) Incubate 4°C for 1hr and 16°C overnight

13. PCR reaction (results Figure 2)

a) Set up the following mix:

Ligated DNA (step 4)	2ul
50pmol NBam24	1ul
5mM dNTP	2ul
2mM MgCl2	2ul
10X PCR Buffer	5ul
H_2O	38ul

10X PCR buffer: 100 mM Tris-HCl, 500 mM KCl (pH 8.3)

b) Heat at 72°C for 3 minutes

c) Add 0.5ul Taq DNA polymerase (5U/ul)

d) Run cycle:

72°C for 5minutes

(94°C for 1 minute, 72°C for 3 minutes) X 40

hold at 4°C

e) Run 10ul and 40ul of product on 1.0% EtBr gel (leave a well between them to make purification easier)

14. Cloning PCR product

a) Cut out sections of smeared region from gel as a lot of the dominant bands can be contaminating sequence from the products used in the methods, rather than the actual sample. Bands can also be hard to see if they are in the smeared regions.

b) Clean up DNA from agarose using the Minielute Gel Extraction Kit (Qiagen)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colourless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

4. After the gel slice has dissolved completely, check that the colour of the mixture is yellow (similar to Buffer QG without dissolved agarose). Note: If the colour of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The colour of the mixture will turn to yellow.

5. Add 1 gel volume of isopropanol to the sample and mix by inverting the tube several times.

6. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.

7. To bind DNA, apply the sample to the MinElute column, and centrifuge for 1 min.

8. Discard the flow-through and place the MinElute column back in the same collection tube.

9. Add 500 µl of Buffer QG to the spin column and centrifuge for 1 min.

10. Discard the flow-through and place the MinElute column back in the same collection tube.

11. To wash, add 750 µl of Buffer PE to the MinElute column and centrifuge for 1 min.

12. Discard the flow-through and centrifuge the MinElute column for an additional 1 min at ≥10,000 x g (~13,000 rpm).

13. Place the MinElute column into a clean 1.5 ml microcentrifuge tube.

14. To elute DNA, add 10 µl of Buffer EB (10 mM Tris.Cl, pH 8.5) or H_2O to the centre of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.

c) For ligations and cloning use Invitrogen TA Cloning® Kit Version V 7. Set up the 10 µl ligation reaction as follows:

Fresh PCR product	6 µl
10X Ligation Buffer	1 µl
pCR®2.1 vector (25 ng/µl)	2 µl
T4 DNA Ligase (4.0 Weiss units)	1 µl

Incubate the ligation reaction at 14°C overnight, or at -20°C until you are ready for transformation.

d) Transform One Shot® Competent Cells.

1. Centrifuge vials containing the ligation reactions briefly and place them on ice.

2. Thaw on ice one 50 µl vial of frozen One Shot® Competent Cells (enough for 2 ligations).

3. Pipette 2 µl of each ligation reaction into 25ul of competent cells and mix by stirring gently with the pipette tip.

4. Incubate the vials on ice for 30 minutes. Store the remaining ligation mixtures at -20°C.

5. Heat shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the vials to ice.

6. Add 125 µl of room temperature SOC medium to each vial.

7. Shake the vials horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.

8. Spread 50µl to 100 µl from each transformation vial on LB agar plates containing ~ 80mg/ml X-Gal and 100 µg/ml ampicillin.

9. Incubate plates overnight at 37°C. Place plates at 4°C for 2-3 hours to allow for proper colour development.

15. Screening colonies for inserts and sequencing (results Figure 3)

a) Use HotStarTaqMaster Mix (50ul/well of plate):

1X	110X (sufficient for one plate)
25ul HotStarTaqMaster Mix (vortex)	2750ul
12.5ul M13-20f (50pmol)	1375ul
12.5ul M13-20f (50pmol)	1375ul
add 50ul per well of the plate	

To make the M13-20f (50pmol) and M13r (50pmol) stocks : mix 0.5ul of 100uM primer with 12ul of water i.e 500ul of 100uM stock primer + 1200ul water (from HotStarTaq Kit).

b) Place sterile aluminium foil over the plate containing the HotStar TaqMaster Mix. Stab through the foil to make a hole, and then stab a bacterial colony into each well of the plate.

c) Take off aluminium foil and add strip caps to seal plate.

d) Run PCR protocol:

95°C for 15min
(94°C for 30s, 50°C for 30s, 72°C for 1min) X30
72°C for 1min
4°C hold.

e) Run 5-10ul of PCR on gel

f) Use Qiagen Mini elute to clean up the remaining PCR product to sequence.

Example 2

Enzyme Linked Immunosorbent Assay to detect antibodies to PMC virus

1. Clone and express the PMC virus protein of interest (eg E2, NS3) in baculovirus and purify the expressed protein. This purified protein can be used as an antigen to detect specific antibodies to the PMC virus proteins of interest.

2. Coat 'medium binding' 96 well microplates (50 uL per well) with antigen diluted in carbonate buffer (0.05M Carbonate buffer 1x (pH 9.6): Na_2CO_3 (1.59 gm); $NaHCO_3$ (2.93 gm) water to 1L). Hold overnight at room temperature (18-25°C).

3. Dilute samples and controls (Negative, High and Low Positive) 1/100 in sample diluent (phosphate buffered saline (pH 7.3) solution containing 1% skim milk powder and 0.05% Tween 20).

4. Wash plates 5 times with PBS-Tween and tap to dry.

5. Transfer diluted samples and controls to the ELISA plate in duplicate: 50 uL to each well.

6. Incubate at 37°C for 1 hr in a humidified container.

7. Wash plates 5 times with PBS-Tween, rotate and wash 5 more times, then tap to dry.

8. Dilute conjugate (antiporcine IgG, horseradish peroxidase conjugated) in sample diluent and add 50 uL to each well.

9. Incubate at 37°C for 1 hr in a humidified container.

10. Wash plates 10 times with PBS-Tween, then 5 times with purified water.

11. Develop by adding 100 uL of TMB substrate to each well. Incubate at 37°C in the dark for about 10 min until target OD is achieved for controls. A commercially available TMB substrate can be used (eg. Boehringer Mannheim Corp., Pierce Chemical Co., and Kirkegaard & Perry Laboratories).

12. Stop by adding 100 uL of 1M sulphuric acid.

13. Read OD values at 450nm.

14. Calculate results.

Example 3

Enzyme Linked Immunosorbent Assay to detect antigens of PMC virus

It should be noted that working solutions of the detector reagent and enzyme conjugate reagents should be made within approximately 1 hour of anticipated use and then stored at 4^0 C.

- Materials

ELISA Wash Buffer--10X concentrate: 1 M Tris; HCl (6.25 Normal) for pH adjustment; 0.01% Thimerosal; and 5% Tween 20.

Detector Reagent--10X concentrate: 25% Ethylene Glycol, 0.01% Thimerosal, approximately 5% biotinylated goat anti-PMC virus antibody, and 0.06% yellow food colouring in PBS (pH 7.4). The working Detector Reagent is prepared by mixing 1 part of the Detector Reagent--10X concentrate, 1 part of NSB Reagent 10X concentrate, and 8 parts of Reagent Diluent Buffer. This working agent should be prepared within approximately 1 hour of anticipated use.

NSB Reagent--10X concentrate: 25% Ethylene Glycol, 0.01% Thimerosal, 0.2% Mouse IgG, 0.06% red food colouring in PBS (pH 7.4).

Reagent Diluent Buffer: 2.5% Bovine Serum Albumin, 0.01% Thimerosal, and 1.0% bovine gamma globulin in PBS (pH 7.4).

Enzyme Conjugate Reagent--10X concentrate: 25% Ethylene Glycol, 0.01% Thimerosal, streptavidin-biotinylated horseradish peroxidase complex (dilution approximately 1 to 700), 0.1% rabbit albumin, and 0.02% rabbit gamma globulin in PBS (pH7.4). Working Enzyme Conjugate Reagent should be prepared by mixing 1 part of Enzyme Conjugate Reagent--10X concentrate, 1 part of NSB Reagent--10X concentrate, and 8 parts of Reagent Diluent buffer. This working reagent should be prepared within approximately 1 hour of anticipated use.

Negative Control: 1% Igepal, and 0.01% Thimerosal in PBS (pH 7.4).

Positive Control: 1% Igepal, 0.01% Thimerosal, 1% Bovine Serum Albumin , PMC virus culture (dilution approximately 1:20) and 50 μM phenyl methyl sulfonyl fluoride in PBS (pH7.4).

- Method

1. Prepare specimens by standard methods. For samples containing cells (tissues, white blood cells) homogenise the tissue and add sample lysis buffer (1% NP40). Allow at least 1 hour for antigen extraction and mix continually.

2. Clarify specimens by centrifuging for 15 minutes at approximately 2000g;

3. Coat 96 well microplates with purified polyclonal antiserum raised against PMC virus antigens (100uL/well). Alternatively, a mixture of anti-PMC virus monoclonal antibodies may be used. Each 96-well tray is coated overnight at room temperature with 0.1 ml per well of a solution containing purified antibody at 5 μg/ml and bovine serum albumin at 10 μg/ml in carbonate buffer (pH9.6). Following the coating, each tray is washed three times with ELISA wash buffer and allowed to dry overnight at 4^0 C. A foil pouch is used to encase each tray after drying, and a desiccant is included inside each pouch to remove moisture.

4. Wash ELISA plates 3 times by pipetting 0.2 ml of ELISA Wash Buffer into each well and tap or pipette dry prior to the addition of sample.

5. Block ELISA plates with Blocking solution 1 (200uL/well) for 30 min at 37°C in a humidified container.

6. Transfer 100uL of each specimen (including controls) to the ELISA plate;

7. Incubate plates for 60 min at 37°C in a humidified container;

8. Wash ELISA plates 5 times with ELISA Wash Solution;

9. Block ELISA plates with Blocking Solution 2 (150uL) for 30 min at 37°C in a humidified container;

10 Wash ELISA plates 5 times;

11. Add Detector Reagent containing biotinylated anti-PMC virus monoclonal antibody (100uL) to all wells;

12 Incubate plates for 60 min at 37°C in a humidified container;

13 Wash plates 5 times;

14. Add Enzyme Conjugate Reagent containing streptavidin-biotinylated horseradish peroxidase complex and add 100uL to all wells;

15. Incubate plates for 30 min at 37°C in a humidified container;

16. Wash plates 10 times;

17. Prepare and add 100uL of TMB substrate solution to all wells. A commercially available TMB substrate may be used (eg. Boehringer Mannheim Corp, Pierce Chemical Co, and Kirkegaard & Perry Laboratories).

18. Incubate plates for approx 10 min at room temperature in the dark;

19. Stop reaction with 1M sulphuric acid (100uL per well);

20. Read ODs on ELISA plate reader at 450nm;

21. Calculate results.

Example 4

Detection of PMC virus RNA by reverse transcriptase (RT) polymerase chain reaction (PCR)

a) Extract RNA from the test specimen as described in Example 1. Include in all steps of the reactions known positive and negative controls and a 'blank'.

b) Reverse transcribe (RT) the RNA as follows:

1. Mix together the following:

random hexamers (50pmol)	1ul
RNA (in H2O)	9ul

2. Heat at 90°C for 3 minutes, spin and put on ice

3. On ice add:

1st strand buffer	4ul
0.1M dTT	2ul
5mM dNTP	2ul
SSIII (200U)	1ul

4. Mix, spin heat at 45°C for 60 minutes.

5. Heat inactivate at 70°C for 10 minutes

6. Place on ice.

c) Set up 1st round PCR

1. Mix together the following PCR reagents

RT	5ul
Forward primer 4uM	1ul
Reverse primer 4uM	1ul
Hotstart PCR mix (Qiagen)	12.5ul
Water	5.5ul

(see Table 3 for 1st reaction PCR primers)

2. Cycle the PCR machine at:

95°C for 15 minutes
(94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min) x 40
72°C for 1 min
4°C hold

d) Set up Nested PCR

1. Mix together the following PCR reagents:

1st PCR product	1ul
Forward nested primer 20uM	1ul
Reverse nested primer 20uM	1ul
Hotstart PCR mix (Qiagen)	12.5ul
Water	9.5ul

(see Table 3 for nested PCR primers. If no nested primer is listed, use 1st PCR primer)

2. Cycle the PCR machine at

95°C for 15 minutes
(94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min) x 25
72°C for 1 min
4°C hold

e) Run 5ul of nested PCR product on a 1.5% ethidium bromide gel for 1 hour. Depending on the primers used, the expected size of the product is as listed in Table 1.

Table 3. Primers for PCR detection of PMC virus

Clone	Virus	*Primer name	Primer Sequence (5' to 3')	Nested Product size
CR3 9	Pestivirus	CR39F (63) CR39R (190) CR39FN (87)	CACATCTAGCAGCAGACTATGA GTACCAGTTGCACCACCC TGAAAAGGATTCACGG	103bp
ER5 10	Pestivirus	ER510F (7) ER510R (213) ER510FN (68) ER510RN (182)	AAACCGACGAAGTAGACC AGACGAGAACATAGTGGC GAAACAGTAAAGCCAACG CTGGTAATCGGAAACATC	114bp
ER6 2	Pestivirus	ER62F (203) ER62FN (373) ER62R (637) ER62RN (516)	GGGACCGAGGGATACGA AGAGGTAATTGGGTAT CAGCAGGTTGATTTCTTCAT TTGCCAAGTTTCAC	98bp
ER5 5	Pestivirus	ER55F (31) ER55R (214) ER55FN (64) ER55RN (162)	AAACCGCCGAAGTAAACC CTGGAGCCCTGGTAATGG GACGGGAATGGGTTCA TAGGTGCTTCTTATTGGTAT	143bp

*F = forward primer, R = reverse primer, FN = forward nested primer, RN = reverse nested primer

Example 5

Determination of Full length viral sequence

Once the authenticity of the presence of PMC virus sequence has been confirmed in a sample by PCR, the entire viral sequence can be acquired by designing PCR primers to span the gaps between the clones (refer to Table 4). RT-PCR was carried out as either a two step (RT then PCR) or one step RT-PCR reaction.

1. RT reaction:

a) Mix together the following:

1ul random hexamers (50pmol)
4ul RNA
4ul Rnase free water

b) Heat 70°C 10 minutes, spin and put on ice

c) On ice add

4ul 1st strand buffer

2ul 0.1M dTT
2ul 5mM dNTP
2ul SSIII (400U)

d) Mix, spin heat at 42°C for 60 minutes.

e) Heat inactivate at 70°C for 10 minutes

f) Place on ice

2. PCR Reaction:

a) Mix together the following PCR reagents

RT	1ul
Forward primer 20uM	1ul
Reverse primer 20uM	1ul
Hotstart PCR mix (Qiagen)	12.5ul
Water	13.5ul

(see Table 4 for PCR primers)

b) Cycle the PCR machine at:

95°C for 15 minutes
(94°C for 30 sec, 47°C for 30 sec, 72°C for 2 min) x 40
72°C for 1 min
4°C - hold

3. One Step RT-PCR Method

a) Mix together the following reagents from the SSIII RT-PCR Kit

2x reaction mix	25ul
Forward primer 30uM	1ul
Reverse primer 30uM	1ul
SSIII RT/Platinum mix	2ul for products 2.5kb or less 4ul for products 2.5kb or more
Water	15.8ul for products 2.5kb or less 13.8ul for products 2.5kb or more

(see Table 4 for PCR primers)

b) Cycle the PCR machine at:

50°C for 50 minutes
94°C for 2min
(94°C for 15 sec, 50°C for 30 sec, 68°C for 1min/kb)x40
68°C for 5 min
4°C - hold

RT-PCR product of interest was PCR spin cleaned and cloned into the Invitrogen TA cloning vector PCR2.1 (see Example 1). Positive clones were then identified and sent for sequencing, as described in Example 1.

The primers used for sequencing were M13r, m13-20, primers in Table 4 and primers designed specifically for sequencing (see Table 5).

Plasmid sequence, PCR primers and poor sequence reads were removed from the sequence before being used in the program Bioedit (Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.). Bioedit allowed the construction of contigs and the production of the full length consensus sequence for the virus.

Table 4. Primers designed to PCR the gaps between the SISPA clones sequences

Region to PCR	*Primer names	Primer Sequence (5' to 3')	Product size
5'UTR-Erns	JFP1F JFRR3R	CATGCCCATAGTAGGAC ACCAGTTRCACCAMCCAT	1338bp
Erns-P7	CR39-Er55PCRF ER55-510-512 R	AGGGCTCTCACATGGTTGTC CCATTACCAGGGCTCCAG	1810bp
Erns-NS5A	CR39F(63) ER55RN(162)	CACATCTAGCAGCAGACTATGA TAGGTGCTTCTTATTGGTAT	2349bp
P7-NS5A	ER55-510-512 F CR316-CR24R	CGTTGGCTTTACTGTTTCATTG TCCCCGAAGCTTGGTTTAAT	5560bp
NS3-NS5A	NS3F CR316-CR24R	GTCAGGCCTGCCTATCTTTG TCCCCGAAGCTTGGTTTAAT	4431bp
NS5A-NS5B	CR316-CR24F ER62-ER63R	CGGGACCATTAAACCAAGC CAGGGGGTTCCAAGAATACA	2440bp

* F = forward primer, R= reverse primer

Table 5. Primers designed for sequencing

Protein location of primer	*Primer names	Primer Sequence (5' to 3')
5 UTR	5utr (140)R	GGTGTACTCACCGCTTAGCC
NPRO	NPRO(630)RS	TTGCTACAATCGCCCTTCTT
NPRO	NPRO(779)FS	AGGGAGAATGACAGGGTCTG
Capsid	capsid(927)FS	ACAAAGGAGCAAAACCCAAG
ERNS	EO(1365)RS	GTCACGTTGGTGGACCCTAC
E1	E1(2402)RS	AGCCAGAAATGCCACAGC
E1	E1(2606)FS	ACCTGTGTGGGTGCTAACAT
E2	E2(3086)RS	TTACTTTGTCTTCCCGTTGC
NS2	NS2(4409)FS	CCAAGAAACTTCCCCATACG
NS2	ns2(4460)RS	TTCCACATCCTCTTTCTTCTTTT
NS3	NS3(5170)RS	GCTGGCCCTCGAATGATCCA
NS3	NS3(5468)FS	GTTCCCTGTGTCCTTGCTGA
NS3	NS3(5670)RS	TGTTTTTGTCTTGGCACTGG
NS3	NS3(6296)FS	GAGCACAACAGGGCAGAAAT
NS3	NS3(6479)RS	CCATCTTCCTTGTAGGCACA
NS3	NS3F(6525)F	GTCAGGCCTGCCTATCTTTG
NS3	NS3(7153)FS	GGAGAAGTCACTGACGCACA
NS3	NS3(7241)RS	GCCATTTCAATCCCAGTATG
NS4B	NS4B(7715)FS	GGGGTCCACACAGCATTGTA
NS4B	NS4B (7893)RS	CCCTTGATACTCACGCCTGT
NS4B	NS4B(8532)FS	GCCGACTCAAAATGGAGAAA
NS5A	NS5A(8810)RS	GCCACCCTATTCTTGGATCTC
NS5B	NS5B(10889)FS	AAATGAGAAGAGGGCAGTGG
NS5B	NS5BF-10936	AAGGCCACCACTCAAATCAC
NS5B	NS5BR-12039	AGGCTTCTGCTTGACCCAGT

* FS = forward primer, RS= reverse primer

NOTE: Numbers in brackets are estimated locations on Reference pestivirus strain NADL.

Example 6

UTR Sequences

5'RACE and 3'Race were used to aquire the 5'UTR and 3'UTR sequences.

1. 5' RACE Method

Sequence data from the complete 5' untranslated region (UTR) was generated using rapid amplification of cDNA ends (RACE, BD), as described by BD Biosciences Clonetech with the following modifications. PMC virus -specific primer CR24R (5'TCCCCGAAGCTTGGTTTAAT 3') was used to generate the cDNA. Hotstart PCR (Qiagen) was carried out with primers CR39R (Table 3) and BD Universal primer A mix (5'CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT3' and 5'CTAATACGACTCACTATAGGGC3') with an annealing temperature of 67°C and extension time of 2minutes. The PMC virus specific primer N^{Pro}(630)RS (Table 5) and BD nested Universal Primer A (5'AAGCAGTGGTATCAACGCAGAT3') were used for the Hotstart Nested PCR, with an annealing temperature of 55 °C and an extension time 2 minutes. Nested PCR products were cleaned, cloned and sequenced.

2. 3' RACE Method

Sequence data from the complete 3' untranslated region was generated by first adding a poly (A) tail to the viral RNA, using Epicentre's A-Plus Ploy(A) polymerase tailing Kit for 8 minutes. This was followed by rapid amplification of cDNA ends (RACE, BD), as described by BD Biosciences Clonetech with the following modifications. Hotstart PCR (Qiagen) was carried out with primers ER62F (Table 3) and BD Universal primer A mix (5'CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT3' and 5'CTAATACGACTCACTATAGGGC3') with an annealing temperature of 65°C and extension time of 2minutes. The PMC virus specific primer NS5B(12100)F (Table 5) and BD nested Universal Primer A

(5'AAGCAGTGGTATCAACGCAGAT3') were used for the Hotstart Nested PCR, with an annealing temperature of 65 °C and an extension time 2 minutes. Nested PCR products were cleaned, cloned and sequenced.

Example 7

Real Time PCR

The following primers and a matching probe based on Taqman® technology were developed:

Forward primer: CAGTTGGTGTGATCCATGATCCT

Reverse primer: GGCCTCACCCTGCAACTTT

Probe: 6FAMAAGTCTTCAGCAGTTAACTMGBNFQ

Similar primer/probe combinations may be developed for other segments of the PMC genome.

A Real Time PCR assay was carried out using the following steps:

a) Extract RNA from the test specimen. Include in all steps of the reactions known positive and negative controls and a 'blank'.

b) Prepare reaction mixture (volumes per sample) as follows:

2x Mastermix (Roche)	12.5uL
40x Multiscribe	0.625uL
Forward primer	1uL
Reverse Primer	1uL
Taqman Probe	1uL
Template (sample)	2uL
Water	6.875uL

c) Set up cycling conditions for the PCR cycler available (the cycles below are appropriate for a Cepheid Smartcycler)

Cycle the PCR machine at:

Stage 1: Repeat 1x

48°C for 30 min

95°C for 10 min

Stage 2: Repeat 45x

95°C for 15 secs

58°C for 30 secs each

d) Determine results using the Smartcycler software using cycle-threshold (CT) values. A CT value of <35 is considered to be positive. Values between 35-40 are suspicious and values >40 are negative.

Example 8

Production of recombinant baculoviruses and expression of recombinant PMC virus proteins

1. Cloning of PCR fragments

PCR products are purified with PCR SPINCLEAN™ columns (Progen Industries, Limited), according to the manufacturer's instructions. If the PCR reaction produces non-specific bands in addition to the required product, or subcloning from another plasmid was necessary, the DNA can be further purified by elution from a 0.8% agarose gel, using a modification of the method described by Heery (1990).

Purified PCR fragments are digested and ligated into pBlueBacHis A, B or C baculovirus transfer vectors (MaxBac Baculovirus Expression System, Invitrogen Corporation) containing compatible cohesive overhangs, using standard cloning protocols (Sambrook et al., 1989; Current Protocols in Molecular Biology, 1991).

A, B or C vectors provide three different reading frames to achieve protein expression in the baculovirus expression system.

2. Transformation of baculovirus plasmids with the PCR fragments

The ligations are transformed into competent *E. coli* strain Top 10 (Invitrogen Corporation), Genotype: F⁻mcrA D(mrr-hsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL endA1 nupG, and/or Sure® *E. coli* (Stratagene), Genotype : e14⁻(McrA⁻)D (mcrCB-hsdSMR-mrr) 171 endA1 supE44 thi-1 gyrA96 rel A1 lac recB recJ sbcc umuc::Tn5 (kan^r) uurC[F' proAB lacI^{q}Z D m15 Tn10(Tet^r)]^c. Protocols for the preparation of competent cells and transformation of the bacteria are taken from the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8.

Screening bacterial clones for plasmid containing PCR fragment and plasmid purification for transfection

Bacterial clones containing pBlueBacHis + PCR fragment are identified by growing colonies, extracting the plasmids using the boiling miniprep method described in Sambrook, et al. (1989), and then undertaking restriction digests of the plasmids to verify those containing the correct-sized insert. Recombinant plasmids are purified to a level suitable for transfection reactions using plasmid purification kits (QIAGEN Pty Ltd., tip-20 or tip-100 columns), according to the manufacturer's instructions.

3. Production of purified recombinant baculoviruses by Cationic liposome transfection of Sf9 cells to produce recombinant baculoviruses

Recombinant baculoviruses are produced by co-transfecting linearised wild-type Autographa californica nuclear polyhedrosis virus (AcMNPV) DNA and baculovirus transfer vector containing PCR fragment into Sf9 cells, by the technique of cationic liposome mediated transfection. This is carried out according to the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8.

4. Plaque purifying recombinant baculoviruses

Recombinant virus is plaque purified three times before virus master stocks are prepared, ensuring the virus is cloned from a single particle and no wild-type virus is present. Plaque assays are set up according the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8.

After each round of plaque purification, the recombinant viruses are screened using a modified Pestivirus antigen-capture ELISA (PACE) (Shannon et al., 1991). The modified method involves supernatant + cells (50 µl/well) being added directly to a blocked, washed ELISA plate, and the plate incubated for 1 hr at 37°C. Antibody solution (50 µl/well) is then added. The antibody used is either biotinylated goat anti-pestivirus antiserum or individual anti-PMC virus monoclonal antibodies (mAbs). The plate is incubated overnight at 22°C, then developed as described by Shannon et al. (1991), omitting the incubation with biotinylated anti-mouse IgG for samples that are reacted with the biotinylated goat antiserum.

5. Recombinant baculovirus master, seed and working stocks

The master virus stock for each of the recombinant baculoviruses constructed are made according the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8. The titre of the stock is determined by a plaque assay, as described above, except that the cells are overlaid with 1.5% carboxymethylcellulose (CMC, BDH; 6% CMC in deionised water, diluted 1 in 4 with complete TC100 + X-gal [125µg/ml, Boehringer Mannheim]). After 7 days, the blue plaques are counted to give the virus titre.

The seed and working stock are made from the master and seed stock, respectively using a low MOI of 0.1 to 0.5pfu/ml. All virus stocks are stored at 4°C for use in vaccine production. For long term storage of Master, Seed and Working stocks, each recombinant virus is ampouled and frozen at -80°C.

6. Optimisation of recombinant protein production

Sf9 insect-cell suspensions, adapted to Sf-900 II Serum Free Media according to the protocol described by Gibco BRL (1995), are used to optimise recombinant protein expression. Two conical flasks, containing 50ml cells ($1.5x10^6$ cells per ml), are infected with recombinant baculovirus at a high and low MOI, between 0.1 and 5.0. A third flask acts as an uninfected control culture. The 3 flasks are incubated with shaking at 28°C, and 5ml aliquots removed at 24 hr intervals for up to 7 days.

The samples are centrifuged at room temperature (RT) for 10 min at 900 x g, and the supernatants carefully removed. The pellets and supernatants are stored at -20°C until daily sampling is completed. The amount of specific, recombinant pestivirus protein in the samples is then determined using the modified PACE described above. The cell pellets are reconstituted in 200µl or 250µl NP-40 (1% [v/v] in PBS), vortexed and centrifuged at RT for 10 min at 900 x g. Serial dilutions of the pellet extract (in 1% [v/v] NP40) are assayed. The culture supernatants are assayed undiluted, as well as serially diluted (in 1% [v/v] NP40). If cell viability is reduced at a higher rate of infection, then an MOI or 0.1 to 2 is more appropriate.

Modifications of the above-described modes of carrying out the various embodiments of this invention will be apparent to those skilled in the art based on the above teachings related to the disclosed invention. The above embodiments of the invention are merely exemplary and should not be construed to be in any way limiting.

We Claim:

1) An isolated PMC virus RNA or DNA molecule that binds PMC nucleic acid under stringent hybridization conditions characterized in that said isolated PMC virus RNA or DNA molecule consists of:
   a) SEQ ID NO: 6, wherein when the isolated RNA or DNA molecule is an RNA molecule, thymidine (t) nucleotides are substituted with uridine (u) nucleotides;
   b) an RNA or DNA molecule having at least 90% sequence identity over at least 100 contiguous nucleotides with the nucleotide sequence set forth in SEQ ID NO: 6; or
   c) an RNA or DNA molecule comprising the complement of the nucleotide sequence of (a), or (b).

2) A method for detecting the presence or absence of PMC virus nucleic acids in a biological sample, the method including the steps of:
   a) bringing the biological sample containing nucleic acid into contact with a polynucleotide probe or primer comprising an isolated RNA or DNA molecule according to claim 1, under stringent hybridisation conditions; and
   b) detecting any duplex formed between the probe or primer and nucleic acids in the sample.

3) A method for the detection of PMC virus nucleic acids present in a biological sample, the method including the steps of:
   a) amplifying the nucleic acids with at least one primer comprising an isolated RNA or DNA molecule according to claim 1, and
   b) detecting the amplified nucleotide sequences.

4) A method for the detection of PMC virus nucleic acids present in a biological sample, the method including the steps of:
   a) hybridizing the nucleic acids of the biological sample under stringent hybridisation conditions with one or more probes comprising an isolated RNA or DNA molecule according to claim 1,
   b) washing under appropriate conditions, and
   c) detecting the hybrids formed.

5) A method for screening the tissue of subjects for PMC virus nucleic acids, the method comprising the steps of:
   a) extracting DNA from tissue ex vivo;
   b) restriction enzyme cleavage of said DNA;
   c) electrophoresis of the fragments; and
   d) Southern blotting of genomic DNA from tissues and subsequent hybridization under stringent hybridization conditions with a labeled cloned DNA molecule according to claim 1.

Figure 1

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gtataacgac agtagttcaa gtgtcgttat gcatcattgg ccataacaaa ttatctaatt      60
tggaataggg acctgcgacc tgtacgaagg ccgagcgtcg gtagccattc cgactagtag     120
gactagtaca aataggtcaa ctggttgagc aggtgagtgt gctgcagcgg ctaagcggtg     180
agtacaccgt attcgtcaac aggtgctact ggaaaggatc acccactagc gatgcctgtg     240
tggacgagga catgtccaag ccaatgttat cagtagcggg ggtcgttact gagaaagctg     300
cccagaatgg gtagttgcac atacagtctg ataggatgcc ggcggatgcc ctgtattttg     360
accagtataa atattatccg ttgtaaagca tatgaatact tttactttta atacatatgg     420
agggagtgag gaaggaaaca tgttctttag aactgcaccc acgccgccac cagggtgcca     480
agaaccggtt tacacaagca caatgagacc aatttttggc gaaccccatc cacctctaca     540
caaacacagc acgttaaaat tgccacattg gaggggggatc aaaacaatta gagttaagaa     600
gagagaattg ccaaagaagg gcgattgtag caactcaaca acagctccca cttcgggggt     660
gtacgttgaa ttaggggctg tgttctataa agattacacg ggcacggtat accatcgtgt     720
accgctagaa ctttgtacaa accaagagag gtgcgaggga tccaagtgtg tagggagaat     780
gacagggtct gatggcaggt tgtacaacgt tttagtatgt ccggacgatt gtatcctctt     840
tgagagacac tgtagaggtc aaacagtcgt cctgaaatgg atttccaacc ccttgacatc     900
accactttgg gtccagagtt gttctgacga caaaggagca aaacccaagg tgaaaccaaa     960
agacgacagg atgaagcaag gaaaaatagt gacaaagcct aaagagactg aagcagatca    1020
aaaaactaga ccaccagatg ccacgatagt ggttgacggg cagaagtatc aggtgaggaa    1080
gaaggggaaa gcgaaaccca agactcaaga cggcttatac cacaacaaga acaaaccaga    1140
agcgtccagg aagaagcttg agaaggcctt gctagcatgg gcaatattag cctgcctatt    1200
ggtggtaccg gtagggtcca ccaacgtgac acaatggaac ttatgggaca ataaaagtac    1260
tacagacata catagcgtca tgttttctag agggattaaa aggagtctgc atggaatttg    1320
gcccacacaa atctgcaaag ggatccctac acatctagca gcagactatg aactgaaaag    1380
gattcacggg atggtggatg caagccccat gaccaacttc acatgttgta ggctacagag    1440
acatgagtgg aacaagcatg ggtggtgcaa ctggtacaat atagagccgt ggatcaatct    1500
catgaataat acccaaggac tattaaacac tggagacaat ttcactgagt gcgcagtcac    1560
atgcaggtat gatgcagact taggggtgaa tatagtgact caagccagga ctactccaac    1620
tatcctgact ggctgtaaga aagggcacaa cttctctttc tcaggggagg tcagggcctc    1680
accctgcaac tttgagttaa ctgctgaaga cttgctcagg atcatggatc acaccaactg    1740
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Figure 2

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ITKKGSVYTI NLSTWWESER LASLEPLFRE LLSKCRPVDR ETYKNCHFAT AAQLAGGNWV    3240
PVAPVVHLGE IPVKKKKTLP YEAYKLLKEM VDSEKEFHKP VSREKHQWIL NKVKTGGDLG    3300
LKNLVCPGRV GEPILREKKK FNIYNKRITS TMLSVGIRPE KLPVVRAQTS TKEFHEAIRD    3360
KIDKKANTQT PGLHKELLEI FNSICAIPEL RNTYKEVDWD VLTSGINRKG AAGYFEKMNI    3420
GEIIDSDKKS VEQLIKRMKS GLEFNYYETA IPKNEKRAVV DDWMEGDYVE EKRPRVIQYP    3480
```

EAKMRLAITK VMYNWVKQKP IVIPGYEGKT PLFHVFDKVH KEWKNFNSPV AVSFDTKAWD 3540
TQVTPKDLLL ISEIQKYYYK KEYHRFIDNL TEKMVEVPVV CEDGNVYIRE GQRGSGQPDT 3600
SAGNSMLNVL TMIYAFCKAN SIPYSAFHRV AKIHVCGDDG FLITEKSFGE AFAIKGPQIL 3660
MEAGKPQKLI GEFGLKLAYK FDDIEFCSHT PIKVRWADNN TSYMPGRDTA TILAKMATRL 3720
DSSGERGTEG YELAVAFSFL LMYSWNPLVR RICLLVMSTI DTKEASQNNT IYTFRGDPIG 3780
AYTEVIGYRL DQLKQTEFSK LAQLNLSMAI LQIYNKNTTK RLIEDCVKLG NQNKQILVNA 3840
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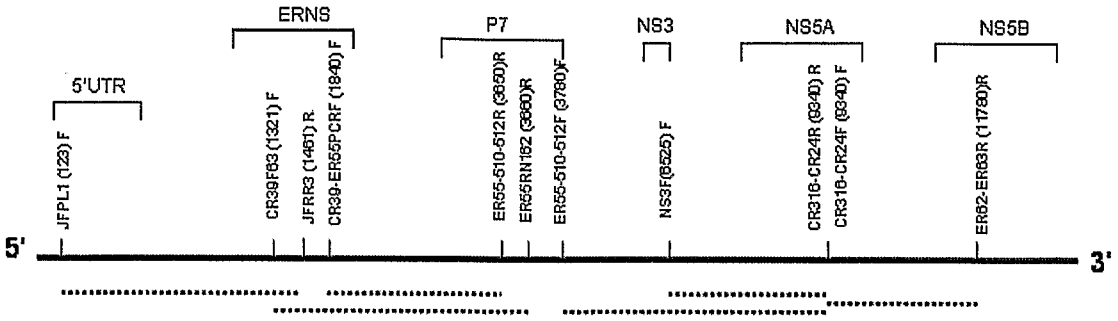
Figure 3



Keylist :

PCR Product

NADL Reference Sequence gi 9626849

.....{...}F or R Primers (...) indicates bp locations based on NADL sequence
R indicates reverse primer
F indicates forward primer

Figure 4

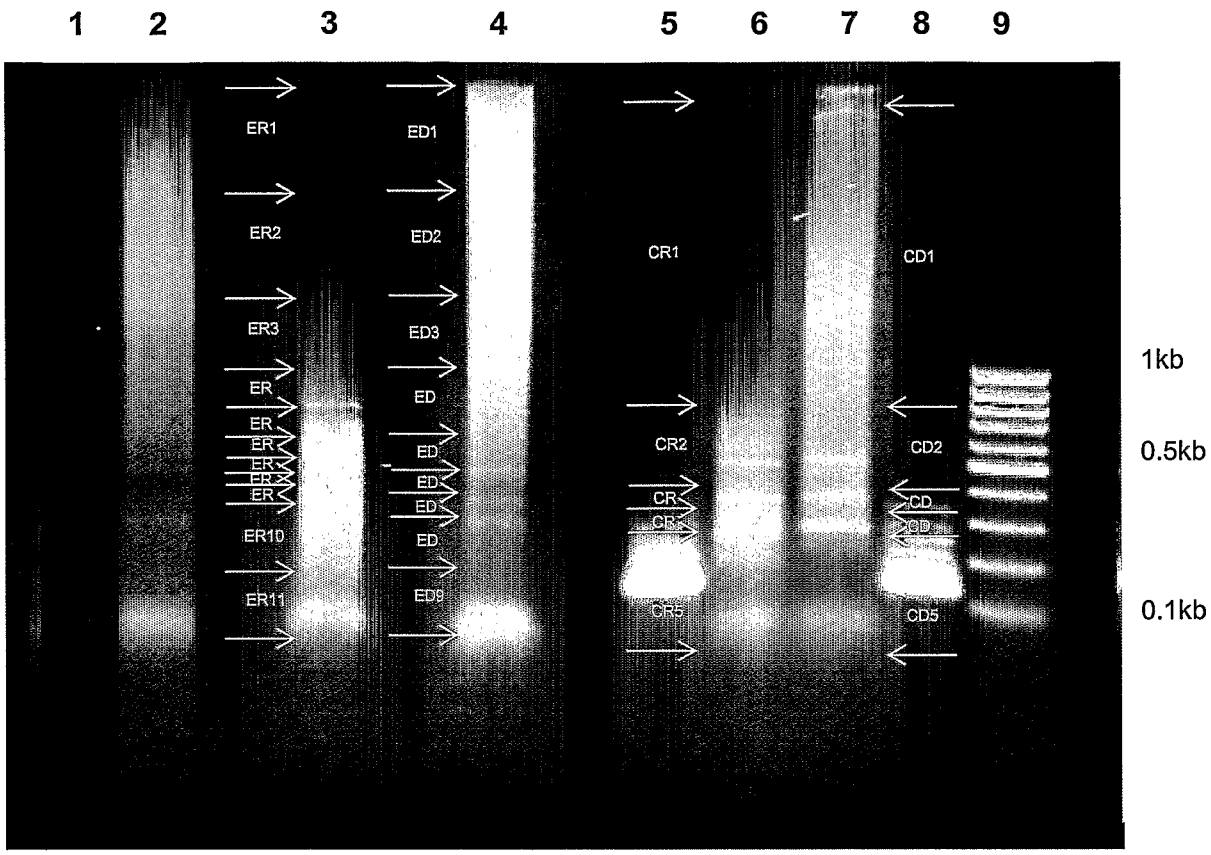

1
2
3
4
5
6
7
8
9
ER1
ER2
ER3
ER
ER
ER
ER
ER
ER
ER10
ER11
ED1
ED2
ED3
ED
ED
ED
ED
ED
ED9
CR1
CR2
CR
CR
CR5
CD1
CD2
CD
CD
CD5
1kb
0.5kb
0.1kb

Figure 5A

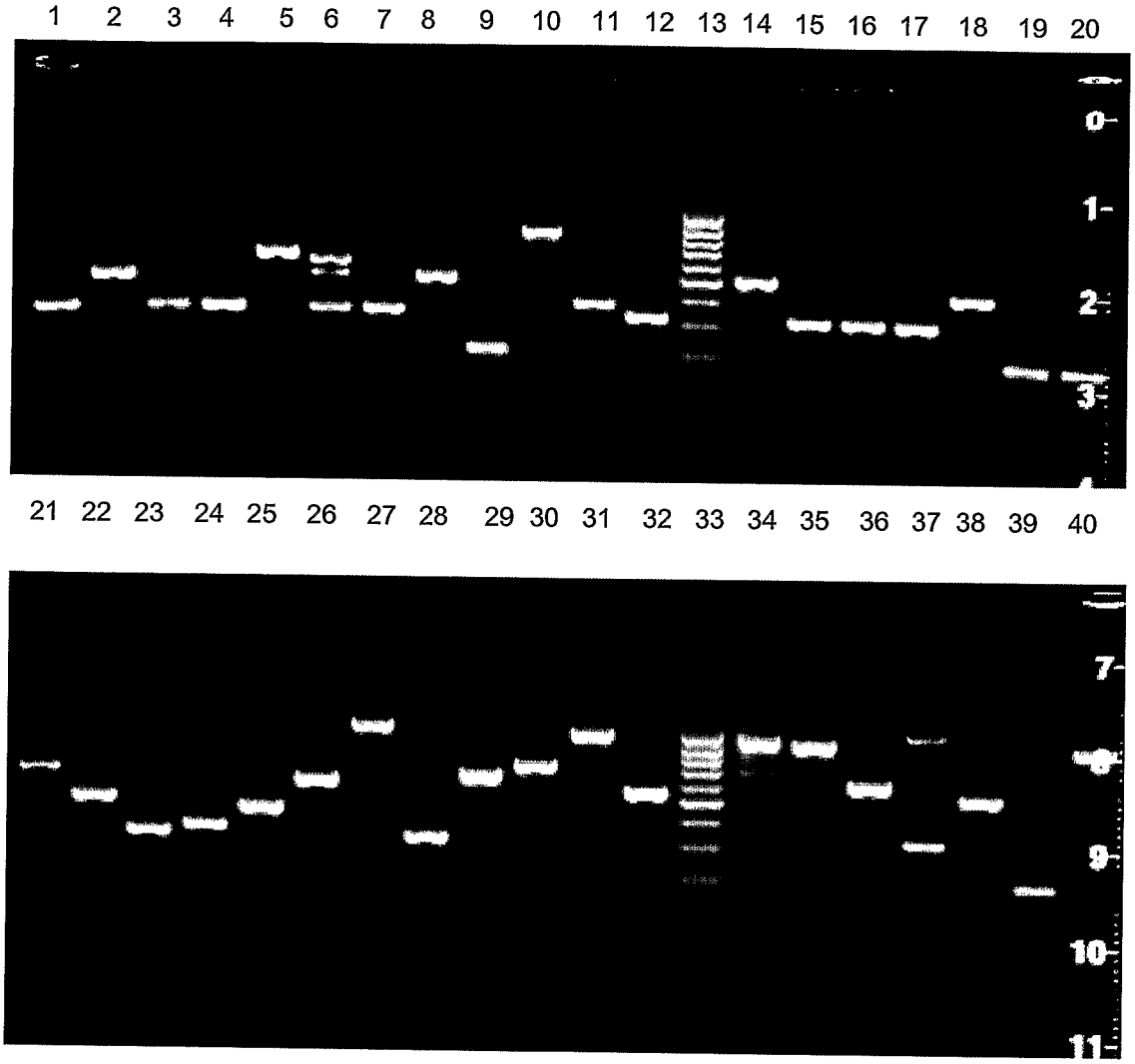

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
0
1
2
3
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
7
8
9
10
11

Figure 5B

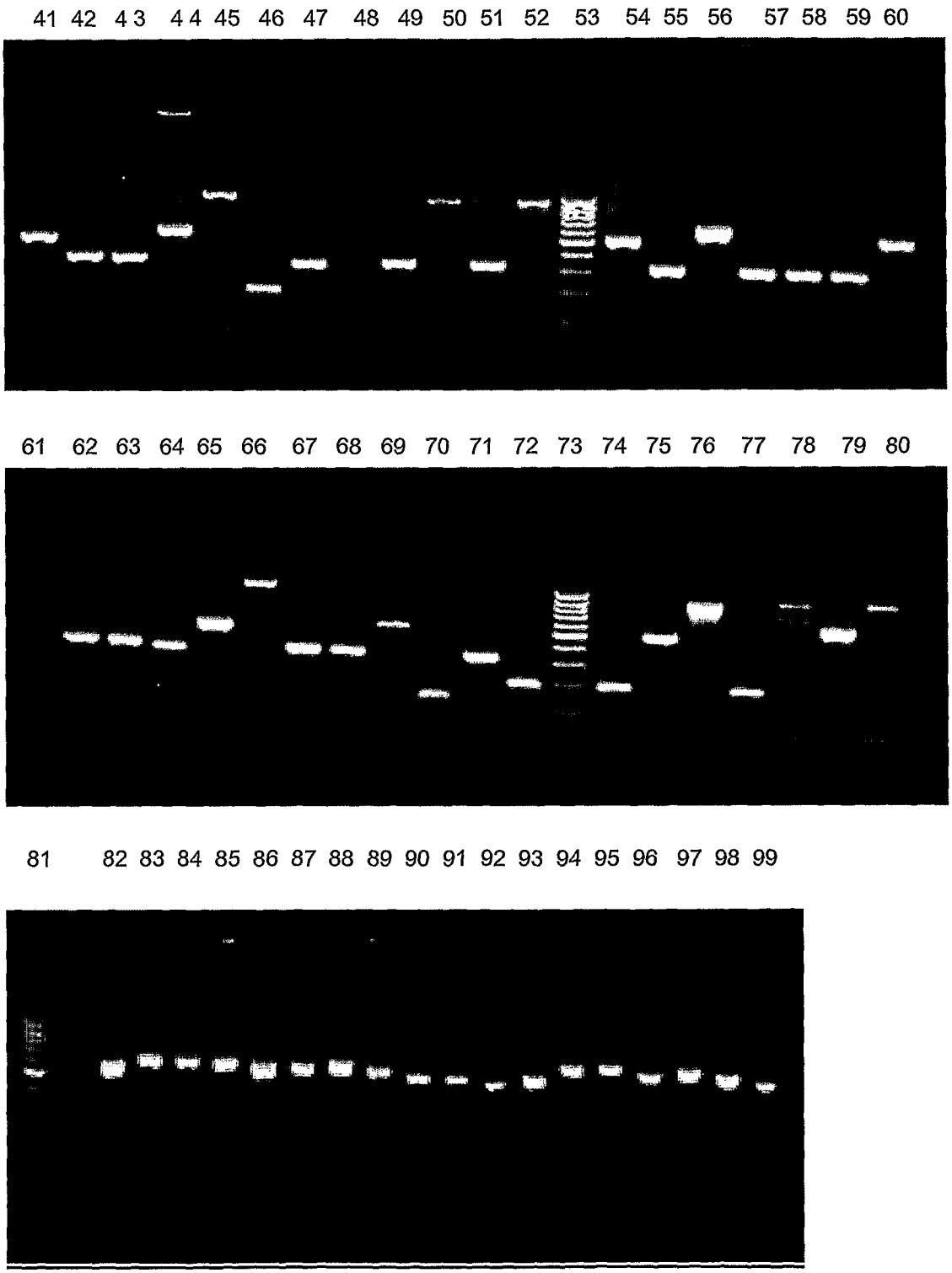

41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80
81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99

Figure 6A

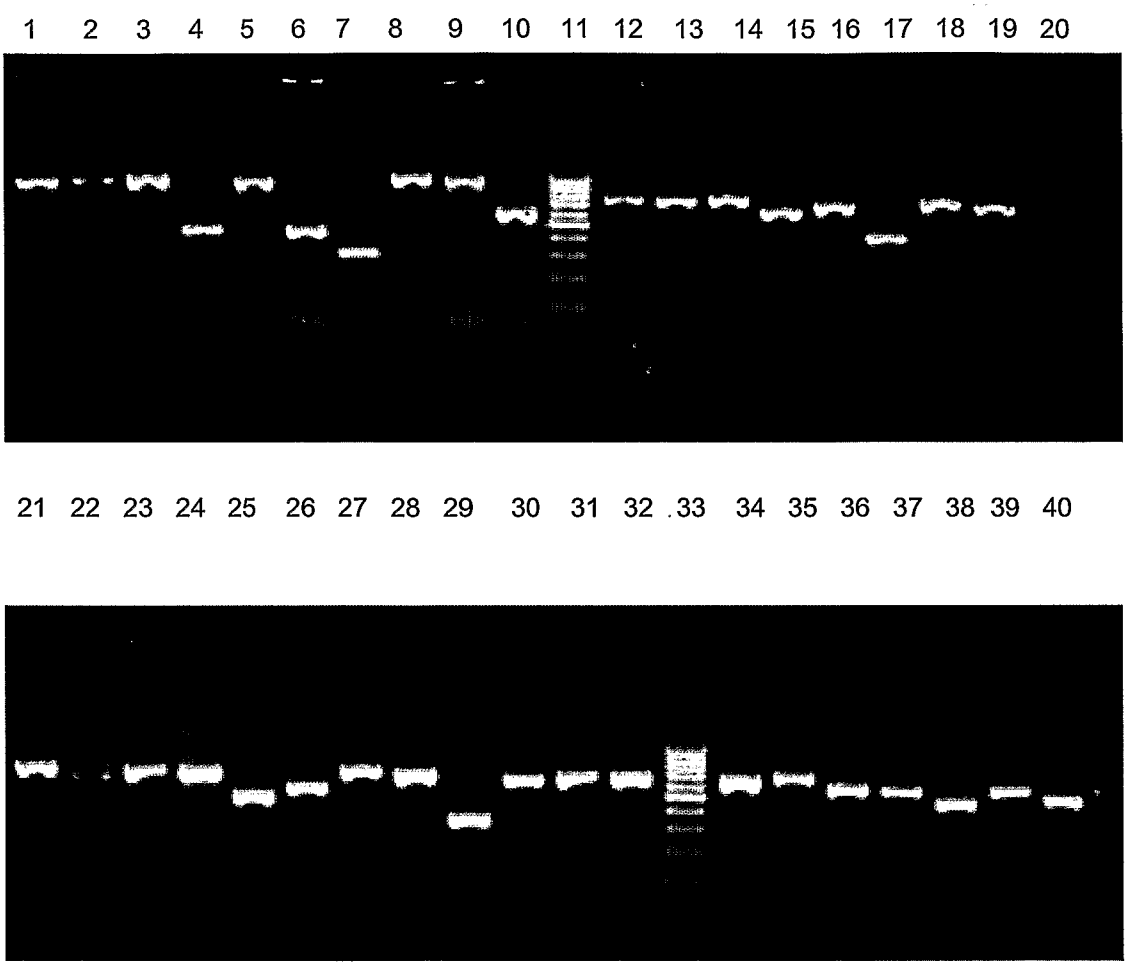
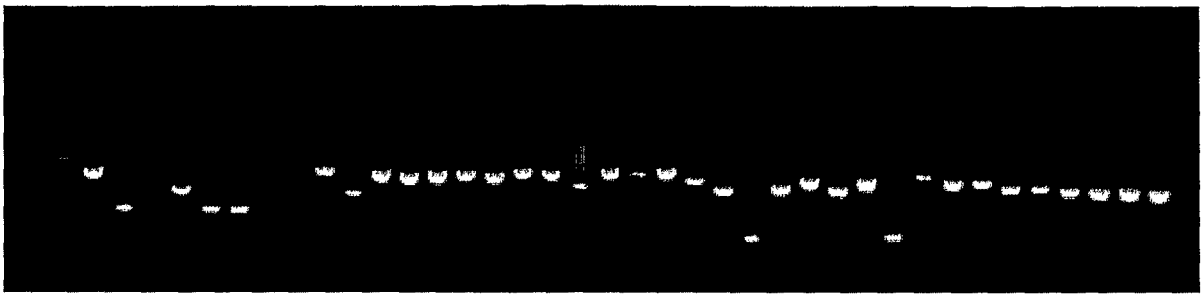

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

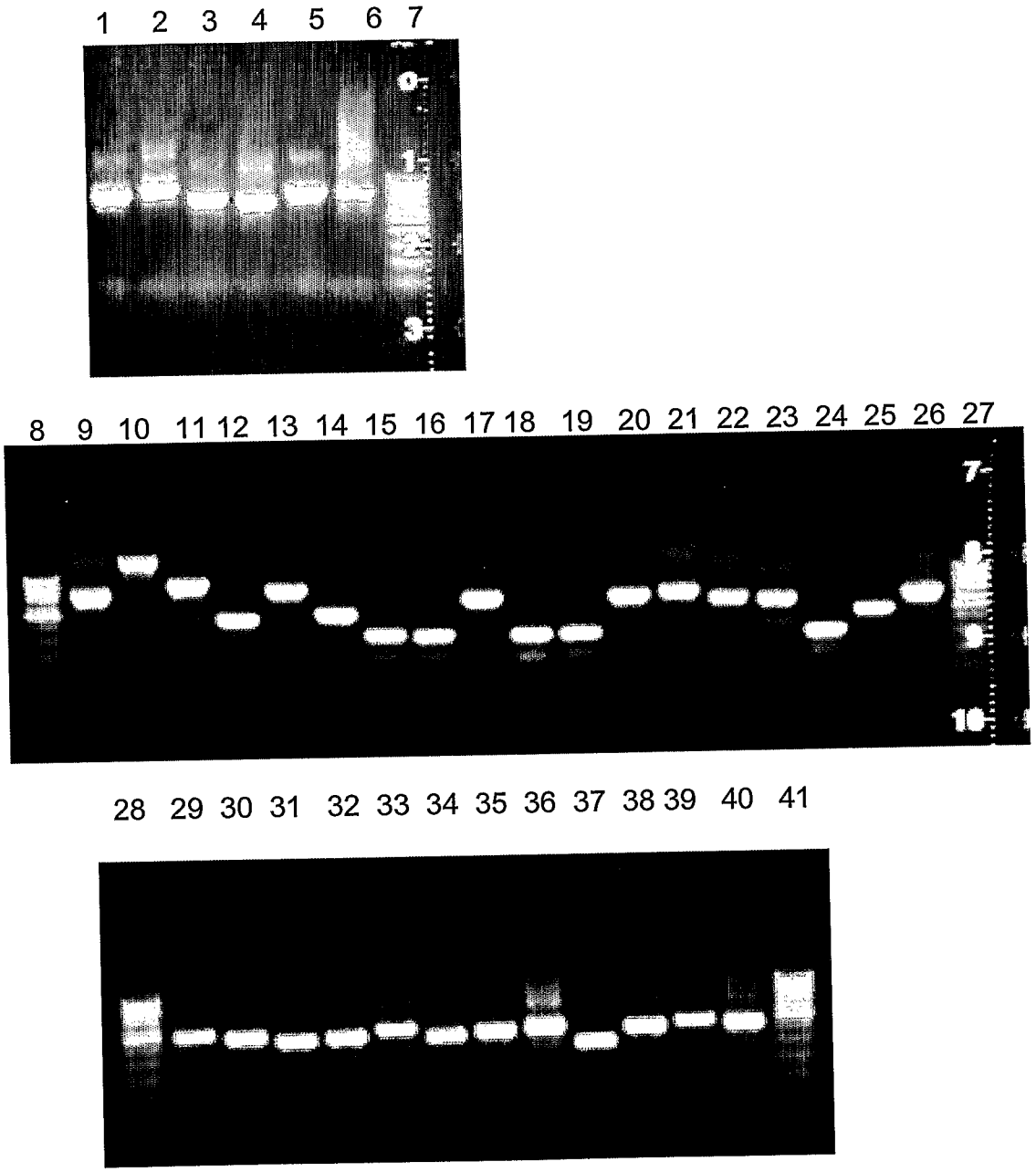
Figure 6B

41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80



81 82 83 84 85 86 87

Figure 7A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28 29 30 31 32 33 34 35 36 37 38 39 40 41

Figure 7B

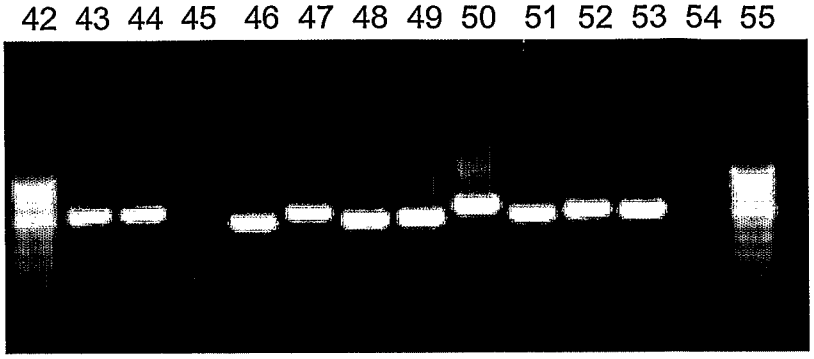
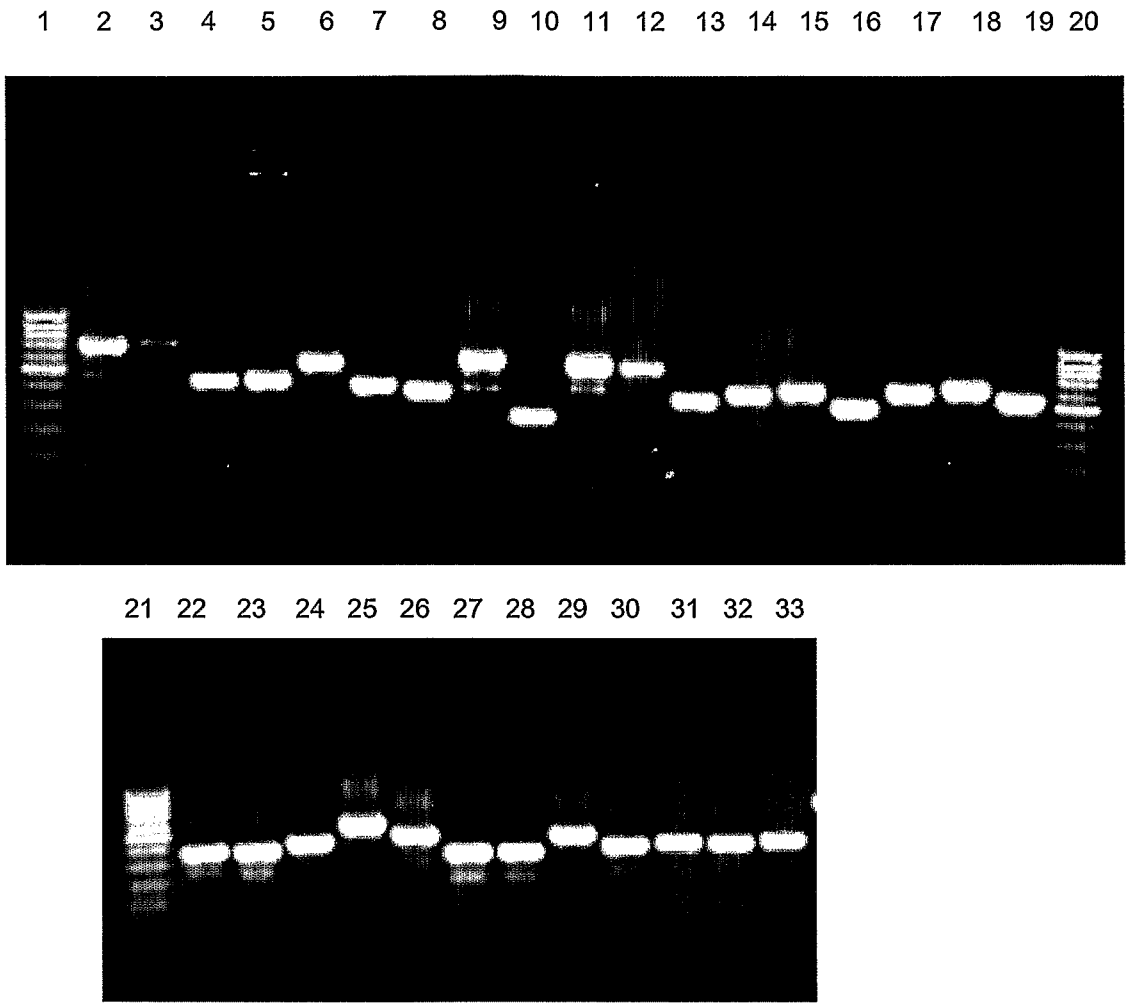


Figure 8A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21 22 23 24 25 26 27 28 29 30 31 32 33

Figure 8B

34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53

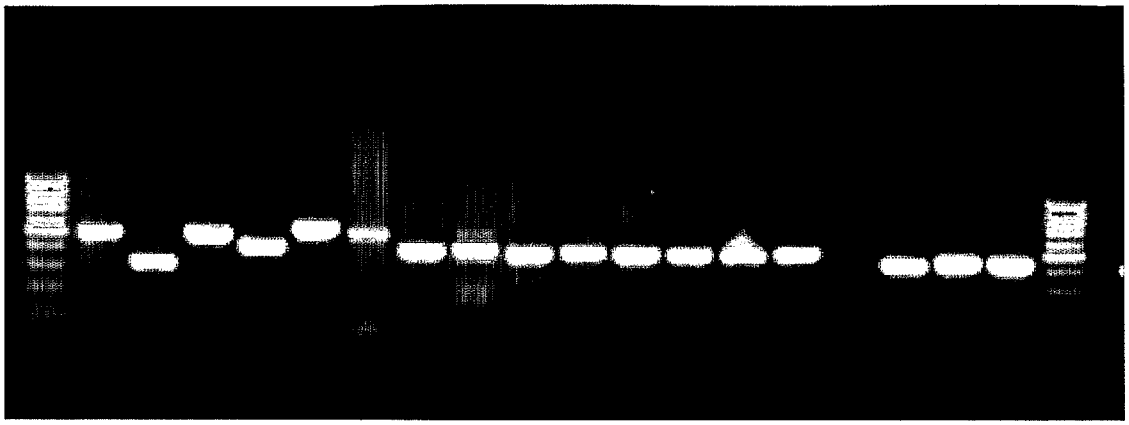


Figure 9

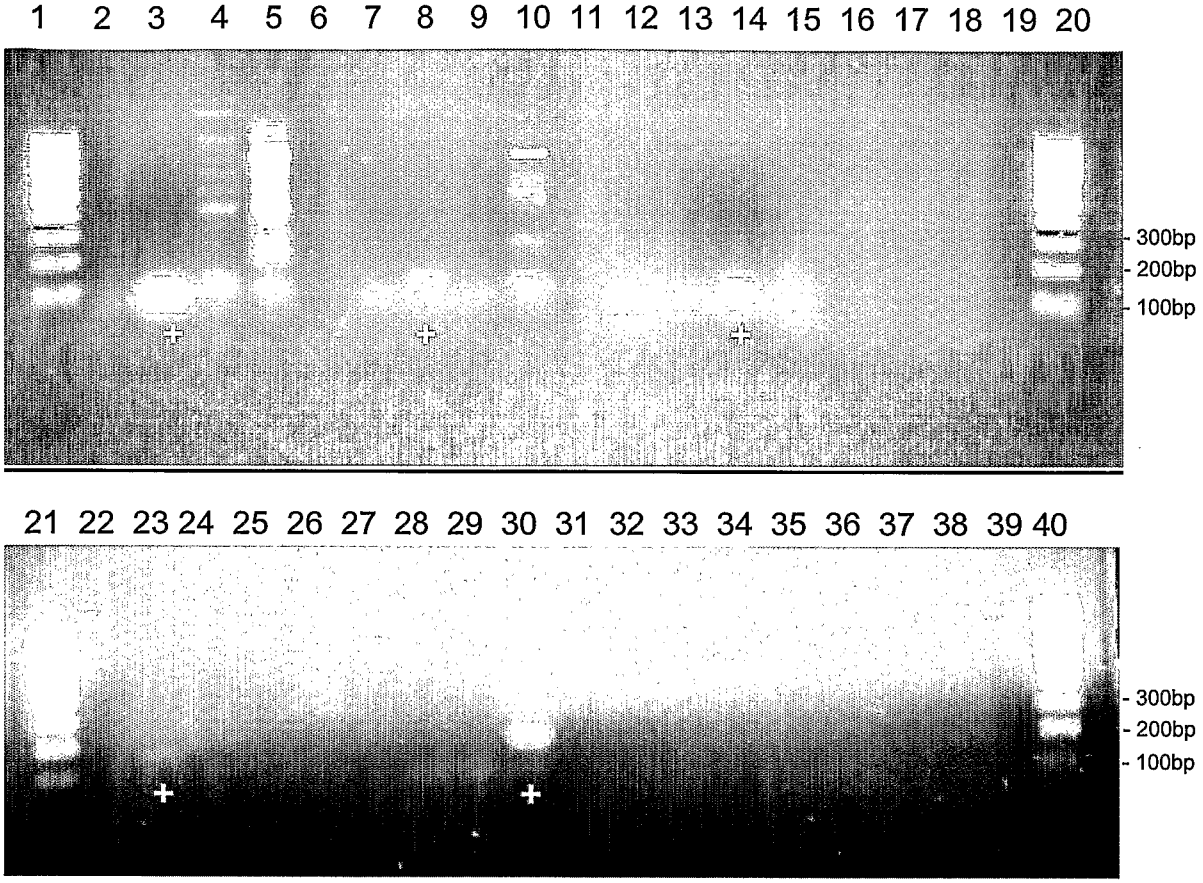
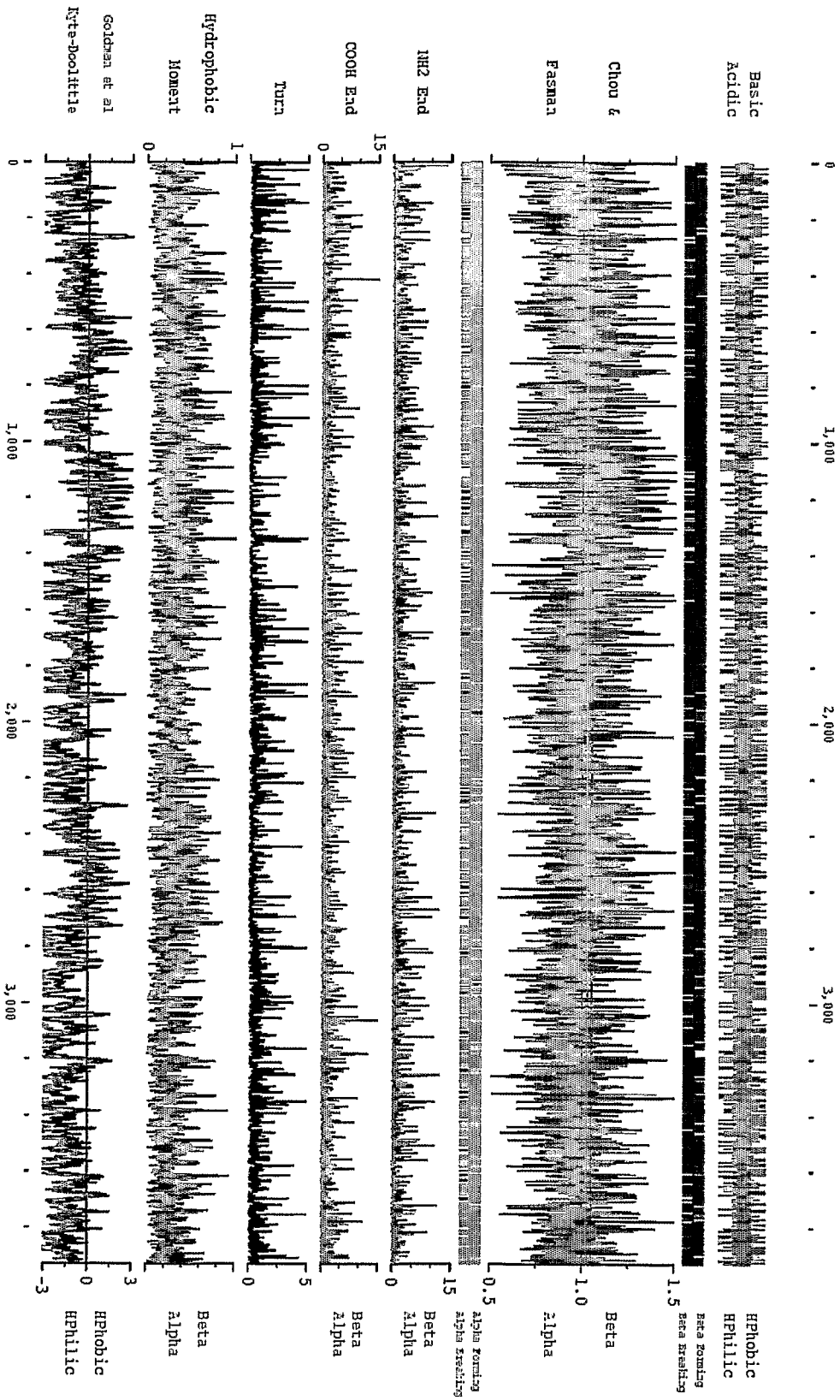

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
300bp
200bp
100bp
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
300bp
200bp
100bp

Figure 10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Basic
Acidic
Chou &
Fasman
NH2 End
COOH End
Turn
Hydrophobic
Moment
Goldman et al
Kyte-Doolittle
HPhobic
HPhilic
Beta Forming
Beta Breaking
Beta
Alpha
alpha Forming
alpha Breaking
1,000
2,000
3,000