VACCINE AGAINST BOVINE VIRAL DIARRHEA VIRUS

FIELD OF THE INVENTION

[0001] This invention is in the field of cattle vaccines, particularly vaccines against Bovine Viral Diarrhea Virus (BVDV).

BACKGROUND

[0002] BVDV virions are 40 to 60 nm in diameter. The nucleocapsid of BVDV consists of a single molecule of RNA and the capsid protein C. The nucleocapsid is surrounded by a lipid membrane with two glycoproteins anchored in it, E1 and E2. A third glycoprotein, E^{rns}, is loosely associated to the envelope. The genome of BVDV is approximately 12.5 kb in length, and contains a single open reading frame located between the 5' and 3' non-translated regions (NTRs). A polyprotein of approximately 438 kD is translated from this open reading frame, and is processed by cellular and viral proteases into at least eleven viral structural and nonstructural (NS) proteins (Tautz, et al., J. Virol. 71:5415-5422 (1997); Xu, et al., J. Virol. 71:5312-5322 (1997); Elbers, et al., J. Virol. 70:4131-4135 (1996); and Wiskerchen, et al., Virology 184:341-350 (1991)). The genomic order of BVDV is p20/N^{pro}, p14/C, gp48/E^{rns}, gp25/E1, gp53/E2, p54/NS2, p80/NS3, p10/NS4A, p32/NS4B, p58/NS5A and p75/NS5B. The three envelope proteins, gp48/E^{rns}, gp25/E1 and gp53/E2, are heavily glycosylated. E^{rns} (formerly referred to as E0 or gp48) forms homodimers, covalently linked by disulfides. The absence of a hydrophobic membrane anchor region suggests that E^{rns} is loosely associated with the envelope. E^{rns} induces high antibody titers in infected cattle, but the antisera has limited virus-neutralizing activity.

[0003] According to BVDV virus growth studies in cultured cells, two viral biotypes have been classified: viruses that induce a cytopathic effect (cp) and viruses that do not induce a cytopathic effect (ncp) in infected cells (Lee et al., Am. J. Vet. Res. 18: 952-953; Gillespie et al., Cornell Vet. 50: 73-79, 1960). Cp variants can arise from the persistently infected (PI) animals pre-infected with ncp viruses (Howard et al., Vet. Microbiol. 13: 361-369, 1987; Corapi et al., J. Virol. 62: 2823-2827, 1988).

[0004] Based on the genetic diversity of the 5' non-translated-region (NTR) and the antigenic differences in the virion surface glycoprotein E2 of BVD viruses, two major genotypes have

been proposed: type I and II. BVDV type 1 represents classical or traditional virus strains which usually produce only mild diarrhea in immunocompetent animals, whereas BVDV type 2 are emerging viruses with high virulence which can produce thrombocytopenia, hemorrhages and acute fatal disease (Corapi et al., J. Virol. 63: 3934-3943; Bolin et al., Am. J. Vet. Res. 53: 2157-2163; Pellerin et al., Virology 203: 260-268, 1994; Ridpath et al., Virology 205: 66-74, 1994; Carman et al., J. Vet. Diagn. Invest. 10: 27-35, 1998). Type I and II BVDV viruses have distinct antigenicity determined by a panel of monoclonal antibodies (Mabs) and by cross-neutralization using virus-specific antisera raised in animals (Corapi et al., Am. J. Vet. Res. 51: 1388-1394, 1990). Viruses of either genotype may exist as one of the two biotypes, cp or ncp virus.

[0005] BVDV Types 1 and 2 have been implicated in a variety of clinical syndromes. Studies have established that the virus causes severe primary respiratory disease; that PI cattle are a major source of infection for susceptible calves; and that BVDV preferentially infects white blood cell (lymphocytes) causing profound and broad-based deficits in the immune system. Ellis et al. (1996) JAVMA 208:393-400; Baum et al. (1993) The Compendium Collection: Infectious Disease in Food Animal Practice. Trenton, N.J. Veterinary Learning Systems-113-121; Meyling et al. (1987) Agric Pestivirus Infect Rumin 225-231. Abortion or fetal mummification can result when pregnant cattle become infected especially during the first trimester. Bolin et al. (1989) Am J. Vet Res 52:1033-1037. Mucosal disease, another often fatal manifestation of bovine viral diarrhea (BVD), results from early fetal infection with a ncpBVDV biotype, development of immunotolerance to the virus, birth of a PI calf, and subsequent superinfection with a cpBVDV biotype. Bolin et al. (1989) Am J. Vet Res 52:1033-1037. BVDV Type 2, once recognized chiefly as a hemorrhagic BVDV isolate mostly in dairy herds, has become the predominant strain isolated in most regions of the United States from both BVD-related abortions and respiratory cases. Van Oirschot et al. (1999) Vet Micro 64:169-183.

[0006] BVDV is classified in the pestivirus genus and Flaviviridae family. It is closely related to viruses causing border disease in sheep and classical swine fever. Infected cattle exhibit "mucosal disease" which is characterized by elevated temperature, diarrhea, coughing and ulcerations of the alimentary mucosa (Olafson, et al., Cornell Vet. 36:205-213 (1946); Ramsey,

et al., North Am. Vet. 34:629-633 (1953)). The BVD virus is capable of crossing the placenta of pregnant cattle and may result in the birth of PI calves (Malmquist, J. Am. Vet. Med. Assoc. 152:763-768 (1968); Ross, et al., J. Am. Vet. Med. Assoc. 188:618-619 (1986)). These calves are immunotolerant to the virus and persistently viremic for the rest of their lives. They provide a source for outbreaks of mucosal disease (Liess, et al., Dtsch. Tieraerztl. Wschr. 81:481-487 (1974) and are highly predisposed to infection with microorganisms causing diseases such as pneumonia or enteric disease (Barber, et al., Vet. Rec. 117:459-464 (1985).

[0007] Studies from BVD virus infected animals suggest that exposure to BVD viruses induce both antigen-specific B-cell and T-cell responses in animals (Donis et al., Virology 158: 168-173, 1987; Larsson et al., Vet. Microbiol. 31: 317-325, 1992; Howard et al., Vet. Immunol. Immunopathol. 32: 303-314, 1992; Lambot et al., J. Gen. Virol. 78: 1041-1047, 1997; Beer et al., Vet. Microbiology. 58: 9-22, 1997).

[0008] A number of BVDV vaccines have been developed using either modified live or chemically inactivated BVD viral isolates (Fernelius et al., Am. J. Vet. Res. 33: 1421-1431, 1972; Kolar et al., Am. J. Vet. Res. 33: 1415-1420, 1972; McClurkin et al., Arch. Virol. 58: 119, 1978). Multiple doses are required for the inactivated viral vaccines to achieve primary immunization. A single-dose anti-BVDV vaccine has been described (see, e.g., 20070298053) but there is still a need in the art for more efficient anti-BVDV vaccines.

SUMMARY OF INVENTION

[0009] In one aspect, provided is a vaccine comprising: an antigen component comprising a BVDV antigen, and an adjuvant component comprising a triterpenoid saponin, an immunostimulatory nucleotide containing CpG, a phospholipid, and a sterol, wherein said vaccine is a water-in-oil emulsion and wherein said vaccine is effective as a single-dose vaccine.

[0010] In different embodiments, the BVDV antigen may contain a BVDV-1 antigen and a BVDV-2 antigen. In certain embodiments, the BVDV antigen may be a cytopathic (cp) BVDV virus or a noncytopathic (ncp) BVDV virus.

[0011] In certain embodiments, the BVDV antigen may be a recombinant antigen. Preferably, such recombinant antigen comprises a marker allowing for an assay differentiating between vaccinated and infected animals (DIVA).

[0012] In certain embodiments, the triterpenoid saponin is Quil A or a purified fraction thereof.

[0013] In certain embodiments, the phospholipid is phosphatidylcholine.

[0014] In certain embodiments, the sterol is cholesterol.

[0015] In certain embodiments, the triterpenoid saponin is Quil A or a purified fraction thereof, and/or the CpG comprises SEQ ID NO: 8.

[0016] In a second aspect, the invention provides a method of vaccinating a bovine in need thereof against BVDV, the method comprising administering to said bovine the vaccine according to any of the embodiments described above.

[0017] In a third aspect, applicable to the vaccines containing a marked antigen, the invention provides a method of differentiating between the vaccinated and infected animals, the method comprising vaccinating the bovines in need thereof with the vaccine according to any of the embodiments described above, with a proviso that the vaccine comprises the marked BVDV antigen, obtaining samples from said vaccinated bovines, assaying said samples for the presence or absence of the antibodies to the BVDV and the marker; identifying the animal having said antibodies both against the BVDV and against the marker as having been vaccinated with said vaccine; and identifying the animal having said antibodies against the BVDV and lacking said antibodies against the marker as having been infected with the wild type BVDV.

DETAILED DESCRIPTION

Definitions

[0018] "About" or "approximately," when used in connection with a measurable numerical variable, refers to the indicated value of the variable and to all values of the variable that are within the experimental error of the indicated value (e.g., within the 95% confidence interval for the mean) or within 10 percent of the indicated value, whichever is greater, unless about is used in reference to time intervals in weeks where "about 3 weeks," is 17 to 25 days, and about 2 to about 4 weeks is 10 to 40 days.

[0019] "Antigen" or "immunogen" refers to any substance that is recognized by the animal's immune system and generates an immune response. The term includes killed, inactivated, attenuated, or modified live bacteria, viruses, or parasites. The term "antigen" also includes polynucleotides, polypeptides, recombinant proteins, synthetic peptides, protein extract, cells

(including tumor cells), tissues, polysaccharides, or lipids, or fragments thereof, individually or in any combination thereof. The term antigen also includes antibodies, such as anti-idiotype antibodies or fragments thereof, and to synthetic peptide mimotopes that can mimic an antigen or antigenic determinant (epitope).

[0020] "Buffer" means a chemical system that prevents change in the concentration of another chemical substance, e.g., proton donor and acceptor systems serve as buffers preventing marked changes in hydrogen ion concentration (pH). A further example of a buffer is a solution containing a mixture of a weak acid and its salt (conjugate base) or a weak base and its salt (conjugate acid).

[0021] “Consisting essentially” as applied to the adjuvant formulations refers to formulation which does not contain unrecited additional adjuvanting or immunomodulating agents in the amounts at which said agent exerts measurable adjuvanting or immunomodulating effects.

[0022] The reference to a composition or vaccine being “effective as a single-dose vaccine” refers to a vaccine which, upon a single administration to a bovine, provides duration of immunity of at least six months (preferably, at least seven months, eight months, nine months, ten months, eleven months, or at least one year) against BVDV challenge of the same type as the antigen (e.g., if the vaccine comprises a BVDV-1 antigen, then the single dose vaccine would provide at least a one-year protection against BVDV-1 challenge). In this application, at least one year DOI indicates at least 75% (preferably, at least 80%, or at least 85% or at least 90% or at least 95% or at least 99%) of fetal protection when the vaccinated cows or heifers were exposed to natural challenge twelve months after a single vaccination**.**

[0023] The term “emulsifier” is used broadly in the instant disclosure. It includes substances generally accepted as emulsifiers, e.g., different products of TWEEN® or SPAN® product lines (fatty acid esters of polyethoxylated sorbitol and fatty-acid-substituted sorbitan surfactants, respectively), and different solubility enhancers such as PEG-40 Castor Oil or another PEGylated hydrogenated oil. For the purposes of this disclosure, phospholipids, such as posphatidylcholine and more specifically, lecithin, are excluded from the “emulsifiers” as described herein.

[0024] "Immunologically protective amount" or "immunologically effective amount" or "effective amount to produce an immune response" of an antigen is an amount effective to induce an immunogenic response in the recipient. The immunogenic response may be sufficient for diagnostic purposes or other testing, or may be adequate to prevent signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with a disease agent. Either humoral immunity or cell-mediated immunity or both may be induced. The immunogenic response of an animal to an immunogenic composition may be evaluated, e.g., indirectly through measurement of antibody titers, lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild type strain, whereas the protective immunity conferred by a vaccine can be evaluated by measuring, e.g., reduction in clinical signs such as mortality, morbidity, temperature number, overall physical condition, and overall health and performance of the subject. The immune response may comprise, without limitation, induction of cellular and/or humoral immunity.

[0025] "Immunogenic" means evoking an immune or antigenic response. Thus an immunogenic composition would be any composition that induces an immune response.

[0026] "Lipids" refers to any of a group of organic compounds, including the fats, oils, waxes, sterols, and triglycerides that are insoluble in water but soluble in nonpolar organic solvents, are oily to the touch, and together with carbohydrates and proteins constitute the principal structural material of living cells.

[0027] “Pharmaceutically acceptable" refers to substances, which are within the scope of sound medical judgment, suitable for use in contact with the tissues of subjects without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit-to-risk ratio, and effective for their intended use.

The antigens

[0028] Multiple BVDV antigens are suitable for the vaccines of this invention. For example, the antigen component may contain whole BVDV viruses (e.g., modified live or inactivated viruses), or surface proteins from the viruses. The antigens may be selected from different types of BVDV, such as, for example, BVDV-1 (including both BVDV-1a, -1b and/or -1c) and BVDV-2. The viruses may of either cytopathic variant or non-cytopathic variant of BVDV, or a combination of

the two. For example, the BVDV Type 2 component is cytopathic (cpBVD-2 strain 53637-ATCC No. PTA-4859) and the BVDV Type 1 component is cytopathic 5960 (cpBDV-1 strain 5960-National Animal Disease Center, United States Department of Agriculture, Ames, Iowa). The antigen component may comprise BVDV-1c strains, e.g., Bega strain (GenBank reference AF049221) or Trangie strain (GenBank reference AF049222). These strains are present in PESTIGARD® vaccine (adjuvanted with ISCOM). The present invention also contemplates non-cytopathic BVDV Type 1 and Type 2 strains.

[0029] Additionally or alternatively, the antigen component of the present vaccine may comprise a "marked" BVDV antigen. Such “marked” vaccine could either contain an additional antigenic determinant which is not present in the wild-type virus, or lack an antigenic determinant which is present in the wild-type virus. With respect to the former, vaccinated animals mount an immune response to the "marker" immunogenic determinant, while non-vaccinated animals do not. Through the use of an immunological assay directed against the marker determinant, vaccinated animals could be differentiated from non-vaccinated, naturally-infected animals by the presence of antibodies to the marker determinant. In the case of the latter strategy, animals infected with the wild-type virus mount an immune response to the marker determinant, while non-infected, vaccinated animals do not, as a result of the determinant not being present in the marked vaccine.

[0030] Through the use of an immunological assay directed against the marker determinant, infected animals could be differentiated from vaccinated, non-infected animals. For example, E^{rns} gene of BVDV may be replaced with E^{rns} gene of a related pestivirus, e.g., a reindeer pestivirus, a giraffe pestivirus, and a pronghorn antelope pestivirus. The BVDV/giraffe chimeric pestivirus is the strain deposited as UC 25547 with American Type Culture Collection (ATCC®), 10801 University Boulevard, Manassas, Va. 20110-2209, USA, and given the ATCC® deposit designation of PTA-9938. In one embodiment, the BVDV/pronghorn antelope chimeric pestivirus is the strain deposited as UC 25548 with ATCC® and given the ATCC® deposit designation of PTA-9939. In one embodiment, the BVDV/reindeer chimeric pestivirus is the strain deposited as UC 25549 with ATCC® and given the ATCC® deposit designation of PTA-9940. This strategy is described in details in, e.g., US 20170151321.

[0031] The methods of making BVDV antigens suitable for the compositions disclosed herein are well known in the art. For example, the viruses of the present invention can be propagated in cells, cell lines and host cells. Said cells, cell lines or host cells may be for example, but not limited to, mammalian cells and non-mammalian cells, including insect and plant cells. Cells, cell lines and host cells in which the BVDV virus of the present invention may be propagated are readily known and accessible to those of ordinary skill in the art.

[0032] The BVDV viruses of the present invention can be attenuated or inactivated prior to use in an immunogenic composition or vaccine. Methods of attenuation and inactivation are well known to those skilled in the art. Methods for attenuation include, but are not limited to, serial passage in cell culture on a suitable cell line, ultraviolet irradiation, and chemical mutagenesis. Methods for inactivation include, but are not limited to, treatment with formalin, betapropriolactone (BPL) or binary ethyleneimine (BEI), or other methods known to those skilled in the art.

[0033] Inactivation by formalin can be performed by mixing the virus suspension with 37% formaldehyde to a final formaldehyde concentration of 0.05%. The virus-formaldehyde mixture is mixed by constant stirring for approximately 24 hours at room temperature. The inactivated virus mixture is then tested for residual live virus by assaying for growth on a suitable cell line.

[0034] Inactivation by BEI can be performed by mixing the virus suspension of the present invention with 0.1 M BEI (2-bromo-ethylamine in 0.175 N NaOH) to a final BEI concentration of 1 mM. The virus-BEI mixture is mixed by constant stirring for approximately 48 hours at room temperature, followed by the addition of 1.0 M sodium thiosulfate to a final concentration of 0.1 mM. Mixing is continued for an additional two hours. The inactivated virus mixture is tested for residual live virus by assaying for growth on a suitable cell line.

[0035] Purified viruses can be used directly in an immunogenic composition or vaccine, or can be further attenuated, or inactivated. Typically, an immunogenic composition or vaccine contains between about 1×10^2 and about 1×10^{12} virus particles, or between about 1×10^3 and about 1×10^{11} virus particles, or between about 1×10^4 and about 11×10^{10} virus particles, or between about 1×10^5 and about 1×10^9 virus particles, or between about 1×10^6 and about 1 x

10^8 virus particles. The precise amount of a virus in an immunogenic composition or vaccine effective to provide a protective effect can be determined by a skilled artisan.

[0036] Antigen component may also contain membrane fractions or purified immunogenic proteins of the strains disclosed above. Methods of fractionation and/or purification and/or recombinant techniques for production of the immunogenic proteins are well known in the art.

[0037] The antigen component may comprise a vectored vaccine containing a vector (e.g., a viral vector such as, for example, a canarypox vector or a vaccinia vector) carrying a nucleic acid sequence encoding immunogenic protein capable of eliciting protective immune response to a challenge with BVDV strain(s) or types disclosed herein.

Sterols

[0038] Sterols share a common chemical core, which is a steroid ring structure[s], having a hydroxyl (OH) group, usually attached to carbon-3. The hydrocarbon chain of the fatty-acid substituent varies in length, usually from 16 to 20 carbon atoms, and can be saturated or unsaturated. Sterols commonly contain one or more double bonds in the ring structure and also a variety of substituents attached to the rings. Sterols and their fatty-acid esters are essentially water insoluble. In view of these chemical similarities, it is thus likely that the sterols sharing this chemical core would have similar properties when used in the vaccine compositions of the instant invention. Sterols are well known in the art and can be purchased commercially. For example cholesterol is disclosed in the Merck Index, 12th Ed., p. 369. Suitable sterols include, without limitations, β-sitosterol, stigmasterol, ergosterol, ergocalciferol, and cholesterol.

Phospholipids

[0039] The adjuvants disclosed herein further contain phospholipids. Particularly preferable phospholipids are phosphatidylcholines. Phosphatidylcholines are phospholipids that incorporate choline as a head group. They are a major component of biological membranes and can be easily obtained from a variety of readily available sources, such as egg yolk or soybeans, from which they are mechanically or chemically extracted using hexane. They are also a member of the lecithin group of yellow-brownish fatty substances occurring in animal and plant tissues.

[0040] Lecithin is a phosphatidylcholine that is particularly suitable for the adjuvant described herein. Lecithin can be obtained as a mixture of phosphatides and triglycerides by water-washing crude vegetable oils, and separating and drying the resulting hydrated gums. A refined product can be obtained by fractionating the mixture for acetone insoluble phospholipids and glycolipids remaining after removal of the triglycerides and vegetable oil by acetone washing. Alternatively, lecithin can be obtained from various commercial sources.

[0041] Other suitable phospholipids include phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidic acid, cardiolipin, phosphatidylethanolamine and lysolecithins (e.g., lysophosphatidylcholine). The phospholipids may be isolated from natural sources or conventionally synthesized.

[0042] In certain aspects, the sterol and the phospholipid are used in weight ratios from 1:5 to 1:20, sterol: phospholipid or a subrange thereof. For example, weight ratios of sterol to phosphatidylcholine may be 1:7 to 1:15, or 1:8 to 1:13 or 1:9 to 1:11, or about 1:10.

[0043] The total weight of the sterol and phospholipid per dose of the vaccine may be between about 70 mg and about 220 mg, e.g., about 80 to about 200 mg, or about 90 to about 180 mg, or about 100 to about 160 mg or about 110 to about 140 mg, or about 130 mg.

Immunostimulatory oligonucleotides containing CpG.

[0044] The adjuvant also comprises immunostimulatory oligonucleotides containing CpG, which are oligonucleotides which include at least one unmethylated CpG dinucleotide. An oligonucleotide containing at least one unmethylated CpG dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e., "CpG DNA" or DNA containing a 5' cytosine followed by 3' guanine and linked by a phosphate bond) and activates the immune system. The entire CpG oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must be unmethylated CpG. The terms CpG oligonucleotide or CpG nucleic acid as used herein refer to an immunostimulatory CpG oligonucleotide or a nucleic acid unless otherwise indicated.

[0045] In aspects of the invention, immunostimulatory oligonucleotides containing CpG include, but are not limited to, oligonucleotides that are A-Class, B-Class, C-Class, T-Class, P-Class or any Class with an E modification.

[0046] A-Class oligonucleotides are potent for inducing IFN-α and NK cell activation but are relatively weak at stimulating B cells. The A-Class oligonucleotides typically have stabilized poly-G sequences at 5' and 3' ends and a palindromic phosphodiester CpG dinucleotide-containing sequence of at least 6 nucleotides and form multimeric structures. A-Class oligonucleotides have been described in U.S. Pat. No. 6,949,520, issued Sep. 27, 2005 and published PCT application no. PCT/US00/26527 (WO 01/22990), published on Apr. 5, 2001.

[0047] B-Class oligonucleotides are potent at activating B cells but are relatively weak in inducing IFN-alpha and NK cell activation. The B-Class oligonucleotides are monomeric and may be fully stabilized with a wholly phosphorothioate backbone. B-Class oligonucleotides may also have some native phosphodiester linkages, for example, between the C and G of the CpG, in which case they are referred to as semi-soft. B-Class oligonucleotides have been described in U.S. Pat. Nos. 6,194,388 B1 and 6,239,116 B1, issued on Feb. 27, 2001 and May 29, 2001 respectively, and in published PCT application no. WO/1996/002555, published on Feb. 1, 1996 and published PCT application no. WO/1998/018810, published on May 7, 1998. C-Class oligonucleotides have both a traditional "stimulatory" CpG sequence and a "GC-rich" or "B-cell neutralizing" motif. C-Class CpG oligonucleotides have properties intermediate to A- and B-Classes so activate B cells and NK cells and induce IFN-alpha (Krieg A M et al. (1995) Nature 374:546-9; Ballas Z K et al. (1996) J Immunol 157:1840-5; Yamamoto S et al. (1992) J Immunol 148:4072-6).

[0048] The C-Class oligonucleotides, contain a single palindrome such that they can form secondary structures such as stem-loops or tertiary structures such as dimmers. The backbone of C-Class oligonucleotides may have a fully stabilized, chimeric or semi-soft backbone. C-Class oligonucleotides include a B-Class-type sequence and a GC-rich palindrome or near-palindrome. This Class has been described in US published application no. 20030148976, published on Aug. 7, 2003 and in published PCT application no. WO2008/068638, published on Jun. 12, 2008.

[0049] The P-Class oligonucleotides have the ability in some instances to induce much higher levels of IFN-alpha secretion than the C-Class oligonucleotides. The P-Class oligonucleotides have the ability to spontaneously self-assemble into concatamers either in vitro and/or in vivo.

[0050] These oligonucleotides are, in a strict sense, single-stranded, but the presence of palindromes allows for formation of concatamers or possibly stem-and-loop structures. The overall length of P- class immunostimulatory oligonucleotides is between 19 and 100 nucleotides, e.g., 19-30 nucleotides, 30-40 nucleotides, 40-50 nucleotides, 50-60 nucleotides, 60-70 nucleotides, 70-80 nucleotides, 80-90 nucleotides, 90-100 nucleotides.

[0051] In one aspect of the invention the immunostimulatory oligonucleotide contains a 5' TLR activation domain and at least two palindromic regions, one palindromic region being a 5' palindromic region of at least 6 nucleotides in length and connected to a 3' palindromic region of at least 8 nucleotides in length either directly or through a spacer.

[0052] The P-class immunostimulatory oligonucleotides may be modified according to techniques known in the art. For example, J-modification refers to iodo-modified nucleotides. E-modification refers to ethyl-modified nucleotide(s). Thus, E-modified P-class immunostimulatory oligonucleotides are P-class immunostimulatory oligonucleotides, wherein at least one nucleotide (preferably 5' nucleotide) is ethylated. Additional modifications include attachment of 6-nitro-benzimidazol, O-Methylation, modification with proynyl-dU, inosine modification, 2-bromovinyl attachment (preferably to uridine).

[0053] The P-class immunostimulatory oligonucleotides may also contain a modified internucleotide linkage including, without limitations, phosphodiesther linkages and phosphorothioate linkages. The oligonucleotides of the instant invention may be synthesized or obtained from commercial sources.

[0054] P-Class oligonucleotides and modified P-class oligonucleotides are further disclosed in published PCT application no. WO2008/068638, published on Jun. 12, 2008. Suitable non-limiting examples of modified P-class immunostiumulatory oligonucleotides are provided below (In SEQ ID NOs 1-10, "*" refers to a phosphorothioate bond and "_" refers to a phosphodiester bond).

SEQ ID NO: 1	5' T*C_G*T*C_G*A*C_G*A*T*C_G*G*C*G*C_G*C*G*C*C*G 3'
SEQ ID NO: 2	5' T*C_G*A*C*G*T*C*G*A*T*C*G*G*C*G*C*G*C*C*G*C*G 3'
SEQ ID NO: 3	5' T*C*G*A*C*G*T*C*G*A*T*C*G*G*C*G*C*G*C*C*G*T 3'
SEQ ID NO: 4	5' JU*C_G*A*C*G*T*C*G*A*T*C*G*G*C*G*C*G*C*G*C*C*G 3'
SEQ ID NO: 5	5' JU*C_G*A*C*G*T*C*G*A*T*C*G*G*C*G*C*G*C*G*C*C* G*T 3'

SEQ ID NO: 6	5' JU*C*G*A*C*G*T*C*G*A*T*C*G*G*C*G*C*G*C*G*C*C* G*T 3'
SEQ ID NO: 7	5' EU*C_G*A*C*G*T*C*G*A*T*C*G*G*C*G*C*G*C*G*C*C*G 3'
SEQ ID NO: 8	5' JU*C_G*T*C*G*A*C*G*A*T*C*G*G*C*G*G*C*C*G*C*C* G*T 3'
SEQ ID NO: 9	5' JU*C*G*T*C*G*A*C*G*A*T*C*G*G*C*G*G*C*C*G*C*C* G*T 3'
SEQ ID NO: 10	5' T*C_G*T*C_G*A*C_G*A*T*C_G*G*C*G*C_G*C*G*C*C*G 3'

[0055] The amount of P-class immunostimulatory oligonucleotide for use in the adjuvant compositions described herein ranges between about 50 μg and 500 μg, e.g., between 100 and 400 μg, or 200 and 300 μg, or 250-300 μg per dose, for application to a cattle animal.

Triterpenoid saponins

[0056] Ttriterpenoid saponins a group of surface-active glycosides of plant origin and share common chemical core composed of a hydrophilic region (usually several sugar chains) in association with a hydrophobic region of either steroid or triterpenoid structure. Because of these similarities, the saponins sharing this chemical core are likely to have similar adjuvanting properties. Triterpenoids suitable for use in the adjuvant compositions can come from many sources, either plant derived or synthetic equivalents, including but not limited to, Quillaja saponaria, tomatine, ginseng extracts, mushrooms, and an alkaloid glycoside structurally similar to steroidal saponins.

[0057] If a triterpenoid saponin is used, the adjuvant compositions generally contain an immunologically active saponin fraction from the bark of *Quillaja saponaria*. The saponin may be, for example, Quil A or another purified or partially purified saponin preparation, which can be obtained commercially. Thus, saponin extracts can be used as mixtures or purified individual components such as QS-7, QS-17, QS-18, and QS-21. In one embodiment the Quil A is at least 85% pure. In other embodiments, the Quil A is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% pure.

[0058] Generally, the triterpenoid saponin such as Quil A or a purified fraction thereof, is present in the amount of between about 400 and about 2000 μg per dose (e.g., 500-1600 μg, or 750-1300 μg per dose, or about 1000 μg per dose).

Oil and emulsifiers

[0059] The vaccines disclosed herein are provided in the form of water-in-oil (W/O emulsions) that contain a continuous oil phase with aqueous droplets interspersed therein.

[0060] Multiple oils and combinations thereof are suitable for use of the instant invention. These oils include, without limitations, animal oils, vegetable oils, as well as non-metabolizable oils. Non-limiting examples of vegetable oils suitable in the instant invention are corn oil, peanut oil, soybean oil, coconut oil, and olive oil. Non-limiting example of animal oils is squalane. Suitable non-limiting examples of non-metabolizable oils include light mineral oil, straight chained or branched saturated oils, and the like. Commercially available oil-based adjuvants are also suitable for use with the vaccines described herein. Such commercially available adjuvants include MONTANIDE™ oils from Seppic (Paris, France), including, without limitation MONTANIDE™ ISA50V2, which is a water-in -oil emulsion adjuvanted with TWEEN®85 and SPAN®85 for use in cattle and sheep.

[0061] In certain embodiments, applicable to all adjuvants/vaccines of the instant invention, the volume percentage of the oil and the oil-soluble emulsifier together is at least 50%, e.g., 50% to 95% by volume; or, in the amount from 50% to 85%; or, in the amount from 5% to 70%, or, in the amount from 70% to 95%; or, in the amount from 70% to 90%; or, in the amount from 80% to 95%.

[0062] Emulsifiers suitable for use in the present emulsions include natural biologically compatible emulsifiers and non-natural synthetic surfactants.

[0063] Non-natural, synthetic emulsifiers suitable for use in the adjuvant formulations of the present invention include sorbitan-based non-ionic surfactants, e.g. fatty-acid-substituted sorbitan surfactants (commercially available under the name SPAN® or ARLACEL®), fatty acid esters of polyethoxylated sorbitol (TWEEN®), polyethylene glycol esters of fatty acids from sources such as castor oil (EMULFOR®); polyethoxylated fatty acid (e.g., stearic acid available under the name SIMULSOL® M-53), polyethoxylated isooctylphenol/formaldehyde polymer (TYLOXAPOL®), polyoxyethylene fatty alcohol ethers (BRIJ®); polyoxyethylene nonphenyl ethers (TRITON® N), polyoxyethylene isooctylphenyl ethers (TRITON® X). Preferred synthetic

surfactants are the surfactants available under the name SPAN® and TWEEN®, such as TWEEN®-80 (Polyoxyethylene (20) sorbitan monooleate) and SPAN®-80 (sorbitan monooleate).

[0064] Generally speaking, the emulsifier(s) may be present in the vaccine composition in an amount of 0.01% to 40% by volume, preferably, 0.1% to 15%, more preferably 2% to 10%.

Methods of making the vaccine

[0065] Several methods exist for preparation of the vaccine composition according to the instant invention. For example, a first subsolution may be prepared by admixing the sterol and the phospholipid. The resulting admixture is heated if needed, and sterile-filtered. The sterile-filtered admixture is then mixed with an aqueous solution comprising the immunostimulatory oligonucleotide containing CpG, antigen component, the triterpenoid saponin and, optionally, water-soluble emulsifier and/or excipients. The resulting solution is then combined with oil and, optionally, lipid-soluble emulsifier and homogenized, thereby forming the W/O emulsion. The resulting emulsion may be freeze dried and reconstituted before use.

[0066] In another method, the sterol, the phospholipid, the oil, and the optional oil-soluble emulsifier are heated, if needed, homogenized, and sterile-filtered. Another subsolution, comprising the aqueous component of the vaccine (the antigen, the triterpenoid saponin, the CpG, and optional excipients in an aqueous buffer) is homogenized. Then the aquesous subsolution is added to the oil phase and further homogenized to produce a water-in-oil emulsion

Use

[0067] The vaccines described herein are useful as single-dose vaccines (or single dose products) for protection of bovines in need thereof against infection with BVDV. Suitable bovine subjects include calves, steers, bulls, heifers and cows and belong to species within the genus *Bos*, including, without limitation *B. taurus* and *B indicus*.

[0068] The vaccine may be administered by injection, e.g., intradermally, subcutaneously, or intramuscularly.

[0069] The vaccine may further comprise different pharmaceutically acceptable excipients, e.g., buffers, pH and/or osmolarity adjusters, and/or preservatives.

[0070] The vaccines described herein can be administered directly into the bloodstream, into muscle, or into an internal organ. Suitable means for parenteral administration include, intraperitoneal, intrathecal, intraurethral, intramuscular and subcutaneous. Suitable devices for parenteral administration include needle (including microneedle) injectors, needle-free injectors and infusion techniques.

[0071] Parenteral formulations are typically aqueous solutions which can contain excipients such as salts, carbohydrates and buffering agents (preferably to a pH of from about 3 to about 9, or from about 4 to about 8, or from about 5 to about 7.5, or from about 6 to about 7.5, or about 7 to about 7.5), but, for some applications, they can be more suitably formulated as a sterile non-aqueous solution or as a dried form to be used in conjunction with a suitable vehicle such as sterile, pyrogen-free water.

[0072] The preparation of parenteral formulations under sterile conditions, for example, by lyophilization, can readily be accomplished using standard pharmaceutical techniques well known to those skilled in the art.

[0073] The vaccines of the present invention can also be administered topically to the skin or mucosa, that is, dermally or transdermally. Typical formulations for this purpose include gels, hydrogels, lotions, solutions, creams, ointments, dusting powders, dressings, foams, films, skin patches, wafers, implants, sponges, fibers, bandages and microemulsions. Liposomes can also be used. Typical carriers include alcohol, water, mineral oil, liquid petrolatum, white petrolatum, glycerin, polyethylene glycol and propylene glycol. Penetration enhancers can be incorporated. See, for example, Finnin and Morgan, J. Pharm Sci, 88 (10):955-958 (1999).

[0074] Other means of topical administration include delivery by electroporation, iontophoresis, phonophoresis, sonophoresis and microneedle or needle-free (e.g. Powderject^{TM}, Bioject^{TM}, etc.) injection.

[0075] The vaccines described herein can also be administered intranasally or by inhalation, typically in the form of a dry powder (either alone or as a mixture, for example, in a dry blend with lactose, or as a mixed component particle, for example, mixed with phospholipids, such as phosphatidylcholine) from a dry powder inhaler or as an aerosol spray from a pressurized container, pump, spray, atomizer (preferably an atomizer using electrohydrodynamics to

produce a fine mist), or nebulizer, with or without the use of a suitable propellant, such as 1,1,1,2-tetrafluoroethane or 1,1,1,2,3,3,3-heptafluoropropane. For intranasal use, the powder can comprise a bioadhesive agent, for example, chitosan or cyclodextrin.

[0076] The pressurized container, pump, spray, atomizer, or nebulizer contains a solution or suspension of the compound(s) of the invention comprising, for example, ethanol, aqueous ethanol, or a suitable alternative agent for dispersing, solubilizing, or extending release of the active, a propellant(s) as solvent and an optional surfactant, such as sorbitan trioleate, oleic acid, or an oligolactic acid.

[0077] Prior to use in a dry powder or suspension formulation, the drug product is generally micronized to a size suitable for delivery by inhalation (typically less than about 5 microns). This can be achieved by any appropriate comminuting method, such as spiral jet milling, fluid bed jet milling, supercritical fluid processing to form nanoparticles, high pressure homogenization, or spray drying.

[0078] Capsules (made, for example, from gelatin or hydroxypropylmethylcellulose), blisters and cartridges for use in an inhaler or insufflator can be formulated to contain a powder mix of the compound of the invention, a suitable powder base such as lactose or starch and a performance modifier such as l-leucine, mannitol, or magnesium stearate. The lactose can be anhydrous or in the form of the monohydrate. Other suitable excipients include dextran, glucose, maltose, sorbitol, xylitol, fructose, sucrose and trehalose.

[0079] In the case of dry powder inhalers and aerosols, the dosage unit is generally determined by means of a valve which delivers a metered amount. Units in accordance with the invention are typically arranged to administer a metered dose or "puff" containing from about 10 ng to about 100 μg of the compound of the invention. In another embodiment, the amount of compound administered in a metered dose is from about 50 ng to about 75 μg, or from about 100 ng to about 50 μg, or from about 500 ng to about 25 μg, or from about 750 ng to about 10 μg, or from about 1 μg to about 5 μg.

[0080] The vaccines of the present invention can also be administered orally or perorally, that is into a subject's body through or by way of the mouth and involves swallowing or transport through the oral mucosa (e.g., sublingual or buccal absorption) or both. Suitable flavors, such as

menthol and levomenthol, or sweeteners, such as saccharin or saccharin sodium, can be added to those formulations of the invention intended for oral or peroral administration.

[0081] Immunogenic compositions and vaccines of the present invention can be administered rectally or vaginally, for example, in the form of a suppository, pessary, or enema. Cocoa butter is a traditional suppository base, but various alternatives can be used as appropriate.

[0082] The vaccines of the present invention can also be administered directly to the eye or ear, typically in the form of drops of a micronized suspension or solution in isotonic, pH-adjusted, sterile saline. Other formulations suitable for ocular and aural administration include ointments, biodegradable (e.g. absorbable gel sponges, collagen) and non-biodegradable (e.g. silicone) implants, wafers, lenses and particulate or vesicular systems, such as niosomes or liposomes. A polymer such as crossed-linked polyacrylic acid, polyvinylalcohol, hyaluronic acid, a cellulosic polymer, for example, hydroxypropylmethylcellulose, hydroxyethylcellulose, or methyl cellulose, or a heteropolysaccharide polymer, for example, gelan gum, can be incorporated together with a preservative, such as benzalkonium chloride. Such formulations can also be delivered by iontophoresis.

[0083] In a further aspect, suitable for vaccines where the BVDV antigen is marked, the present invention provides method of vaccinating an animal, wherein a marked BVDV vaccine is administered to said animal, and wherein said marked vaccine comprises the chimeric pestivirus. For example, said chimeric pestivirus may lack at least one E^{rns} epitope which is present in wild-type bovine viral diarrhea virus. Thus, if such BVDV with altered E^{rns} protein is administered the present invention provides method of differentiating between an animal vaccinated with a vaccine comprising the chimeric pestivirus as described above and an animal infected with wild type bovine viral diarrhea virus, wherein the animal vaccinated with said vaccine generates antibodies to at least one E^{rns} epitope which is present in the chimeric pestivirus of said vaccine, but which is not present in wild-type bovine viral diarrhea virus, said method comprising the steps of: a) obtaining a serum sample from the animals; b) assaying said samples for the presence or absence of the anti - E^{rns} antibodies; c) identifying the animal having said antibodies as having been vaccinated with said vaccine; and d) identifying the animal lacking said antibodies as having been infected with the wild type BVDV.

[0084] The examples below are presented as illustrative embodiments, but should not be taken as limiting the scope of the invention. Many changes, variations, modifications, and other uses and applications of this invention will be apparent to those skilled in the art.

EXAMPLES

Example 1

[0085] The objective of this study was to evaluate, by serology, the suitability of different formulations for a single shot BVDV vaccine with at least 6 months duration of immunity (DOI).

[0086] Seventy (70), BVDV free (both antigen and antibody negative (VNT<1:4)), Brahman heifers were enrolled in the study and allocated to the six treatment groups (T01-T06) according to a completely randomized design. Fifteen animals were allocated to T01 and eleven animals each to T02-T06.

[0087] The vaccine compositions were adjuvanted as follows for the various treatment groups:

[0088] T01 – negative control (not vaccinated)

[0089] T02: CpG containing oligonucleotide (SEQ ID NO: 8, 85% homogeneity) – 250 ug; Quil A – 1mg; Lipoid S 100 (phosphatidylcholine) – 120 mg, cholesterol – 12 mg, thiomersal – 0.01%, and Minimum Essential Media (qs).

[0090] T03: DEAE Dextran – 100 mg, CpG containing oligonucleotide (SEQ ID NO: 8, 85% homogeneity) – 100 μg, mineral oil-45% v/v, SPAN®80 - 6.3% v/v, TWEEN®80 - 1.45% v/v, thiomersal – 0.01% w/v water (qs)

[0091] T04: DEAE Dextran – 100 mg, CpG containing oligonucleotide (SEQ ID NO: 8, 85% homogeneity) – 100 μg, mineral oil-45% v/v, SPAN®80 - 6.3% v/v, TWEEN®80 - 1.45% v/v, thiomersal – 0.01% w/v, REHYDRAGEL® - 2.5% w/v, water (qs).

[0092] T05: Quil A – 1mg, Cholesterol- 1 mg, DDA (dimethyl dioctadecyl ammonium bromide)- 0.5 mg, CpG containing oligonucleotide (SEQ ID NO: 8, 85% homogeneity) – 250 μg, CARBOPOL®(polyacrylic acid polymer) - 0.0375%, BAY®R1005 – 1 mg, thiomersal – 0.01%, and phosphate buffer (qs).

[0093] BVDV Trangie 1c (10^8 $TCID_{50}$/dose) was used as antigen for each of the formulations T02-T05.

[0094] Animals in T06 were vaccinated with PESTIGUARD® vaccine. This vaccine contains Bega and Trangie strains, both of which were licensed from the Elizabeth Macarthur Agricultural Institute. PESTIGUARD® vaccine is adjuvanted with immune stimulating complex (ISCOM).

[0095] Animals in group T06 were vaccinated on Day 0 and Day 28 and animals in T02-T05 (single dose IVP groups) were vaccinated on Day 28. Animals in T01 were unvaccinated controls.

[0096] Blood samples were collected on days 28, 42, 56, 86, 116, 148, 227 for the measurement of virus neutralizing antibodies to BVDV.

[0097] Safety was assessed by monitoring clinical observations and local injection site reactions.

[0098] All statistical tests were two-sided at the 5% level of significance. The experimental unit was the individual animal.

[0099] Antibody data in the control groups (T01) were used as the criterion for a valid test.

[0100] The proportion of animals showing a ≥ 4x fold increase in titer compared to Day 0 was summarized for each treatment group, at each time point post-vaccination up to Day 227.

[0101] Antibody titers were log-transformed and analyzed using a general linear mixed model for repeated measures, with terms including the fixed effects of treatment group, time point and the interaction of these effects, and the random effect of animal. Least squares means and 95% confidence intervals were presented for each treatment group at each time point, following back-transformation to the original scale. Where the main effect of treatment group or the treatment group by time point interaction term were significant at the 5% level, differences between treatment groups were presented for each time point. Treatment groups and/or time points where no detectable titers were recorded were omitted from the analysis.

[0102] BVDV virus neutralizing titers reported as <2 were set to 1 for the analysis. At the higher end, there were a large number of non-end-pointed results, with various upper values, e.g. ≥4871, ≥23170.

[0103] For consistency across time points, and to avoid fluctuations across time purely due to the varying upper values, any values ≥4871 were set to 4871 for the repeated measures analysis (this included all end-pointed and non-end-pointed values ≥4871). The resulting

geometric means would be an obvious underestimate for a number of groups, but this is unavoidable with non-end-pointed data. Laboratory data from Day 211 was not end-pointed to a high level (highest values ≥91), so this time point was excluded from the repeated measures analysis; these data were still included in the assessment of response and the assessment of the first criterion for a valid test. The proportion of animals with injection site reactions, the proportion of animals with visible injection site reactions and the number of animals with discharging injection sites were summarized for each treatment group at each time point and also across time points post-vaccination.

[0104] Injection site volumes were calculated from the measurements of length, width and depth using the formula for an ellipsoid. These volumes were log-transformed and analyzed using a general linear mixed model for repeated measures, with terms including the fixed effects of treatment group, time point and the interaction of these effects, and the random effect of animal. Least squares means and 95% confidence intervals were presented for each treatment group at each time point, following back-transformation to the original scale.

[0105] Where the main effect of treatment group or the treatment group by time point interaction term were significant at the 5% level, differences between treatment groups were presented for each time point. Treatment groups and/or time points where no (non-zero) volumes were recorded were omitted from the analysis.

[0106] All animals in the control group (T01) had consistently low titers (mostly <2, nothing above 3) from Day 0 to Day 227, and no injection site reactions throughout the study.

Virus Neutralizing Titers (VNT)

[0107] VNT for treatment groups T01, T02 and T05 were measured throughout the entire study. Treatment groups T04 and T06 only had consistent antibody data up to Day 148.

[0108] Data for animals B43 (T04) and B25 (T05) with very high titers on Day 0 and/or Day 28 prior to vaccination were excluded from all the summaries, analyses and figures. For all animals included in the analysis (n=15 in T01, n=11 in T02, T03 and T06, n=10 in T04 and T05), all titers on Day 0 were <2.

[0109] The data for groups T01-T06 are summarized in Tables 1-6, respectively.

Table 1 – Virus Neutralizing Titer for Treatment T01

Study Day	N	Geometric Mean	Lower 95% confidence limit	Upper 95% confidence limit	Min	Max
28	15	1	0.9	1.1	1	1
42	15	1	0.7	1.5	1	1
56	15	1	0.7	1.9	1	3
86	15	1	0.6	1.6	1	1
116	15	1	0.6	1.6	1	1
148	15	1	0.6	1.7	1	1
227	11	1	0.4	2.6	1	1

Table 2– Virus Neutralizing Titer for Treatment T02

Study Day	N	Geometric Mean	Lower 95% confidence limit	Upper 95% confidence limit	Min	Max
28	11	1	0.9	1.2	1	1
42	11	15.8	10	25	1	91
56	11	46.1	26.2	81	3	256
86	11	90.5	52.9	154.6	16	256
116	11	114.6	64.6	203.2	19	256
148	11	104.3	55.2	197	16	304
227	11	150.1	53.1	424.2	19	4871

Table 3– Virus Neutralizing Titer for Treatment T03

Study Day	N	Geometric Mean	Lower 95% confidence limit	Upper 95% confidence limit	Min	Max
28	11	1	0.9	1.2	1	1
42	11	1.8	1.1	2.8	1	10
56	11	12.4	7.1	21.8	1	215
86	11	50.6	29.6	86.5	2	304
116	11	45.4	25.6	80.5	1	256
148	11	54	28.6	102.1	3	215
227	11	78.1	27.6	220.8	3	4871

Table 4– Virus Neutralizing Titer for Treatment T04

Study Day	N	Geometric Mean	Lower 95% confidence limit	Upper 95% confidence limit	Min	Max
28	10	1	0.9	1.2	1	1
42	10	1.1	0.7	1.7	1	2
56	10	1.6	0.9	2.9	1	8
86	10	3.8	2.2	6.7	1	27
116	10	3.4	1.9	6.3	1	27
148	10	4.4	2.2	8.5	1	76

Table 5– Virus Neutralizing Titer for Treatment T05

Study Day	N	Geometric Mean	Lower 95% confidence limit	Upper 95% confidence limit	Min	Max
28	10	1	0.9	1.2	1	1
42	10	5.9	3.6	9.5	2	38
56	10	32.3	17.9	58.4	5	152
86	10	93.8	53.5	164.6	38	304
116	10	85.8	47	156	38	362
148	10	93.7	48.1	182.6	38	431
227	10	46.9	15.8	139.4	16	304

Table 6– Virus Neutralizing Titer for Treatment T06

Study Day	N	Geometric Mean	Lower 95% confidence limit	Upper 95% confidence limit	Min	Max
28	11	46.5	40	54	13	128
42	11	4871	3075.4	7714.9	4871	4871
56	11	4646.1	2644.7	8162.3	4096	4871
86	11	3133.4	1833.4	5355.3	1448	4871
116	11	1722.1	970.9	3054.8	512	4096
148	11	1425.2	754.3	2692.6	215	3444

[0110] All animals in groups T02, T03, T05 and T06 demonstrated a response to vaccination (≥4 x rise compared to Day 0), with most responses occurring by Day 56; one animal in each of groups T02 and T05 and three animals in T03 did not respond until Day 86, and an additional animal in T03 did not respond until Day 211. Only 4 animals (40%) in T04 showed a response to vaccination, and all titers in these groups were ≤76. It should be noted that the antibody titers in the PESTIGUARD® group (T06) were markedly higher than those seen in the single dose IVP

groups (e.g. all ≥4871 in T06 on Day 42, compared with all ≤431 in groups T02-T05 up to Day 148).

[0111] Geometric mean titers were significantly higher in T06 compared to other groups from Day 28 to Day 148 (the last time point included in the analysis for groups T04 and T06). Geometric mean titers were lower in group T04 compared with the other single dose groups (T02, T03 and T05), with significant differences in most cases from Day 42 to Day 148.

Injection site reactions

[0112] Following the vaccinations on Day 28, palpable, visible reactions were recorded for 82% of animals in T02, 64% in T03, 80% in T04, 40% in T05 and 55% in T06. No discharging reactions were recorded. The largest measurement recorded was 60 x 80 x 30 mm (animal B08 in T02 on Day 36), a calculated volume of 75.4 cm^3. There were a few significant pairwise differences in terms of geometric mean volumes, mainly due to higher volumes in T02 and T04 compared to other groups on Days 36 and 56; mean volumes in T03 were also higher compared to T05 and T06 at these time points, and mean volumes were also higher in T04 compared to T05 and T06 on Day 86.

[0113] Formulations T02 and T05 were selected for further development, based on the serological responses to vaccination with 6 month DOI and the safety profile. The rest of the formulations were not considered for further development.

Example 2

[0114] The aim of this study was to determine DOI of BVDV vaccines generally described in the previous example.

[0115] Sixty Bovine/*Bos Indicus* (Brahman and crossbred Brahman) heifers from a commercial herd in Northern Australia were used in this study. The heifers were seronegative (VNT <1:4) and antigen free from BVDV at the time of enrolment. Initial body weight of the animals was between 266 and 382 kg.

[0116] The animals were enrolled in the study and randomly allocated to the three treatment groups. There were 20 animals per group at the start of the study, with allocation to groups based on a randomized, generalized block design with a block size of 6 and blocking based on weight.

[0117] The first group (T01) was treated with 0.9% saline.

[0118] The second group (T02) was treated with a vaccine adjuvanted with Essential Media (qs) (40% of volume for Antigen and buffer), CpG (250 µg), Quil A (1 mg), Lipoid (120 mg), S100/Cholesterol (10:1), Montanide ISA50V2® oil (50%) and Thiomersal (0.01%).

[0119] The third group (T03) was adjuvanted with Phosphate (qs) (75% of volume for Antigen and buffer), Quil A (1 mg), Cholesterol (1 mg), DDA (0.5 mg), CpG (250 µg), CARBOPOL® (0.0375%), R1005 (1mg) and Thiomersal (0.01%).

[0120] $10^{8.0}$ $TCID_{50}$ of the Inactivated Trangie strain of BVDV 1c was used as the antigen for vaccines of groups T02 and T03. The volume of each treatment (T01-T03) was 2 ml, administered subcutaneously.

Table 7

Group	N	Vaccination Day	Blood sample collection, day	Injection site monitoring, day	Pregnancy testing	PI Challenge[#]	Necropsy & Fetal Sample Collection
T01	20	0	-43, 0^, 28, 56, 86, 107, 167, 230, 269, 335, 358, 372^, 386 & 419	0, 8, 28, 56, 86, 230	335, 351	358-400	400-422
T02	20	0	-43, 0^, 28, 56, 86, 107, 167, 230, 269, 335, 358, 372^, 386 & 419	0, 8, 28, 56, 86, 230	335, 351	358-400	400-422
T03	20	0	-43, 0^, 28, 56, 86, 107, 167, 230, 269, 335, 358, 372^, 386 & 419	0, 8, 28, 56, 86, 230	335, 351	358-400	400-422

- 14 pregnant heifers from each treatment group were randomly selected for the challenge. The remaining animals not selected for the challenge phase were removed from the study.
* Blood samples on Days post 344 were only collected from animals selected for the challenge.
^ blood samples collected on Day 0 were not analyzed and Day 372 samples were analyzed but not reported as the results were not end-pointed.

[0121] Persistently infected (PI) cattle were used for the challenge. Prior to the challenge, nasal swabs, blood samples and ear notch samples were collected from 10 suspected BVDV infected cattle. Sourced cattle were confirmed as being persistently BVDV infected based on presence of BVDV antigen in ear notches and by virus isolation (VI) from nasal swabs. Following the initial confirmation, all PI calves were tested 3 times prior to the challenge to confirm PI status by

virus isolation from nasal swabs. Blood or nasal swabs were used to determine the genotype of the BVDV in the PI animals.

[0122] The experimental design is provided in table 7 above.

[0123] *Testing of bulls and PIs.* Bulls: nasal swabs, blood, serum and ear notch samples were collected from 9 bulls on 3 occasions prior to the selection for natural breeding. Nasal swabs and blood samples were tested by qRT-PCR and ELISA respectively for the detection of BVDV antigens. Serum samples were tested for the measurement of BVDV antibodies by ELISA test. Ear notch samples were tested for BVDV antigen using the IDEXX in house test. All bulls were negative to BVDV antigens and antibodies and only 3 bulls were randomly selected for the breeding program.

[0124] PIs: approximately 11 calves persistently infected (PI) with BVDV were recruited from 3 different farms in Victoria prior to the challenge phase. All PIs were tested and confirmed to be persistently infected with BVDV on at least 3 occasions and the last PI tests were conducted on Day 335. Ear notch samples and nasal swabs were collected at each time point and tested for BVDV by IDEXX in house test and qRT-PCR at EMAI respectively. The BVDV subtyping was also performed on the nasal swabs at EMAI. Based on the BVDV subtyping, 8 PI calves infected with BVDV type 1a were selected for the challenge.

[0125] *Reproductive examination*. All heifers were examined by ultrasound examination for the detection of corpus luteum (CL) on two occasions on Days 256 and 269 prior to joining. Three BVDV free bulls were introduced with the heifers on Day 272 and the bulls were removed on Day 328. Pregnancy examination was performed by ultrasound on Days 335 and 351. Based on the pregnancy examination results on Day 351, 14 pregnant heifers were randomly selected for the challenge.

[0126] *Challenge*. Fourteen (14) heifers selected from each treatment group (42 study animals in total) were placed into a 0.2 ha confined paddock at the study site. On day 358, eight (8) selected PIs were introduced into the challenge paddock and comingled with the pregnant heifers for 42 days. Two separate hay bales and one water source were provided in the paddock to enhance BVDV exposure between animals. On Day 400, the 6 week long challenge phase was completed and the 8 PI calves were removed from the paddock. The pregnant heifers remained

in the same paddock until necropsy (Days 420-422). The estimated gestational age of the foetuses at the start of the challenge on Day 358 was 6 to 12 weeks. The gestational age of the foetuses during the challenge period was 42 to 126 days.

[0127] *Necropsy*. Pregnant heifers for the foetal harvest were randomly allocated to day of necropsy. Necropsy was conducted over a period of 3 days from Days 420 to 422. Prior to euthanasia and necropsy, selected pregnant heifers were transported to the abattoir by truck.

[0128] *Foetal harvest*. Each foetus was removed from the uterus and dissected surgically under sterile conditions. Duplicate individual swabs were collected from foetal thymus, lungs, liver, spleen and lymph nodes into sterile containers and submitted to EMAI for qRT-PCR assays. A second set of tissue samples from foetal thymus, lungs, liver, spleen, lymph nodes and skin were collected into individual sterile containers containing media supplied by EMAI and stored at the study site. Pericardial fluid in sterile containers and heart blood in plain serum tubes were also collected and stored at EMAI.

[0129] *qRT-PCR*. The qRT-PCR on the individual tissue swabs were performed using validated methods. Each qRT-PCR test was run for at least 45 cycles and the cycle threshold (Ct) reading was set as 40. Any values below 40 were reported as positive to BVDV and values of ≥40 were reported as negative to BVDV. If any one of the 5 tissue swab samples showed a positive result to qRT-PCR (i.e, Ct values ≤ 40), the entire foetus was reported as positive to BVDV in this study.

[0130] All statistical tests were two-sided at the 5% level of significance. The experimental unit was the individual animal. Antibody data prior to challenge and laboratory results from foetuses were used to assess the criteria for a valid test.

[0131] The proportion of animals with foetal positive results, as measured by PCR in each treatment group following challenge, was summarized for each treatment group. This primary outcome was analyzed as a binary variable (positive or negative result) using a generalized linear model with the fixed effect of treatment group and the random effect of block. Contrasts were used to make pairwise comparisons between treatment groups, with the primary comparisons being between the IVP treatment groups (T02 and T03) and the negative control group (T01).

[0132] BVDV antibody levels were log-transformed and analyzed using a general linear mixed model for repeated measures, with terms including the fixed effects of treatment group, time point and the interaction of these effects, and the random effects of block and animal.

[0133] The proportion of animals with visible injection site reactions, the proportion of animals with palpable injection site reactions and the proportion of animals with discharging injection site reactions were summarized for each treatment group at each time point, and across time points post-vaccination.

[0134] Injection site volumes were calculated from the measurements of length, width and depth using the formula for an ellipsoid. These volumes were log-transformed and analyzed using a general linear mixed model for repeated measures, with terms including the fixed effects of treatment group, time point and the interaction of these effects, and the random effects of block and animal. Treatment groups and/or time points where no (non-zero) volumes were measured were excluded from the analysis.

[0135] For all repeated measures analyses, least squares means have been presented for each treatment group at each time point separately, following back-transformation to the original scale. Where either the overall effect of treatment group, or the treatment by time point interaction term were significant at the 5% level, pairwise comparisons between treatment groups at each time point have also been presented.

[0136] The criteria for a valid test were met in this study as all the heifers in T01 remained seronegative to BVDV prior to the challenge on Day 358 and more than 80% foetuses from heifers in the negative control group were positive to BVDV.

[0137] One heifer in group T01 was euthanized due to chronic pneumonia, pericarditis and preuritis on day 283. No other significant adverse effects were observed in course of this study.

[0138] Virus Neutralizing Titers (Geometric Mean Titers) are provided in Table 8.

Table 8

Study Day	T01	T02	T03
-43	2.0	2.0	2.0
28	2.0	5.5	3.0
56	2.0	25.1	21.1
86	2.0	28.8	19.0
107	2.0	42.2	26.9
167	2.0	36.8	26.0
230	2.0	32.0	19.7
269	2.0	46.9	33.1
335	2.0	32.0	24.3
358	2.0	30.9	24.4
386	25.0	6490.4	8397.9
419	231.9	2939.3	4245.9

From Day 28 to 335, 20 animals per each treatment group, except on Day 335, T01 only had 19 heifers. From Days 358 to 419, all treatment groups had 14 animals, except on Day 419, T03 had data for only 13 heifers.

[0139] Table 9 summarizes Geometric mean and range of virus neutralizing antibody titers at pre- and post-vaccination and challenge.

Table 9

Group	Pre-vaccination (Day -43)	Post vaccination Day 28 Range (G mean)	Pre-Challenge (day 358) Range (G mean)	Post-Challenge (day 386) Range (G mean)
T01	<4	<4	<4	8-128 (25.0)
T02	<4	<4 – 64 (5.5)	4 – 256 (30.9)	1024-65536 (6490.4)
T03	<4	<4 – 8 (3.0)	4 – 128 (4.4)	2048-16384 (8397.9)

[0140] All heifers in T02 and T03 had detectable titers post-vaccination, with all heifers having a positive titer by Day 56 (though one animal in T02 had a titer <4 on Day 86, back to 8 by Day 107). However, the titers were generally low prior to challenge, with geometric means below 50 in both groups. One heifer in T03 had titers ≤32 to Day 230 but titers of 4096 and 2048 on Days 269 and 335 and this heifer was not selected for challenge. The reason for this unusual result is unknown.

[0141] Following challenge, heifers in T01 showed a rise in titer to 8-128 on Day 386 and 64-512 on Day 419 whereas heifers in T02 and T03 showed a much stronger antibody response to

challenge, with all titers on Days 386 and 419 in the range 512-65536 and geometric means between 2939 and 8398, respectively.

[0142] Based on the repeated measures analysis, geometric mean VN titers were significantly higher in T02 compared with T01 from Day 28 onwards and significantly higher in T03 compared with T01 from Day 56 onwards. There were no significant differences in geometric mean titers between T02 and T03.

[0143] In T02, the majority of animals (80%) had a palpable reaction, with most animals (70%) having a visible reaction at one or more time points. All reactions were apparent by Day 8 and the largest volumes were recorded at this time: two lumps of 60 x 60 x 30 mm (57 cm^3) and one of 60 x 70 x 30 mm (66 cm^3). All ISR volumes reduced to below 16 cm^3 by Day 28. By Day 230 (the last time point measured), 9 animals still had palpable reactions (8 visible) though the size of these lumps had reduced to below 5 cm^3 in all cases.

[0144] In T03, 9 animals (45%) had a palpable reaction. All reactions were visible and apparent by Day 8, but the largest volume recorded was only 25 x 25 x 20 mm (6.5 cm^3). All volumes reduced to below 2.1 cm^3 by Day 28 and largely resolved by Day 56; a different animal had a reaction of 0.3 cm^3 on each of Days 56, 86 and 230.

[0145] At day 56, 95% of the animals in group T02 and 95% of the animals in group T03 had no visible injection site reactions. At day 86, 95% of the animals in group T02 and 100% of the animals in group T03 had no visible injection site reactions. At day 230, 30% of the animals in group T02 and 95% of the animals in group T03 had no visible injection site reactions.

[0146] Table 10 summarizes geometric mean injection site volumes (cm^3) in groups T02 and T03.

Table 10

Day	T02	T03
8	5.23	0.83
28	1.22	0.18
56	0.03	0.01
86	0.03	0.01
230	0.47	0.01

[0147] Geometric mean volumes were significantly higher in T02 compared to T03 on Days 8, 28 and 230. No animals had a discharging reaction.

[0148] Twelve months after vaccination with the single dose vaccines, BVDV fetal infection was determined by qRT-PCR.

[0149] BVDV fetal transmission was detected in 11/13 (84.6%) foetuses in T01, 2/14 (14.3%) in T02 and 7/14 (50%) in T03. Based on the foetal protection efficacy calculated using Formula I, treatment T02 resulted in 83.1% efficacy whereas treatment T03 resulted in 40.9% efficacy against the natural BVDV Type 1a challenge derived from BVDV persistently infected calves (see Table 11).

Efficacy = (%of T01 infected – % of [T02 or T03] infected)/% of T01 infected

Formula I

Table 11

Group	Number of BVDV positive fetuses by qRT-PCR	Percentage of Fetal Protection
T01	11/13^	-
T02	2/14	83.1
T03	7/14	40.9

^ One heifer was empty at necropsy. Uterine tissue was tested negative to BVDV by qRT-PCR.

[0150] The proportion of animals with foetal positive results was significantly higher in the control group (T01) compared to group T02 ($p=0.003$), but there were no significant differences between groups T01 and T03 ($p=0.09$) or between groups T02 and T03 ($p=0.06$).

[0151] All publications cited in the specification, both patent publications and non-patent publications, are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein fully incorporated by reference to the same extent as if each individual publication were specifically and individually indicated as being incorporated by reference.

[0152] Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the spirit and scope of the present invention as defined by the following claims.

CLAIMS

1. A vaccine comprising:
    a) an antigen component comprising a BVDV antigen
    b) an adjuvant component comprising a triterpenoid saponin, an immunostimulatory nucleotide containing CpG, a phospholipid, and a sterol,

wherein said vaccine is a water-in-oil emulsion and wherein said vaccine is effective as a single-dose vaccine.

2. The vaccine of claim 1 wherein the BVDV antigen comprises a BVDV-1 antigen and a BVDV-2 antigen.

3. The vaccine of claim 1 or 2, wherein the BVDV antigen comprises a cytopathic virus.

4. The vaccine of any one of claims 1-3, wherein the BVDV antigen comprises a noncytopathic virus.

5. The vaccine of any one of claims 1-4, wherein said BVDV antigen is a recombinant antigen.

6. The vaccine of claim 5, wherein said antigen comprises a marker for differentiation between vaccinated and infected animals.

7. The vaccine of any one of claims 1-6, wherein the phospholipid is phosphatidylcholine.

8. The vaccine of any one of claims 1-7, wherein the phospholipid is present in the amount of about 120 mg per dose.

9. The vaccine of any one of claims 1-8 wherein the immunostimulatory nucleotide containing CpG comprises SEQ ID NO: 8.

10. The vaccine of any one of claims 1-9, wherein the immunostimulatory nucleotide containing CpG is present in the amount of about 250 μg per dose.

11. The vaccine of any one of claims 1-10 wherein the triterpenoid saponin is Quil A or a purified fraction thereof.

12. The vaccine of any one of claims 1-11 wherein the triterpenoid saponin is present in the amount of about 1000 μg per dose.

13. The vaccine of any one of claims 1-12 wherein the sterol is cholesterol.

14. The vaccine of any one of claims 1-13, wherein the oil is a mineral oil.

15. The vaccine of any one of claims 1-13, wherein the sterol and the phospholipid are present in mass ratio of about 1:8 to about 1:12.

16. A method of vaccinating against BVDV to a bovine in need thereof, the method comprising administering to said bovine the vaccine according to any one of claims 1-14.

17. The method of claim 16 wherein only a single dose of said vaccine is administered.

18. The method of claim 16 or claim 17, wherein said animal has not been previously vaccinated against BVDV.

19. A method of differentiating between the vaccinated and infected animals, the method comprising
    a) vaccinating the bovines in need thereof with the vaccine comprising a vaccine according to any one of claims 6-15,
    b) obtaining samples from said vaccinated bovines,
    c) assaying said samples for the presence or absence of the antibodies to the BVDV and the marker;
    d) identifying the animal having said antibodies both against the BVDV and against the marker as having been vaccinated with said vaccine; and
    e) identifying the animal having said antibodies against the BVDV and lacking said antibodies against the marker as having been infected with the wild type BVDV.

20. The vaccine of claim 1, substantially as herein described with reference to any one of the Examples thereof.