

MULTIVALENT VACCINES AGAINST MAJOR SWINE VIRAL DISEASES

SEQUENCE LISTING

[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577254PCTSequenceListing.txt, created on 4 May 2017 and is 23 kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to the field of vaccines. More specifically, the present invention relates to a multivalent vaccine against major swine viral diseases. In one embodiment, the multivalent vaccine is a recombinant pseudorabies viral vector.

[0003] The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the Bibliography.

[0004] Pseudorabies virus (PRV) belonging to the Alphaherpesvirinae subfamily of the Herpesviridae family, is the causative agent of Aujeszky's disease. PRV is prevalent in the developing countries and cause substantial economic losses in the pig industry. Live attenuated PRV vaccines have played a key role for prevention and eradication of PRV (Pomeranz et al., 2005). PRV Bartha-K61 is an attenuated PRV vaccine strain with gE and part of gI genes have been deleted (Klupp et al., 2012; Dong et al., 2014). Also, several previous reports have described live attenuated PRV Bartha-K61 is an excellent vaccine vector for expressing heterologous antigenic genes (Nie et al., 2011; Li et al., 2008; Song et al., 2007; Xu et al., 2004; Jiang et al., 2007). Hence, we utilized live attenuated PRV Bartha-K61 vaccine strain, as a vector to express multiple antigenic genes of major swine viral diseases.

[0005] Porcine circovirus (PCV), a member of the *family Circoviridae*, is a single-stranded circular DNA genome. PCV2 is the primary cause of post-weaning multisystemic wasting syndrome (PMWS) that developed in infected pigs. The infection is characterized by diarrhea, decreased weight gain, sudden death, enlarged lymph nodes, respiratory distress and multinucleated giant cell formation (Liu et al., 2005). ORF2 is one of the major open reading frames within the PCV2 genome. It encodes the capsid protein (Cap) which is the immunodominant viral protein that can be served as an ideal target for vaccine development in pigs.

[0006] Classical swine fever (CSF) virus is a highly contagious and economically important viral disease that affects domestic pigs and wild boars. CSF virus (CSFV) is a positive sense single-stranded RNA virus and a member of the genus Pestivirus within the family Flaviviridae. The envelope glycoprotein E2 contains four antigenic domains and it can induce protective immunity against CSFV in pig population. Therefore, the E2 glycoprotein is a potential candidate for CSFV vaccine design (Zhang et al., 2014). Commercially available whole inactivated vaccines based on C-strain are effective and provide complete protection against the three groups of CSFV but could not offer differentiability of infected from vaccinated animals (Zhang et al., 2014).

[0007] Porcine reproductive and respiratory syndrome virus (PRRSV) is classified in the family *Arteriviridae* of the order *Nidovirales*. It is an enveloped, positive strand RNA virus that leads to the development of Porcine Reproductive and Respiratory Syndrome (PRRS). Infection of PRRSV can cause late-term abortion in sows, massive reproductive failure, and respiratory disorder in piglets. PRRSV genome consists of nine open reading frame encoding 7 structural proteins and 14 non-structural proteins. Among those, glycoprotein (GP) 5 is the major envelope protein encoded by ORF5. It is one of the key immunogenic protein of PRRSV which is essential for viral assembly, infectivity and the induction of neutralizing antibodies. The ORF5 is one of the most variable region of PRRSV genome and it has been widely use to study the molecular epidemiology of PRRSV. GP5 is also leading target in vaccine development against PRRSV infection (Li et al., 2012).

[0008] Currently available vaccines and vaccination programs are complicated and it is difficult to immunize against all four diseases separately, resulting in multi-injections and multi-boosts. Hence, the development of a multivalent vaccine for the four diseases would be time consuming and cost effective, and could also lead to a more effective vaccine.

SUMMARY OF THE INVENTION

[0009] The present invention relates to the field of vaccines. More specifically, the present invention relates to a multivalent vaccine against major swine viral diseases. In one embodiment, the multivalent vaccine is a recombinant pseudorabies viral vector.

[0010] The present invention describes the utilization of established live attenuated pseudorabies (PRV) vaccine strain, such as the Bartha-K61 strain, as a vaccine vector to express multi immunogens from major swine viral diseases of PCV2, CSF and PRRSV. The PRV genome is approximately 140 kb and composed of a unique long (UL) region, a unique short

(US) region, large inverted repeat sequences, internal repeats (IRs), and terminal repeats (TRs). Half of the genome is considered nonessential regions such as protein kinase (PK), thymidine kinases (TK), gE, gG and gI, thus permitting modification or insertion of foreign genes without affecting virus replication. Also, its well-documented safety and protective efficacy profiles of PRV Bartha-K61 vaccine strain, has been used for decades to successfully control Aujeszky's disease. In accordance with the present invention, a tetravalent PRV vaccine against PRRSV, PCV2, CSF and PRV and a trivalent PRV vaccine against PCV2, CSF and PRV have been generated.

[0011] Thus, in one aspect, the present invention provides a tetravalent PRV vaccine against PRRSV, PCV2, CSF and PRV. In accordance with this aspect, a nucleic acid construct is prepared which comprises a gene of PRRSV, a gene of PCV2 and a gene of CSF. In some embodiments, the PRRSV gene is a PRRSV ORF5 gene. In some embodiments, the PRRSV ORF5 gene is derived from the highly pathogenic PRRSV Chinese strain (HP-HRRSV-JXA1). In some embodiments, the PCV2 gene is a PCV2 ORF2 gene. In some embodiments, the PCV2 gene is derived from the PCV2 Indonesian strain (genotype 2B) (PCV2 TLL-Indo, GenBank Accession No. KX130941). In some embodiments, the CFS gene is a CFS gene is a CFS E2 gene. In some embodiments, the CFS E2 gene is derived from a new emergence of a sub-genotype 2.1 of a CSF Indonesian strain) (CSF TLL-Indo, GenBank Accession No. KX130940).

[0012] In some embodiments, each gene is operatively linked to a promoter active in mammalian cells. Any suitable promoter active in mammalian cells may be used in accordance with the present invention. In some embodiments, the promoter is a cytomegalovirus (CMV) promoter. In some embodiments, the CMV promoter is a CMV intermediate early (CMV ie) promoter. In some embodiments, the promoter is the elongation factor 1 alpha (EF1 α) promoter.

[0013] In some embodiments, each gene is operatively linked to a terminator sequence operative in mammalian cells. Any suitable terminator active in mammalian cells may be used in accordance with the present invention. In some embodiments, the terminator sequence is a bovine growth hormone polyadenylation (BGH polyA) sequence. In some embodiments, the terminator sequence is an SV40 virus polyadenylation (SV40 polyA) sequence.

[0014] In some embodiments, a nucleic acid construct is provided which comprises the PRRSV gene, PCV2 gene and CSF gene described herein. In some embodiments, the nucleic acid construct further comprises the promoters and/or terminators described herein. In some embodiments, the nucleic acid construct further comprises gG sequences of PRV. In some

embodiments, the PRV is the PRV Bartha-K61 strain. In some embodiments, the nucleic acid construct comprises the nucleotide sequence set forth in SEQ ID NO:1.

[0015] In some embodiments, a PRV strain is provided in which the PRV strain is modified to contain a nucleic acid construct described herein. In some embodiments, the modified PRV is a modified PRV Bartha-K61 strain. In some embodiments, the nucleic acid construct in the modified PRV strain is stable with no mutations or deletions after five passages in pig kidney 15 (PK-15) cells. In some embodiments, the PK-15 cells are PK-15 cells ATCC[®] CCL-33[™] cells obtained from the American Type Culture Collection.

[0016] In some embodiments, a cell line is provided which is transfected with the nucleic acid construct described herein in a manner suitable for producing a virus useful for a tetravalent vaccine. In some embodiments, the nucleic acid construct described herein is inserted into a PRV strain described herein by homologous recombination in the transfected cell line. In some embodiments, the cell line is the PK-15 cells described herein.

[0017] In a second aspect, the present invention provides a trivalent PRV vaccine against PCV2, CSF and PRV. In accordance with this aspect, a nucleic acid construct is prepared which comprises a gene of PCV2 and a gene of CSF. In some embodiments, the PCV2 gene and the CSF gene are as described herein.

[0018] In some embodiments, each gene is operatively linked to a promoter active in mammalian cells. In some embodiments, the promoter is as described herein.

[0019] In some embodiments, each gene is operatively linked to a terminator sequence operative in mammalian cells. In some embodiments, the terminator sequence is as described herein.

[0020] In some embodiments, a nucleic acid construct is provided which comprises the PCV2 gene and CSF gene described herein. In some embodiments, the nucleic acid construct further comprises the promoters and/or terminators described herein. In some embodiments, the nucleic acid construct further comprises gG sequences of PRV. In some embodiments, the PRV is the PRV Bartha-K61 strain. In some embodiments, the nucleic acid construct comprises the nucleotide sequence set forth in SEQ ID NO:2.

[0021] In some embodiments, a PRV strain is provided in which the PRV strain is modified to contain a nucleic acid construct described herein. In some embodiments, the modified PRV strain is as described herein. In some embodiments, the nucleic acid construct in the modified PRV strain is stable with no mutations or deletions after five passages in pig kidney 15 (PK-15) cells. In some embodiments, the PK-15 cells are as described herein.

[0022] In some embodiments, a cell line is provided which is transfected with the nucleic acid construct described herein in a manner suitable for producing a virus useful for a trivalent vaccine. In some embodiments, the nucleic acid construct described herein is inserted into a PRV strain described herein by homologous recombination in the transfected cell line. In some embodiments, the cell line is the PK-15 cells described herein.

[0023] The invention also provides a kit for immunization of a subject with a recombinant PRV described herein. The kit comprises a recombinant PRV described herein, a pharmaceutically acceptable carrier, an applicator, and an instructional material for the use thereof.

[0024] The invention also provides methods of use. In one embodiment, the invention provides a method of eliciting a protective immune response in a subject, for example swine, comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of a recombinant PRV described herein. In another embodiment, the invention provides a method of preventing a subject from becoming afflicted with PRV, PRRSV, PCV and CSF or PRV, PCV and CSF comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of a recombinant PRV described herein.

BRIEF DESCRIPTION OF THE FIGURES

[0025] Figure 1 shows a strategy for construction of rPRV according to one embodiment of the invention. Pseudorabies virus (PRV) genome: unique long region (U_L), internal repeat (IR), unique short region (U_S), and terminal repeat (TR). Tetravalent PRV expressing PRRSV-ORF5, PCV2-ORF2, CSF-E2; Trivalent PRV expressing PCV2-ORF2, CSF-E2. Abbreviations: N-gG = N (amino) terminus of glycoprotein G; C-gG = Carboxyl-terminus of glycoprotein; PK = protein kinase; CMV ie = immediate early promoter of human cytomegalovirus; EF1 α promoter = elongation factor 1 alpha promoter; terminators SV40 polyA, bovine growth hormone polyadenylation (BGH polyA).

[0026] Figures 2A and 2B shows immunofluorescence assays of PK15 cells infected with multivalent vaccine. Fig. 2A: Immuno-fluorescence assay of PK15 cells infected with bivalent PRV (ORF2-PRV) or trivalent PRV (ORF2-E2-PRV) or tetravalent PRV (ORF5-ORF2-E2-PRV) for 36 h at 37° C with 5% CO₂. Anti-ORF2 (mouse hyperimmune sera) signal was seen in all the constructs. Fig. 2B: Immuno-fluorescence assay of PK15 cells infected with trivalent PRV (ORF2-E2-PRV) or tetravalent PRV (ORF5-ORF2-E2-PRV) for 36 h at 37° C with 5% CO₂. Anti-E2 (mouse hyperimmune sera) signal was seen in all the constructs.

[0027] Figure 3 shows groups of mice (n=8/group) immunized with trivalent or tetravalent PRV vaccine on day 0 and 21. Sera were collected on day 20 and 42. Serum PRV/CSF/PCV2/PRRSV specific IgG antibody levels on day 20 & 42 by indirect ELISA.

[0028] Figure 4 shows groups of mice (n=8/group) immunized with trivalent or tetravalent PRV vaccine on day 0 and 21. Sera were collected on day 20 and 42. The results show serum PCV2-ORF2 or CSF-E2 specific antibody titers on 42 (21 days after second immunization)

DETAILED DESCRIPTION OF THE INVENTION

[0029] The present invention relates to the field of vaccines. More specifically, the present invention relates to a multivalent vaccine against major swine viral diseases. In one embodiment, the multivalent vaccine is a recombinant pseudorabies viral vector.

[0030] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

[0031] The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence.

[0032] As used herein, "gene" refers to a nucleic acid sequence that encompasses the coding region of the gene product.

[0033] "Introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct) into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid fragment into a yeast or fungi cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

[0034] "Operable linkage" or "operably linked" or "operatively linked" as used herein is understood as meaning, for example, the sequential arrangement of a promoter and the nucleic acid to be expressed and, if appropriate, further regulatory elements such as, for example, a terminator, in such a way that each of the regulatory elements can fulfill its function in the recombinant expression of the nucleic acid to make the desired product. This does not necessarily require direct linkage in the chemical sense. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are somewhat distant, or indeed from other DNA molecules (cis or trans localization).

Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned downstream of the sequence which acts as promoter, so that the two sequences are covalently bonded with one another. Regulatory or control sequences may be positioned on the 5' side of the nucleotide sequence or on the 3' side of the nucleotide sequence as is well known in the art.

[0035] The terms "polynucleotide," "nucleic acid" and "nucleic acid molecule" are used interchangeably herein to refer to a polymer of nucleotides which may be a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, including deoxyribonucleic acid, ribonucleic acid, and derivatives thereof. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. Unless otherwise indicated, nucleic acids or polynucleotide are written left to right in 5' to 3' orientation. Nucleotides are referred to by their commonly accepted single-letter codes. Numeric ranges are inclusive of the numbers defining the range.

[0036] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Amino acids may be referred to by their commonly known three-letter or one-letter symbols. Amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range.

[0037] "Promoter" refers to a nucleic acid fragment capable of controlling transcription of another nucleic acid fragment.

[0038] "Promoter active in a mammalian cell" is a promoter capable of controlling transcription in mammalian cells whether or not its origin is from a mammalian cell.

[0039] "Recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques. "Recombinant" also includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or a cell derived from a cell so modified, but does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

[0040] "Recombinant DNA construct" refers to a combination of nucleic acid fragments that are not normally found together in nature. Accordingly, a recombinant DNA construct may

comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that normally found in nature. The terms “recombinant DNA construct” and “recombinant construct” are used interchangeably herein. In several embodiments described herein, a recombinant DNA construct may also be considered an “over expression DNA construct.” The term “nucleic acid construct” may also be used interchangeably with “recombinant DNA construct.”

[0041] “Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences. The terms “regulatory sequence” and “regulatory element” are used interchangeably herein.

[0042] Sequence alignments and percent identity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the Megalign® program of the LASERGENE® bioinformatics computing suite (DNASTAR® Inc., Madison, WI). Unless stated otherwise, multiple alignment of the sequences provided herein were performed using the Clustal V method of alignment (Higgins and Sharp (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal V method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences, using the Clustal V program, it is possible to obtain “percent identity” and “divergence” values by viewing the “sequence distances” table on the same program; unless stated otherwise, percent identities and divergences provided and claimed herein were calculated in this manner.

[0043] Alternatively, the Clustal W method of alignment may be used. The Clustal W method of alignment (described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989); Higgins, D. G. et al., *Comput. Appl. Biosci.* 8:189-191 (1992)) can be found in the MegAlign™ v6.1 program of the LASERGENE® bioinformatics computing suite (DNASTAR® Inc., Madison, Wis.). Default parameters for multiple alignment correspond to GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergent Sequences=30%, DNA Transition Weight=0.5,

Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB. For pairwise alignments the default parameters are Alignment=Slow-Accurate, Gap Penalty=10.0, Gap Length=0.10, Protein Weight Matrix=Gonnet 250 and DNA Weight Matrix=IUB. After alignment of the sequences using the Clustal W program, it is possible to obtain "percent identity" and "divergence" values by viewing the "sequence distances" table in the same program.

[0044] The term "under stringent conditions" means that two sequences hybridize under moderately or highly stringent conditions. More specifically, moderately stringent conditions can be readily determined by those having ordinary skill in the art, e.g., depending on the length of DNA. The basic conditions are set forth by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, third edition, chapters 6 and 7, Cold Spring Harbor Laboratory Press, 2001 and include the use of a prewashing solution for nitrocellulose filters 5xSSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization conditions of about 50% formamide, 2xSSC to 6xSSC at about 40-50 °C (or other similar hybridization solutions, such as Stark's solution, in about 50% formamide at about 42 °C) and washing conditions of, for example, about 40-60 °C, 0.5-6xSSC, 0.1% SDS. Preferably, moderately stringent conditions include hybridization (and washing) at about 50 °C and 6xSSC. Highly stringent conditions can also be readily determined by those skilled in the art, e.g., depending on the length of DNA.

[0045] Generally, such conditions include hybridization and/or washing at higher temperature and/or lower salt concentration (such as hybridization at about 65 °C, 6xSSC to 0.2xSSC, preferably 6xSSC, more preferably 2xSSC, most preferably 0.2xSSC), compared to the moderately stringent conditions. For example, highly stringent conditions may include hybridization as defined above, and washing at approximately 65-68 °C, 0.2xSSC, 0.1% SDS. SSPE (1xSSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, and 1.25 mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15 M NaCl and 15 mM sodium citrate) in the hybridization and washing buffers; washing is performed for 15 minutes after hybridization is completed.

[0046] It is also possible to use a commercially available hybridization kit which uses no radioactive substance as a probe. Specific examples include hybridization with an ECL direct labeling & detection system (Amersham). Stringent conditions include, for example, hybridization at 42 °C for 4 hours using the hybridization buffer included in the kit, which is supplemented with 5% (w/v) Blocking reagent and 0.5 M NaCl, and washing twice in 0.4% SDS, 0.5xSSC at 55 °C for 20 minutes and once in 2xSSC at room temperature for 5 minutes.

[0047] As used herein, the term "substantially homologous" or "substantial homology", with reference to a nucleic acid sequence, includes a nucleotide sequence that hybridizes under

stringent conditions to a referenced SEQ ID NO:, or a portion or complement thereof, are those that allow an antiparallel alignment to take place between the two sequences, and the two sequences are then able, under stringent conditions, to form hydrogen bonds with corresponding bases on the opposite strand to form a duplex molecule that is sufficiently stable under conditions of appropriate stringency, including high stringency, to be detectable using methods well known in the art. Substantially homologous sequences may have from about 70% to about 80% sequence identity, or more preferably from about 80% to about 85% sequence identity, or most preferable from about 90% to about 95% sequence identity, to about 99% sequence identity, to the referent nucleotide sequences as set forth the sequence listing, or the complements thereof. Alternatively, substantially homologous sequences include those which hybridize under stringent conditions to the target regions of introns of plant genes. For stringency conditions, see the description herein and see also U.S. Patent Nos. 8,455,716 and 8,536,403.

[0048] “PRRSV ORF5 gene” or “ORF5 gene” refers to an ORF5 gene derived from a highly pathogenic PRRSV Chinese strain (HP-HRRSV-JXA1). In some embodiments, the ORF5 gene has the sequence set forth in nucleotides 1041-1643 of SEQ ID NO:1. In other embodiments, the ORF5 gene has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V or Clustal W method of alignment, when compared to nucleotides 1041-1643 of SEQ ID NO:1.

[0049] “PCV ORF2 gene” or “ORF2 gene” refers to an ORF2 gene derived from PCV2 Indonesian strain (genotype 2B) (PCV2 TLL-Indo, GenBank Accession No. KX130941). In some embodiments, the ORF2 gene has the sequence set forth in nucleotides 3063-3767 of SEQ ID NO:1. In other embodiments, the ORF2 gene has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V or Clustal W method of alignment, when compared to nucleotides 3063-3767 of SEQ ID NO:1.

[0050] “CSF E2 gene” or “E2 gene” refers to an E2 gene derived from a new emergence of a sub-genotype 2.1 of a CSF Indonesian strain (CSF TLL-Indo, GenBank Accession No. KX130940). In some embodiments, the E2 gene has the sequence set forth in nucleotides 4729-5916 of SEQ ID NO:1. In other embodiments, the E2 gene has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V or Clustal W method of alignment, when compared to nucleotides 4729-5916 of SEQ ID NO:1.

[0051] In one aspect, the present invention provides a tetravalent PRV vaccine against PRRSV, PCV2, CSF and PRV. In accordance with this aspect, a nucleic acid construct is prepared which comprises a gene of PRRSV, a gene of PCV2 and a gene of CSF. In some embodiments, the PRRSV gene is a PRRSV ORF5 gene. In some embodiments, the PRRSV ORF5 gene is derived from the highly pathogenic PRRSV Chinese strain (HP-HRRSV-JXA1). In some embodiments, the ORF5 gene has the nucleotide sequence described herein. In some embodiments, the PCV2 gene is a PCV2 ORF2 gene. In some embodiments, the PCV2 gene is derived from the PCV2 Indonesian strain (genotype 2B). In some embodiments, the ORF2 gene has the nucleotide sequence described herein. In some embodiments, the CFS gene is a CFS gene is a CFS E2 gene. In some embodiments, the CFS E2 gene is derived from a new emergence of a sub-genotype 2.1 of a CSF Indonesian strain. In some embodiments, the E2 gene has the nucleotide sequence described herein.

[0052] In some embodiments, each gene is operatively linked to a promoter active in mammalian cells. Any suitable promoter active in mammalian cells may be used in accordance with the present invention. In some embodiments, the promoter is a cytomegalovirus (CMV) promoter. In some embodiments, the CMV promoter is a CMV intermediate early (CMV ie) promoter. In some embodiments the CMV ie promoter has the sequence set forth nucleotides 445-1034 of SEQ ID NO:1. In some embodiments, the CMV promoter has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V or Clustal W method of alignment, when compared to nucleotides 445-1034 of SEQ ID NO:1. In some embodiments, the promoter is the elongation factor 1 alpha (EF1 α) promoter. In some embodiments the EF1 α promoter has the sequence set forth nucleotides 1869-3056 of SEQ ID NO:1. In some embodiments, the EF1 α promoter has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V or Clustal W method of alignment, when compared to nucleotides 1869-3056 of SEQ ID NO:1.

[0053] In some embodiments, each gene is operatively linked to a terminator sequence operative in mammalian cells. Any suitable terminator active in mammalian cells may be used in accordance with the present invention. In some embodiments, the terminator sequence is a bovine growth hormone polyadenylation (BGH polyA) sequence. In some embodiments the BGH polyA sequence has the sequence set forth nucleotides 1644-1868 of SEQ ID NO:1. In some embodiments, the BGH polyA sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the

Clustal V or Clustal W method of alignment, when compared to nucleotides 1644-1868 of SEQ ID NO:1. In some embodiments, the terminator sequence is an SV40 virus polyadenylation (SV40 polyA) sequence. In some embodiments the SV40 polyA sequence has the sequence set forth nucleotides 5925-6192 of SEQ ID NO:1. In some embodiments, the SV40 polyA sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V or Clustal W method of alignment, when compared to nucleotides 5925-6192 of SEQ ID NO:1.

[0054] In some embodiments, a nucleic acid construct is provided which comprises the PRRSV gene, PCV2 gene and CSF gene described herein. In some embodiments, the nucleic acid construct further comprises the promoters and/or terminators described herein. In some embodiments, the nucleic acid construct further comprises gG sequences of PRV. In some embodiments, the PRV is the PRV Bartha-K61 strain. In some embodiments, the nucleic acid construct comprises the nucleotide sequence set forth in SEQ ID NO:1.

[0055] In some embodiments, a PRV strain is provided in which the PRV strain is modified to contain a nucleic acid construct described herein. In some embodiments, the modified PRV is a modified PRV Bartha-K61 strain. In some embodiments, the nucleic acid construct in the modified PRV strain is stable with no mutations or deletions after five passages in pig kidney 15 (PK-15) cells. In some embodiments, the PK-15 cells are PK-15 cells ATCC[®] CCL-33[™] cells obtained from the American Type Culture Collection.

[0056] In some embodiments, a cell line is provided which is transfected with the nucleic acid construct described herein in a manner suitable for producing a virus useful for a tetravalent vaccine. In some embodiments, the nucleic acid construct described herein is inserted into a PRV strain described herein by homologous recombination in the transfected cell line. In some embodiments, the cell line is the PK-15 cells described herein.

[0057] In a second aspect, the present invention provides a trivalent PRV vaccine against PCV2, CSF and PRV. In accordance with this aspect, a nucleic acid construct is prepared which comprises a gene of PCV2 and a gene of CSF. In some embodiments, the PCV2 gene and the CSF gene are as described herein.

[0058] In some embodiments, each gene is operatively linked to a promoter active in mammalian cells. In some embodiments, the promoter is as described herein.

[0059] In some embodiments, each gene is operatively linked to a terminator sequence operative in mammalian cells. In some embodiments, the terminator sequence is as described herein.

[0060] In some embodiments, a nucleic acid construct is provided which comprises the PCV2 gene and CSF gene described herein. In some embodiments, the nucleic acid construct further comprises the promoters and/or terminators described herein. In some embodiments, the nucleic acid construct further comprises gG sequences of PRV. In some embodiments, the PRV is the PRV Bartha-K61 strain. In some embodiments, the nucleic acid construct comprises the nucleotide sequence set forth in SEQ ID NO:2.

[0061] In some embodiments, a PRV strain is provided in which the PRV strain is modified to contain a nucleic acid construct described herein. In some embodiments, the modified PRV strain is as described herein. In some embodiments, the nucleic acid construct in the modified PRV strain is stable with no mutations or deletions after five passages in pig kidney 15 (PK-15) cells. In some embodiments, the PK-15 cells are as described herein.

[0062] In some embodiments, a cell line is provided which is transfected with the nucleic acid construct described herein in a manner suitable for producing a virus useful for a trivalent vaccine. In some embodiments, the nucleic acid construct described herein is inserted into a PRV strain described herein by homologous recombination in the transfected cell line. In some embodiments, the cell line is the PK-15 cells described herein.

[0063] In preparing the nucleic acid construct, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g. transitions and transversions may be involved.

[0064] Nucleic acids of the present invention may also be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences, by methods known in the art. Thus, all or a portion of the nucleic acids of the present invention may be synthesized using codons preferred by a selected host. Species-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a particular host species. Other modifications of the nucleotide sequences may result in mutants having slightly altered activity.

[0065] Tetravalent recombinant PRV or trivalent recombinant TRV are prepared by transfecting a suitable cell line, such as a PK-15 cell line described herein. In some embodiments, the cell line is transfected with the nucleic acid construct described herein and

with PRV nucleocapsid DNA. In some embodiments, the PRV nucleocapsid DNA is modified to containing a nucleic acid construct for the expression of red fluorescent protein (RFP). The tetravalent recombinant PRV or trivalent recombinant TRV is passaged in PK-15 cells for a number of passages to ensure stable recombinant viruses with no mutations or deletions in the viral nucleic acid. In some embodiments, five passages are sufficient to produce stable recombinant viruses.

[0066] Once prepared, the recombinant TRV viruses can be replicated in suitable cell lines. In some embodiments, the cell line is a PK-15 cell line (cells) as described herein. In some embodiments, the cell line (cells) is a baby hamster kidney 21 (BHK21) cell line. In some embodiments, the BHK21 cell line is BHK21 (clone 13). In some embodiments, the BHK21 (clone 13) cell line is ATCC® CCL-10™ and is available from the American Type Culture Collection.

[0067] Porcine Reproductive and Respiratory Syndrome virus (PRRSV) and pseudorabies virus (PRV) are the main causes of infectious reproductive failure in pigs and cause significant losses to the pig industry worldwide. Also, PCV2 and Classical Swine Fever cause devastating diseases and have a severe impact on animal welfare and the economies of many Asian countries. Development of multivalent vaccine approach could potentially reduce the administration of vaccine to a one dose schedule to cover all four viruses, greatly aiding the current situation for protecting pigs against these four diseases. We selected live attenuated PRV Bartha-K61 as vector for multivalent vaccine development. The genome structure and genetic background of PRV Bartha-K61 strain is relatively well defined and reported multiplication and stable expression of foreign genes does not affect the stability of the virus itself (Boldogkoi , Nogradi, 2003).

[0068] As described herein, tetravalent PRV expressing PCV2-ORF2, CSF-E2 and PRRSV-ORF5 and trivalent PRV expressing PCV2-ORF2 and CSF-E2 are generated by homologous recombination into gG locus of PRV Bartha K-61 vaccine strain. The replication assay and stability test revealed that the recombinant PRV either trivalent or tetravalent PRV are stable and replicate in vitro as efficiently as PRV Bartha K-61, demonstrating that insertion of foreign genes in the PK and gG locus of PRV does not affect the replication of PRV. Further, immunogenicity study in mice experiment demonstrated that tetravalent PRV induced high level of serum-specific humoral antibodies against PCV2-ORF2, CSF-E2 and PRRSV-ORF5 antigens, similarly trivalent PRV induced serum specific humoral antibodies against PCV2-ORF2 and CSF-E2. These results suggest that expression of multivalent immune genes from

major swine virus in PRV Bartha K-61 is a novel approach towards the development of a multivalent vaccine against PCV2, CSF or PRRSV and including pseudorabies.

[0069] The recombinant pseudorabies viral vector of the present invention comprising ORF2 of PCV2, E2 of CSF and ORF5 of PRRSV effectively induced antibodies against all four diseases which allows the cost-effective production of large quantities in a single manufactured product.

[0070] According to the present invention, it was found that both trivalent and tetravalent recombinant vaccines are stable after 5 sequential passages in PK15/BHK21 cell lines. Insertion of two or three genes in a single insertion site are very stable and show neither genetic instability nor modifications in the transcription of the inserted genes.

[0071] According to the present invention, it was found that insertion of multiple genes in a single insertion site of gG locus of PRV-Bartha did not affect viral titers.

[0072] The invention also provides methods of use. In one embodiment, the invention provides a method of eliciting a protective immune response in a subject, for example swine, comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of a recombinant PRV described herein. In another embodiment, the invention provides a method of preventing a subject from becoming afflicted with PRV, PRRSV, PCV and CSF or PRV, PCV and CSF comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of a recombinant PRV described herein.

[0073] As used herein, "administering" means delivering using any of the various methods and delivery systems known to those skilled in the art. Administering can be performed, for example, intraperitoneally, intracerebrally, intravenously, orally, transmucosally, subcutaneously, transdermally, intradermally, intramuscularly, topically, parenterally, via implant, intrathecally, intralymphatically, intralesionally, pericardially, or epidurally. An agent or composition may also be administered in an aerosol, such as for pulmonary and/or intranasal delivery. Administering may be performed, for example, once, a plurality of times, and/or over one or more extended periods.

[0074] Eliciting a protective immune response in a subject can be accomplished, for example, by administering a primary dose of a vaccine to a subject, followed after a suitable period of time by one or more subsequent administrations of the vaccine. A suitable period of time between administrations of the vaccine may readily be determined by one skilled in the art, and is usually on the order of several weeks to months. The present invention is not limited, however, to any particular method, route or frequency of administration.

[0075] A "prophylactically effective dose" or "a immunologically effective dose" is any amount of a vaccine that, when administered to a subject prone to viral infection or prone to affliction with a virus-associated disorder, induces in the subject an immune response that protects the subject from becoming infected by the virus or afflicted with the disorder. "Protecting" the subject means either reducing the likelihood of the subject's becoming infected with the virus, or lessening the likelihood of the disorder's onset in the subject, by at least two-fold, preferably at least ten-fold. For example, if a subject has a 1% chance of becoming infected with a virus, a two-fold reduction in the likelihood of the subject becoming infected with the virus would result in the subject having a 0.5% chance of becoming infected with the virus. Most preferably, a "prophylactically effective dose" induces in the subject an immune response that completely prevents the subject from becoming infected by the virus or prevents the onset of the disorder in the subject entirely.

[0076] Certain embodiments of any of the instant immunization and therapeutic methods may further comprise administering to the subject at least one adjuvant. An "adjuvant" shall mean any agent suitable for enhancing the immunogenicity of an antigen and boosting an immune response in a subject. Numerous adjuvants, including particulate adjuvants, suitable for use with both protein- and nucleic acid-based vaccines, and methods of combining adjuvants with antigens, are well known to the skilled artisan. Adjuvants suitable for use with protein immunization include, but are not limited to, alum, Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), alum adjuvants, saponin-based adjuvants, such as Quil A, and QS-21, and the like.

[0077] It should be understood that a virus strain described herein, where used to elicit a protective immune response in a subject or to prevent a subject from becoming afflicted with a virus-associated disease, is administered to the subject in the form of a composition additionally comprising one or more a physiologically or pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are well known to the skilled artisan and include, but are not limited to, one or more of 0.01 M - 0.1 M and preferably 0.05 M phosphate buffer, phosphate-buffered saline (PBS), or 0.9% saline. Such carriers also include aqueous or non-aqueous solutions, suspensions, and emulsions. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils.

Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Solid compositions may comprise nontoxic solid carriers such as, for example, glucose, sucrose, mannitol, sorbitol, lactose, starch, magnesium stearate, cellulose or cellulose derivatives, sodium carbonate and magnesium carbonate. For administration in an aerosol, such as for pulmonary and/or intranasal delivery, an agent or composition is preferably formulated with a nontoxic surfactant, for example, esters or partial esters of C6 to C22 fatty acids or natural glycerides, and a propellant. Additional carriers such as lecithin may be included to facilitate intranasal delivery. Pharmaceutically acceptable carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives and other additives, such as, for example, antimicrobials, antioxidants and chelating agents, which enhance the shelf life and/or effectiveness of the active ingredients. The instant compositions can, as is well known in the art, be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to a subject.

[0078] The invention also provides a kit for immunization of a subject with a recombinant PRV described herein. The kit comprises a recombinant PRV described herein, a pharmaceutically acceptable carrier, an applicator, and an instructional material for the use thereof. The invention includes other embodiments of kits that are known to the skilled artisan. The instructions can provide any information that is useful for directing the administration of the of a stable cold-adapted temperature sensitive virus strain described herein, or inactivated form thereof.

[0079] the present invention provides vaccine technology associated with the virus strains described herein. In one embodiment, the virus strains described herein are used in a method of making a vaccine. In another embodiment, a nucleic acid construct described herein is used in a method of making a vaccine. In an additional embodiment, the virus strains described herein are used for vaccine development. In a further embodiment, a a nucleic acid construct described herein is used for vaccine development.

[0080] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and

Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire *et al.*, *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley-VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0081] The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Methods

[0082] *Selection of immunogens*: ORF2 gene from PCV2 Indonesian strain (genotype 2B) (PCV2 TLL-Indo, GenBank Accession No. KX130941) was selected based on the phylogenetic analysis with circulating strains. Also, PCV2 genotype 2B is highly predominant genotype in pig

population (Opriessnig et al., 2013). E2 gene was selected from new emergence of sub-genotype 2.1 of CSF Indonesian strain (CSF TLL-Indo, GenBank Accession No. KX130940). ORF5 gene was selected from HP-PRRSV (Highly pathogenic PRRSV-JXA1) Chinese strain, which is high similarity with currently circulating PRRSV strains (Yin et al., 2012; Jantafong et al., 2015)).

[0083] *Construction of recombinant transfer plasmids and synthetic genes:* ORF2 gene from PCV2 strain and E2 from CSF strain were amplified and individually cloned into the XhoI/ApaI and HindIII/BamHI restriction sites, respectively, of the intermediate pcDNA3.1+ vector (Invitrogen).

[0084] Synthetic genes comprised of combinations of ORF5 of *PRRSV strain*, bovine growth hormone (BGH) sequence and elongation factor 1 α promoter (EF1 α) sequence (ORF5-BGH-EF1 α) were synthesized (Genscript, USA). One such synthetic gene has the sequence shown in SEQ ID NO:3. The BglII and AgeI restriction sites are included.

[0085] For construction of tetravalent rPRV vaccine, the ORF5-BGH-EF1 fragment was cloned into the BglII/AgeI restriction sites of the transfer plasmid pUC-gG-MCS (Figure 1) (kindly provided by Prof. Enquist, Princeton University, USA). pUC-gG-MCS is a pUC57 plasmid with an insertion of a synthetic construct which consists of gG locus of PRV Bartha. The ORF2-BGH fragment from pcDNA3.1+ was amplified using primers AgeI-ORF2 F: 5'-CTGACCGGTATGACGTATCCAAGGAGGCG-3' (SEQ ID NO:4; AgeI site underlined) and ORF2-R-XhoI: 5'-CGGCTCGAGCCATAGAGCCCACCGCATC-3' (SEQ ID NO:5; XhoI site underlined) and cloned into the AgeI/XhoI restriction sites of the transfer plasmid pUC-gG-MCS (Figure 1). Similarly, the CMV-E2 fragment from pcDNA3.1+ was amplified using primers SalI-CMV+E2-F: 5'-CGCGTCGACGTTGACATTGATTATTGAC-3' (SEQ ID NO:6; SalI site underlined) and NotI-E2-R: 5'-TAAAGCGGCGCCGACACCAGCGGCGAGTG TTCTG-3' (SEQ ID NO:7; NotI site underlined) and cloned into the SalI/NotI restriction sites of the transfer plasmid pUC-gG-MCS (Figure 1). The recombinant transfer plasmid pUC-gG-ORF5-ORF2-E2 was verified by PCR and sequencing. The sequence of a nucleic acid comprising gG-CMV-ORF5-BGH-EF1-orf2-BGH-CMV-E2-SV40-gG is set forth in SEQ ID NO:1.

[0086] For construction of trivalent rPRV vaccine, the ORF2-BGH fragment from pcDNA3.1+ was amplified using primers AgeI-ORF2 F: 5'-CTGACCGGTATGACGTATCCAAGGAGGCG-3' (SEQ ID NO:4; AgeI site underlined) and ORF2-R-XhoI: 5'-CGGCTCGAGCCATAGAGCCCACCGCATC-3' (SEQ ID NO:5; XhoI site underlined) and cloned into the AgeI/XhoI restriction sites of the transfer plasmid pUC-gG-MCS (Figure 1). Synthetic EF1 α promoter and amplified E2 fragment were cloned into the SalI/NotI restriction sites, respectively

of the transfer plasmid pUC-gG-MCS (Figure 1) using primers SalI-EF1 F PCR: 5'-GCGTCG ACCGTGAGGCTCCGGT 3' (SEQ ID NO:8; SalI site underlined) and NotI-E2-R: 5'-TAAA GCGGCCGCACCAGCGGCGAGTTGTTCTG 3' (SEQ ID NO:7; NotI site underlined). The recombinant transfer plasmid pUC-gG- ORF2-E2 was verified by PCR and sequencing. The sequence of a nucleic acid comprising gG-CMV-ORF2-BGH-EF1-E2-SV40-gG is set forth in SEQ ID NO:2.

[0087] *Generation of tetravalent and trivalent recombinant PRV:* Nucleocapsid DNA of PRV Bartha expressing red fluorescent protein (RFP) (kindly provided by Prof. Enquist, Princeton University, USA) was linearized by EcoRI enzyme for recombination. The transfer plasmids pUC-gG-ORF5-ORF2-E2 or pUC-gG-ORF2-E2 was digested with HindIII to release the construct for combination. Briefly, PK-15 cell was seeded in 6 well plate at 1×10^6 cell per well. 3ug of digested construct DNA gG-ORF5-ORF2-E2 (tetravalent PRV vaccine) or gG-ORF2-E2 (trivalent PRV vaccine) was co-transfected with 5ug of linearized PRV-RFP nucleocapsid DNA into PK-15 cells using Lipofectamine 2000. After occurrence of cytopathic effect, transfection progenies were plated into PK-15 cells for plaque purification.

[0088] *Plaque assay:* The transfection supernatant (after freeze-thawed) containing of recombinant virus was titrated from dilution 10^{-1} to 10^{-6} and incubated with PK-15 cell culture for 1 hour at 37° C supplied with 5% CO₂. Supernatant was removed and replaced with 1% agarose overlay. Viral plaques without showing fluorescence signal were selected after 48 hours. After, 3 to 4 rounds of plaque purification, the selected plaques were passaged on PK-15 cells and recombinant viruses were sequenced to confirm presence of introduced genes (ORF2, E2 and ORF5) and the absence of mutations.

[0089] *Expression analysis by indirect immunofluorescence assay:* Trivalent or tetravalent rPRV infected cells were analysed by immunofluorescence staining using CSF-E2 specific polyclonal or PCV2-ORF2 specific monoclonal antibodies. Briefly, PK15 cells were infected with Trivalent or tetravalent rPRV for 36 h at 37° C with 5% CO₂. After fixation, cells were permeated with 0.1% Triton X-100 and incubated with anti-E2 or anti-ORF2 polyclonal antibody for 1 h at 37° C. The cells were then incubated with FITC-conjugated rabbit anti-mouse antibody (DAKO Cytomation, Copenhagen, Denmark). The fluorescence signal was detected with an inverted fluorescence microscope (Olympus, Essex, UK) and the images were captured by a digital imaging system (Nikon, Tokyo, Japan).

[0090] *Replication kinetics of recombinant PRV vaccines in PK15 and BHK21 cell lines:* To investigate virus replication in different cell lines, PK15 or BHK21 cells were infected with

PRV-Bartha or trivalent rPRV or tetravalent rPRV at an MOI of 5. After 1h at 37 C. Cells were washed twice with phosphate buffered saline (PBS) and 1 mL of medium containing 2% FBS was added. The culture plates were incubated at 37 C for 24 h and 48 h. At these time points, cells were lysed by three freeze and thaw cycles and the supernatant containing viruses were stored at -80 C. Virus titration was performed in triplicate on PK15 cells with each cell type and time point replicated three times. The viral titers were calculated and expressed as 50% tissue culture-infectious doses per volume (TCID₅₀/mL) using the Reed and Muench method (Reed and Muench, 1938).

[0091] *Genetic stability of recombinant PRV vaccine constructs:* Recombinant PRV vaccine constructs were serially passaged up to 5 times in PK15 cells. After 5 sequential passages, trivalent rPRV or tetravalent rPRV vaccine constructs were verified by PCR and sequencing to confirm the absence of mutations or deletions.

[0092] *Immunogenicity study in mouse model:* Six to seven week-old female BALB/c mice (n=8/group) were intramuscularly vaccinated with 10⁶ TCID₅₀ of attenuated PRV Bartha (Negative control), bivalent rPRV (PRV & ORF2), trivalent rPRV (PRV, ORF2 & E2), tetravalent (PRV, ORF2, E2 & ORF5) and PBS control on days 0 and 21. Sera were collected on days 20 and 42. The immunogenicity of recombinant vaccine constructs were evaluated by serum PRV specific antibody titer, PCV2-ORF2, CSF-E2 and PRRSV-ORF5 specific antibody titers by indirect ELISA on days 20 and 42.

[0093] *Measurement of specific antibody titers by indirect ELISA:* Serum specific antibody titers against PRV, PCV2-ORF2, CSF-E2 and PRRSV-ORF5 antigens were tested by indirect ELISA. Briefly, microtiter well ELISA plates were coated with purified PRV viral antigen or PCV2-ORF2 or CSF-E2 or PRRSV-ORF5 antigens in coating buffer (0.1 mol/liter carbonate-bicarbonate, pH 9.6). Serum samples (1:10 diluted) were 2-fold serially in 3% nonfat dry milk in PBS containing 0.05% Tween 20 were added to the plates in triplicates. After three washes with PBS-T, 1000 times diluted horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulins (DAKO) were added into each well. The reaction was developed by 100 ml TMB substrate (3, 3', 5', 5'-tetramethylbenzidine) and then terminated by 50 ml of 2 M H₂SO₄. The optical densities at 450 nm were determined using a microwell plate absorbance reader (Tecan, Switzerland).

EXAMPLE 2

Construction and Characterizations of Recombinant PRV Vaccines

[0094] Recombinant trivalent PRV vector expressing PCV2-ORF2 and CSF-E2 (trivalent PRV-ORF2-E2) and tetravalent PRV vector expressing PCV2-ORF2, CSF-E2 and PRRSV-ORF5 (tetravalent PRV-ORF5-ORF2-E2) were generated by integration of linear transfer plasmids (pUC-gG-tetravalent/pUC-gG-trivalent) cassettes into the PRV Bartha DNA by homologous recombination (Figure 1). After transfection, resulting recombinants were plaque-purified on PK15 cells by selection of non-red fluorescent plaques. Positive recombinants were re-screened. After 3-4 times plaque purification, the positive recombinants were analyzed by sequencing and titrated in PK15 cells. Also, the immunofluorescence assay against individual ORF2 or E2-specific antibodies demonstrated efficient expression of ORF2 or E2 proteins by a single PRV vector (Figures 2A and 2B). In contrast, no fluorescent cells were seen for the PRV negative control. Also, stability testing revealed that recombinant vaccines (tetravalent rPRV or trivalent rPRV) were stable and absence of mutations or deletions after five sequential passages in PK15 cells.

EXAMPLE 3

Replication in PK15 and BHK21 Cells

[0095] To investigate whether insertion or expression of foreign transgenes at gG locus has an effect on replication properties, one-step growth kinetics of recombinant PRV vaccine constructs trivalent, tetravalent rPRV and parental PRV Bartha strain were compared in PK15 and BHK21 cells. The viral titers of both trivalent and tetravalent PRV vaccine constructs showed 10^8 TCID₅₀ and $10^{8.3}$ TCID₅₀ in PK15 and BHK21 cells, respectively and the replication titers were comparable with PRV-Bartha (TCID₅₀ $10^{8.5-8.7}$) parental strain.

EXAMPLE 4

Immunogenicity Study in a Mouse Model

[0096] The results showed that mice immunized with tetravalent PRV vaccine showed serum specific antibody levels against PRV, E2, ORF2 and PRRSV. Also, mouse vaccinated with trivalent PRV vaccine induced serum specific antibody levels against PRV, ORF2 and E2 antigens (Figure 3). The antibody titers results showed that mice immunized with Bivalent rPRV (ORF2) showed antibody titers of >260 against PCV2-ORF2 antigen. Also, mice immunized with trivalent PRV and tetravalent PRV vaccine showed antibody titers of 240 and 180 against

ORF2 antigen, respectively. Moreover, both trivalent and tetravalent PRV vaccines showed >256 antibody titers against classical swine fever E2 glycoproteins (Figure 4).

[0097] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0098] Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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1. A nucleic acid construct comprising the nucleotide sequence set forth in SEQ ID NO: 2, wherein the nucleic acid construct comprises;
 - a pseudorabies virus (PRV) gG gene containing inserted therein:
 - (a) a first promoter active in a mammalian cell operatively linked to a porcine circovirus (PCV2) ORF2 gene operatively linked to a first terminator active in a mammalian cell and
 - (b) a second promoter active in a mammalian cell operatively linked to a classical swine fever (CSF) virus E2 gene operatively linked to a second terminator active in a mammalian cell,
 - wherein the first promoter active in a mammalian cell is one of a cytomegalovirus (CMV) intermediate early (ie) promoter and the second promoter active in a mammalian cell is the elongation factor 1 alpha (EF1 α) promoter and wherein the first terminator active in a mammalian cell is one of a bovine growth hormone (BGH) poly adenylation sequence (polyA) and the second terminator active in a mammalian cell is the simian virus 40 (SV40) polyA.
2. The nucleic acid construct of claim 1, wherein the PCV2 ORF2 gene has the sequence set forth in nucleotides 3063-3767 of SEQ ID NO:1 and the CSF E2 gene has the sequence set forth in nucleotides 4729-5916 of SEQ ID NO:1.
3. A vector comprising the nucleic acid construct of any one of claims 1-2.
4. A mammalian cell line transfected with the nucleic acid construct of any one of claims 1-2 and a linearized PRV nucleocapsid nucleic acid.
5. The mammalian cell line of claim 4 which is a PK-15 cell line.
6. The mammalian cell line of claim 4 or 5, wherein the PRV is PRV Bartha-K61.
7. A trivalent recombinant PRV comprising the nucleic acid construct of any one of claims 1-2.

8. The trivalent recombinant PRV of claim 7, wherein the PRV is PRV Bartha-K61.
9. A vaccine comprising the trivalent recombinant PRV of claim 7 or 8 and a physiologically acceptable carrier.
10. Use of the trivalent recombinant PRV of claim 7 or 8 for the manufacture of a medicament for eliciting a protective immune response in swine against PRV, PCV2 and CSF virus.
11. Use of the trivalent recombinant PRV of claim 7 or 8 for the manufacture of a medicament for preventing swine from becoming infected with PRV, PCV2 and CSF virus.
12. Use of the trivalent recombinant PRV of claim 7 or 8 for development and production of a vaccine.
13. A trivalent recombinant PRV of claim 7 or 8 for use in vaccine development.
14. A nucleic acid construct comprising the nucleotide sequence set forth in SEQ ID NO: 1, wherein the nucleic acid construct comprises;
 - a pseudorabies virus (PRV) gG gene containing inserted therein:
 - (a) a first promoter active in a mammalian cell operatively linked to a porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 gene operatively linked to a first terminator active in a mammalian cell.
 - (b) a second promoter active in a mammalian cell operatively linked to a porcine circovirus (PCV2) ORF2 gene operatively linked to a second terminator active in a mammalian cell; and
 - (c) a third promoter active in a mammalian cell operatively linked to a classical swine fever (CSF) virus E2 gene operatively linked to a third terminator active in a mammalian cell,
 - wherein the first promoter active in a mammalian cell is one of a cytomegalovirus (CMV) intermediate early (ie) promoter and the second promoter active in a mammalian cell is the elongation factor 1 alpha (EF1 α) promoter, wherein the third promoter active in

a mammalian cell is one of a cytomegalovirus (CMV) intermediate early (ie) promoter, wherein the first terminator active in a mammalian cell is one of a bovine growth hormone (BGH) poly adenylation sequence (polyA) and the second terminator active in a mammalian cell is one of a bovine growth hormone (BGH) poly adenylation sequence (polyA) and wherein the third terminator active in a mammalian cell is one of simian virus 40 (SV40) polyA.

15. The nucleic acid construct of claim 14, wherein the PCV2 ORF2 gene has the sequence set forth in nucleotides 3063-3767 of SEQ ID NO:1, the CSF virus E2 gene has the sequence set forth in nucleotides 4729-5916 of SEQ ID NO:1 and the PRRSV ORF5 has the sequence set forth in nucleotides 1041-1643 of SEQ ID NO:1.
16. A vector comprising the nucleic acid construct of any one of claims 14-15.
17. A mammalian cell line transfected with the nucleic acid construct of any one of claims 14-15 and a linearized PRV nucleocapsid nucleic acid.
18. The mammalian cell line of claim 17 which is a PK-15 cell line.
19. The mammalian cell line of claim 17 or 18, wherein the PRV is PRV Bartha-K61.
20. A tetravalent recombinant PRV comprising the nucleic acid construct of any one of claims 14-15.
21. The tetravalent recombinant PRV of claim 20, wherein the PRV is PRV Bartha-K61.
22. A vaccine comprising the tetravalent recombinant PRV of claim 20 or 21 and a physiologically acceptable carrier.
23. Use of the tetravalent recombinant PRV of claim 20 or 21 for the manufacture of a medicament for eliciting a protective immune response in swine against PRV, PRRSV, PCV2 and CSF virus.

24. Use of the tetravalent recombinant PRV of any claim 20 or 21 for the manufacture of a medicament for preventing swine from becoming infected with PRV, PRRSV, PCV2 and CSF virus.
25. Use of the tetravalent recombinant PRV of claim 20 or 21 for development and production of a vaccine.
26. The tetravalent recombinant PRV of claim 20 or 21 for use in vaccine development.