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(57) **Abrégé/Abstract:**

The present invention provides safe vaccines and methods of preparing such vaccines. The vaccines of the present invention contain at least two live mutant viruses of the same family or nucleic acid molecules encoding such viruses, wherein each of the two viruses or the encoding nucleic acids contains a mutation that confers a desirable phenotype and the mutations in the viruses reside in the same genomic site such that the mutant viruses cannot recombine with each other to eliminate the mutations.

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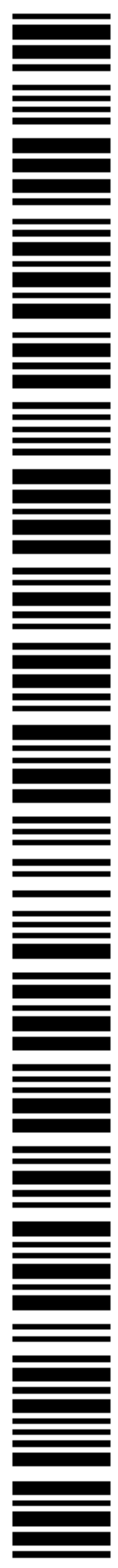
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(54) Title: SAFE MUTANT VIRAL VACCINES

(57) Abstract: The present invention provides safe vaccines and methods of preparing such vaccines. The vaccines of the present invention contain at least two live mutant viruses of the same family or nucleic acid molecules encoding such viruses, wherein each of the two viruses or the encoding nucleic acids contains a mutation that confers a desirable phenotype and the mutations in the viruses reside in the same genomic site such that the mutant viruses cannot recombine with each other to eliminate the mutations.



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SAFE MUTANT VIRAL VACCINES

5 Field Of The Invention

The present invention relates generally to vaccines suitable for administration to animals against viral infections. More specifically, the present invention relates to safe vaccines and methods of preparing such vaccines. The vaccines of the present invention contain at least two live mutant viruses of the same family or nucleic acid molecules
10 encoding such viruses, wherein each of the viruses or the encoding nucleic acids contains a mutation that confers a desirable phenotype and the mutations in the viruses reside in the same genomic site such that the mutant viruses cannot recombine with each other to eliminate the mutations.

15 Background Of The Invention

The virus family *Flaviviridae* consists of the genera *Pestivirus*, *Flavivirus* and *Hepacivirus*. The genus *Pestivirus* is represented by the species Bovine viral diarrhea virus 1 (BVDV-1), BVDV-2, classical swine fever virus, and Border disease virus. The virions of the family members encapsulate positive-strand RNA genomes of about 9.5 to 12.3 kb. The
20 genomic RNAs contain contiguous long open reading frames (ORFs), which are translated into polyproteins that are processed by cellular and viral proteases to give rise to the mature viral proteins. For members of *Pestivirus*, the ORF encodes a polyprotein of about 3900 amino acids, which is cotranslationally and posttranslationally processed to the following mature viral proteins (from 5' to 3'): N^{pro}, C, E^{ns}, E1, E2, NS2-3, NS4A, NS4B, NS5A, and
25 NS5B.

Two biotypes are found among some members of *Pestivirus* based on their effect on tissue culture cells, namely cytopathogenic (cytopathic or cp) and noncytopathogenic (noncytopathic or ncp). Genome analyses revealed insertions of cellular sequences, sometimes accompanied by duplication of viral sequences, genomic rearrangements, and/or
30 deletions of viral sequences in the genomes of cp pestiviruses, but not in the RNAs of the corresponding ncp pestiviruses. This suggests that cp pestiviruses are evolved from ncp pestiviruses by RNA recombination.

BVDV is a widely distributed pathogen of cattle. BVDV-1 usually produces only mild diarrhea in immunocompetent animals, whereas BVDV-2 can produce thrombocytopenia, hemorrhages and acute fatal disease. BVDV is capable of crossing the placenta of pregnant
35 cattle and may result in the birth of persistently infected (PI) calves (Malmquist, *J. Am. Vet. Med. Assoc.* 152:763-768 (1968); Ross, et al., *J. Am. Vet. Med. Assoc.* 188:618-619 (1986)). Viremic 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)). Viruses of either genotype may exist as one of the two biotypes, cp or ncp. The cp phenotype correlates with the expression of NS3, since cells infected with either cp or ncp BVDV both express NS2-3, whereas NS3 is detected only after infection with cp BVDV. NS3 is colinear to the C-terminal part of NS2-3. The expression of NS3 appears to be a result of genomic alterations observed for cp BVDV.

Presently available viral vaccines include killed or attenuated live viral vaccines, live-vectored vaccines, subunit vaccines, and DNA or RNA vaccines. See Roth et al., "New Technology For Improved Vaccine Safety And Efficacy", *Veterinary Clinics North America: Food Animal Practice* 17(3): 585-597 (2001). Attenuation of viruses can be achieved by UV irradiation, chemical treatment, or high serial passage *in vitro*. The number, position and nature of mutations induced by these methods are unknown absent genomic sequence analyses. Attenuation can also be achieved by making defined genetic alterations, for example, specific deletion of viral sequences known to confer virulence, or insertion of sequences into the viral genome. One concern with respect to the use of attenuated live viral vaccines is that attenuated mutant viruses have the potential to recombine *in vivo* to eliminate the attenuating mutation(s) thereby restoring virulence. For example, in the presence of a virulent (wild type) field strain, attenuated viruses having deletions in the viral genome have the potential to recombine with the virulent strain to restore the deleted sequence. See, e.g., Roth et al., *supra*. Cytopathic pestiviruses having cellular insertions have also been observed to give rise to noncytopathic viruses in cell culture by deletion of the cellular sequences, possibly through RNA recombination. See, e.g., Baroth et al., "Insertion of cellular NEDD8 coding sequences in a pestivirus", *Virology*. 278(2): 456-66, (2000), and Becher et al., "RNA recombination between persisting pestivirus and a vaccine strain: generation of cytopathogenic virus and induction of lethal disease", *Journal of Virology* 75(14): 6256-64 (2001). Where it is desired to include two attenuated mutant viruses from the same species, genus or family in a vaccine composition, there is a concern that the two viruses may recombine in the vaccinated animal thereby eliminating the attenuating mutations. See, e.g., Glazenburg et al., "Genetic recombination of pseudorabies virus: evidence that homologous recombination between insert sequences is less frequent than between autologous sequences", *Archives of Virology*, 140(4): 671-85 (1995).

There remains a need to develop safe and effective vaccines that protect animals against viral infections.

Summary Of The Invention

The present invention provides safe vaccines which contain at least two live mutant viruses of the same family or nucleic acid molecules encoding such viruses, wherein each virus or the encoding nucleic acid contains a mutation that confers a desirable phenotype,

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and the mutations in the viruses reside in the same genomic site such that the mutant viruses cannot recombine with each other to eliminate the mutations.

The present invention also provides a method of preparing a safe viral vaccine by selecting or constructing two or more live mutant viruses of the same family, genus or species, wherein each virus contains a mutation that confers a desirable phenotype, and the mutations in the viruses reside in the same genomic site such that the mutant viruses can not undergo homologous recombination to eliminate the mutations.

The present invention further provides a method of protecting an animal against viral infections by administering to the animal a vaccine composition of the present invention.

Brief Description Of The Drawings

Figure 1. Alignment of the cellular insertions and flanking viral sequences from the NS2-3 regions of BVDV-1 strain NADL and BVDV-2 strain 53637.

Detailed Description Of The Invention

It has been uniquely recognized in accordance with the present invention that live mutant viruses of the same family, which contain mutations at the same genomic site of the viruses, cannot recombine with one another to eliminate the mutations.

Accordingly, in one embodiment, the present invention provides safe vaccine compositions containing at least two, i.e., two or more, live mutant viruses of the same family, or nucleic acid molecules encoding such viruses, wherein the mutations in the viruses reside in the same genomic site such that the mutant viruses cannot recombine with each other to eliminate the mutations.

In an embodiment, the invention provides a vaccine composition comprising at least two live mutant viruses of the same family, wherein each virus contains a mutation in the viral genome, and the mutations in the viruses reside in

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the same genomic site such that the mutant viruses cannot recombine with each other to eliminate the mutations, and wherein two of the live mutant viruses consist of mutant Bovine Viral Diarrhea Viruses (BVDV).

In another embodiment the present invention provides a method of preparing a safe viral vaccine, as described hereinabove. Specifically, a safe vaccine is prepared by selecting or constructing two or more live mutant viruses of the same family, genus or species, wherein each virus contains a mutation that confers a desirable phenotype (for example attenuation of virulence, alteration of cellular tropism or biotype, alteration of species tropism, or expression of a foreign gene cassette), and the mutations in the viruses reside in the same genomic site such that the mutant viruses can not undergo homologous recombination with each other to eliminate the mutations.

In an embodiment, the invention provides a method of preparing a safe viral vaccine comprising selecting or constructing two live mutant viruses of the same family, wherein each virus contains a mutation and the mutations in the viruses reside in the same genomic site such that the mutant viruses can not undergo homologous recombination to eliminate the mutations, and wherein the viruses consist of mutant Bovine Viral Diarrhea Viruses (BVDV).

The term "vaccine" or "vaccine composition" refers to a composition containing live mutant viruses which, upon inoculation into an animal, induces a complete or partial immunity to the pathogenic version of the viruses, or alleviates the symptoms of diseases caused by the pathogenic versions of the viruses. The protective effects of a vaccine composition against a virus are normally achieved by inducing in the subject an immune response, either a cell-mediated or a humoral immune response, or a combination of both. Generally speaking, abolished or reduced incidences of viral infection, amelioration of the

symptoms, or accelerated elimination of the viruses from the infected subjects, are indicative of the protective effects of the vaccine composition.

By "animal" is meant to include birds, for example, chickens, turkeys, domestic waterfowl, and any mammal, for example, cattle, sheep, swine, goats, dogs, cats, and
5 horses.

The term "viruses", "viral isolates" or "viral strains" as used herein refer to viral particles or virions that contain viral genomic DNA or RNA, associated proteins, and other chemical constituents (such as lipids).

By "nucleic acid molecule encoding a virus" or "nucleic acid molecule of a virus" is
10 meant the genomic nucleic acid molecule of the virus, either in the form of RNA or DNA.

By "mutation" is meant to include deletion, insertion or substitution of one or more nucleotides, or a combination thereof. In accordance with the present invention, the mutation preferably confers a desirable phenotype, for example attenuation of virulence, alteration of cellular tropism or biotype, alteration of species tropism, or expression of a
15 foreign gene cassette. Especially preferred mutations are mutations that confer attenuated virulence.

By "attenuation" is meant that the virus has lost some or all of its ability to proliferate and/or cause disease in an animal infected with the virus. For example, an attenuated virus can be a virus that is unable to replicate at all or is limited to one or a few rounds of
20 replication, or restricted in cell or tissue tropism, when present in an animal in which a wild type pathogenic version of the attenuated virus can replicate.

An attenuated virus may have one or more mutations in a gene or genes that are involved in pathogenicity of the virus. Such mutations are also referred to herein as "attenuating mutation(s)". An attenuated virus can be produced from the wild type,
25 pathogenic virus by UV irradiation, chemical treatment, or high serial passage of the wild type, pathogenic virus *in vitro*. Alternatively, an attenuated virus can be produced from the wild type, pathogenic virus by making specific deletion of viral sequences known to confer virulence, insertion of sequences into the viral genome, or making one or more point mutations in the viral genome. An attenuated virus can be a viral isolate obtained from an
30 animal, which isolate is derived from the wild type, pathogenic version of the virus through events other than artificial means, e.g., events that have occurred in a host animal such as recombination.

The two or more live mutant viruses present in the vaccine compositions of the present invention contain mutations that reside in the same genomic site. By "same genomic
35 site" is meant that when the genomic nucleotide sequences of the viruses are aligned, the mutations in the viral genomes overlap with one another such that there is no opportunity for homologous recombination between and among the viral genomes to eliminate the mutations. In other words, when the genomic nucleotide sequences of the viruses are aligned, there is at least one contiguous portion of the aligned sequences where the

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sequences in the aligned viral genomes are mutant sequences. There are a number of computer programs that compare and align nucleic acid sequences which one skilled in the art may use. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in a nucleic acid sequence for optimal alignment with a second nucleic acid sequence). For example, the NBLAST and XBLAST programs as described in Altschul, et al., 1990, *J. Mol. Biol.* 215:403-410, the Gapped BLAST program as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402, and the PSI-Blast program as described in Altschul et al., 1997, *supra*. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

Generally speaking, the concept of the present invention, i.e., including in the same vaccine composition two or more live mutant viruses of the same family having mutations at the same genomic site, applies to mutant viruses from any family where the viral genomes have sufficient sequence identity to permit homologous recombination. It has been shown that a nucleotide identity as short as 15 nucleotides can lead to efficient homologous recombination (Nagy and Bujarski, *J. Virol.* 69:131-140, 1995).

The present invention applies especially to viruses of the *Flaviviridae* family. The *Flaviviridae* family consists of the genera *Pestivirus*, *Flavivirus* and *Hepacivirus*. The virions of the *Flaviviridae* family members encapsulate positive-strand RNA genomes of about 9.5 to 12.3 kb. The genomic RNAs containing contiguous long open reading frames, which are translated into polyproteins that are processed by cellular and viral proteases to give rise to the mature viral proteins.

Preferably, the mutant viruses of the vaccine composition of the present invention are from the same genus, either the same or different species.

In a preferred embodiment, the vaccine composition of the present invention contains two or more live mutant viruses from the *Pestivirus* genus. The genus *Pestivirus* is represented by the species Bovine Viral Diarrhea Virus Type 1 (BVDV-1), Bovine Viral Diarrhea Virus Type 2 (BVDV-2), classical swine fever virus, and Border disease virus. The ORF encodes a polyprotein of about 3900 amino acids, which is co-translationally and post-translationally processed to the following mature viral proteins (from 5' to 3'): N^{pro}, C, E^{ns}, E1, E2, NS2-3, NS4A, NS4B, NS5A, and NS5B.

Ordinarily, BVDV has a genome in the form of RNA. RNA can be reverse-transcribed into DNA for use in cloning. Thus, references made herein to nucleic acid and BVD viral sequences encompass both viral RNA sequences and DNA sequences derived from the viral RNA sequences. For convenience, genomic sequences of BVDV as depicted in the SEQUENCE LISTING hereinbelow only refer to the DNA sequences. The corresponding RNA sequence for each is readily apparent to those of skill in the art.

In a more preferred embodiment, the vaccine composition of the present invention contains a cytopathic BVDV-1 and a cytopathic BVDV-2, wherein the mutations in both

viruses associated with the cytopathic biotype reside in the same genomic site such that the two mutant viruses cannot recombine to eliminate the mutations.

BVDV-1 and BVDV-2 represent two closely related genotypes of BVDV. The nucleotide sequences of the two viruses share about 70% identity over the entire genome, and slightly higher percent identity within the NS2-3 region. It is believed that the percent identity between the viral genomes of BVDV-1 and BVDV-2, at least in the NS2-3 region, is sufficient to permit homologous recombination.

BVDV-1 usually produce only mild diarrhea in animals, whereas BVDV-2 are 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). The two types of viruses have distinct antigenicity determined by a panel of 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, cytopathogenic (cytopathic or cp) or noncytopathogenic (noncytopathic or ncp). Cp viruses induce cytopathic effects (e.g., cell lysis) on cultured cells, while noncytopathic viruses do not.

It is desirable to prepare vaccines that provide protection against both BVDV-1 and BVDV-2. However, because of the high degree of sequence identity between the two viruses, there is a possibility that a live cytopathic BVDV-1 and a live cytopathic BVDV-2 included in the same vaccine composition, could recombine with each other in the vaccinated animal to yield noncytopathic viruses. Recombination between BVDV-1 and BVDV-2 has been documented. See, e.g., Ridpath et al., *Virology* 212: 259-262 (1995). Infection of the fetus in pregnant cattle with ncp viruses before immunocompetence develops can result in the fetus remaining viremic through the period of gestation and the subsequent birth of a calf that remains persistently viremic. Such a calf can die of mucosal disease upon superinfection with a cp BVDV. Accordingly, the vaccine compositions provided by the present invention, which contain live cp BVDV-1 and live cp BVDV-2 having mutations at the same genomic site, are especially desirable for protecting animals against both BVDV-1 and BVDV-2.

In one embodiment, BVDV cp isolates obtained from animals can be used in the vaccine composition of the present invention. Cp isolates of both BVDV-1 and BVDV-2 have been reported and are available to those skilled in the art, e.g., BVDV-1 NADL (ATCC# VR1422 or VR-534), BVDV-2 53637 strain (deposited with the ATCC as PTA-4859), and type 2 field isolates such as those described by Ridpath and Neill, *J. Virol* 74:8771-8774, (2000). Cp isolates reported so far typically contain an insertion of a heterologous sequence, e.g., an ubiquitin coding sequence (Genbank accession number M96687 or De Moerlooze et al., *J. Gen. Virol.* 74:1433-1438, (1993)), a bovine NEDD8 coding sequence (Baroth et al.,

supra), or a *Bos taurus* DnaJ1 coding sequence (as described in the Examples hereinbelow), among others.

In another embodiment, a cp BVDV is generated by making defined alterations in the BVDV genome, e.g., by deleting specific viral sequences, inserting sequences into a specific viral genomic site, or making one or more substitutions, or combinations thereof.

Where a cp BVDV is generated by inserting a heterologous (i.e., foreign to the virus) sequence into a specific genomic site, the nature of the sequence to be inserted is generally not critical to the present invention. In addition, the insertion is not limited to any particular site so long as the insertion results in an attenuated phenotype. As heterologous sequences in cp isolates are often found in the NS2-3 region, the NS2-3 region, especially the part surrounding the putative NS2-3 cleavage site which corresponds to, e.g., amino acid residues # 1679 to #1680 of the BVDV-1 NADL strain (the numbering is based on the published genomic sequence Genbank accession No. M31182, SEQ ID NO: 4), is a preferred location for insertions.

An cp BVDV-1 can be generated by making a defined genomic alteration that mimics the mutation identified in a cp BVDV-2 isolate obtained from an animal, such that these viruses have mutations associated with the cp biotype in the same genomic site. Similarly, a cp BVDV-2 can be generated by way of making a defined genomic alteration that mimics the mutation identified in a cp BVDV-1 isolate obtained from an animal.

In a preferred embodiment, the vaccine composition of the present invention contains NADL (a cp BVDV-1 isolate), and BVDV-2 53637 (a cp BVDV-2 isolate), where the two cp isolates each contain a mutation at the same genomic site which results in the cytopathic biotype. The genomic sequence of the BVDV-1 NADL strain is set forth in SEQ ID NO: 4, and the BVDV-2 53637 strain was deposited with the ATCC as PTA-4859. Both isolates contain an insertion in the NS2-3 region. The attenuated cp BVDV-1 contains an insertion of a *Bos taurus* DnaJ1 coding sequence 3' of the thymidine at nucleotide position # 4993 (NADL sequence numbering), which is the third nucleotide of the codon encoding the glycine residue at amino acid position 1536. The attenuated cp BVDV-2 contains an insertion of a *Bos taurus* DnaJ1 coding sequence at the same genomic site.

According to the present invention, the cp BVDV isolates employed in the present vaccine composition have been attenuated and are therefore nonpathogenic. Methods of attenuation are known to those skilled in the art and are also described hereinbelow.

In another embodiment, the vaccine composition of the present invention contains an attenuated BVDV-1 and an attenuated BVDV-2, wherein the attenuating mutations in both viruses reside in the same genomic site such that the two mutant viruses cannot recombine to eliminate the attenuating mutations.

An attenuated BVDV is generated by UV irradiation, chemical treatment, or high serial passage of the pathogenic version of the virus *in vitro*. Sequence analysis can be conducted in order to determine the nature and genomic location of mutations generated by

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these methods. The mutation can be in the form of a deletion, insertion or substitution of one or more nucleotides, or a combination thereof. Alternatively, an attenuated BVDV is generated by making defined alterations in the BVDV genome, e.g., by deleting specific viral sequences, inserting sequences into a specific viral genomic site, or making one or more
 5 substitutions, or combinations thereof.

As described above, the live mutant viruses for use in the vaccine composition of the present invention can be from the same family, genus or species, where the viral genomes have sufficient sequence identity to permit homologous recombination. Additional examples of combinations of viruses appropriate for use in the vaccine composition of the present
 10 invention include, but are not limited to, combinations of different types of poliovirus, combinations of multiple live mutant strains of infectious bronchitis virus, combinations of multiple live mutant strains of Newcastle disease virus, combinations of Canine adenovirus - 1 and canine adenovirus-2, combinations of equine herpesvirus-1 and equine herpesvirus-4, combinations of multiple live mutant strains of influenza virus, combinations of multiple live
 15 attenuated strains of Feline calicivirus, combinations of multiple serotypes of Rotavirus, combinations of multiple serotypes of Rhinovirus, combinations of multiple serotypes of Foot and Mouth Disease virus, combinations of the European and North American genotypes of Porcine reproductive and respiratory syndrome virus, combinations of standard and variant strains of infectious bursal disease virus.

In accordance with the present invention, although viral particles are the preferred form for use in the vaccines, nucleic acid molecules encoding mutant viruses of the same family, genus or species, can be used directly in vaccines as well. The DNA or RNA molecule can be present in a "naked" form or it can be combined with an agent which facilitates cellular uptake (e.g., liposomes or cationic lipids). Vaccines and vaccination
 25 procedures that utilize nucleic acids (DNA or mRNA) have been well described in the art, e.g., U.S. Patent No. 5,703,055, U.S. Patent No. 5,580,859, U.S. Patent No. 5,589,466, International Patent Publication WO 98/35562, and by Ramsay et al., 1997, *Immunol. Cell Biol.* 75:360-363; Davis, 1997, *Cur. Opinion Biotech.* 8: 635-640; Manickan et al., 1997, *Critical Rev. Immunol.* 17: 139-154; Robinson, 1997, *Vaccine* 15(8): 785-787; Robinson et
 30 al., 1996, *AIDS Res. Hum. Retr.* 12(5): 455-457; Lai and Bennett, 1998, *Critical Rev. Immunol.* 18:449-484; and Vogel and Sarver, 1995, *Clin. Microbiol. Rev.* 8(3): 406-410.

In addition to two or more live mutant viruses from the same family, genus or species, the vaccine compositions can include other antigenic component. Other antigenic
 35 components appropriate for use in accordance with the present invention include, but are not limited to, antigens prepared from pathogenic bacteria such as *Mycoplasma hyopneumonia*, *Haemophilus somnus*, *Haemophilus parasuis*, *Bordetella bronchiseptica*, *Bacillus anthracis*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Mannheimia haemolytica*, *Mycoplasma bovis*, *Mycoplasma galanacium*, *Mycoplasma gallisepticum*, *Mycobacterium*

bovis, *Mycobacterium paratuberculosis*, *Clostridial spp.*, *Streptococcus uberis*, *Streptococcus suis*, *Staphylococcus aureus*, *Erysipelothrix rhusopathiae*, *Campylobacter spp.*, *Fusobacterium necrophorum*, *Escherichia coli*, *Lawsonia intracellularis*, *Listeria monocytogenes*, *Rickettsia rickettsii*, *Borrelia spp.*, *Ehrlichia spp.*, *Chlamydia spp.*, *Brucella spp.*, *Vibrio spp.*, *Salmonella enterica* serovars, *Leptospira spp.*; pathogenic fungi such as *Candida*; protozoa such as *Cryptosporidium parvum*, *Neospora canium*, *Toxoplasma gondii*, *Eimeria spp.*, *Babesia spp.*, *Giardia spp.*; helminths such as *Ostertagia*, *Cooperia*, *Haemonchus*, *Fasciola*; either in the form of an inactivated whole or partial cell preparation, or in the form of antigenic molecules obtained by genetic engineering techniques or chemical synthesis. Additional antigens include pathogenic viruses such as Marek's disease virus, infectious bursal disease virus, Newcastle's disease virus, chicken anemia virus, fowlpox virus, avian leukosis virus, infectious laryngotracheitis virus, reticuloendothelial virus, canine parvovirus, canine distemper virus, canine herpesvirus, canine coronavirus, canine parainfluenza-5, feline panleukopenia virus, feline herpes virus, feline calicivirus, feline immunodeficiency virus, feline infectious peritonitis virus, equine herpesvirus, equine arteritis virus, equine infectious anemia virus, Eastern equine encephalitis virus, Western equine encephalitis virus, Venezuelan equine encephalitis virus, West Nile virus, transmissible gastroenteritis virus, bovine coronavirus, Bovine herpesviruses-1,3,6, Bovine parainfluenza virus, Bovine respiratory syncytial virus, bovine leukosis virus, rinderpest virus, foot and mouth disease virus, rabies virus, African swine fever virus, Porcine parvovirus, PRRS virus, Porcine circovirus, influenza virus, swine vesicular disease virus, Techen fever virus, Pseudorabies virus, either in the form of modified live (attenuated) viral preparation, an inactivated whole or partial virus preparation, or in the form of antigenic molecules obtained by genetic engineering techniques or chemical synthesis. When additional attenuated live viruses are used, such additional viruses should preferably be from a family different from that of the two principal attenuated viruses, as described above.

In a preferred embodiment, the present invention provides a vaccine composition which contains an attenuated cp BVDV-1 derived from the BVDV-1 NADL strain, an attenuated cp BVDV-2 derived from the BVDV-2 53637 strain, where the two cp isolates each contain a mutation associated with the cp biotype at the same genomic site, and at least one (i.e., one or more) of the following antigenic component, either in inactivated or modified live form: bovine herpesvirus-1, bovine respiratory syncytial virus, parainfluenza virus-3, *Campylobacter fetus*, *Leptospira canicola*, *Leptospira grippotyphosa*, *Leptospira hardjo*, *Leptospira icterohaemorrhagiae*, *Leptospira pomona*, or *Mannheimia haemolytica*.

In addition, the vaccine compositions of the present invention can include one or more veterinarily-acceptable carriers. As used herein, "a veterinarily-acceptable carrier" includes any and all solvents, dispersion media, coatings, adjuvants, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like. Diluents can include water, saline, dextrose, ethanol, glycerol,

and the like. Isotonic agents can include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin, among others. The vaccine compositions can further include one or more other immunomodulatory agents such as, e.g., interleukins, interferons, or other cytokines

5 Adjuvants suitable for use in the vaccine compositions include, but are not limited to, the RIBI adjuvant system (Ribi inc.), alum, aluminum hydroxide gel, oil-in water emulsions, water-in-oil emulsions such as, e.g., Freund's complete and incomplete adjuvants, Block co polymer (CytRx, Atlanta GA), SAF-M (Chiron, Emeryville CA), AMPHIGEN® adjuvant, saponin, Quil A, cholesterol, QS-21 (Cambridge Biotech Inc., Cambridge MA), or other
10 saponin fractions, monophosphoryl lipid A, Avridine lipid-amine adjuvant, heat-labile enterotoxin from *E. coli* (recombinant or otherwise), cholera toxin, or muramyl dipeptide, among many others.

 Typically, a live mutant virus is present in a vaccine at an amount of about 1×10^6 and about 1×10^8 virus particles per dose, with a veterinarily acceptable carrier, in a volume
15 of between about 0.5 and about 5 ml. The precise amount of a virus in a vaccine composition effective to provide a protective effect can be determined by a skilled veterinarian. Where the DNA or RNA molecule of the virus is used in the vaccine, the amount of the nucleic acids should generally be between about 0.1 µg/ml and about 5.0 mg/ml.

20 The vaccine compositions of the present invention can be made in various forms depending upon the route of administration. For example, the vaccine compositions can be made in the form of sterile aqueous solutions or dispersions suitable for injectable use, or made in lyophilized forms using freeze-drying techniques. Lyophilized compositions are typically maintained at about 4°C, and can be reconstituted in a stabilizing solution, e.g.,
25 saline or and HEPES, with or without adjuvant.

 The vaccine compositions of the present invention can be administered to an animal for treating or preventing a disease caused by the pathogenic versions of the viruses in the vaccine compositions. Therefore, methods of vaccinating an animal against a disease caused by a virus are also provided by the present invention.

30 In practicing the present methods, a vaccine composition of the present invention is administered to an animal preferably via parenteral routes, although other routes of administration can be used as well, such as e.g., by oral, intranasal, intramuscular, intra-lymph node, intradermal, intraperitoneal, subcutaneous, rectal or vaginal administration, or by a combination of routes. Boosting regimens may be required and the dosage regimen
35 can be adjusted to provide optimal vaccination.

 The present invention is further illustrated by, but by no means limited to, the following examples.

EXAMPLE IDetermination Of The Position Of The Cellular Insertion In BVDV2 Strain 53637

A portion of the sequence of the NS2-3 region from BVDV2-53637 was determined, in order to identify and map the location of any cellular insertions in the region. A 670 base RT-PCR product was amplified from viral RNA, using forward primer 53637U1 (5'-CGTCCACAGATGGTTTGGT-3'; SEQ ID NO: 1) and reverse primer 53637L (5'-GGCTATGTATTGGACGTAACCC-3'; SEQ ID NO: 2). The RT-PCR product was purified and submitted for sequence analysis (SEQ ID NO: 3). When aligned with BVDV1-NADL (Genbank accession number M31182, SEQ ID NO: 4), striking similarities were observed (FIGURE 1). Both viruses contain an in-frame insertion derived from the *Bos taurus DnaJ1* gene. In the case of NADL, the insertion is 90 amino acids (270 nucleotides) in length and is located between glycine-1536 and proline-1627 in the NADL polyprotein. These coordinates correspond to glycine-1536 and proline-1537 in non-cytopathic BVDV1 strains such as SD-1 (Genbank accession number AAA42860, SEQ ID NO: 6), indicating that the genome alteration in NADL is a simple insertion with no concomitant deletion or duplication of flanking viral sequences. Like BVDV1-NADL, there is an insertion of a portion of the *Bos taurus DnaJ1* gene in BVDV2-53637. The cellular insertion is longer (131 amino acids, 393 nucleotides), being extended in both directions relative to the insertion in BVDV1-NADL. The location of the cellular insertion within the NS2-3 region is identical in the two viruses. Unlike BVDV1-NADL, the BVDV2-53637 insertion is accompanied by a deletion of 5 amino acids (15 nucleotides) of flanking viral sequences. Three amino acid residues are absent flanking the 5' end of the insertion, while two amino acids residues are absent flanking the 3' end of the insertion. Because the cellular insertions are at the same genome position in the two vaccine viruses, they cannot undergo homologous recombination to delete the insertion to generate a non-cytopathic chimeric virus.

Example IIAttempts To Detect Non-Cytopathic BVDV Viruses
In Co-Passaged BVDV1-NADL / BVDV2-53637 Cultures

In order to determine whether the two vaccine viruses are capable of recombining to generate detectable levels of non-cytopathic BVDV, the viruses were co-cultivated on susceptible cells and a sensitive hemi-nested RT-PCR assay was used to detect potential non-cytopathic viruses from among an excess of longer cytopathic products that still contain the cellular insert. To increase the probability of intertypic recombination *in vitro*, each virus was inoculated simultaneously onto confluent BK-6 cells in 6-well plates at a multiplicity of infection of 2-4 (12 replicates per experiment). After 2 - 3 days of co-cultivation the cells were frozen and thawed twice, and cell debris was removed by low speed centrifugation. The resulting supernatant fluid was then used as inoculum for the next passage. A total of

seven serial passages were conducted in several studies. During the passages BVDV1-NADL grew more rapidly than BVDV2-53637, but the type II virus was still detectable after seven passages using nested RT-PCR. A sensitive hemi-nested RT-PCR assay was employed in an attempt to detect any non-cytopathic virus.

5 In first round RT-PCR, forward primers 53637U1 (SEQ ID NO: 1) or NADL4744 (5'-CGTGGCTTCTTGGTACGGG-3', SEQ ID NO: 7) were used in conjunction with reverse primers 53637L (SEQ ID NO: 2) or NADL5305 (5'- AGCGGTATATTGTACAAAGCCA-3', SEQ IDNO: 8). All four combinations of forward and reverse primers were used in order to detect BVDV1, BVDV2, and intertypic recombinants. The expected size of RT-PCR product
10 was 562 bp for cytopathic BVDV1-NADL and 670 bp for cytopathic BVDV2-53637. Non-cytopathic viruses, if present at detectable levels, would be expected to yield first round products of 292 bp (BVDV1-NADL) or 277 bp (BVDV2-53637). Intertypic recombinants should be similar in size to one of the parents, or of intermediate length, depending on the location of the recombination site. Non-cytopathic BVDVs were never detected following first
15 round RT-PCR.

To increase the sensitivity of detecting non-cytopathic BVDV in the presence of a large excess of cytopathic BVDV, a restriction enzyme digestion step was included before the nested PCR to destroy the larger NS2-3 templates derived from the cytopathic viruses. A combination of *MspI* and *DraI* was selected based on the observation that they cut within
20 the *Bos taurus DnaJ1* insert but do not cut the flanking viral sequences. In second round (hemi-nested) PCR, forward primers 53637U2 (5'-TGCACGATCTGTGAAGGGAAAGAA -3', SEQ ID NO: 9) or NADL4844 (5'- TGCACTGTATGTGAGGGCCGAGAG -3', SEQ ID NO: 10) were used in conjunction with the same two reverse primers 53637L or NADL5305. Appropriate primer combinations were used to attempt to detect intertypic recombinants as
25 well as BVDV1 and BVDV2. The expected size of RT-PCR product is 462 bp for cytopathic BVDV1-NADL and 570 bp for cytopathic BVDV2-53637 (present at low levels due to incomplete digestion of the cytopathic BVDV RT-PCR products). Non-cytopathic viruses, if present at detectable levels, would be expected to yield second round products of 192 bp (BVDV1-NADL) or 177 bp (BVDV2-53637). Intertypic recombinants should be similar in size
30 to one of the parents, or of intermediate length, depending on the location of the recombination site. Non-cytopathic BVDVs were never detected following second round PCR. In a few individual reactions, aberrant bands of various sizes were seen. All bands between 100 and 300 bp were considered to be potential non-cytopathic products and were submitted for DNA sequence analysis. In every case the aberrant band was the result of
35 false priming during PCR. There was no evidence of non-cytopathic virus in any of the studies.

SEQUENCE LISTING

<110> Welch, Siao-Kun Wan
Calvert, Jay Gregory
O'Hara, Michael K.
Cao, Xuemei K.

<120> SAFE MUTANT VIRAL VACCINES

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<213> Bovine Viral Diarrhea Virus 1 strain NADL

<400> 5

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```

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Pro Val Gly Val Glu Glu Pro Val Tyr Asp Gln Ala Gly Asp Pro Leu
20           25           30

```

```

Phe Gly Glu Arg Gly Ala Val His Pro Gln Ser Thr Leu Lys Leu Pro
35           40           45

```

```

His Lys Arg Gly Glu Arg Asp Val Pro Thr Asn Leu Ala Ser Leu Pro
50           55           60

```

```

Lys Arg Gly Asp Cys Arg Ser Gly Asn Ser Arg Gly Pro Val Ser Gly
65           70           75           80

```

```

Ile Tyr Leu Lys Pro Gly Pro Leu Phe Tyr Gln Asp Tyr Lys Gly Pro
85           90           95

```

```

Val Tyr His Arg Ala Pro Leu Glu Leu Phe Glu Glu Gly Ser Met Cys
100          105          110

```

```

Glu Thr Thr Lys Arg Ile Gly Arg Val Thr Gly Ser Asp Gly Lys Leu
115          120          125

```

```

Tyr His Ile Tyr Val Cys Ile Asp Gly Cys Ile Ile Ile Lys Ser Ala
130          135          140

```

```

Thr Arg Ser Tyr Gln Arg Val Phe Arg Trp Val His Asn Arg Leu Asp
145          150          155          160

```

```

Cys Pro Leu Trp Val Thr Thr Cys Ser Asp Thr Lys Glu Glu Gly Ala
165          170          175

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Thr	Lys	Lys	Lys	Thr	Gln	Lys	Pro	Asp	Arg	Leu	Glu	Arg	Gly	Lys	Met	180	185	190	
Lys	Ile	Val	Pro	Lys	Glu	Ser	Glu	Lys	Asp	Ser	Lys	Thr	Lys	Pro	Pro	195	200	205	
Asp	Ala	Thr	Ile	Val	Val	Glu	Gly	Val	Lys	Tyr	Gln	Val	Arg	Lys	Lys	210	215	220	
Gly	Lys	Thr	Lys	Ser	Lys	Asn	Thr	Gln	Asp	Gly	Leu	Tyr	His	Asn	Lys	225	230	235	240
Asn	Lys	Pro	Gln	Glu	Ser	Arg	Lys	Lys	Leu	Glu	Lys	Ala	Leu	Leu	Ala	245	250	255	
Trp	Ala	Ile	Ile	Ala	Ile	Val	Leu	Phe	Gln	Val	Thr	Met	Gly	Glu	Asn	260	265	270	
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Glu	Lys	Ile	Cys	Thr	Gly	Val	Pro	Ser	His	Leu	Ala	Thr	Asp	Ile	Glu	305	310	315	320
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Leu	Gly	Lys	Lys	Leu	Glu	Asn	Lys	Ser	Lys	Thr	Trp	Phe	Gly	Ala	Tyr	485	490	495	
Ala	Ala	Ser	Pro	Tyr	Cys	Asp	Val	Asp	Arg	Lys	Ile	Gly	Tyr	Ile	Trp	500	505	510	

Tyr	Thr	Lys	Asn	Cys	Thr	Pro	Ala	Cys	Leu	Pro	Lys	Asn	Thr	Lys	Ile	515	520	525
Val	Gly	Pro	Gly	Lys	Phe	Gly	Thr	Asn	Ala	Glu	Asp	Gly	Lys	Ile	Leu	530	535	540
His	Glu	Met	Gly	Gly	His	Leu	Ser	Glu	Val	Leu	Leu	Leu	Ser	Leu	Val	545	550	555
Val	Leu	Ser	Asp	Phe	Ala	Pro	Glu	Thr	Ala	Ser	Val	Met	Tyr	Leu	Ile	565	570	575
Leu	His	Phe	Ser	Ile	Pro	Gln	Ser	His	Val	Asp	Val	Met	Asp	Cys	Asp	580	585	590
Lys	Thr	Gln	Leu	Asn	Leu	Thr	Val	Glu	Leu	Thr	Thr	Ala	Glu	Val	Ile	595	600	605
Pro	Gly	Ser	Val	Trp	Asn	Leu	Gly	Lys	Tyr	Val	Cys	Ile	Arg	Pro	Asn	610	615	620
Trp	Trp	Pro	Tyr	Glu	Thr	Thr	Val	Val	Leu	Ala	Phe	Glu	Glu	Val	Ser	625	630	635
Gln	Val	Val	Lys	Leu	Val	Leu	Arg	Ala	Leu	Arg	Asp	Leu	Thr	Arg	Ile	645	650	655
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 Asn Leu Gly Glu Asp Leu His Asn Cys Ile Leu Gly Gly Asn Trp Thr
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1205						1210					1215			
Gln	Cys	Ile	Leu	Ser	Leu	Val	Ser	Ala	Val	Phe	Leu	Ile	Arg	Ser
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1295						1300					1305			
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1310						1315					1320			
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1340						1345					1350			
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1355						1360					1365			
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Gly	Leu	Lys	Lys	Phe	Tyr	Leu	Leu	Ser	Gly	Arg	Leu	Arg	Asn	Leu
1430						1435					1440			
Ile	Ile	Lys	His	Lys	Val	Arg	Asn	Glu	Thr	Val	Ala	Ser	Trp	Tyr
1445						1450					1455			
Gly	Glu	Glu	Glu	Val	Tyr	Gly	Met	Pro	Lys	Ile	Met	Thr	Ile	Ile
1460						1465					1470			
Lys	Ala	Ser	Thr	Leu	Ser	Lys	Ser	Arg	His	Cys	Ile	Ile	Cys	Thr
1475						1480					1485			

Val	Cys	Glu	Gly	Arg	Glu	Trp	Lys	Gly	Gly	Thr	Cys	Pro	Lys	Cys
1490						1495					1500			
Gly	Arg	His	Gly	Lys	Pro	Ile	Thr	Cys	Gly	Met	Ser	Leu	Ala	Asp
1505						1510					1515			
Phe	Glu	Glu	Arg	His	Tyr	Lys	Arg	Ile	Phe	Ile	Arg	Glu	Gly	Asn
1520						1525					1530			
Phe	Glu	Gly	Met	Cys	Ser	Arg	Cys	Gln	Gly	Lys	His	Arg	Arg	Phe
1535						1540					1545			
Glu	Met	Asp	Arg	Glu	Pro	Lys	Ser	Ala	Arg	Tyr	Cys	Ala	Glu	Cys
1550						1555					1560			
Asn	Arg	Leu	His	Pro	Ala	Glu	Glu	Gly	Asp	Phe	Trp	Ala	Glu	Ser
1565						1570					1575			
Ser	Met	Leu	Gly	Leu	Lys	Ile	Thr	Tyr	Phe	Ala	Leu	Met	Asp	Gly
1580						1585					1590			
Lys	Val	Tyr	Asp	Ile	Thr	Glu	Trp	Ala	Gly	Cys	Gln	Arg	Val	Gly
1595						1600					1605			
Ile	Ser	Pro	Asp	Thr	His	Arg	Val	Pro	Cys	His	Ile	Ser	Phe	Gly
1610						1615					1620			
Ser	Arg	Met	Pro	Phe	Arg	Gln	Glu	Tyr	Asn	Gly	Phe	Val	Gln	Tyr
1625						1630					1635			
Thr	Ala	Arg	Gly	Gln	Leu	Phe	Leu	Arg	Asn	Leu	Pro	Val	Leu	Ala
1640						1645					1650			
Thr	Lys	Val	Lys	Met	Leu	Met	Val	Gly	Asn	Leu	Gly	Glu	Glu	Ile
1655						1660					1665			
Gly	Asn	Leu	Glu	His	Leu	Gly	Trp	Ile	Leu	Arg	Gly	Pro	Ala	Val
1670						1675					1680			
Cys	Lys	Lys	Ile	Thr	Glu	His	Glu	Lys	Cys	His	Ile	Asn	Ile	Leu
1685						1690					1695			
Asp	Lys	Leu	Thr	Ala	Phe	Phe	Gly	Ile	Met	Pro	Arg	Gly	Thr	Thr
1700						1705					1710			
Pro	Arg	Ala	Pro	Val	Arg	Phe	Pro	Thr	Ser	Leu	Leu	Lys	Val	Arg
1715						1720					1725			
Arg	Gly	Leu	Glu	Thr	Ala	Trp	Ala	Tyr	Thr	His	Gln	Gly	Gly	Ile
1730						1735					1740			
Ser	Ser	Val	Asp	His	Val	Thr	Ala	Gly	Lys	Asp	Leu	Leu	Val	Cys
1745						1750					1755			
Asp	Ser	Met	Gly	Arg	Thr	Arg	Val	Val	Cys	Gln	Ser	Asn	Asn	Arg
1760						1765					1770			
Leu	Thr	Asp	Glu	Thr	Glu	Tyr	Gly	Val	Lys	Thr	Asp	Ser	Gly	Cys
1775						1780					1785			
Pro	Asp	Gly	Ala	Arg	Cys	Tyr	Val	Leu	Asn	Pro	Glu	Ala	Val	Asn
1790						1795					1800			

Ile	Ser	Gly	Ser	Lys	Gly	Ala	Val	Val	His	Leu	Gln	Lys	Thr	Gly
1805						1810					1815			
Gly	Glu	Phe	Thr	Cys	Val	Thr	Ala	Ser	Gly	Thr	Pro	Ala	Phe	Phe
1820						1825					1830			
Asp	Leu	Lys	Asn	Leu	Lys	Gly	Trp	Ser	Gly	Leu	Pro	Ile	Phe	Glu
1835						1840					1845			
Ala	Ser	Ser	Gly	Arg	Val	Val	Gly	Arg	Val	Lys	Val	Gly	Lys	Asn
1850						1855					1860			
Glu	Glu	Ser	Lys	Pro	Thr	Lys	Ile	Met	Ser	Gly	Ile	Gln	Thr	Val
1865						1870					1875			
Ser	Lys	Asn	Arg	Ala	Asp	Leu	Thr	Glu	Met	Val	Lys	Lys	Ile	Thr
1880						1885					1890			
Ser	Met	Asn	Arg	Gly	Asp	Phe	Lys	Gln	Ile	Thr	Leu	Ala	Thr	Gly
1895						1900					1905			
Ala	Gly	Lys	Thr	Thr	Glu	Leu	Pro	Lys	Ala	Val	Ile	Glu	Glu	Ile
1910						1915					1920			
Gly	Arg	His	Lys	Arg	Val	Leu	Val	Leu	Ile	Pro	Leu	Arg	Ala	Ala
1925						1930					1935			
Ala	Glu	Ser	Val	Tyr	Gln	Tyr	Met	Arg	Leu	Lys	His	Pro	Ser	Ile
1940						1945					1950			
Ser	Phe	Asn	Leu	Arg	Ile	Gly	Asp	Met	Lys	Glu	Gly	Asp	Met	Ala
1955						1960					1965			
Thr	Gly	Ile	Thr	Tyr	Ala	Ser	Tyr	Gly	Tyr	Phe	Cys	Gln	Met	Pro
1970						1975					1980			
Gln	Pro	Lys	Leu	Arg	Ala	Ala	Met	Val	Glu	Tyr	Ser	Tyr	Ile	Phe
1985						1990					1995			
Leu	Asp	Glu	Tyr	His	Cys	Ala	Thr	Pro	Glu	Gln	Leu	Ala	Ile	Ile
2000						2005					2010			
Gly	Lys	Ile	His	Arg	Phe	Ser	Glu	Ser	Ile	Arg	Val	Val	Ala	Met
2015						2020					2025			
Thr	Ala	Thr	Pro	Ala	Gly	Ser	Val	Thr	Thr	Thr	Gly	Gln	Lys	His
2030						2035					2040			
Pro	Ile	Glu	Glu	Phe	Ile	Ala	Pro	Glu	Val	Met	Lys	Gly	Glu	Asp
2045						2050					2055			
Leu	Gly	Ser	Gln	Phe	Leu	Asp	Ile	Ala	Gly	Leu	Lys	Ile	Pro	Val
2060						2065					2070			
Asp	Glu	Met	Lys	Gly	Asn	Met	Leu	Val	Phe	Val	Pro	Thr	Arg	Asn
2075						2080					2085			
Met	Ala	Val	Glu	Val	Ala	Lys	Lys	Leu	Lys	Ala	Lys	Gly	Tyr	Asn
2090						2095					2100			
Ser	Gly	Tyr	Tyr	Tyr	Ser	Gly	Glu	Asp	Pro	Ala	Asn	Leu	Arg	Val
2105						2110					2115			

Val	Thr	Ser	Gln	Ser	Pro	Tyr	Val	Ile	Val	Ala	Thr	Asn	Ala	Ile
2120						2125					2130			
Glu	Ser	Gly	Val	Thr	Leu	Pro	Asp	Leu	Asp	Thr	Val	Ile	Asp	Thr
2135						2140					2145			
Gly	Leu	Lys	Cys	Glu	Lys	Arg	Val	Arg	Val	Ser	Ser	Lys	Ile	Pro
2150						2155					2160			
Phe	Ile	Val	Thr	Gly	Leu	Lys	Arg	Met	Ala	Val	Thr	Val	Gly	Glu
2165						2170					2175			
Gln	Ala	Gln	Arg	Arg	Gly	Arg	Val	Gly	Arg	Val	Lys	Pro	Gly	Arg
2180						2185					2190			
Tyr	Tyr	Arg	Ser	Gln	Glu	Thr	Ala	Thr	Gly	Ser	Lys	Asp	Tyr	His
2195						2200					2205			
Tyr	Asp	Leu	Leu	Gln	Ala	Gln	Arg	Tyr	Gly	Ile	Glu	Asp	Gly	Ile
2210						2215					2220			
Asn	Val	Thr	Lys	Ser	Phe	Arg	Glu	Met	Asn	Tyr	Asp	Trp	Ser	Leu
2225						2230					2235			
Tyr	Glu	Glu	Asp	Ser	Leu	Leu	Ile	Thr	Gln	Leu	Glu	Ile	Leu	Asn
2240						2245					2250			
Asn	Leu	Leu	Ile	Ser	Glu	Asp	Leu	Pro	Ala	Ala	Val	Lys	Asn	Ile
2255						2260					2265			
Met	Ala	Arg	Thr	Asp	His	Pro	Glu	Pro	Ile	Gln	Leu	Ala	Tyr	Asn
2270						2275					2280			
Ser	Tyr	Glu	Val	Gln	Val	Pro	Val	Leu	Phe	Pro	Lys	Ile	Arg	Asn
2285						2290					2295			
Gly	Glu	Val	Thr	Asp	Thr	Tyr	Glu	Asn	Tyr	Ser	Phe	Leu	Asn	Ala
2300						2305					2310			
Arg	Lys	Leu	Gly	Glu	Asp	Val	Pro	Val	Tyr	Ile	Tyr	Ala	Thr	Glu
2315						2320					2325			
Asp	Glu	Asp	Leu	Ala	Val	Asp	Leu	Leu	Gly	Leu	Asp	Trp	Pro	Asp
2330						2335					2340			
Pro	Gly	Asn	Gln	Gln	Val	Val	Glu	Thr	Gly	Lys	Ala	Leu	Lys	Gln
2345						2350					2355			
Val	Thr	Gly	Leu	Ser	Ser	Ala	Glu	Asn	Ala	Leu	Leu	Val	Ala	Leu
2360						2365					2370			
Phe	Gly	Tyr	Val	Gly	Tyr	Gln	Ala	Leu	Ser	Lys	Arg	His	Val	Pro
2375						2380					2385			
Met	Ile	Thr	Asp	Ile	Tyr	Thr	Ile	Glu	Asp	Gln	Arg	Leu	Glu	Asp
2390						2395					2400			
Thr	Thr	His	Leu	Gln	Tyr	Ala	Pro	Asn	Ala	Ile	Lys	Thr	Asp	Gly
2405						2410					2415			
Thr	Glu	Thr	Glu	Leu	Lys	Glu	Leu	Ala	Ser	Gly	Asp	Val	Glu	Lys
2420						2425					2430			

Ile	Met	Gly	Ala	Ile	Ser	Asp	Tyr	Ala	Ala	Gly	Gly	Leu	Glu	Phe
2435						2440					2445			
Val	Lys	Ser	Gln	Ala	Glu	Lys	Ile	Lys	Thr	Ala	Pro	Leu	Phe	Lys
2450						2455					2460			
Glu	Asn	Ala	Glu	Ala	Ala	Lys	Gly	Tyr	Val	Gln	Lys	Phe	Ile	Asp
2465						2470					2475			
Ser	Leu	Ile	Glu	Asn	Lys	Glu	Glu	Ile	Ile	Arg	Tyr	Gly	Leu	Trp
2480						2485					2490			
Gly	Thr	His	Thr	Ala	Leu	Tyr	Lys	Ser	Ile	Ala	Ala	Arg	Leu	Gly
2495						2500					2505			
His	Glu	Thr	Ala	Phe	Ala	Thr	Leu	Val	Leu	Lys	Trp	Leu	Ala	Phe
2510						2515					2520			
Gly	Gly	Glu	Ser	Val	Ser	Asp	His	Val	Lys	Gln	Ala	Ala	Val	Asp
2525						2530					2535			
Leu	Val	Val	Tyr	Tyr	Val	Met	Asn	Lys	Pro	Ser	Phe	Pro	Gly	Asp
2540						2545					2550			
Ser	Glu	Thr	Gln	Gln	Glu	Gly	Arg	Arg	Phe	Val	Ala	Ser	Leu	Phe
2555						2560					2565			
Ile	Ser	Ala	Leu	Ala	Thr	Tyr	Thr	Tyr	Lys	Thr	Trp	Asn	Tyr	His
2570						2575					2580			
Asn	Leu	Ser	Lys	Val	Val	Glu	Pro	Ala	Leu	Ala	Tyr	Leu	Pro	Tyr
2585						2590					2595			
Ala	Thr	Ser	Ala	Leu	Lys	Met	Phe	Thr	Pro	Thr	Arg	Leu	Glu	Ser
2600						2605					2610			
Val	Val	Ile	Leu	Ser	Thr	Thr	Ile	Tyr	Lys	Thr	Tyr	Leu	Ser	Ile
2615						2620					2625			
Arg	Lys	Gly	Lys	Ser	Asp	Gly	Leu	Leu	Gly	Thr	Gly	Ile	Ser	Ala
2630						2635					2640			
Ala	Met	Glu	Ile	Leu	Ser	Gln	Asn	Pro	Val	Ser	Val	Gly	Ile	Ser
2645						2650					2655			
Val	Met	Leu	Gly	Val	Gly	Ala	Ile	Ala	Ala	His	Asn	Ala	Ile	Glu
2660						2665					2670			
Ser	Ser	Glu	Gln	Lys	Arg	Thr	Leu	Leu	Met	Lys	Val	Phe	Val	Lys
2675						2680					2685			
Asn	Phe	Leu	Asp	Gln	Ala	Ala	Thr	Asp	Glu	Leu	Val	Lys	Glu	Asn
2690						2695					2700			
Pro	Glu	Lys	Ile	Ile	Met	Ala	Leu	Phe	Glu	Ala	Val	Gln	Thr	Ile
2705						2710					2715			
Gly	Asn	Pro	Leu	Arg	Leu	Ile	Tyr	His	Leu	Tyr	Gly	Val	Tyr	Tyr
2720						2725					2730			
Lys	Gly	Trp	Glu	Ala	Lys	Glu	Leu	Ser	Glu	Arg	Thr	Ala	Gly	Arg
2735						2740					2745			

Asn	Leu	Phe	Thr	Leu	Ile	Met	Phe	Glu	Ala	Phe	Glu	Leu	Leu	Gly
2750						2755					2760			
Met	Asp	Ser	Gln	Gly	Lys	Ile	Arg	Asn	Leu	Ser	Gly	Asn	Tyr	Ile
2765						2770					2775			
Leu	Asp	Leu	Ile	Tyr	Gly	Leu	His	Lys	Gln	Ile	Asn	Arg	Gly	Leu
2780						2785					2790			
Lys	Lys	Met	Val	Leu	Gly	Trp	Ala	Pro	Ala	Pro	Phe	Ser	Cys	Asp
2795						2800					2805			
Trp	Thr	Pro	Ser	Asp	Glu	Arg	Ile	Arg	Leu	Pro	Thr	Asp	Asn	Tyr
2810						2815					2820			
Leu	Arg	Val	Glu	Thr	Arg	Cys	Pro	Cys	Gly	Tyr	Glu	Met	Lys	Ala
2825						2830					2835			
Phe	Lys	Asn	Val	Gly	Gly	Lys	Leu	Thr	Lys	Val	Glu	Glu	Ser	Gly
2840						2845					2850			
Pro	Phe	Leu	Cys	Arg	Asn	Arg	Pro	Gly	Arg	Gly	Pro	Val	Asn	Tyr
2855						2860					2865			
Arg	Val	Thr	Lys	Tyr	Tyr	Asp	Asp	Asn	Leu	Arg	Glu	Ile	Lys	Pro
2870						2875					2880			
Val	Ala	Lys	Leu	Glu	Gly	Gln	Val	Glu	His	Tyr	Tyr	Lys	Gly	Val
2885						2890					2895			
Thr	Ala	Lys	Ile	Asp	Tyr	Ser	Lys	Gly	Lys	Met	Leu	Leu	Ala	Thr
2900						2905					2910			
Asp	Lys	Trp	Glu	Val	Glu	His	Gly	Val	Ile	Thr	Arg	Leu	Ala	Lys
2915						2920					2925			
Arg	Tyr	Thr	Gly	Val	Gly	Phe	Asn	Gly	Ala	Tyr	Leu	Gly	Asp	Glu
2930						2935					2940			
Pro	Asn	His	Arg	Ala	Leu	Val	Glu	Arg	Asp	Cys	Ala	Thr	Ile	Thr
2945						2950					2955			
Lys	Asn	Thr	Val	Gln	Phe	Leu	Lys	Met	Lys	Lys	Gly	Cys	Ala	Phe
2960						2965					2970			
Thr	Tyr	Asp	Leu	Thr	Ile	Ser	Asn	Leu	Thr	Arg	Leu	Ile	Glu	Leu
2975						2980					2985			
Val	His	Arg	Asn	Asn	Leu	Glu	Glu	Lys	Glu	Ile	Pro	Thr	Ala	Thr
2990						2995					3000			
Val	Thr	Thr	Trp	Leu	Ala	Tyr	Thr	Phe	Val	Asn	Glu	Asp	Val	Gly
3005						3010					3015			
Thr	Ile	Lys	Pro	Val	Leu	Gly	Glu	Arg	Val	Ile	Pro	Asp	Pro	Val
3020						3025					3030			
Val	Asp	Ile	Asn	Leu	Gln	Pro	Glu	Val	Gln	Val	Asp	Thr	Ser	Glu
3035						3040					3045			
Val	Gly	Ile	Thr	Ile	Ile	Gly	Arg	Glu	Thr	Leu	Met	Thr	Thr	Gly
3050						3055					3060			

Val Thr Pro Val Leu Glu Lys	Val Glu Pro Asp Ala Ser Asp Asn
3065	3070 3075
Gln Asn Ser Val Lys Ile Gly	Leu Asp Glu Gly Asn Tyr Pro Gly
3080	3085 3090
Pro Gly Ile Gln Thr His Thr	Leu Thr Glu Glu Ile His Asn Arg
3095	3100 3105
Asp Ala Arg Pro Phe Ile Met	Ile Leu Gly Ser Arg Asn Ser Ile
3110	3115 3120
Ser Asn Arg Ala Lys Thr Ala	Arg Asn Ile Asn Leu Tyr Thr Gly
3125	3130 3135
Asn Asp Pro Arg Glu Ile Arg	Asp Leu Met Ala Ala Gly Arg Met
3140	3145 3150
Leu Val Val Ala Leu Arg Asp	Val Asp Pro Glu Leu Ser Glu Met
3155	3160 3165
Val Asp Phe Lys Gly Thr Phe	Leu Asp Arg Glu Ala Leu Glu Ala
3170	3175 3180
Leu Ser Leu Gly Gln Pro Lys	Pro Lys Gln Val Thr Lys Glu Ala
3185	3190 3195
Val Arg Asn Leu Ile Glu Gln	Lys Lys Asp Val Glu Ile Pro Asn
3200	3205 3210
Trp Phe Ala Ser Asp Asp Pro	Val Phe Leu Glu Val Ala Leu Lys
3215	3220 3225
Asn Asp Lys Tyr Tyr Leu Val	Gly Asp Val Gly Glu Leu Lys Asp
3230	3235 3240
Gln Ala Lys Ala Leu Gly Ala	Thr Asp Gln Thr Arg Ile Ile Lys
3245	3250 3255
Glu Val Gly Ser Arg Thr Tyr	Ala Met Lys Leu Ser Ser Trp Phe
3260	3265 3270
Leu Lys Ala Ser Asn Lys Gln	Met Ser Leu Thr Pro Leu Phe Glu
3275	3280 3285
Glu Leu Leu Leu Arg Cys Pro	Pro Ala Thr Lys Ser Asn Lys Gly
3290	3295 3300
His Met Ala Ser Ala Tyr Gln	Leu Ala Gln Gly Asn Trp Glu Pro
3305	3310 3315
Leu Gly Cys Gly Val His Leu	Gly Thr Ile Pro Ala Arg Arg Val
3320	3325 3330
Lys Ile His Pro Tyr Glu Ala	Tyr Leu Lys Leu Lys Asp Phe Ile
3335	3340 3345
Glu Glu Glu Glu Lys Lys Pro	Arg Val Lys Asp Thr Val Ile Arg
3350	3355 3360
Glu His Asn Lys Trp Ile Leu	Lys Lys Ile Arg Phe Gln Gly Asn
3365	3370 3375

Leu	Asn	Thr	Lys	Lys	Met	Leu	Asn	Pro	Gly	Lys	Leu	Ser	Glu	Gln
3380						3385					3390			
Leu	Asp	Arg	Glu	Gly	Arg	Lys	Arg	Asn	Ile	Tyr	Asn	His	Gln	Ile
3395						3400					3405			
Gly	Thr	Ile	Met	Ser	Ser	Ala	Gly	Ile	Arg	Leu	Glu	Lys	Leu	Pro
3410						3415					3420			
Ile	Val	Arg	Ala	Gln	Thr	Asp	Thr	Lys	Thr	Phe	His	Glu	Ala	Ile
3425						3430					3435			
Arg	Asp	Lys	Ile	Asp	Lys	Ser	Glu	Asn	Arg	Gln	Asn	Pro	Glu	Leu
3440						3445					3450			
His	Asn	Lys	Leu	Leu	Glu	Ile	Phe	His	Thr	Ile	Ala	Gln	Pro	Thr
3455						3460					3465			
Leu	Lys	His	Thr	Tyr	Gly	Glu	Val	Thr	Trp	Glu	Gln	Leu	Glu	Ala
3470						3475					3480			
Gly	Val	Asn	Arg	Lys	Gly	Ala	Ala	Gly	Phe	Leu	Glu	Lys	Lys	Asn
3485						3490					3495			
Ile	Gly	Glu	Val	Leu	Asp	Ser	Glu	Lys	His	Leu	Val	Glu	Gln	Leu
3500						3505					3510			
Val	Arg	Asp	Leu	Lys	Ala	Gly	Arg	Lys	Ile	Lys	Tyr	Tyr	Glu	Thr
3515						3520					3525			
Ala	Ile	Pro	Lys	Asn	Glu	Lys	Arg	Asp	Val	Ser	Asp	Asp	Trp	Gln
3530						3535					3540			
Ala	Gly	Asp	Leu	Val	Val	Glu	Lys	Arg	Pro	Arg	Val	Ile	Gln	Tyr
3545						3550					3555			
Pro	Glu	Ala	Lys	Thr	Arg	Leu	Ala	Ile	Thr	Lys	Val	Met	Tyr	Asn
3560						3565					3570			
Trp	Val	Lys	Gln	Gln	Pro	Val	Val	Ile	Pro	Gly	Tyr	Glu	Gly	Lys
3575						3580					3585			
Thr	Pro	Leu	Phe	Asn	Ile	Phe	Asp	Lys	Val	Arg	Lys	Glu	Trp	Asp
3590						3595					3600			
Ser	Phe	Asn	Glu	Pro	Val	Ala	Val	Ser	Phe	Asp	Thr	Lys	Ala	Trp
3605						3610					3615			
Asp	Thr	Gln	Val	Thr	Ser	Lys	Asp	Leu	Gln	Leu	Ile	Gly	Glu	Ile
3620						3625					3630			
Gln	Lys	Tyr	Tyr	Tyr	Lys	Lys	Glu	Trp	His	Lys	Phe	Ile	Asp	Thr
3635						3640					3645			
Ile	Thr	Asp	His	Met	Thr	Glu	Val	Pro	Val	Ile	Thr	Ala	Asp	Gly
3650						3655					3660			
Glu	Val	Tyr	Ile	Arg	Asn	Gly	Gln	Arg	Gly	Ser	Gly	Gln	Pro	Asp
3665						3670					3675			
Thr	Ser	Ala	Gly	Asn	Ser	Met	Leu	Asn	Val	Leu	Thr	Met	Met	Tyr
3680						3685					3690			

Gly	Phe	Cys	Glu	Ser	Thr	Gly	Val	Pro	Tyr	Lys	Ser	Phe	Asn	Arg
3695						3700					3705			
Val	Ala	Arg	Ile	His	Val	Cys	Gly	Asp	Asp	Gly	Phe	Leu	Ile	Thr
3710						3715					3720			
Glu	Lys	Gly	Leu	Gly	Leu	Lys	Phe	Ala	Asn	Lys	Gly	Met	Gln	Ile
3725						3730					3735			
Leu	His	Glu	Ala	Gly	Lys	Pro	Gln	Lys	Ile	Thr	Glu	Gly	Glu	Lys
3740						3745					3750			
Met	Lys	Val	Ala	Tyr	Arg	Phe	Glu	Asp	Ile	Glu	Phe	Cys	Ser	His
3755						3760					3765			
Thr	Pro	Val	Pro	Val	Arg	Trp	Ser	Asp	Asn	Thr	Ser	Ser	His	Met
3770						3775					3780			
Ala	Gly	Arg	Asp	Thr	Ala	Val	Ile	Leu	Ser	Lys	Met	Ala	Thr	Arg
3785						3790					3795			
Leu	Asp	Ser	Ser	Gly	Glu	Arg	Gly	Thr	Thr	Ala	Tyr	Glu	Lys	Ala
3800						3805					3810			
Val	Ala	Phe	Ser	Phe	Leu	Leu	Met	Tyr	Ser	Trp	Asn	Pro	Leu	Val
3815						3820					3825			
Arg	Arg	Ile	Cys	Leu	Leu	Val	Leu	Ser	Gln	Gln	Pro	Glu	Thr	Asp
3830						3835					3840			
Pro	Ser	Lys	His	Ala	Thr	Tyr	Tyr	Tyr	Lys	Gly	Asp	Pro	Ile	Gly
3845						3850					3855			
Ala	Tyr	Lys	Asp	Val	Ile	Gly	Arg	Asn	Leu	Ser	Glu	Leu	Lys	Arg
3860						3865					3870			
Thr	Gly	Phe	Glu	Lys	Leu	Ala	Asn	Leu	Asn	Leu	Ser	Leu	Ser	Thr
3875						3880					3885			
Leu	Gly	Val	Trp	Thr	Lys	His	Thr	Ser	Lys	Arg	Ile	Ile	Gln	Asp
3890						3895					3900			
Cys	Val	Ala	Ile	Gly	Lys	Glu	Glu	Gly	Asn	Trp	Leu	Val	Lys	Pro
3905						3910					3915			
Asp	Arg	Leu	Ile	Ser	Ser	Lys	Thr	Gly	His	Leu	Tyr	Ile	Pro	Asp
3920						3925					3930			
Lys	Gly	Phe	Thr	Leu	Gln	Gly	Lys	His	Tyr	Glu	Gln	Leu	Gln	Leu
3935						3940					3945			
Arg	Thr	Glu	Thr	Asn	Pro	Val	Met	Gly	Val	Gly	Thr	Glu	Arg	Tyr
3950						3955					3960			
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<211> 3898

<212> PRT

<213> Bovine Viral Diarrhea Virus 1

<400> 6

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          20          25          30

Phe Gly Glu Arg Gly Ala Ile His Pro Gln Ser Thr Leu Lys Leu Pro
          35          40          45

His Lys Arg Gly Glu Arg Asn Val Pro Thr Ser Leu Ala Ser Leu Pro
          50          55          60

Lys Arg Gly Asp Cys Arg Ser Gly Asn Ser Lys Gly Pro Val Ser Gly
65          70          75          80

Ile Tyr Leu Lys Pro Gly Pro Leu Phe Tyr Gln Asp Tyr Lys Gly Pro
          85          90          95

Val Tyr His Arg Ala Pro Leu Glu Leu Phe Glu Glu Gly Ser Met Cys
          100          105          110

Glu Thr Thr Lys Arg Ile Gly Arg Val Thr Gly Ser Asp Gly Lys Leu
          115          120          125

Tyr His Ile Tyr Ile Cys Ile Asp Gly Cys Ile Thr Val Lys Ser Ala
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Thr Arg Ser His Gln Arg Val Leu Arg Trp Val His Asn Arg Leu Asp
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Cys Pro Leu Trp Val Thr Ser Cys Ser Asp Thr Lys Glu Glu Gly Ala
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Thr Lys Lys Lys Gln Gln Lys Pro Asp Arg Leu Glu Lys Gly Arg Met
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Lys Ile Val Pro Lys Glu Ser Glu Lys Asp Ser Lys Thr Lys Pro Pro
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Asp Ala Thr Ile Val Val Asp Gly Val Lys Tyr Gln Val Lys Lys Lys
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Gly Lys Val Lys Ser Lys Asn Thr Gln Asp Gly Leu Tyr His Asn Lys
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Asn Lys Pro Pro Glu Ser Arg Lys Lys Leu Glu Lys Ala Leu Leu Ala
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Trp Ala Ile Leu Ala Val Val Leu Ile Glu Val Thr Met Gly Glu Asn
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Ile Thr Gln Trp Asn Leu Gln Asp Asn Gly Thr Glu Gly Ile Gln Arg
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Ala Met Phe Gln Arg Gly Val Asn Arg Ser Leu His Gly Ile Trp Pro
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Glu Lys Ile Cys Thr Gly Val Pro Ser His Leu Ala Thr Asp Val Glu
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Leu Lys Thr Ile His Gly Met Met Asp Ala Ser Glu Lys Thr Asn Tyr
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 Asn Trp Tyr Asn Ile Glu Pro Trp Ile Leu Ile Met Asn Arg Thr Gln
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 Ala Asn Leu Thr Glu Gly Gln Pro Pro Arg Glu Cys Ala Val Thr Cys
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 Arg Tyr Asp Arg Asp Ser Asp Leu Asn Val Val Thr Gln Ala Arg Asp
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 Asp Val Leu Phe Lys Glu His Glu Cys Thr Gly Val Phe Gln Asp Thr
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 Pro Gly Ser Val Trp Asn Leu Gly Lys Tyr Val Cys Ile Arg Pro Asp
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 Trp Trp Pro Tyr Glu Thr Ala Ala Val Leu Ala Phe Glu Glu Val Gly
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 Gln Val Val Lys Ile Val Leu Arg Ala Leu Arg Asp Leu Thr Arg Ile
 645 650 655

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Glu	Glu	Ser	Lys	Pro	Thr	Lys	Ile	Met	Ser	Gly	Ile	Gln	Thr	Val
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Ser	Lys	Asn	Thr	Ala	Asp	Leu	Thr	Glu	Met	Val	Lys	Lys	Ile	Thr
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Val	Val	Ile	Leu	Ser	Thr	Thr	Ile	Tyr	Lys	Thr	Tyr	Leu	Ser	Ile
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 Gly Ala Glu Thr Asn Pro Val Met Gly Val Gly Thr Glu Arg Tyr
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24

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CLAIMS:

1. A vaccine composition comprising at least two live mutant viruses of the same family, wherein each virus contains a mutation in the viral genome, and the mutations in the viruses reside in the same genomic site such that the mutant
5 viruses cannot recombine with each other to eliminate the mutations, and wherein two of the live mutant viruses consist of mutant Bovine Viral Diarrhea Viruses (BVDV).
2. The vaccine composition of claim 1, wherein the two mutant live viruses consist of a mutant Bovine Viral Diarrhea Virus Type 1 (BVDV-1) and a
10 mutant Bovine Viral Diarrhea Virus Type 2 (BVDV-2).
3. The vaccine composition of claim 2, wherein the two mutant live viruses consist of a cytopathic (cp) BVDV-1 and a cp BVDV-2, and are both attenuated.
4. The vaccine composition of claim 3, wherein the cp BVDV-1 and the
15 cp BVDV-2 both comprise a mutation in the NS2-3 region that results in a cytopathic biotype.
5. The vaccine composition of claim 4, wherein the cp BVDV-1 is BVDV-1 NADL, and the cp BVDV-2 is BVDV-2 53637.
6. The vaccine composition of any one of claims 1 to 5, further
20 comprising at least one of bovine herpesvirus-1, bovine respiratory syncytial virus, parainfluenza virus-3, *Campylobacter fetus*, *Leptospira canicola*, *Leptospira grippotyphosa*, *Leptospira hardjo*, *Leptospira icterohaemorrhagiae*, *Leptospira pomona*, or *Mannhemia haemolytica*.
7. The vaccine composition of any one of claims 1 to 6, further
25 comprising a veterinarily-acceptable carrier.
8. A method of preparing a safe viral vaccine comprising selecting or constructing two live mutant viruses of the same family, wherein each virus contains a mutation and the mutations in the viruses reside in the same genomic

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site such that the mutant viruses can not undergo homologous recombination to eliminate the mutations, and wherein the viruses consist of mutant Bovine Viral Diarrhea Viruses (BVDV).

9. The method of claim 8, wherein said mutation is selected from a deletion, an insertion, a substitution, or a combination thereof.
10. The method of claim 8, wherein said mutation confers a phenotype selected from attenuation of virulence, alteration of cellular tropism or biotype, alteration of species tropism, expression of a foreign gene cassette, or a combination thereof.
- 10 11. The method of any one of claims 8 to 10, wherein the two mutant live viruses consist of a mutant Bovine Viral Diarrhea Virus Type 1 (BVDV-1) and a mutant Bovine Viral Diarrhea Virus Type 2 (BVDV-2).
12. The composition of claim 1, wherein the two BVDV both comprise a mutation in the NS2-3 region that results in a cytopathic biotype.
- 15 13. The composition of claim 1, wherein the mutation is an insertion.
14. The method of claim 9, wherein the mutation is an insertion.

Figure 1

