USE OF A PCV2 IMMUNOGENIC COMPOSITION FOR LESSENING CLINICAL SYMPTOMS IN PIGS

SEQUENCE LISTING

This application contains a sequence listing in paper format and in computer readable format, the teachings and content of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the use of an immunogenic composition comprising a porcine circovirus type 2 (PCV2) antigen for treatment of several clinical manifestations (diseases). Preferably, those clinical manifestations are associated with a PCV2 infection. More particularly, the present invention is concerned with an immunological composition effective for providing an immune response that reduces, or lessens the severity, of the clinical symptoms associated with PCV2 infection. Preferably, the immunological composition comprises a recombinantly produced antigen of PCV2. More preferably, the PCV2 antigen is a recombinantly produced protein encoded by one of the open reading frames (ORFs) in the PCV2 genome. Still more preferably, the antigen is PCV2 ORF2 protein. Most particularly, the present invention is concerned with an immunological composition effective for treatment of clinical sypmtoms associated with PCV2 infections in swine receiving the immunological composition, and wherein the composition comprises the protein expressed by ORF2 of PCV2. Another aspect of the present invention is the use of any of the compositions provided herewith as a medicament, preferably as a veterinary medicament, even more preferably as a vaccine. Moreover, the present invention also relates

to the use of any of the compositions described herein, for the preparation of a medicament for reducing or lessening the severity of clinical symptoms associated with PCV2 infection. Preferably, the medicament is for the prevention of a PCV2 infection, even more preferably in swine. A further aspect of the present invention relates to a process for the production of a medicament, comprising an immunogenic composition of PCV2 for the treatment of several clinical manifestations.

Description of the Prior Art

Porcine circovirus type 2 (PCV2) is a small (17 -22 nm in diameter), icosahedral, non-enveloped DNA virus, which contains a single-stranded circular genome. PCV2 shares approximately 80% sequence identity with porcine circovirus type 1 (PCV1). However, in contrast with PCV1, which is generally non-virulent, swine infected with PCV2 exhibit a syndrome commonly referred to as Post-weaning Multisystemic Wasting Syndrome (PMWS). PMWS is clinically characterized by wasting, paleness of the skin, unthriftiness, respiratory distress, diarrhea, icterus, and jaundice. In some affected swine, a combination of all symptoms will be apparent while other affected swine will only have one or two of these symptoms. During necropsy, microscopic and macroscopic lesions also appear on multiple tissues and organs, with lymphoid organs being the most common site for lesions. A strong correlation has been observed between the amount of PCV2 nucleic acid or antigen and the severity of microscopic lymphoid lesions. Mortality rates for swine infected with PCV2 can approach 80%. In addition to PMWS, PCV2 has been associated with several other infections including pseudorabies, porcine reproductive and respiratory syndrome (PRRS), Glasser's disease, streptococcal meningitis, salmonellosis, postweaning colibacillosis, dietetic hepatosis, and suppurative bronchopneumonia. However, research thus far has not confirmed

whether any of these clinical symptoms are in fact, the direct result of a PCV2 infection. Moreover, it is not yet known whether any of these clinical symptoms can be effectively reduced or cured by an active agent directed against PCV2.

Current approaches to treat PCV2 infections include DNA-based vaccines, such as those described in U.S. Patent No. 6,703,023. However, such vaccines have been ineffective at conferring protective immunity against PCV2 infection or reducing, lessening the severity of, or curing any clinical symptoms associated therewith. Moreover, vaccines described in the prior art were focused solely on the prevention of PCV2 infections in swine, but did not consider any further medical use.

Accordingly, what is needed in the art is an immunogenic composition for the treatment of several clinical manifestations. Further, what is needed in the art is an immunological composition which confers protective immunity against PCV2 infection but which can also be used to treat existing clinical symptoms associated with PCV2 infection.

DISCLOSURE OF THE INVENTION

The present disclosure overcomes the problems inherent in the prior art and provides a distinct advance in the state of the art. The present disclosure provides a medicinal use(s) of immunogenic composition(s) comprising PCV2 antigen.

In general no adverse events or injection site reactions were noted for any of the PCV2 antigen immunogenic compositions as used herein. Thus, the immunogenic compositions used herein appear to be safe when administered to young pigs, preferably to pigs not older than 15 weeks of age, more preferably not older than 6 weeks of age, even more preferably not older than 3 weeks, most preferably not older than 2 weeks. Alternatively, it is preferred that the administration of the immunogenic compositions of the

present disclosure occur within at least 2 and preferably within at least 3 weeks of exposure to virulent PCV. According to a further embodiment, the immunogenic compositions used herein for any medicinal use described herein, is adminstered to pigs of 3 weeks of age or older, preferably of 2 weeks of age or older, most preferably but not older than 15 weeks of age.

Unexpectedly, it was found that the therapeutic use of the immunogenic compositions described below, is effective for lessening the severity of various clinical symptoms in swine. In particular, it was discovered that the therapeutic use of the immunogenic compositions of the present disclosure, and specifically compositions comprising PCV2 ORF2 antigen, is effective for reducing or lessening lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes in swine infected with PCV2. Moreover, the therapeutic use of an antigenic composition, as provided herewith, and that comprises PCV2 antigen, preferably ORF2 antigen, reduces the overall circovirus load and its immunosuppressive impact, thereby resulting in a higher level of general disease resistance and a reduced incidence of PCV-2 associated diseases and symptoms.

In a first aspect, there is provided the use of an immunogenic composition capable of eliciting or enhancing an immune response against porcine circovirus type 2 (PCV) and comprising recombinantly produced PCV2 ORF2 protein in the manufacture of a medicament for preventing one or more of the following symptoms associated with PCV2 infection: nasal shedding, increased mortality rate, decreased average daily weight gain, wherein the PCV2 ORF2 protein is to be administered once to a pig.

Thus the present disclosure relates to the use of an immunogenic composition comprising PCV2 antigen, preferably recombinant PCV2 antigen, and more preferably PCV2 ORF2 protein as provided herewith, for the preparation of a medicament for the prevention, lessening and/or reduction of lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes in swine. Preferably, said medicament is effective for the prevention, lessening and/or reduction of lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes associated with PCV2 infections in swine. Still more preferably, said medicament is effective for the prevention, lessening and/or reduction of lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes associated with

PCV2 infections in pigs, when administered to pigs not older than 15 weeks of age, more preferably not older than 6 weeks of age, even more preferably not older than 3 weeks, and most preferably not older than 2 weeks. Alternatively, it is preferred that the administration of the immunogenic compositions of the present disclosure occur within at least 2 and preferably within at least 3 weeks of exposure to virulent PCV.

The present disclosure also relates to a method for the treatment of lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes in swine, comprising the administration of an immunogenic composition as provided herewith, to a pig, said immunogenic composition comprising a PCV2 antigen, preferably a recombinant PCV2 antigen, and morepreferably PCV2 ORF2 protein. The present disclosure also provides a method for the treatment of lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes associated with a PCV2 infection in swine, comprising the administration of an immunogenic composition as provided herewith, to a pig, said immunogenic composition comprising a PCV2 antigen, preferably a recombinant PCV2 antigen and more preferably PCV2 ORF2 protein. Preferably, said treatment results in the lessening, reduction, prevention, and/or cure of the lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes in swine receiving said immunogenic composition. The methods for treatment may further comprise the administration of said immunogenic composition to pigs not older than 15 weeks of age, more preferably not older than 6 weeks of age, even more preferably not older than 3 weeks, and most preferably not older than 2 weeks. Alternatively, it is preferred that the administration of the immunogenic compositions of the present disclosure occur within at least 2 and preferably within at least 3 weeks of exposure to virulent PCV.

It was further discovered that the therapeutic use of an immunogenic composition comprising PCV2 antigen, preferably a recominant PCV2 antigen, and most prefereably PCV2 ORF2 protein, as provided herewith, can reduce or lessen lymphadenopathy in combination with one or a multiple of the following symptoms in affected swine: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers, (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc.

The present disclosure also relates to the use of an immunogenic composition comprising PCV2 antigen, preferably a recombinant PCV2 antigen and more preferably, PCV2 ORF2 protein as provided herewith, for the preparation of a medicament for the prevention, lessening and/or reduction of lymphadenopathy in combination with one or a multiple of the following symptoms in pigs: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers, (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc., in pigs. Preferably, said medicament is effective for the prevention, lessening and/or reduction of lymphadenopathy in combination with one or a multiple of the following symptoms associated with PCV2 infection in pigs: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers , (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc. The medicament may be effective for the prevention, lessening and/or reduction of lymphadenopathy in combination with one or a multiple of the following symptoms in pigs: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers , (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc., in pigs, when administered to pigs not older than 15 weeks of age,

more preferably not older than 6 weeks of age, even more preferably not older than 3 weeks, and most preferably not older than 2 weeks. Alternatively, it is preferred that the administration of the immunogenic compositions of the present disclosure occur within at least 2 and preferably within at least 3 weeks of exposure to virulent PCV..

Moreover, the present disclosure also relates to a method for the treatment of lymphadenopathy in combination with one or a multiple of the following symptoms in pigs: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers , (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc., said method comprising the administration of an immunogenic composition comprising PCV2 antigen, preferably a recombinant PCV2 antigen, and more preferably PCV2 ORF2 protein as provided herewith. Preferably, the present disclosure also relates to a method for the treatment of lymphadenopathy in combination with one or a multiple of the following symptoms associated with PCV2 infection in pigs: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers, (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc., said method comprising the administration of an immunogenic composition comprising PCV2 antigen, prefereably recombinant PCV2 antigen and more preferably PCV2 ORF2 protein, as provided herewith, to a pig. Preferably, said treatment results in the lessening or reduction of the lymphadenopathy, and one or multiple of the following symptoms associated with PCV2 infection in pigs: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers , (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc. The methods for treatment may further comprise administration of the immunogenic composition comprising PCV2 antigen, preferably recombinant PCV2

antigen and more preferably PCV2 ORF2 protein, as provided herein, to pigs not older than 15 weeks of age, more preferably not older than 6 weeks of age, even more preferably not older than 3 weeks, and most preferably not older than 2 weeks. Alternatively, it is preferred that the administration of the immunogenic compositions of the present disclosure occur within at least 2 and preferably within at least 3 weeks of exposure to virulent PCV.

It was also unexpectedly found that the therapeutic use of an immunogenic composition comprising PCV antigen, preferably recombinant PCV2 antigen and more preferably PCV2 ORF2 protein as provided herewith, can also reduce or lessen Pia like lesions, normally known to be associated with Lawsonia intracellularis infections (Ileitis).

The present disclosure may relate to the use of an immunogenic composition comprising PCV2 antigen, preferably recombinant PCV2 antigen and more preferably PCV2 ORF2 protein as provided herewith, for the preparation of a medicament for the prevention, lessening the severity of and/or reduction of Pia like lesions, normally known to be associated with Lawsonia intracellularis infections in swine. The medicament may be effective for the prevention, lessening of the severity of and/or reduction of Pia like lesions, normally known to be associated with Lawsonia intracellularis infections, when administered to pigs not older than 15 weeks of age, more preferably not older than 6 weeks of age, even more preferably not older than 3 weeks, and most preferably not older than 2 weeks. Alternatively, it is preferred that the administration of the immunogenic compositions of the present disclosure occur within at least 2 and preferably within at least 3 weeks of exposure to virulent PCV.

Moreover, the present disclosure also relates to a method for the treatment of Pia like lesions, normally known to be associated with Lawsonia intracellularis infections, said method comprising the administration of an immunogenic composition comprising PCV2

antigen, preferably recombinant PCV2 antigen and more preferably PCV2 ORF2 protein as provided herein, to a pig. Preferably, said treatment results in the lessening or reduction of the Pia like lesions, normally known to be associated with Lawsonia intracellularis infections. The methods for treatment described above may further comprise the administration of the immunogenic composition comprising PCV2 antigen, preferably recombiant PCV2 antigen, and more preferably PCV2 ORF2 protein as provided herein, to pigs not older than 15 weeks of age, more preferably not older than 6 weeks of age, even more preferably not older than 3 weeks, and most preferably not older than 2 weeks. Alternatively, it is preferred that the administration of the immunogenic compositions of the present disclosure occur within at least 2 and preferably within at least 3 weeks of exposure to virulent PCV.

The immunogenic composition

The immunogenic composition as used herein is effective for inducing an immune response against PCV2 and preventing, reducing and/or lessening the severity of the clinical symptoms associated with PCV2 infection. The composition generally comprises at least one PCV2 antigen.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. The term "immunogenic composition" as used herein refers to any pharmaceutical composition containing a PCV2 antigen, which composition can be used to prevent or treat a PCV2 infection-associated disease or condition in a subject. A preferred immunogenic composition can induce, stimulate or enhance the immune response against PCV2. The term

thus encompasses both subunit immunogenic compositions, as described below, as well as compositions containing whole killed, or attenuated and/or inactivated PCV2.

The term "subunit immunogenic composition" as used herein refers to a composition containing at least one immunogenic polypeptide or antigen, but not all antigens, derived from or homologous to an antigen from PCV2. Such a composition is substantially free of intact PCV2. Thus, a "subunit immunogenic composition" is prepared from at least partially purified or fractionated (preferably substantially purified) immunogenic polypeptides from PCV2, or recombinant analogs thereof. A subunit immunogenic composition can comprise the subunit antigen or antigens of interest substantially free of other antigens or polypeptides from PCV2, or in fractionated from. A preferred immunogenic subunit composition comprises the PCV2 ORF2 protein as described below.

An "immunological or immune response" to a composition or vaccine is the development in the host of a cellular and/ or antibody-mediated immune response to the composition or vaccine of interest. Usually, an "immune response" includes but is not limited to one or more of the following effects: the production or activation of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or yd T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective immunological response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction in number or severity of, or lack of one or more of the symptoms associated with PCV2 infections as described above.

The terms "immunogenic" protein or polypeptide or “antigen” as used herein refer to an amino acid sequence which elicits an immunological response as described above. An

"immunogenic" protein or polypeptide, as used herein, includes the full-length sequence of any PCV2 proteins, analogs thereof, or immunogenic fragments thereof. The term "immunogenic fragment" refers to a fragment of a protein which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) Proc. Natl. Acad. Sci. USA 81:3998-4002; Geysen et al. (1986) Molec. Immunol. 23:709-715. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols, supra.

Synthetic antigens are also included within the definition, for example, polyepitopes, flanking epitopes, and other recombinant or synthetically derived antigens. See, e.g., Bergmann et al. (1993) Eur. J. Immunol. 23:2777-2781; Bergmann et al. (1996), J. Immunol. 157:3242-3249; Suhrbier, A. (1997), Immunol. and Cell Biol. 75:402-408; Gardner et al., (1998) 12th World AIDS Conference, Geneva, Switzerland, June 28-July 3, 1998.

In a preferred embodiment of the present invention, an immunogenic composition that induces an immune response and, more preferably, confers protective immunity against the clinical signs of PCV2 infection, is provided. The composition most preferably comprises the

polypeptide, or a fragment thereof, expressed by ORF2 of PCV2, as the antigenic component of the composition. PCV2 ORF2 DNA and protein, used herein for the preparation of the compositions and within the processes provided herein is a highly conserved domain within PCV2 isolates and thereby, any PCV2 ORF2 would be effective as the source of the PCV ORF2 DNA and/or polypeptide as used herein. A preferred PCV2 ORF2 protein is that of SEQ ID NO. 11. A preferred PCV ORF2 polypeptide is provided herein as SEQ ID NO. 5, but it is understood by those of skill in the art that this sequence could vary by as much as 6-10% in sequence homology and still retain the antigenic characteristics that render it useful in immunogenic compositions. The antigenic characteristics of an immunological composition can be, for example, estimated by the challenge experiment as provided by Example 4. Moreover, the antigenic characteristic of a modified antigen is still retained, when the modified antigen confers at least 70%, preferably 80%, more preferably 90% of the protective immunity as compared to the PCV2 ORF 2 protein, encoded by the polynucleotide sequence of SEQ ID NO:3 or SEQ ID NO:4. An "immunogenic composition" as used herein, means a PCV2 ORF2 protein which elicits an "immunological response" in the host of a cellular and/or antibody-mediated immune response to PCV2 ORF2 protein. Preferably, this immunogenic composition is capable of eliciting or enhancing an immune response against PCV2 thereby confering protective immunity against PCV2 infection and a reduction in the incidence of, severity of, or prevention of one or more, and preferably all of the clinical signs associated therewith.

In some forms, immunogenic portions of PCV2 ORF2 protein are used as the antigenic component in the composition. The term "immunogenic portion" as used herein refers to truncated and/or substituted forms, or fragments of PCV2 ORF2 protein and/or polynucleotide, respectively. Preferably, such truncated and/or substituted forms, or

fragments will comprise at least 6 contiguous amino acids from the full-length ORF2 polypeptide. More preferably, the truncated or substituted forms, or fragments will have at least 10, more preferably at least 15, and still more preferably at least 19 contiguous amino acids from the full-length ORF2 polypeptide. Two preferred sequences in this respect are provided herein as SEQ ID NOs. 9 and 10. It is further understood that such sequences may be a part of larger fragments or truncated forms.

A further preferred PCV2 ORF2 polypeptide provided herein is encoded by the nucleotide sequences of SEQ ID NO: 3 or SEQ ID NO: 4. However, it is understood by those of skill in the art that this sequence could vary by as much as 6-20% in sequence homology and still retain the antigenic characteristics that render it useful in immunogenic compositions. In some forms, a truncated or substituted form, or fragment of this PVC2 ORF2 polypeptide is used as the antigenic component in the composition. Preferably, such truncated or substituted forms, or fragments will comprise at least 18 contiguous nucleotides from the full-length ORF2 nucleotide sequence, e.g. of SEQ ID NO: 3 or SEQ ID NO: 4. More preferably, the truncated or substituted forms, or fragments, will have at least 30, more preferably at least 45, and still more preferably at least 57 contiguous nucleotides of the full-length ORF2 nucleotide sequence, e.g. SEQ ID NO: 3 or SEQ ID NO: 4.

"Sequence Identity" as it is known in the art refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences are

"identical" at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by known methods, including but not limited to, those described in Computational Molecular Biology, Lesk, A. N., ed., Oxford University Press, New York (1988), Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinge, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988), the teachings of which are incorporated herein by reference. Preferred methods to determine the sequence identity are designed to give the largest match between the sequences tested. Methods to determine sequence identity are codified in publicly available computer programs which determine sequence identity between given sequences. Examples of such programs include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research, 12(1):387 (1984)), BLASTP, BLASTN and FASTA (Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al., NCVI NLM NIH Bethesda, MD 20894, Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990), the teachings of which are incorporated herein by reference). These programs optimally align sequences using default gap weights in order to produce the highest level of sequence identity between the given and reference sequences. As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 85%, preferably 90%, even more preferably 95% "sequence

identity" to a reference nucleotide sequence, it is intended that the nucleotide sequence of the given polynucleotide is identical to the reference sequence except that the given polynucleotide sequence may include up to 15, preferably up to 10, even more preferably up to 5 point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, in a polynucleotide having a nucleotide sequence having at least 85%, preferably 90%, even more preferably 95% identity relative to the reference nucleotide sequence, up to 15%, preferably 10%, even more preferably 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 15%, preferably 10%, even more preferably 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having a given amino acid sequence having at least, for example, 85%, preferably 90%, even more preferably 95% sequence identity to a reference amino acid sequence, it is intended that the given amino acid sequence of the polypeptide is identical to the reference sequence except that the given polypeptide sequence may include up to 15, preferably up to 10, even more preferably up to 5 amino acid alterations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a given polypeptide sequence having at least 85%, preferably 90%, even more preferably 95% sequence identity with a reference amino acid sequence, up to 15%, preferably up to 10%, even more preferably up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 15%, preferably up to 10%, even more preferably up to 5% of the total number of amino acid

residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or the carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in the one or more contiguous groups within the reference sequence. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. However, conservative substitutions are not included as a match when determining sequence identity.

"Sequence homology", as used herein, refers to a method of determining the relatedness of two sequences. To determine sequence homology, two or more sequences are optimally aligned, and gaps are introduced if necessary. However, in contrast to "sequence identity", conservative amino acid substitutions are counted as a match when determining sequence homology. In other words, to obtain a polypeptide or polynucleotide having 95% sequence homology with a reference sequence, 85%, preferably 90%, even more preferably 95% of the amino acid residues or nucleotides in the reference sequence must match or comprise a conservative substitution with another amino acid or nucleotide, or a number of amino acids or nucleotides up to 15%, preferably up to 10%, even more preferably up to 5% of the total amino acid residues or nucleotides, not including conservative substitutions, in the reference sequence may be inserted into the reference sequence. Preferably the homolog sequence comprises at least a stretch of 50, even more preferably at least 100, even more preferably at least 250, and even more preferably at least 500 nucleotides.

A "conservative substitution" refers to the substitution of an amino acid residue or nucleotide with another amino acid residue or nucleotide having similar characteristics or properties including size, hydrophobicity, etc., such that the overall functionality does not change significantly.

"Isolated" means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

Thus, the immunogenic composition as used herein also refers to a composition that comprises PCV2 ORF2 protein, wherein said PCV2 ORF2 protein is anyone of those, described above. Preferably, said PCV2 ORF2 protein is

i) a polypeptide comprising the sequence of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11;

ii) any polypeptide that is at least 80% homologous to the polypeptide of i),

iii) any immunogenic portion of the polypeptides of i) and/or ii)

iv) the immunogenic portion of iii), comprising at least 10 contiguous amino acids included in the sequences of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11,

v) a polypeptide that is encoded by a DNA comprising the sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

vi) any polypeptide that is encoded by a polynucleotide that is at least 80% homologous to the polynucleotide of v),

vii) any immunogenic portion of the polypeptides encoded by the polynucleotide of v) and/or vi)

viii) the immunogenic portion of vii), wherein polynucleotide coding for said immunogenic portion comprises at least 30 contiguous nucleotides included in the sequences of SEQ ID NO: 3, or SEQ ID NO: 4.

Preferably any of those immunogenic portions have the immunogenic characteristics of PCV2 ORF2 protein that is encoded by the sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

According to a further aspect, PCV2 ORF2 protein is provided in the immunological composition at an antigen inclusion level effective for inducing the desired immune response, namely reducing the incidence of, lessening the severity of, or preventing one or more clinical signs resulting from PCV2 infection. Preferably, the PCV2 ORF2 protein inclusion level is at least 0.2 μg antigen / ml of the final immunogenic composition (μg/ml), more preferably from about 0.2 to about 400 μg/ml, still more preferably from about 0.3 to about 200 μg/ml, even more preferably from about 0.35 to about 100 μg/ml, still more preferably from about 0.4 to about 50 μg/ml, still more preferably from about 0.45 to about 30 μg/ml, still more preferably from about 0.6 to about 15 μg /ml, even more preferably from about 0.75 to about 8 μg/ml, even more preferably from about 1.0 to about 6 μg/ml, still more preferably from about 1.3 to about 3.0 μg/ml, even more preferably from about 1.4 to about 2.5 μg/ml, even more preferably from about 1.5 to about 2.0 μg/ml, and most preferably about 1.6 μg/ml.

According to a further aspect, the ORF2 antigen inclusion level is at least 0.2 μg/ PCV2 ORF2 protein as described above per dose of the final antigenic composition (μg/dose), more preferably from about 0.2 to about 400 μg/dose, still more preferably from about 0.3 to about 200 μg/dose, even more preferably from about 0.35 to about 100 μg/dose, still more preferably from about 0.4 to about 50 μg/dose, still more preferably from about 0.45 to about 30 μg/dose, still more preferably from about 0.6 to about 15 μg/dose, even more preferably from about 0.75 to about 8 μg/dose, even more preferably from about 1.0 to about 6 μg/dose, still more preferably from about 1.3 to about 3.0 μg/dose, even more preferably from about 1.4 to about 2.5 μg/dose, even more preferably from about 1.5 to about 2.0 μg/dose, and most preferably about 1.6 μg/dose.

The PCV2 ORF2 polypeptide used in the immunogenic composition in accordance with the present invention can be derived in any fashion including isolation and purification of PCV2 ORF2, standard protein synthesis, and recombinant methodology. Preferred methods for obtaining PCV2 ORF2 polypeptide are provided in U.S. Patent Application Serial No. 11/034,797, the teachings and content of which are hereby incorporated by reference. Briefly, susceptible cells are infected with a recombinant viral vector containing PCV2 ORF2 DNA coding sequences, PCV2 ORF2 polypeptide is expressed by the recombinant virus, and the expressed PCV2 ORF2 polypeptide is recovered from the supernate by filtration and inactivated by any conventional method, preferably using binary ethylenimine, which is then neutralized to stop the inactivation process.

The immunogenic composition as used herein also refers to a composition that comprises **i)** any of the PCV2 ORF2 protein described above, preferably in concentrations described above, and **ii)** at least a portion of the viral vector expressing said PCV2 ORF2 protein, preferably of a recombinant baculovirus. Moreover, the immunogenic composition can comprise **i)** any of the PCV2 ORF2 proteins described above, preferably in concentrations described above, **ii)** at least a portion of the viral vector expressing said PCV2 ORF2 protein, preferably of a recombinant baculovirus, and **iii)** a portion of the cell culture supernate.

The immunogenic composition as used herein also refers to a composition that comprises **i)** any of the PCV2 ORF2 proteins described above, preferably in concentrations described above, **ii)** at least a portion of the viral vector expressing said PCV2 ORF2 protein, preferably of a recombinant baculovirus, and **iii)** a portion of the cell culture; wherein about 90% of the components have a size smaller than 1 μm.

The immunogenic composition as used herein also refers to a composition that comprises **i)** any of the PCV2 ORF2 proteins described above, preferably in concentrations

described above, **ii)** at least a portion of the viral vector expressing said PCV2 ORF2 protein, **iii)** a portion of the cell culture, **iv)** and inactivating agent to inactivate the recombinant viral vector preferably BEI, wherein about 90% of the components i) to iii) have a size smaller than 1 μm. Preferably, BEI is present in concentrations effective to inactivate the baculovirus. Effective concentrations are described above.

The immunogenic composition as used herein also refers to a composition that comprises **i)** any of the PCV2 ORF2 proteins described above, preferably in concentrations described above, **ii)** at least a portion of the viral vector expressing said PCV2 ORF2 protein, **iii)** a portion of the cell culture, **iv)** an inactivating agent to inactivate the recombinant viral vector preferably BEI, and **v)** an neutralization agent to stop the inactivation mediated by the inactivating agent, wherein about 90% of the components i) to iii) have a size smaller than 1 μm. Preferably, if the inactivating agent is BEI, said composition comprises sodium thiosulfate in equivalent amounts to BEI.

The polypeptide is incorporated into a composition that can be administered to an animal susceptible to PCV2 infection. In preferred forms, the composition may also include additional components known to those of skill in the art (see also Remington's Pharmaceutical Sciences. (1990). 18th ed. Mack Publ., Easton). Additionally, the composition may include one or more veterinary-acceptable carriers. As used herein, "a veterinary-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. In a preferred embodiment, the immunogenic composition comprises PCV2 ORF2 protein as provided herewith, preferably in concentrations described above, which is mixed with an adjuvant, preferably Carbopol, and physiological saline.

Those of skill in the art will understand that the composition used herein may incorporate known injectable, physiologically acceptable sterile solutions. For preparing a ready-to-use solution for parenteral injection or infusion, aqueous isotonic solutions, such as e.g. saline or corresponding plasma protein solutions, are readily available. In addition, the immunogenic and vaccine compositions of the present invention can include diluents, isotonic agents, stabilizers, or adjuvants. 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 and alkali salts of ethylendiamintetracetic acid, among others.

"Adjuvants" as used herein, can include aluminum hydroxide and aluminum phosphate, saponins e.g., Quil A, QS-21 (Cambridge Biotech Inc., Cambridge MA), GPI-0100 (Galenica Pharmaceuticals, Inc., Birmingham, AL), water-in-oil emulsion, oil-in-water emulsion, water-in-oil-in-water emulsion. The emulsion can be based in particular on light liquid paraffin oil (European Pharmacopea type); isoprenoid oil such as squalane or squalene oil resulting from theoligomerization of alkenes, in particular of isobutene or decene; esters of acids or of alcohols containing a linear alkyl group, more particularly plant oils, ethyl oleate, propylene glycol di-(caprylate/caprate), glyceryl tri-(caprylate/caprate) or propylene glycol dioleate; esters of branched fatty acids or alcohols, in particular isostearic acid esters. The oil is used in combination with emulsifiers to form the emulsion. The emulsifiers are preferably nonionic surfactants, in particular esters of sorbitan, of mannide (e.g. anhydromannitol oleate), of glycol, of polyglycerol, of propylene glycol and of oleic, isostearic, ricinoleic or hydroxystearic acid, which are optionally ethoxylated, and polyoxypropylene-polyoxyethylene copolymer blocks, in particular the Pluronic products, especially L121. See Hunter et al., The Theory and Practical Application of Adjuvants (Ed.Stewart-Tull, D. E.

S.). JohnWiley and Sons, NY, pp51-94 (1995) and Todd et al., Vaccine 15:564-570 (1997).

For example, it is possible to use the SPT emulsion described on page 147 of "Vaccine Design, The Subunit and Adjuvant Approach" edited by M. Powell and M. Newman, Plenum Press, 1995, and the emulsion MF59 described on page 183 of this same book.

A further instance of an adjuvant is a compound chosen from the polymers of acrylic or methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative. Advantageous adjuvant compounds are the polymers of acrylic or methacrylic acid which are cross-linked, especially with polyalkenyl ethers of sugars or polyalcohols. These compounds are known by the term carbomer (Phameuropa Vol. 8, No. 2, June 1996). Persons skilled in the art can also refer to U. S. Patent No. 2,909,462 which describes such acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than 8, the hydrogen atoms of at least three hydroxyls being replaced by unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents, such as methyl. The products sold under the name Carbopol ; (BF Goodrich, Ohio, USA) are particularly appropriate. They are cross-linked with an allyl sucrose or with allyl pentaerythritol. Among them, there may be mentioned Carbopol 974P, 934P and 971P. Most preferred is the use of Carbopol, in particular the use of Carbopol 971P, preferably in amounts of about 500 μg to about 5 mg per dose, even more preferred in an amount of about 750 μg to about 2.5 mg per dose and most preferred in an amount of about 1 mg per dose.

Further suitable adjuvants include, but are not limited to, the RIBI adjuvant system (Ribi Inc.), Block co-polymer (CytRx, Atlanta GA), SAF-M (Chiron, Emeryville CA),

monophosphoryl lipid A, Avridine lipid-amine adjuvant, heat-labile enterotoxin from E. coli (recombinant or otherwise), cholera toxin, IMS 1314, or muramyl dipeptide among many others.

Preferably, the adjuvant is added in an amount of about 100 μg to about 10 mg per dose. Even more preferably, the adjuvant is added in an amount of about 100 μg to about 10 mg per dose. Even more preferably, the adjuvant is added in an amount of about 500 μg to about 5 mg per dose. Even more preferably, the adjuvant is added in an amount of about 750 μg to about 2.5 mg per dose. Most preferably, the adjuvant is added in an amount of about 1 mg per dose.

Additionally, the composition can include one or more pharmaceutical-acceptable carriers. As used herein, "a pharmaceutical-acceptable carrier" includes any and all solvents, dispersion media, coatings, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like. Most preferably, the composition provided herewith, contains PCV2 ORF2 protein recovered from the supernate of *in vitro* cultured cells, wherein said cells were infected with a recombinant viral vector containing PCV2 ORF2 DNA and expressing PCV2 ORF2 protein, and wherein said cell culture was treated with about 2 to about 8 mM BEI, preferably with about 5 mM BEI to inactivate the viral vector, and an equivalent concentration of a neutralization agent, preferably sodium thiosulfate solution to a final concentration of about 2 to about 8 mM, preferably of about 5 mM.

The present invention also relates to an immunogenic composition that comprises **i)** any of the PCV2 ORF2 proteins described above, preferably in concentrations described above, **ii)** at least a portion of the viral vector expressing said PCV2 ORF2 protein, **iii)** a portion of the cell culture, **iv)** an inactivating agent to inactivate the recombinant viral vector

preferably BEI, and v) an neutralization agent to stop the inactivation mediated by the inactivating agent, preferably sodium thiosulfate in equivalent amounts to BEI; and **vi)** a suitable adjuvant, preferably Carbopol 971 in amounts described above; wherein about 90% of the components i) to iii) have a size smaller than 1 μm. According to a further aspect, this immunogenic composition further comprises a pharmaceutical acceptable salt, preferably a phosphate salt in physiologically acceptable concentrations. Preferably, the pH of said immunogenic composition is adjusted to a physiological pH, meaning between about 6.5 and 7.5.

The immunogenic composition as used herein also refers to a composition that comprises per one ml **i)** at least 1.6 μg of PCV2 ORF2 protein described above, **ii)** at least a portion of baculovirus expressing said PCV2 ORF2 protein **iii)** a portion of the cell culture, **iv)** about 2 to 8 mM BEI, v) sodium thiosulfate in equivalent amounts to BEI; and **vi)** about 1 mg Carbopol 971, and **vii)** phosphate salt in a physiologically acceptable concentration; wherein about 90% of the components i) to iii) have a size smaller than 1 μm and the pH of said immunogenic composition is adjusted to about 6.5 to 7.5.

The immunogenic compositions can further include one or more other immunomodulatory agents such as, e. g., interleukins, interferons, or other cytokines. The immunogenic compositions can also include Gentamicin and Merthiolate. While the amounts and concentrations of adjuvants and additives useful in the context of the present invention can readily be determined by the skilled artisan, the present invention contemplates compositions comprising from about 50 μg to about 2000 μg of adjuvant and preferably about 250 μg/ ml dose of the vaccine composition. Thus, the immunogenic composition as used herein also refers to a composition that comprises from about 1ug/ml to about 60 μg/ml of antibiotics, and more preferably less than about 30 μg/ml of antibiotics.

The immunogenic composition as used herein also refers to a composition that comprises **i)** any of the PCV2 ORF2 proteins described above, preferably in concentrations described above, **ii)** at least a portion of the viral vector expressing said PCV2 ORF2 protein, **iii)** a portion of the cell culture, **iv)** an inactivating agent to inactivate the recombinant viral vector preferably BEI, and **v)** an neutralization agent to stop the inactivation mediated by the inactivating agent, preferably sodium thiosulfate in equivalent amounts to BEI; **vi)** a suitable adjuvant, preferably Carbopol 971 in amounts described above; **vii)** a pharmaceutical acceptable concentration of a saline buffer, preferably of a phosphate salt, and **viii)** an anti-microbiological active agent; wherein about 90% of the components i) to iii) have a size smaller than 1 µm.

It has been surprisingly found, that the immunogenic composition comprising the PCV2 ORF2 protein was highly stable over a period of 24 months. It has also been found the immunogenic compositions are very effective in reducing the clinical symptoms associated with PCV2 infections. It was also discovered, that the immunogenic compositions comprising the recombinant baculovirus expressed PCV2 ORF2 protein as described above, are surprisingly more effective than an immunogenic composition comprising the whole PCV2 virus in an inactivated form, or isolated viral PCV2 ORF2 antigen. In particular, it has been surprisingly found, that the recombinant baculovirus expressed PCV2 ORF2 protein is effective in very low concentrations, which means in concentrations up to 0.25 µg/dose. This unexpected high immunogenic potential of the PCV2 ORF2 protein is increased Carbopol. Examples 1 to 3 disclose in detail the production of PCV2 ORF2 comprising immunogenic compositions.

The immunogenic composition as used herein also refers to CircoFLEX®, (Boehringer Ingelheim Vetmedica Inc, St Joseph, MO, USA), CircoVac® (Merial SAS,

Lyon, France), CircoVent (Intervet Inc., Millsboro, DE, USA), or Suvaxyn PCV-2 One Dose® (Fort Dodge Animal Health, Kansas City, KA, USA).

Administration of the immunogenic composition

The composition according to the invention may be applied intradermally, intratracheally, or intravaginally. The composition preferably may be applied intramuscularly or intranasally, most preferably intramuscularlly. In an animal body, it can prove advantageous to apply the pharmaceutical compositions as described above via an intravenous or by direct injection into target tissues. For systemic application, the intravenous, intravascular, intramuscular, intranasal, intraarterial, intraperitoneal, oral, or intrathecal routes are preferred. A more local application can be effected subcutaneously, intradermally, intracutaneously, intracardially, intralobally, intramedullarly, intrapulmonarily or directly in or near the tissue to be treated (connective-, bone-, muscle-, nerve-, epithelial tissue). Depending on the desired duration and effectiveness of the treatment, the compositions according to the invention may be administered once or several times, also intermittently, for instance on a daily basis for several days, weeks or months and in different dosages.

Preferably, at least one dose of the immunogenic compositions as described above is intramuscularly administered to the subject in need thereof. According to a further aspect, the PCV-2 antigen or the immunogenic composition comprising any such PCV-2 antigen as described above is formulated and administered in one (1) mL per dose. Thus, according to a further aspect, the present invention also relates to a 1 ml immunogenic composition, comprising PCV-2 antigen as described heren, for reducing or lessening lymphadenopathy, lymphoid depletion and/or multinucleated/giant histiocytes in pigs infected with PCV2.

According to a further aspect, according to a further aspect, the present invention also relates to a 1 ml immunogenic composition, comprising PCV-2 antigen as described herein, for reducing or lessening lymphadenopathy in combination with one or a multiple of the following symptoms in pigs: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers, (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies.

According to a further aspect, at least one further administration of at least one dose of the immunogenic composition as described above is given to a subject in need thereof, wherein the second or any further administration is given at least 14 days beyond the initial or any former administrations. Preferably, the immunogenic composition is administered with an immune stimulant. Preferably, said immune stimulant is given at least twice. Preferably, at least 3 days, more preferably at least 5 days, even more preferably at least 7 days are in between the first and the second or any further administration of the immune stimulant. Preferably, the immune stimulant is given at least 10 days, preferably 15 days, even more preferably 20, even more preferably at least 22 days beyond the initial administration of the immunogenic composition provided herein. A preferred immune stimulant is, for example, keyhole limpet hemocyanin (KLH), preferably emulsified with incomplete Freund's adjuvant (KLH/ICFA). However, it is herewith understood, that any other immune stimulant known to a person skilled in the art can also be used. The term "immune stimulant" as used herein, means any agent or composition that can trigger the immune response, preferably without initiating or increasing a specific immune response, for example the immune response against a specific pathogen. It is further instructed to administer the immune stimulant in a suitable dose.

Moreover, it has also been surprisingly found that the immunogenic potential of the immunogenic compositions used herein, preferably those that comprise recombinant baculovirus expressed PCV2 ORF2 protein, even more preferably in combination with Carbopol, can be further confirmed by the administration of the IngelVac PRRS MLV vaccine (see Example 5). PCV2 clinical signs and disease manifestations are greatly magnified when PRRS infection is present. However, the immunogenic compositions and vaccination strategies as provided herewith lessened this effect greatly, and more than expected. In other words, an unexpected synergistic effect was observed when animals, preferably piglets were treated with any of the PCV2 ORF2 immunogenic compositions, as provided herewith, and the Ingelvac PRRS MLV vaccine (Boehringer Ingelheim).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic flow diagram of a preferred construction of PCV2 ORF2 recombinant baculovirus; and

Figs. 2a and 2b are each schematic flow diagrams of how to produce one of the compositions used in accordance with the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following examples set forth preferred materials and procedures in accordance with the present invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. It is to be understood, however, that these examples are provided by way of illustration only, and nothing therein should be deemed a limitation upon the overall scope of the invention.

EXAMPLE 1

This example compares the relative yields of ORF2 using methods of the present invention with methods that are known in the prior art. Four 1000mL spinner flasks were each seeded with approximately $1.0x10^6$ Sf+ cells/ml in 300mL of insect serum free media, Excell 420 (JRH Biosciences, Inc., Lenexa, KS). The master cell culture is identified as SF+ (*Spodoptera frugiperda*) Master Cell Stock, passage 19, Lot#N112-095W. The cells used to generate the SF+ Master Cell Stock were obtained from Protein Sciences Corporation, Inc., Meriden, CT. The SF+ cell line for this example was confined between passages 19 and 59. Other passages will work for purposes of the present invention, but in order to scale the process up for large scale production, at least 19 passages will probably be necessary and passages beyond 59 may have an effect on expression, although this was not investigated. In more detail, the initial SF+ cell cultures from liquid nitrogen storage were grown in Excell 420 media in suspension in sterile spinner flasks with constant agitation. The cultures were grown in 100 mL to 250mL spinner flasks with 25 to 150 mL of Excell 420 serum-free media. When the cells had multiplied to a cell density of 1.0 - 8.0 x 10^6 cells/mL, they were split to new vessels with a planting density of 0.5 - 1.5 x 10^6 cells/mL. Subsequent expansion cultures were grown in spinner flasks up to 36 liters in size or in stainless steel bioreactors of up to 300 liters for a period of 2-7 days at 25 - 29°C.

After seeding, the flasks were incubated at 27°C for four hours. Subsequently, each flask was seeded with a recombinant baculovirus containing the PCV2 ORF2 gene (SEQ ID NO: 4). The recombinant baculovirus containing the PCV2 ORF2 gene was generated as follows: the PCV2 ORF2 gene from a North American strain of PCV2 was PCR amplified to contain a 5' Kozak's sequence (SEQ ID NO: 1) and a 3' EcoR1 site (SEQ ID NO: 2), and

cloned into the pGEM-T-Easy vector (Promega, Madison, WI). Then, it was subsequently excised and subcloned into the transfer vector pVL1392 (BD Biosciences Pharmingen, San Diego, CA). The subcloned portion is represented herein as SEQ ID NO: 7. The pVL1392 plasmid containing the PCV2 ORF2 gene was designated N47-064Y and then co-transfected with BaculoGold® (BD Biosciences Pharmingen) baculovirus DNA into Sf+ insect cells (Protein Sciences, Meriden, CT) to generate the recombinant baculovirus containing the PCV2 ORF2 gene. The new construct is provided herein as SEQ ID NO: 8. The recombinant baculovirus containing the PCV2 ORF2 gene was plaque-purified and Master Seed Virus (MSV) was propagated on the SF+ cell line, aliquotted, and stored at -70°C. The MSV was positively identified as PCV2 ORF2 baculovirus by PCR-RFLP using baculovirus specific primers. Insect cells infected with PCV2 ORF2 baculovirus to generate MSV or Working Seed Virus express PCV2 ORF2 antigen as detected by polyclonal serum or monoclonal antibodies in an indirect fluorescent antibody assay. Additionally, the identity of the PCV2 ORF2 baculovirus was confirmed by N-terminal amino acid sequencing. The PCV2 ORF2 baculovirus MSV was also tested for purity in accordance with 9 C.F.R. 113.27 (c), 113.28, and 113.55. Each recombinant baculovirus seeded into the spinner flasks had varying multiplicities of infection (MOIs). Flask 1 was seeded with 7.52mL of .088 MOI seed; flask 2 was seeded with 3.01mL of 0.36MOI seed; flask 3 was seeded with 1.5mL of 0.18MOI seed; and flask 4 was seeded with 0.75mL of 0.09MOI seed. A schematic flow diagram illustrating the basic steps used to construct a PCV2 ORF2 recombinant baculovirus is provided herein as Figure 1.

After being seeded with the baculovirus, the flasks were then incubated at 27 ± 2°C for 7 days and were also agitated at 100rpm during that time. The flasks used ventilated caps to allow for air flow. Samples from each flask were taken every 24 hours for the next 7 days.

After extraction, each sample was centrifuged, and both the pellet and the supernatant were separated and then microfiltered through a 0.45-1.0 μm pore size membrane.

The resulting samples then had the amount of ORF2 present within them quantified via an ELISA assay. The ELISA assay was conducted with capture antibody Swine anti-PCV2 Pab IgG Prot. G purified (diluted 1:250 in PBS) diluted to 1:6000 in 0.05M Carbonate buffer (pH 9.6). 100 μL of the antibody was then placed in the wells of the mictrotiter plate, sealed, and incubated overnight at 37°C. The plate was then washed three times with a wash solution which comprised 0.5mL of Tween 20 (Sigma, St. Louis, MO), 100mL of 10X D-PBS (Gibco Invitrogen, Carlsbad, CA) and 899.5mL of distilled water. Subsequently, 250 μL of a blocking solution (5g Carnation Non-fat dry milk (Nestle, Glendale, CA) in 10mL of D-PBS QS to 100mL with distilled water) was added to each of the wells. The next step was to wash the test plate and then add pre-diluted antigen. The pre-diluted antigen was produced by adding 200 μL of diluent solution (0.5mL Tween 20 in 999.5mL D-PBS) to each of the wells on a dilution plate. The sample was then diluted at a 1:240 ratio and a 1:480 ratio, and 100 μL of each of these diluted samples was then added to one of the top wells on the dilution plate (i.e. one top well received 100 μL of the 1:240 dilution and the other received 100 μL of the 1:480 dilution). Serial dilutions were then done for the remainder of the plate by removing 100 μL form each successive well and transferring it to the next well on the plate. Each well was mixed prior to doing the next transfer. The test plate washing included washing the plate three times with the wash buffer. The plate was then sealed and incubated for an hour at 37°C before being washed three more times with the wash buffer. The detection antibody used was monoclonal antibody to PCV ORF2. It was diluted to 1:300 in diluent solution, and 100 μL of the diluted detection antibody was then added to the wells. The plate was then sealed and incubated for an hour at 37°C before being washed three times

with the wash buffer. Conjugate diluent was then prepared by adding normal rabbit serum (Jackson Immunoresearch, West Grove, PA) to the diluent solution to 1% concentration. Conjugate antibody Goat anti-mouse (H+l)-HRP (Jackson Immunoresearch) was diluted in the conjugate diluent to 1:10,000. 100 µL of the diluted conjugate antibody was then added to each of the wells. The plate was then sealed and incubated for 45 minutes at 37°C before being washed three times with the wash buffer. 100 µL of substrate (TMB Peroxidase Substrate, Kirkgaard and Perry Laboratories (KPL), Gaithersberg, MD), mixed with an equal volume of Peroxidase Substrate B (KPL) was added to each of the wells. The plate was incubated at room temperature for 15 minutes. 100 µL of 1N HCL solution was then added to all of the wells to stop the reaction. The plate was then run through an ELISA reader. The results of this assay are provided in Table 1 below:

Table 1

Day	Flask	ORF2 in pellet (µg)	ORF2 in supernatant (µg)
3	1	47.53	12
3	2	57.46	22
3	3	53.44	14
3	4	58.64	12
4	1	43.01	44
4	2	65.61	62
4	3	70.56	32
4	4	64.97	24

5	1	31.74	100
5	2	34.93	142
5	3	47.84	90
5	4	55.14	86
6	1	14.7	158
6	2	18.13	182
6	3	34.78	140
6	4	36.88	146
7	1	6.54	176
7	2	12.09	190
7	3	15.84	158
7	4	15.19	152

These results indicate that when the incubation time is extended, expression of ORF2 into the supernatant of the centrifuged cells and media is greater than expression in the pellet of the centrifuged cells and media. Accordingly, allowing the ORF2 expression to proceed for at least 5 days and recovering it in the supernate rather than allowing expression to proceed for less than 5 days and recovering ORF2 from the cells, provides a great increase in ORF2 yields, and a significant improvement over prior methods.

EXAMPLE 2

This example provides data as to the efficacy of the invention claimed herein. A 1000mL spinner flask was seeded with approximately $1.0x10^6$ Sf+ cells/ml in 300mL of Excell 420 media. The flask was then incubated at 27°C and agitated at 100rpm. Subsequently, the flask was seeded with 10 mL of PCV2 ORF2/Bac p+6 (the recombinant baculovirus containing the PCV2 ORF2 gene passaged 6 additional times in the Sf9 insect cells) virus seed with a 0.1 MOI after 24 hours of incubation.

The flask was then incubated at 27°C for a total of 6 days. After incubation, the flask was then centrifuged and three samples of the resulting supernatant were harvested and inactivated. The supernatant was inactivated by bringing its temperature to 37 ± 2°C. To the first sample, a 0.4M solution of 2-bromoethyleneamine hydrobromide which had been cyclized to 0.2M binary ethlylenimine (BEI) in 0.3N NaOH was added to the supernatant to give a final concentration of BEI of 5mM. To the second sample, 10mM BEI was added to the supernatant. To the third sample, no BEI was added to the supernatant. The samples were then stirred continuously for 48 hrs. A 1.0 M sodium thiosulfate solution to give a final minimum concentration of 5 mM was added to neutralize any residual BEI. The quantity of ORF2 in each sample was then quantified using the same ELISA assay procedure as described in Example 1. The results of this may be seen in Table 2 below:

Table 2

Sample	ORF2 in supernatant (μg)
1	78.71
2	68.75
3	83.33

This example demonstrates that neutralization with BEI does not remove or degrade significant amounts of the recombinant PCV2 ORF2 protein product. This is evidenced by the fact that there is no large loss of ORF2 in the supernatant from the BEI or elevated temperatures. Those of skill in the art will recognize that the recovered ORF2 is a stable protein product.

EXAMPLE 3

This example demonstrates that the present invention is scalable from small scale production of recombinant PCV2 ORF2 to large scale production of recombinant PCV2 ORF2. 5.0×10^5 cells/ml of SF+ cells/ml in 7000mL of ExCell 420 media was planted in a 20000mL Applikon Bioreactor. The media and cells were then incubated at 27°C and agitated at 100RPM for the next 68 hours. At the 68^{th} hour, 41.3mL of PCV2 ORF2 Baculovirus MSV+3 was added to 7000mL of ExCell 420 medium. The resultant mixture was then added to the bioreactor. For the next seven days, the mixture was incubated at 27°C and agitated at 100RPM. Samples from the bioreactor were extracted every 24 hours beginning at day 4, post-infection, and each sample was centrifuged. The supernatant of the samples were preserved and the amount of ORF2 was then quantified using SDS-PAGE densitometry. The results of this can be seen in Table 3 below:

Table 3

Day after infection:	ORF2 in supernatant (μg/mL)
4	29.33
5	41.33
6	31.33

7	60.67

EXAMPLE 4

This example tests the efficacy of seven PCV2 candidate vaccines and further defines efficacy parameters following exposure to a virulent strain of PCV2. One hundred and eight (108) cesarean derived colostrum deprived (CDCD) piglets, 9-14 days of age, were randomly divided into 9 groups of equal size. Table 4 sets forth the General Study Design for this Example.

Table 4. General Study Design

Group	No. Of Pigs	Treatment	Day of Treatment	KLH/ICFA on Day 21 and Day 27	Challenged with Virulent PCV2 on Day 24	Necropsy on Day 49
1	12	PCV2 Vaccine No. 1 - (vORF2 16 µg)	0	+	+	+
2	12	PCV2 Vaccine No. 2 - (vORF2 8 µg)	0	+	+	+
3	12	PCV2 Vaccine No. 3 - (vORF2 4 µg)	0	+	+	+

4	12	PCV2 Vaccine No. 4 - (rORF2 16 μg)	0	+	+	+
5	12	PCV2 Vaccine No. 5 - (rORF2 8 μg)	0	+	+	+
6	12	PCV2 Vaccine No. 6 - (rORF2 4 μg)	0	+	+	+
7	12	PCV2 Vaccine No. 7 - (Killed whole cell virus)	0	+	+	+
8	12	None - Challenge Controls	N/A	+	+	+
9	12	None - Strict Negative Control Group	N/A	+	-	+

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; killed whole cell virus = PCV2 virus grown in suitable cell culture

Seven of the groups (Groups 1 - 7) received doses of PCV2 ORF2 polypeptide, one of the groups acted as a challenge control and received no PCV2 ORF2, and another group acted as the strict negative control group and also received no PCV2 ORF2. On Day 0, Groups 1 through 7 were treated with assigned vaccines. Piglets in Group 7 were given a booster treatment on Day 14. Piglets were observed for adverse events and injection site reactions following vaccination and on Day 19, piglets were moved to the second study site. At the second study site, Groups 1-8 were group housed in one building while Group 9 was housed

in a separate building. All pigs received keyhole limpet hemocyanin (KLH)/incomplete Freund's adjuvant (ICFA) on Days 21 and 27 and on Day 24, Groups 1-8 were challenged with a virulent PCV2.

Pre- and post-challenge, blood samples were collected for PCV2 serology. Post-challenge, body weight data for determination of average daily weight gain (ADWG), and clinical symptoms, as well as nasal swab samples to determine nasal shedding of PCV2, were collected. On Day 49, all surviving pigs were necropsied, lungs were scored for lesions, and selected tissues were preserved in formalin for Immunohistochemistry (IHC) testing at a later date.

Materials and Methods

This was a partially blinded vaccination-challenge feasibility study conducted in CDCD pigs, 9 to 14 days of age on Day 0. To be included in the study, PCV2 IFA titers of sows were $\leq$ 1:1000. Additionally, the serologic status of sows were from a known PRRS-negative herd. Twenty-eight (28) sows were tested for PCV2 serological status. Fourteen (14) sows had a PCV2 titer of $\leq$ 1000 and were transferred to the first study site. One hundred ten (110) piglets were delivered by cesarean section surgeries and were available for this study on Day -4. On Day -3, 108 CDCD pigs at the first study site were weighed, identified with ear tags, blocked by weight and randomly assigned to 1 of 9 groups, as set forth above in table 4. If any test animal meeting the inclusion criteria was enrolled in the study and was later excluded for any reason, the Investigator and Monitor consulted in order to determine the use of data collected from the animal in the final analysis. The date of which enrolled piglets were excluded and the reason for exclusion was documented. Initially, no sows were excluded. A total of 108 of an available 110 pigs were randomly assigned to one

of 9 groups on Day -3. The two smallest pigs (Nos. 17 and 19) were not assigned to a group and were available as extras, if needed. During the course of the study, several animals were removed. Pig 82 (Group 9) on Day -1, Pig No. 56 (Group 6) on Day 3, Pig No. 53 (Group 9) on Day 4, Pig No. 28 (Group 8) on Day 8, Pig No. 69 (Group 8) on Day 7, and Pig No. 93 (Group 4) on Day 9, were each found dead prior to challenge. These six pigs were not included in the final study results. Pig no 17 (one of the extra pigs) was assigned to Group 9. The remaining extra pig, No. 19, was excluded from the study.

The formulations given to each of the groups were as follows: Group 1 was designed to administer 1ml of viral ORF2 (vORF2) containing 16 μg ORF2/ml. This was done by mixing 10.24 ml of viral ORF2 (256 μg/25 μg/ml = 10.24 ml vORF2) with 3.2 ml of 0.5% Carbopol and 2.56 ml of phosphate buffered saline at a pH of 7.4. This produced 16 ml of formulation for group 1. Group 2 was designed to administer 1ml of vORF2 containing 8 μg vORF2/ml. This was done by mixing 5.12 ml of vORF2 (128 μg/25 μg/ml = 5.12 ml vORF2) with 3.2 ml of 0.5% Carbopol and 7.68 ml of phosphate buffered saline at a pH of 7.4. This produced 16 ml of formulation for group 2. Group 3 was designed to administer 1ml of vORF2 containing 4 μg vORF2/ml. This was done by mixing 2.56 ml of vORF2 (64 μg/25 μg/ml = 2.56 ml vORF2) with 3.2 ml of 0.5% Carbopol and 10.24 ml of phosphate buffered saline at a pH of 7.4. This produced 16 ml of formulation for group 3. Group 4 was designed to administer 1ml of recombinant ORF2 (rORF2) containing 16 μg rORF2/ml. This was done by mixing 2.23 ml of rORF2 (512 μg/230 μg/ml = 2.23 ml rORF2) with 6.4 ml of 0.5% Carbopol and 23.37 ml of phosphate buffered saline at a pH of 7.4. This produced 32 ml of formulation for group 4. Group 5 was designed to administer 1ml of rORF2 containing 8μg rORF2/ml. This was done by mixing 1.11 ml of rORF2 (256 μg/230 μg/ml = 1.11 ml rORF2) with 6.4 ml of 0.5% Carbopol and 24.49 ml of phosphate-buffered saline at a pH of

7.4. This produced 32 ml of formulation for group 5. Group 6 was designed to administer 1ml of rORF2 containing 8 μg rORF2/ml. This was done by mixing 0.56 ml of rORF2 (128 μg/230 μg/ml = 0.56 ml rORF2) with 6.4 ml of 0.5% Carbopol and 25.04 ml of phosphate buffered saline at a pH of 7.4. This produced 32 ml of formulation for group 6. Group 7 was designed to administer 2ml of PCV2 whole killed cell vaccine (PCV2 KV) containing the MAX PCV2 KV. This was done by mixing 56 ml of PCV2 KV with 14 ml of 0.5% Carbopol. This produced 70 ml of formulation for group 7. Finally group 8 was designed to administer KLH at 0.5 μg/ml or 1.0 μg/ml per 2 ml dose. This was done by mixing 40.71 ml KLH (7.0 μg protein/ml at 0.5 μg/ml = 570 ml (7.0 μg/ml)(x) = (0.5)(570 ml)), 244.29 ml phosphate buffered saline at a pH of 7.4, and 285 ml Freunds adjuvant. Table 5 describes the time frames for the key activities of this Example.

Table 5. Study Activities

Study Day	Study Activity
-4, 0 to 49	General observations for overall health and clinical symptoms
-3	Weighed; Randomized to groups; Collected blood samples from all pigs
0	Health examination; Administered IVP Nos. 1-7 to Groups 1-7, respectively
0-7	Observed pigs for injection site reactions
14	Boostered Group 7 with PCV2 Vaccine No. 7; Blood samples from all pigs

14-21	Observed Group 7 for injection site reactions
16-19	Treated all pigs with antibiotics (data missing)
19	Pigs transported from the first test site to a second test site
21	Treated Groups 1-9 with KLH/ICFA
24	Collected blood and nasal swab samples from all pigs; Weighed all pigs; Challenged Groups 1-8 with PCV2 challenge material
25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47	Collected nasal swab samples from all pigs
27	Treated Groups 1-9 with KLH/ICFA
31	Collected blood samples from all pigs
49	Collected blood and nasal swab samples from all pigs; Weighed all pigs; Necropsy all pigs; Gross lesions noted with emphasis placed on icterus and gastric ulcers; Lungs evaluated for lesions; Fresh and formalin fixed tissue samples saved; In-life phase of the study completed

Following completion of the in-life phase of the study, formalin fixed tissues were

examined by Immunohistochemistry (IHC) for detection of PCV2 antigen by a pathologist, blood samples were evaluated for PCV2 serology, nasal swab samples were evaluated for PCV2 shedding, and average daily weight gain (ADWG) was determined from Day 24 to Day 49.

Animals were housed at the first study site in individual cages in five rooms from birth to approximately 11 days of age (approximately Day 0 of the study). Each room was identical in layout and consisted of stacked individual stainless steel cages with heated and filtered air supplied separately to each isolation unit. Each room had separate heat and ventilation, thereby preventing cross-contamination of air between rooms. Animals were housed in two different buildings at the second study site. Group 9 (The Strict negative control group) was housed separately in a converted finisher building and Groups 1-8 were housed in converted nursery building. Each group was housed in a separate pen (11-12 pigs per pen) and each pen provided approximately 3.0 square feet per pig. Each pen was on an elevated deck with plastic slatted floors. A pit below the pens served as a holding tank for excrement and waste. Each building had its own separate heating and ventilation systems, with little likelihood of cross-contamination of air between buildings.

At the first study site, piglets were fed a specially formulated milk ration from birth to approximately 3 weeks of age. All piglets were consuming solid, special mixed ration by Day 19 (approximately 4 ½ weeks of age). At the second study site, all piglets were fed a custom non-medicated commercial mix ration appropriate for their age and weight, *ad libitum*. Water at both study sites was also available *ad libitum*.

All test pigs were treated with Vitamin E on Day -2, with iron injections on Day -1 and with NAXCEL® (1.0 mL, IM, in alternating hams) on Days 16, 17, 18 and 19. In addition, Pig No. 52 (Group 9) was treated with an iron injection on Day 3, Pig 45 (Group 6)

was treated with an iron injection on Day 11, Pig No. 69 (Group 8) was treated with NAXCEL® on Day 6, Pig No. 74 (Group 3) was treated with dexamethazone and penicillin on Day 14, and Pig No. 51 (Group 1) was treated with dexamethazone and penicillin on Day 13 and with NAXCEL® on Day 14 for various health reasons.

While at both study sites, pigs were under veterinary care. Animal health examinations were conducted on Day 0 and were recorded on the Health Examination Record Form. All animals were in good health and nutritional status before vaccination as determined by observation on Day 0. All test animals were observed to be in good health and nutritional status prior to challenge. Carcasses and tissues were disposed of by rendering. Final disposition of study animals was records on the Animal Disposition Record.

On Day 0, pigs assigned to Groups 1-6 received 1.0 mL of PCV2 Vaccines 1-6, respectively, IM in the left neck region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x ½" needle. Pigs assigned to Group 7 received 2.0 mL of PCV2 Vaccine No. 7 IM in the left neck region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x ½" needle. On Day 14, pigs assigned to Group 7 received 2.0 mL of PCV2 Vaccine No. 7 IM in the right neck region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x ½" needle.

On Day 21 all test pigs received 2.0 mL of KLH/ICFA IM in the right ham region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x1" needle. On Day 27 all test pigs received 2.0 mL of KLH/ICFA in the left ham region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x 1" needle.

On Day 24, pigs assigned to Groups 1-8 received 1.0 mL of PCV2 ISUVDL challenge material (5.11 $\log_{10}$ $TCID_{50}$/mL) IM in the left neck region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x 1" needle. An additional 1.0 mL of the same material was administered IN to each pig (0.5 mL per nostril) using a sterile 3.0 mL Luer-lock syringe and

nasal canula.

Test pigs were observed daily for overall health and adverse events on Day -4 and from Day 0 to Day 19. Observations were recorded on the Clinical Observation Record. All test pigs were observed from Day 0 to Day 7, and Group 7 was further observed from Day 14 to 21, for injection site reactions. Average daily weight gain was determined by weighing each pig on a calibrated scale on Days -3, 24 and 49, or on the day that a pig was found dead after challenge. Body weights were recorded on the Body Weight Form. Day -3 body weights were utilized to block pigs prior to randomization. Day 24 and Day 49 weight data was utilized to determine the average daily weight gain (ADWG) for each pig during these time points. For pigs that died after challenge and before Day 49, the ADWG was adjusted to represent the ADWG from Day 24 to the day of death.

In order to determine PCV2 serology, venous whole blood was collected from each piglet from the orbital venous sinus on Days -3 and 14. For each piglet, blood was collected from the orbital venous sinus by inserting a sterile capillary tube into the medial canthus of one of the eyes and draining approximately 3.0 mL of whole blood into a 4.0 mL Serum Separator Tube (SST). On Days 24, 31, and 49, venous whole blood from each pig was collected from the anterior vena cava using a sterile 18g x 1 ½" Vacutainer needle (Becton Dickinson and Company, Franklin Lakes, New Jersey), a Vacutainer needle holder and a 13 mL SST. Blood collections at each time point were recorded on the Sample Collection Record. Blood in each SST was allowed to clot, each SST was then spun down and the serum harvested. Harvested serum was transferred to a sterile snap tube and stored at -70± 10° C until tested at a later date. Serum samples were tested for the presence of PCV2 antibodies by BIVI-R&D personnel.

Pigs were observed once daily from Day 20 to Day 49 for clinical symptoms and

clinical observations were recorded on the Clinical Observation Record.

To test for PCV2 nasal shedding, on Days 24, 25, and then every other odd numbered study day up to and including Day 49, a sterile dacron swab was inserted intra nasally into either the left or right nostril of each pig (one swab per pig) as aseptically as possible, swished around for a few seconds and then removed. Each swab was then placed into a single sterile snap-cap tube containing 1.0 mL of EMEM media with 2% IFBS, 500 units/mL of Penicillin, 500 μg/mL of Streptomycin and 2.5 μg/mL of Fungizone. The swab was broken off in the tube, and the snap tube was sealed and appropriately labeled with animal number, study number, date of collection, study day and "nasal swab." Sealed snap tubes were stored at -40 ± 10° C until transported overnight on ice to BIVI-St. Joseph. Nasal swab collections were recorded on the Nasal Swab Sample Collection Form. BIVI-R&D conducted quantitative virus isolation (VI) testing for PCV2 on nasal swab samples. The results were expressed in $\log_{10}$ values. A value of 1.3 logs or less was considered negative and any value greater than 1.3 logs was considered positive.

Pigs that died (Nos. 28, 52, 56, 69, 82, and 93) at the first study site were necropsied to the level necessary to determine a diagnosis. Gross lesions were recorded and no tissues were retained from these pigs. At the second study site, pigs that died prior to Day 49 (Nos. 45, 23, 58, 35), pigs found dead on Day 49 prior to euthanasia (Nos. 2, 43), and pigs euthanized on Day 49 were necropsied. Any gross lesions were noted and the percentages of lung lobes with lesions were recorded on the Necropsy Report Form.

From each of the 103 pigs necropsied at the second study site, a tissue sample of tonsil, lung, heart, liver, mesenteric lymph node, kidney and inguinal lymph node was placed into a single container with buffered 10% formalin; while another tissue sample from the same aforementioned organs was placed into a Whirl-pak (M-Tech Diagnostics Ltd.,

Thelwall, UK) and each Whirl-pak was placed on ice. Each container was properly labeled. Sample collections were recorded on the Necropsy Report Form. Afterwards, formalin-fixed tissue samples and a Diagnostic Request Form were submitted for IHC testing. IHC testing was conducted in accordance with standard ISU laboratory procedures for receiving samples, sample and slide preparation, and staining techniques. Fresh tissues in Whirl-paks were shipped with ice packs to the Study Monitor for storage (-70° ± 10° C) and possible future use. Formalin-fixed tissues were examined by a pathologist for detection of PCV2 by IHC and scored using the following scoring system: 0 = None; 1 = Scant positive staining, few sites; 2 = Moderate positive staining, multiple sites; and 3 = Abundant positive staining, diffuse throughout the tissue. Due to the fact that the pathologist could not positively differentiate inguinal LN from mesenteric LN, results for these tissues were simply labeled as Lymph Node and the score given the highest score for each of the two tissues per animal.

Results

Results for this example are given below. It is noted that one pig from Group 9 died before Day 0, and 5 more pigs died post-vaccination (1 pig from Group 4; 1 pig from Group 6; 2 pigs from Group 8; and 1 pig from Group 9). Post-mortem examination indicated all six died due to underlying infections that were not associated with vaccination or PMWS. Additionally, no adverse events or injection site reactions were noted with any groups.

Average daily weight gain (ADWG) results are presented below in Table 6. Group 9, the strict negative control group, had the highest ADWG (1.06 ± 0.17 lbs/day), followed by Group 5 (0.94± 0.22 lbs/day), which received one dose of 8 μg of rORF2. Group 3, which received one dose of 4 μg of vORF2, had the lowest ADWG (0.49 ± 0.21 lbs/day), followed by Group 7 (0.50± 0.15 lbs/day), which received 2 doses of killed vaccine.

Table 6. Summary of Group Average Daily Weight Gain (ADWG)

Group	Treatment	N	ADWG - lbs/day (Day 24 to Day 49) or adjusted for pigs dead before Day 29
1	vORF2 - 16 μg (1 dose)	12	0.87 ± 0.29 lbs/day
2	vORF2 – 8 μg (1 dose)	12	0.70 ± 0.32 lbs/day
3	vORF2 – 4 μg (1 dose)	12	0.49 ± 0.21 lbs/day
4	rORF2 - 16 μg (1 dose)	11	0.84 ± 0.30 lbs/day
5	rORF2 - 8 μg (1 dose)	12	0.94 ± 0.22 lbs/day
6	rORF2 - 4 μg (1 dose)	11	0.72 ± 0.25 lbs/day
7	KV (2 doses)	12	0.50 ± 0.15 lbs/day
8	Challenge Controls	10	0.76 ± 0.19 lbs/day
9	Strict Negative Controls	11	1.06 ± 0.17 lbs/day

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; killed whole cell virus = PCV2 virus grown in suitable cell culture

PCV2 serology results are presented below in Table 7. All nine groups were seronegative for PCV2 on Day -3. On Day 14, Groups receiving vORF2 vaccines had the highest titers, which ranged from 187.5 to 529.2. Pigs receiving killed viral vaccine had the next highest titers, followed by the groups receiving rORF2 vaccines. Groups 8 and 9 remained seronegative at this time. On Day 24 and Day 31, pigs receiving vORF2 vaccines continued to demonstrate a strong serological response, followed closely by the group that

received two doses of a killed viral vaccine. Pigs receiving rORF2 vaccines were slower to respond serologically and Groups 8 and 9 continued to remain seronegative. On Day 49, pigs receiving vORF2 vaccine, 2 doses of the killed viral vaccine and the lowest dose of rORF2 demonstrated the strongest serological responses. Pigs receiving 16 μg and 8 μg of rORF2 vaccines had slightly higher IFA titers than challenge controls. Group 9 on Day 49 demonstrated a strong serological response.

Table 7. Summary of Group PCV2 IFA Titers

AVERAGE IFA TITER

Group	Treatment	Day -3	Day 14	Day 24	Day 31**	Day 49***
1	vORF2 - 16 μg (1 dose)	50.0	529.2	4400.0	7866.7	11054.5
2	vORF2 - 8 μg (1 dose)	50.0	500.0	3466.7	6800.0	10181.8
3	vORF2 - 4 μg (1 dose)	50.0	187.5	1133.3	5733.3	9333.3
4	rORF2 - 16 μg (1 dose)	50.0	95.5	1550.0	3090.9	8000.0
5	rORF2 - 8 μg (1 dose)	50.0	75.0	887.5	2266.7	7416.7
6	rORF2 - 4 μg (1 dose)	50.0	50.0	550.0	3118.2	10570.0
7	KV (2 doses)	50.0	204.2	3087.5	4620.8	8680.0
8	Challenge Controls	50.0	55.0	50.0	50.0	5433.3
9	Strict Negative Controls	50.0	59.1	59.1	54.5	6136.4

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; killed whole cell virus = PCV2 virus grown in suitable cell culture

*For calculation purposes, a ≤100 IFA titer was designated as a titer of "50"; a ≥6400 IFA

titer was designated as a titer of "12,800".

**Day of Challenge

***Day of Necropsy

The results from the post-challenge clinical observations are presented below in Table 8. This summary of results includes observations for Abnormal Behavior, Abnormal Respiration, Cough and Diarrhea. Table 9 includes the results from the Summary of Group Overall Incidence of Clinical Symptoms and Table 10 includes results from the Summary of Group Mortality Rates Post-challenge. The most common clinical symptom noted in this study was abnormal behavior, which was scored as mild to severe lethargy. Pigs receiving the 2 lower doses of vORF2, pigs receiving 16 μg of rORF2 and pigs receiving 2 doses of KV vaccine had incidence rates of ≥ 27.3%. Pigs receiving 8 μg of rORF2 and the strict negative control group had no abnormal behavior. None of the pigs in this study demonstrated any abnormal respiration. Coughing was noted frequently in all groups (0 to 25%), as was diarrhea (0-20%). None of the clinical symptoms noted were pathognomic for PMWS.

The overall incidence of clinical symptoms varied between groups. Groups receiving any of the vORF2 vaccines, the group receiving 16 μg of rORF2, the group receiving 2 doses of KV vaccine, and the challenge control group had the highest incidence of overall clinical symptoms (≥36.4%). The strict negative control group, the group receiving 8 μg of rORF2 and the group receiving 4 μg of rORF2 had overall incidence rates of clinical symptoms of 0%, 8.3% and 9.1%, respectively.

Overall mortality rates between groups varied as well. The group receiving 2 doses of KV vaccine had the highest mortality rate (16.7%); while groups that received 4 μg of vORF2, 16 μg of rORF2, or 8 μg of rORF2 and the strict negative control group all had 0% mortality rates.

Table 8. Summary of Group Observations for Abnormal Behavior, Abnormal Respiration, Cough, and Diarrhea

Group	Treatment	N	Abnormal Behavior[1]	Abnormal Behavior[2]	Cough[3]	Diarrhea[4]
1	vORF2 - 16 µg (1 dose)	12	2/12 (16.7%	0/12 (0%)	3/12 (25%)	2/12 (16/7%)
2	vORF2 - 8 µg (1 dose)	12	4/12 (33.3%)	0/12 (0%)	1/12 (8.3%	1/12 (8.3%)
3	vORF2 - 4 µg (1 dose)	12	8/12 (66.7%)	0/12 (0%)	2/12 (16.7%)	1/12 (8.3%)
4	rORF2 - 16 µg (1 dose)	11	3/11 (27.3%)	0/11 (0%)	0/11 (0%)	2/11 (18.2%)
5	rORF2 - 8 µg (1 dose)	12	0/12 (0%)	0/12 (0%)	1/12 (8.3%)	0/12 (0%)
6	rORF2 - 4 µg (1 dose)	11	1/11 (9.1%)	0/11 (0%)	0/11 (0%)	0/12 (0%)
7	KV (2 doses)	12	7/12 (58.3)	0/12 (0%)	0/12 (0%)	1/12 (8.3%)
8	Challenge Controls	10	1/10 (10%)	0/10 (0%)	2/10 (20%)	2/10 (20%)

9	Strict Negative Controls	11	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; killed whole cell virus = PCV2 virus grown in suitable cell culture

[1]Total number of pigs in each group that demonstrated any abnormal behavior for at least one day

[2]Total number of pigs in each group that demonstrated any abnormal respiration for at least one day

[3]Total number of pigs in each group that demonstrated a cough for at least one day

[4]Total number of pigs in each group that demonstrated diarrhea for at least one day

Table 9. Summary of Group Overall Incidence of Clinical Symptoms

Group	Treatment	N	Incidence of pigs with Clinical Symptoms[1]	Incidence Rate
1	vORF2 - 16 µg (1 dose)	12	5	41.7%
2	vORF2 - 8 µg (1 dose)	12	5	41.7%
3	vORF2 - 4 µg (1 dose)	12	8	66.7%
4	rORF2 - 16 µg (1 dose)	11	4	36.4%
5	rORF2 - 8 µg (1 dose)	12	1	8.3%
6	rORF2 - 4 µg (1 dose)	11	1	9.1%
7	KV (2 doses)	12	7	58.3%

8	Challenge Controls	10	4	40%
9	Strict Negative Controls	11	0	0%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; killed whole cell virus = PCV2 virus grown in suitable cell culture

[1]Total number of pigs in each group that demonstrated any clinical symptom for at least one day

Table 10. Summary of Group Mortality Rates Post-challenge

Group	Treatment	N	Dead Post-challenge	Mortality Rate
1	vORF2 - 16 µg (1 dose)	12	1	8.3%
2	vORF2 - 8 µg (1 dose)	12	1	8.3%
3	vORF2 - 4 µg (1 dose)	12	0	0%
4	rORF2 - 16 µg (1 dose)	11	0	0%
5	rORF2 - 8 µg (1 dose)	12	0	0%
6	rORF2 - 4 µg (1 dose)	11	1	9.1%
7	KV (2 doses)	12	2	16.7%
8	Challenge Controls	10	1	10%
9	Strict Negative Controls	11	0	0%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; killed whole cell

virus = PCV2 virus grown in suitable cell culture

PCV2 nasal shedding results are presented below in Table 11. Following challenge on Day 24, 1 pig in Group 7 began shedding PCV2 on Day 27. None of the other groups experienced shedding until Day 33. The bulk of nasal shedding was noted from Day 35 to Day 45. Groups receiving any of the three vORF2 vaccines and groups receiving either 4 or 8 μg of rORF2 had the lowest incidence of nasal shedding of PCV2 (≤ 9.1%). The challenge control group (Group 8) had the highest shedding rate (80%), followed by the strict negative control group (Group 9), which had an incidence rate of 63.6%.

Table 11. Summary of Group Incidence of Nasal Shedding of PCV2

Group	Treatment	N	No. Of pigs that shed for at least one day	Incidence Rate
1	vORF2 - 16 μg (1 dose)	12	1	8.3%
2	vORF2 - 8 μg (1 dose)	12	1	8.3%
3	vORF2 - 4 μg (1 dose)	12	1	8.3%
4	rORF2 - 16 μg (1 dose)	11	2	18.2%
5	rORF2 - 8 μg (1 dose)	12	1	8.3%
6	rORF2 - 4 μg (1 dose)	11	1	9.1%
7	KV (2 doses)	12	5	41.7%
8	Challenge Controls	10	8	80%

9	Strict Negative Controls	11	7	63.6%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; killed whole cell virus = PCV2 virus grown in suitable cell culture

The Summary of Group Incidence of Icterus, Group Incidence of Gastric Ulcers, Group Mean Lung Lesion Scores, and Group Incidence of Lung Lesions are shown below in Table12. Six pigs died at the first test site during the post-vaccination phase of the study (Group 4, N=1; Group 6, N=1; Group 8, N=2; Group 9, N=2). Four out of six pigs had fibrinous lesions in one or more body cavities, one pig (Group 6) had lesions consistent with clostridial disease, and one pig (Group 9) had no gross lesions. None of the pigs that died during the post-vaccination phased of the study had lesions consistent with PMWS.

Pigs that died post-challenge and pigs euthanized on Day 49 were necropsied. At necropsy, icterus and gastric ulcers were not present in any group. With regard to mean % lung lesions, Group 9 had lowest mean % lung lesions (0%), followed by Group 1 with 0.40 ± 0.50% and Group 5 with 0.68 ± 1.15%. Groups 2, 3, 7 and 8 had the highest mean % lung lesions (≥ 7.27%). Each of these four groups contained one pig with % lung lesions ≥71.5%, which skewed the results higher for these four groups. With the exception of Group 9 with 0% lung lesions noted, the remaining 8 groups had ≤ 36% lung lesions. Almost all lung lesions noted were described as red/purple and consolidated.

Table 12. Summary of Group Incidence of Icterus, Group Incidence of Gastric Ulcers, Group Mean % Lung Lesion Scores, and Group Incidence of Lung Lesions Noted

Group	Treatment	Icterus	Gastric Ulcers	Mean % Lung Lesions	Incidence of Lung Lesions Noted
1	vORF2 - 16 µg (1 dose)	0/12 (0%)	0/12 (0%)	0.40 ± 0.50%	10/12 (83%)
2	vORF2 - 8 µg (1 dose)	0/12 (0%)	0/12 (0%)	7.41 ± 20.2%	10/12 (83%)
3	vORF2 - 4 µg (1 dose)	0/12 (0%)	0/12 (0%)	9.20 ± 20.9%	10/12 (83%)
4	rORF2 - 16 µg (1 dose)	0/11 (0%)	0/11 (0%)	1.5 ± 4.74%	4/11 (36%)
5	rORF2 - 8 µg (1 dose)	0/12 (0%)	0/12 (0%)	0.68 ± 1.15%	9/12 (75%)
6	rORF2 - 4 µg (1 dose)	0/11 (0%)	0/11 (0%)	2.95 ± 5.12%	7/11 (64%)
7	KV (2 doses)	0/12 (0%)	0/12 (0%)	7.27 ± 22.9%	9/12 (75%)
8	Challenge Controls	0/10 (0%)	0/10 (0%)	9.88 ± 29.2%	8/10 (80%)
9	Strict Negative Controls	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

The Summary of Group IHC Positive Incidence Results is shown in Table 13. Group 1 (vORF2 - 16 μg) and Group 5 (rORF2 - 8 μg) had the lowest rate of IHC positive results (16.7%). Group 8 (Challenge Controls) and Group 9 (Strict Negative Controls) had the highest rate of IHC positive results, 90% and 90.9%, respectively.

Table 13. Summary of Group IHC Positive Incidence Rate

Group	Treatment	N	No. Of pigs that had at least one tissue positive for PCV2	Incidence Rate
1	vORF2 - 16 μg (1 dose)	12	2	16.7%
2	vORF2 - 8 μg (1 dose)	12	3	25.0%
3	vORF2 - 4 μg (1 dose)	12	8	66.7%
4	rORF2 - 16 μg (1 dose)	11	4	36.3%
5	rORF2 - 8 μg (1 dose)	12	2	16.7%
6	rORF2 - 4 μg (1 dose)	11	4	36.4%
7	KV (2 doses)	12	5	41.7%
8	Challenge Controls	10	9	90.0%
9	Strict Negative Controls	11	10	90.9%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

Post-challenge, Group 5, which received one dose of 8 μg of rORF2 antigen, outperformed the other 6 vaccine groups. Group 5 had the highest ADWG (0.94 ± 0.22 lbs/day), the lowest incidence of abnormal behavior (0%), the second lowest incidence of cough (8.3%), the lowest incidence of overall clinical symptoms (8.3%), the lowest mortality rate (0%), the lowest rate of nasal shedding of PCV2 (8.3%), the second lowest rate for mean % lung lesions (0.68 ± 1.15%) and the lowest incidence rate for positive tissues (16.7%). Groups receiving various levels of rORF2 antigen overall outperformed groups receiving various levels of vORF2 and the group receiving 2 doses of killed whole cell PCV2 vaccine performed the worst. Tables 14 and 15 contain summaries of group post-challenge data.

Table 14. Summary of Group Post-Challenge Data - Part 1

Group	N	Treatment	ADWG (lbs/day)	Abnormal Behavior	Cough	Overall Incidence of Clinical Symptoms
1	12	vORF2 - 16 μg (1 dose)	0.87 ± 0.29	2/12 (16.7%)	3/12 (25%)	41.7%
2	12	vORF2 - 8 μg (1 dose)	0.70 ± 0.32	4/12 (33.3%	1/12 (8.3%	41.7%
3	12	vORF2 - 4 μg (1 dose)	0.49 ± 0.21	8/12 (66.7%)	2/12 (16.7%	66.7%

4	11	rORF2 - 16 µg (1 dose)	0.84 ± 0.30	3/11 (27.3%)	0/11 (0%)	36.4%
5	12	rORF2 - 8 µg (1 dose)	0.94 ± 0.22	0/12 (0%)	1/12 (8.3%	8.3%
6	11	rORF2 - 4 µg (1 dose)	0.72 ± 0.25	1/11 (9.1%	0/11 (0%)	9.1%
7	12	KV (2 doses)	0.50 ± 0.15	7/12 (58.3)	0/12 (0%)	58.3%
8	10	Challenge Controls	0.76 ± 0.19	1/10 (10%)	2/10 (20%	40%
9	11	Strict Negative Controls	1.06 ± 0.17	0/11 (0%)	0/11 (0%)	0%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

Table 15. Summary of Group Post-Challenge Data - Part 2

Group	N	Treatment	Mortality Rate	Nasal Shedding	Mean % Lung Lesions	Incidence Rate of at least one tissue IHC positive for PCV2
1	12	vORF2 - 16 µg (1 dose)	8.3%	8.3%	0.40 ± 0.50%	16.7%

2	12	vORF2 - 8 µg (1 dose)	8.3%	8.3%	7.41 ± 20.2%	25.0%
3	12	vORF2 - 4 µg (1 dose)	0%	8.3%	9.20 ± 20.9%	66.7%
4	11	rORF2 - 16 µg (1 dose)	0%	18.2%	1.50 ± 4.74%	36.3%
5	12	rORF2 - 8 µg (1 dose)	0%	8.3%	0.68 ± 1.15%	16.7%
6	11	rORF2 - 4 µg (1 dose)	9.1%	9.1%	2.95 ± 5.12%	36.4%
7	12	KV (2 doses)	16.7%	41.7%	7.27 ± 22.9%	41.7%
8	10	Challenge Controls	10%	80%	9.88 ± 29.2%	90.0%
9	11	Strict Negative Controls	0%	63.6%	0/11 (0%)	90.9%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

Results of this study indicate that all further vaccine efforts should focus on a rORF2 vaccine. Overall, nasal shedding of PCV2 was detected post-challenge and vaccination with a PCV2 vaccine resulted in a reduction of shedding. Immunohistochemistry of selected lymphoid tissues also served as a good parameter for vaccine efficacy, whereas large

differences in ADWG, clinical symptoms, and gross lesions were not detected between groups. This study was complicated by the fact that extraneous PCV2 was introduced at some point during the study, as evidenced by nasal shedding of PCV2, PCV2 seroconversion and positive IHC tissues in Group 9, the strict negative control group.

Discussion

Seven PCV2 vaccines were evaluated in this study, which included three different dose levels of vORF2 antigen administered once on Day 0, three different dose levels of rORF2 antigen administered once on Day 0 and one dose level of killed whole cell PCV2 vaccine administered on Day 0 and Day 14. Overall, Group 5, which received 1 dose of vaccine containing 8 μg of rORF2 antigen, had the best results. Group 5 had the highest ADWG, the lowest incidence of abnormal behavior, the lowest incidence of abnormal respiration, the second lowest incidence of cough, the lowest incidence of overall clinical symptoms, the lowest mortality rate, the lowest rate of nasal shedding of PCV2, the second lowest rate for mean % lung lesions and the lowest incidence rate for positive IHC tissues.

Interestingly, Group 4, which received a higher dose of rORF2 antigen than Group 5, did not perform as well or better than Group 5. Group 4 had a slightly lower ADWG, a higher incidence of abnormal behavior, a higher incidence of overall clinical symptoms, a higher rate of nasal shedding of PCV2, a higher mean % lung lesions, and a higher rate for positive IHC tissues than Group 5. Statistical analysis, which may have indicated that the differences between these two groups were not statistically significant, was not conducted on these data, but there was an observed trend that Group 4 did not perform as well as Group 5.

Post-vaccination, 6 pigs died at the first study site. Four of the six pigs were from Group 8 or Group 9, which received no vaccine. None of the six pigs demonstrated lesions

consistent with PMWS, no adverse events were reported and overall, all seven vaccines appeared to be safe when administered to pigs approximately 11 days of age. During the post-vaccination phase of the study, pigs receiving either of three dose levels of vORF2 vaccine or killed whole cell vaccine had the highest IFAT levels, while Group 5 had the lowest IFAT levels just prior to challenge, of the vaccine groups.

Although not formally proven, the predominant route of transmission of PCV2 to young swine shortly after weaning is believed to be by oronasal direct contact and an efficacious vaccine that reduces nasal shedding of PCV2 in a production setting would help control the spread of infection. Groups receiving one of three vORF2 antigen levels and the group receiving 8 μg of rORF2 had the lowest incidence rate of nasal shedding of PCV2 (8.3%). Expectedly, the challenge control group had the highest incidence rate of nasal shedding (80%).

Gross lesions in pigs with PMWS secondary to PCV2 infection typically consist of generalized lymphadenopathy in combination with one or a multiple of the following: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers , (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc. At necropsy, icterus, hepatitis, nephritis, and gastric ulcers were not noted in any groups and lymphadenopathy was not specifically examined for. The mean % lung lesion scores varied between groups. The group receiving 16 μg of vORF2 antigen had the lowest mean % lung lesion score (0.40 ± 0.50%), followed by the group that received 8 μg of rORF2 (0.68 ± 1.15%). As expected, the challenge control group had the highest mean % lung lesion score (9.88 ± 29.2%). In all four groups, the mean % lung lesion scores were elevated due to one pig in each of these groups that had very high lung lesion scores. Most of the lung lesions were described as red/purple and consolidated. Typically, lung

lesions associated with PMWS are described as tan and non-collapsible with interlobular edema. The lung lesions noted in this study were either not associated with PCV2 infection or a second pulmonary infectious agent may have been present. Within the context of this study, the % lung lesion scores probably do not reflect a true measure of the amount of lung infection due to PCV2.

Other researchers have demonstrated a direct correlation between the presence of PCV2 antigen by IHC and histopathology. Histopathology on select tissues was not conducted with this study. Group 1 (16 μg of vORF2) and Group 5 (8 μg of rORF2) had the lowest incidence rate of pigs positive for PCV2 antigen (8.3%), while Group 9 (the strict negative control group - 90.9%) and Group 8 (the challenge control group - 90.0%) had the highest incidence rates for pigs positive for PCV2 antigen. Due to the non-subjective nature of this test, IHC results are probably one of the best parameters to judge vaccine efficacy on.

Thus, in one aspect of the present invention, the Minimum Portective Dosage (MPD) of a 1 ml/1 dose recombinant product with extracted PCV2 ORF2 (rORF2) antigen in the CDCD pig model in the face of a PCV2 challenge was determined. Of the three groups that received varying levels of rORF2 antigen, Group 5 (8 μg of rORF2 antigen) clearly had the highest level of protection. Group 5 either had the best results or was tied for the most favorable results with regard to all of the parameters examined. When Group 5 was compared with the other six vaccine groups post-challenge, Group 5 had the highest ADWG (0.94 ± 0.22 lbs/day), the lowest incidence of abnormal behavior (0%), the second lowest incidence of cough (8.3%), the lowest incidence of overall clinical symptoms (8.3%), the lowest mortality rate (0%), the lowest rate of nasal shedding of PCV2 (8.3%), the second lowest rate for mean % lung lesions (0.68 ± 1.15%) and the lowest incidence rate for positive IHC tissues (16.7%).

In another aspect of the present invention, the MPD of a 1 ml/1 dose conventional product that is partially purified PCV2 ORF2 (vORF2) antigen in the CDCD pig model in the face of a PCV2 challenge was determined. Of the three groups that received varying levels of vORF2 antigen, Group 1 (16 μg of vORF2) had the highest level of protection. Group 1 outperformed Groups 2 and 3 with respect to ADWG, mean % lung lesions, and IHC. Groups 1 and 2 (8 μg of vORF2 antigen) performed equally with respect to overall incidence of clinical symptoms, Group 3 (4 μg of vORF2 antigen) had the lowest mortality rate, and all three groups performed equally with respect to nasal shedding. Overall, vORF vaccines did not perform as well as rORF vaccines.

In yet another aspect of the present invention, the efficacy of a maximum dose of a 2ml/2 dose Conventional Killed PCV2 vaccine in the CDCD pig model in the face of a PCV2 challenge was determined. Of the seven vaccines evaluated in this study, the killed whole cell PCV2 vaccine performed the worst. Piglets receiving two doses of killed whole cell PCV2 vaccine had the lowest ADWG, the second highest rate of abnormal behavior (58.3%), the second highest overall incidence of clinical symptoms (58.3%), the highest mortality rate (16.7%), the second highest incidence of nasal shedding (41.7%), highest mean % lung lesions (9.88 ± 29.2%), a high incidence of lung lesions noted (75%) and a moderate IHC incidence rate in tissues (41.7%). However, it was still effective at invoking an immune response.

In still another aspect of the present invention, nasal shedding of PCV2 was assessed as an efficacy parameter and the previous PCV2 efficacy parameters from previous studies were reconfirmed. Results from this study indicate that nasal shedding of PCV2 occurs following intra nasal challenge and that PCV2 vaccines reduce nasal shedding of PCV2 post-challenge. Furthermore, results from this study and reports in the literature indicate that IHC

should continue to be evaluated in future PCV2 vaccine trials as well.

Some additional conclusions arising from this study are that lymphadenopathy is one of the hallmarks of PMWS. Another one of the hallmarks of PMWS is lymphoid depletion and multinucleated/giant histiocytes. Additionally, no adverse events or injection site reactions were noted for any of the 7 PCV2 vaccines and all 7 PCV2 vaccines appeared to be safe when administered to young pigs.

EXAMPLE 5

This example tests the efficacy of eight PCV2 candidate vaccines and reconfirms PCV2 challenge parameters from earlier challenge studies following exposure to a virulent strain of PCV2. One hundred and fifty (150) cesarean derived colostrum deprived (CDCD) piglets, 6-16 days of age, were blocked by weight and randomly divided into 10 groups of equal size. Table 16 sets forth the General Study Design for this Example.

Table 16. General Study Design

Group	No. Of Pigs	Treatment	Day of Treatment	KLH/ICFA on Day 22 and Day 28	Challenge with Virulent PCV2 on Day 25	PRRSV MLV on Day 46	Necropsy on Day 50
1	15	PVC2 Vaccine 1 16 µg rORF2 – IMS 1314	0 & 14	+	+	+	+
2	15	PVC2 Vaccine 2 16 µg vORF2 – Carbopol	0 & 14	+	+	+	+
3	15	PCV2 Vaccine 3 16 µg rORF2 – Carbopol	0 & 14	+	+	+	+

4	15	PCV2 Vaccine 2 16 µg vORF2 – Carbopol	0	+	+	+	+
5	15	PVC2 Vaccine 3 4 µg rORF2 – Carbopol	0 & 14	+	+	+	+
6	15	PVC2 Vaccine 3 1 µg rORF2 – Carbopol	0 & 14	+	+	+	+
7	15	PVC2 Vaccine 3 0.25 µg rORF2 – Carbopol	0 & 14	+	+	+	+
8	15	PVC2 Vaccine 4 > 8.0 log KV – Carbopol	0 & 14	+	+	+	+
9	15	Challenge Controls	N/A	+	+	+	+
10	15	None - Strict Negative Control Group	N/A	+	-	+	+

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

The vaccine formulations given to each group were as follows. PCV2 Vaccine No. 1, administered at 1 x 2 ml dose to Group 1, was a high dose (16 ug/2 ml dose) of inactivated recombinant ORF2 antigen adjuvanted with IMS 1314 (16 ug rORF2 – IMS 1314). PCV2 Vaccine No. 2, administered at 1 x 2 ml dose to Group 2, was a high dose (16 ug/2 ml dose) of a partially purified VIDO R-1 generated PCV2 ORF2 antigen adjuvanted with Carbopol (16 ug vORF2 – Carbopol). PCV2 Vaccine No. 3, administered at 1 x 2 ml dose to Group 3, was a high dose (16 ug/2 ml dose) of inactivated recombinant ORF2 antigen adjuvanted with Carbopol (16 ug rORF2 – Carbopol). PCV2 Vaccine No. 4, administered at

1 x 1 ml dose to Group 4, was a high dose (16 ug /1 ml dose) of a partially purified VIDO R-1 generated PCV2 ORF2 antigen adjuvanted with Carbopol (16 ug vORF2 – Carbopol). Vaccine No. 5, administered at 1 x 2 ml dose to Group 5, was a 4 ug/2 ml dose of an inactivated recombinant ORF2 antigen adjuvanted with Carbopol (4 ug rORF2 – Carbopol). PCV2 Vaccine No. 6, administered at 1 x 2 ml dose to Group 6, was a 1 ug/2 ml dose of an inactivated recombinant ORF2 antigen adjuvanted with Carbopol (1 ug rORF2 – Carbopol). PCV2 Vaccine No. 7, administered at 1 x 2 ml dose to Group 7, was a low dose (0.25 ug/2 ml dose) of inactivated recombinant ORF2 antigen adjuvanted with Carbopol (0.25 ug rORF2 – Carbopol). PCV2 Vaccine No. 8, administered at 1 x 2 ml dose to Group 8, was a high dose (pre-inactivation titer > 8.0 log/2 ml dose) Inactivated Conventional Killed VIDO R-1 generated PCV2 Struve antigen adjuvanted with Carbopol (>8.0 log KV – Carbopol). On Day 0, Groups 1-8 were treated with their assigned vaccines. Groups 1-3 and 5-8 received boosters of their respective vaccines again on Day 14. The effectiveness of a single dose of 16 μg of vORF2 – Carbopol was tested on Group 4 which did not receive a booster on Day 14. Piglets were observed for adverse events and injection site reactions following both vaccinations. On Day 21 the piglets were moved to a second study site where Groups 1-9 were group housed in one building and Group 10 was housed in a separate building. All pigs received keyhole limpet hemocyanin emulsified with incomplete Freund's adjuvant (KLH/ICFA) on Days 22 and 28. On Day 25, Groups 1-9 were challenged with approximately 4 logs of virulent PCV2 virus. By Day 46, very few deaths had occurred in the challenge control group. In an attempt to immunostimulate the pigs and increase the virulence of the PCV2 challenge material, all Groups were treated with INGELVAC® PRRSV MLV (Porcine Reproductive and Respiratory Vaccine, Modified Live Virus) on Day 46.

Pre- and post-challenge blood samples were collected for PCV2 serology. Post-challenge, body weight data for determination of average daily weight gain (ADWG) and observations of clinical signs were collected. On Day 50, all surviving pigs were necropsied, gross lesions were recorded, lungs were scored for pathology, and selected tissues were preserved in formalin for examination by Immunohistochemistry (IHC) for detection of PCV2 antigen at a later date.

Materials and Methods

This was a partially-blind vaccination-challenge feasibility study conducted in CDCD pigs, 6 to 16 days of age on Day 0. To be included in the study, PCV2 IFA titers of sows were $\leq$ 1:1000. Additionally, the serologic status of sows were from a known PRRS-negative herd. Sixteen (16) sows were tested for PCV2 serological status and all sixteen (16) had a PCV2 titer of $\leq$ 1000 and were transferred to the first study site. One hundred fifty (150) piglets were delivered by cesarean section surgeries and were available for this study on Day -3. On Day -3, 150 CDCD pigs at the first study site were weighed, identified with ear tags, blocked by weight and randomly assigned to 1 of 10 groups, as set forth above in table 16. Blood samples were collected from all pigs. If any test animal meeting the inclusion criteria was enrolled in the study and was later excluded for any reason, the Investigator and Monitor consulted in order to determine the use of data collected from the animal in the final analysis. The date of which enrolled piglets were excluded and the reason for exclusion was documented. No sows meeting the inclusion criteria, selected for the study and transported to the first study site were excluded. No piglets were excluded from the study, and no test animals were removed from the study prior to termination. Table 17 describes the time frames for the key activities of this Example.

Table 17. Study Activities

Study Day	Actual Dates	Study Activity
-3	4-04-03	Weighed pigs; health exam; randomized to groups; collected blood samples
-3, 0-21	4-04-03 4-07-03 to 5-27-03	Observed for overall health and for adverse events post-vaccination
0	4-07-03	Administered respective IVPs to Groups 1-8
0-7	4-07-03 to 4-14-03	Observed pigs for injection site reactions
14	4-21-03	Boostered Groups 1-3, 5-8 with respective IVPs; blood sampled all pigs
14-21	4-21-03 to 4-28-03	Observed pigs for injection reactions
19-21	4-26-03 to 4-28-03	Treated all pigs with antibiotics
21	4-28-03	Pigs transported from Struve Labs, Inc. to Veterinary Resources, Inc.(VRI)
22-50	4-28-03 to 5-27-03	Observed pigs for clinical signs post-challenge
22	4-29-03	Treated Groups 1-10 with KLH/ICFA
25	5-02-03	Collected blood samples from all pigs; weighed all pigs; challenged Groups 1-9 with PCV2 challenge material
28	5-05-03	Treated Groups 1-10 with KLH/ICFA
32	5-09-03	Collected blood samples from all pigs
46	5-23-03	Administered INGELVAC® PRRS MLV to all groups
50	5-27-03	Collected blood samples, weighed and necropsied all pigs; gross lesions were recorded; lungs were evaluated for lesions; fresh and formalin fixed tissue samples were saved; In-life phase of the study was completed

Following completion of the in-life phase of the study, formalin fixed tissues were examined by Immunohistochemistry (IHC) for detection of PCV2 antigen by a pathologist, blood samples were evaluated for PCV2 serology, and average daily weight gain (ADWG) was determined from Day 25 to Day 50.

Animals were housed at the first study site in individual cages in seven rooms from birth to approximately 11 days of age (approximately Day 0 of the study). Each room was

identical in layout and consisted of stacked individual stainless steel cages with heated and filtered air supplied separately to each isolation unit. Each room had separate heat and ventilation, thereby preventing cross-contamination of air between rooms. Animals were housed in two different buildings at the second study site. Group 10 (The Strict negative control group) was housed separately in a converted nursery building and Groups 1-9 were housed in a converted farrowing building. Each group was housed in a separate pen (14-15 pigs per pen) and each pen provided approximately 2.3 square feet per pig. Groups 2, 4 and 8 were penned in three adjacent pens on one side of the alleyway and Groups 1, 3, 5, 6, 7, and 9 were penned in six adjacent pens on the other side of the alleyway. The Group separation was due to concern by the Study Monitor that vaccines administered to Groups 2, 4, and 8 had not been fully inactivated. Each pen was on an elevated deck with plastic slatted floors. A pit below the pens served as a holding tank for excrement and waste. Each building had its own separate heating and ventilation systems, with little likelihood of cross-contamination of air between buildings.

At the first study site, piglets were fed a specially formulated milk ration from birth to approximately 3 weeks of age. All piglets were consuming solid, special mixed ration by Day 21 (approximately 4 ½ weeks of age). At the second study site, all piglets were fed a custom non-medicated commercial mix ration appropriate for their age and weight, *ad libitum*. Water at both study sites was also available *ad libitum*.

All test pigs were treated with 1.0 mL of NAXCEL®, IM, in alternating hams on Days 19, 20, and 21. In addition, Pig No. 11 (Group 1) was treated with 0.5 mL of NAXCEL® IM on Day 10, Pig No. 13 (Group 10) was treated with 1 mL of Penicillin and 1 mL of PREDEF® 2X on Day 10, Pig No. 4 (Group 9) was treated with 1.0 mL of NAXCEL® IM on Day 11, and Pigs 1 (Group 1), 4 and 11 were each treated with 1.0 mL of

NAXCEL® on Day 14 for various health reasons.

While at both study sites, pigs were under veterinary care. Animal health examinations were conducted on Day -3 and were recorded on the Health Examination Record Form. All animals were in good health and nutritional status before vaccination as determined by observation on Day 0. All test animals were observed to be in good health and nutritional status prior to challenge. Carcasses and tissues were disposed of by rendering. Final disposition of study animals was recorded on the Animal Disposition Record.

On Days 0 and 14, pigs assigned to Groups 1-3 and 5-8 received 2.0 mL of assigned PCV2 Vaccines 1-4, respectively, IM in the right and left neck region, respectively, using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x ½" needle. Pigs assigned to Group 4 received 1.0 mL of PCV2 Vaccine No. 2, IM in the right neck region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x ½" needle on Day 0 only.

On Day 22 all test pigs received 2.0 mL of KLH/ICFA IM in the left neck region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x1" needle. On Day 28 all test pigs received 2.0 mL of KLH/ICFA in the right ham region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x 1" needle.

On Day 25, pigs assigned to Groups 1-9 received 1.0 mL of PCV2 ISUVDL challenge material (3.98 $\log_{10}$ $TCID_{50}$/mL) IM in the right neck region using a sterile 3.0 mL Luer-lock syringe and a sterile 20g x 1" needle. An additional 1.0 mL of the same material was administered IN to each pig (0.5 mL per nostril) using a sterile 3.0 mL Luer-lock syringe and nasal canula.

On Day 46, all test pigs received 2.0 mL INGELVAC® PRRS MLV, IM, in the right neck region using a sterile 3.0 mL Luer0lock syringe and a sterile 20g x 1" needle. The PRRSV MLV was administered in an attempt to increase virulence of the PCV2 challenge

material.

Test pigs were observed daily for overall health and adverse events on Day -3 and from Day 0 to Day 21. Each of the pigs were scored for normal or abnormal behavior, respiration, or cough. Observations were recorded on the Clinical Observation Record. All test pigs were observed from Day 0 to Day 7, and Group 7 was further observed from Day 14 to 21, for injection site reactions. Average daily weight gain was determined by weighing each pig on a calibrated scale on Days -3, 25 and 50, or on the day that a pig was found dead after challenge. Body weights were recorded on the Body Weight Form. Day -3 body weights were utilized to block pigs prior to randomization. Day 25 and Day 50 weight data was utilized to determine the average daily weight gain (ADWG) for each pig during these time points. For pigs that died after challenge and before Day 50, the ADWG was adjusted to represent the ADWG from Day 25 to the day of death.

In order to determine PCV2 serology, venous whole blood was collected from each piglet from the orbital venous sinus on Days -3 and 14. For each piglet, blood was collected from the orbital venous sinus by inserting a sterile capillary tube into the medial canthus of one of the eyes and draining approximately 3.0 mL of whole blood into a 4.0 mL Serum Separator Tube (SST). On Days 25, 32, and 50, venous whole blood from each pig was collected from the anterior vena cava using a sterile 20g x 1 ½" Vacutainer® needle (Becton Dickinson and Company, Franklin Lakes, New Jersey), a Vaccutainer® needle holder and a 13 mL SST. Blood collections at each time point were recorded on the Sample Collection Record. Blood in each SST was allowed to clot, each SST was then spun down and the serum harvested. Harvested serum was transferred to a sterile snap tube and stored at -70± 10° C until tested at a later date. Serum samples were tested for the presence of PCV2 antibodies by BIVI-R&D personnel.

Pigs were observed once daily from Day 22 to Day 50 for clinical symptoms and scored for normal or abnormal behavior, respiration or cough. Clinical observations were recorded on the Clinical Observation Record.

Pigs Nos. 46 (Group 1) and 98 (Groups 9) died at the first study site. Both of these deaths were categorized as bleeding deaths and necropsies were not conducted on these two pigs. At the second study site, pigs that died after challenge and prior to Day 50, and pigs euthanized on Day 50, were necropsied. Any gross lesions were noted and the percentages of lung lobes with lesions were recorded on the Necropsy Report Form.

From each of the pigs necropsied at the second study site, a tissue sample of tonsil, lung, heart, and mesenteric lymph node was placed into a single container with buffered 10% formalin; while another tissue sample from the same aforementioned organs was placed into a Whirl-pak® (M-Tech Diagnostics Ltd., Thelwall, UK) and each Whirl-pak® was placed on ice. Each container was properly labeled. Sample collections were recorded on the Necropsy Report Form. Afterwards, formalin-fixed tissue samples and a Diagnostic Request Form were submitted for IHC testing. IHC testing was conducted in accordance with standard laboratory procedures for receiving samples, sample and slide preparation, and staining techniques. Fresh tissues in Whirl-paks® were shipped with ice packs to the Study Monitor for storage (-70° ± 10° C) and possible future use.

Formalin-fixed tissues were examined by a pathologist for detection of PCV2 by IHC and scored using the following scoring system: 0 = None; 1 = Scant positive staining, few sites; 2 = Moderate positive staining, multiple sites; and 3 = Abundant positive staining, diffuse throughout the tissue. For analytical purposes, a score of 0 was considered "negative," and a score of greater than 0 was considered "positive."

Results

Results for this example are given below. It is noted that Pigs No. 46 and 98 died on days 14 and 25 respectively. These deaths were categorized as bleeding deaths. Pig No. 11 (Group 1) was panting with rapid respiration on Day 15. Otherwise, all pigs were normal for behavior, respiration and cough during this observation period and no systemic adverse events were noted with any groups. No injection site reactions were noted following vaccination on Day 0. Following vaccination on Day 14, seven (7) out of fourteen (14) Group 1 pigs (50.0%) had swelling with a score of "2" on Day 15. Four (4) out of fourteen (14) Group 1 (28.6%) still had a swelling of "2" on Day 16. None of the other groups experienced injection site reactions following either vaccination.

Average daily weight gain (ADWG) results are presented below in Table 18. Pig Nos. 46 and 98 that died from bleeding were excluded from group results. Group 4, which received one dose of 16 ug vORF2 – Carbopol, had the highest ADWG (1.16 ± 0.26 lbs/day), followed by Groups 1, 2, 3, 5, 6, and 10 which had ADWGs that ranged from 1.07 ± 0.23 lbs/day to 1.11 ± 0.26 lbs/day. Group 9 had the lowest ADWG (0.88 ± 0.29 lbs/day), followed by Groups 8 and 7, which had ADWGs of 0.93 ± 0.33 lbs/day and 0.99 ± 0.44 lbs/day, respectively.

Table 18. Summary of Group Average Daily Weight Gains (ADWG)

Group	Treatment	N	ADWG - lbs/day (Day 25 to Day 50) or adjusted for pigs dead before Day 50
1	rORF2 - 16 μg – IMS 1314 2 doses	14	1.08 ± 0.30 lbs/day
2	vORF2 – 16 μg – Carbopol 2 doses	15	1.11 ± 0.16 lbs/day
3	rORF2 – 16 μg – Carbopol 2 doses	15	1.07 ± 0.21 lbs/day

4	vORF2 - 16 μg – Carbopol 1 dose	15	1.16 ± 0.26 lbs/day
5	rORF2 - 4 μg – Carbopol 1 dose	15	1.07 ± 0.26 lbs/day
6	rORF2 - 1 μg – Carbopol 2 doses	15	1.11 ± 0.26 lbs/day
7	rORF2 – 0.25 μg – Carbopol 2 doses	15	0.99 ± 0.44 lbs/day
8	KV > 8.0 log – Carbopol 2 doses	15	0.93 ± 0.33 lbs/day
9	Challenge Controls	14	0.88 ± 0.29 lbs/day
10	Strict Negative Controls	15	1.07 ± 0.23 lbs/day

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

PVC2 serology results are presented below in Table 19. All ten (10) groups were seronegative for PCV2 on Day –3. On Day 14, PCV2 titers remained low for all ten (10) groups (range of 50-113). On Day 25, Group 8, which received the whole cell killed virus vaccine, had the highest PCV2 titer (4617), followed by Group 2, which received 16 ug vORF2 – Carbopol, Group 4, which received as single dose of 16 ug vORF2 – Carbopol, and Group 3, which received 16 ug rORF2 – Carbopol, which had titers of 2507, 1920 and 1503 respectively. On Day 32 (one week post challenge), titers for Groups 1-6 and Group 8 ranged from 2360 to 7619; while Groups 7 (0.25 ug rORF2 – Carbopol), 9 (Challenge Control), and 10 (Strict negative control) had titers of 382, 129 and 78 respectively. On Day 50 (day of necropsy), all ten (10) groups demonstrated high PCV2 titers (≥ 1257).

On Days 25, 32, and 50, Group 3, which received two doses of 16 ug rORF2 – Carbopol, had higher antibody titers than Group 1, which received two doses of 16 ug rORF2 – IMS 1314. On Days 25, 32 and 50, Group 2, which received two doses of 16 ug vORF2, had higher titers than Group 4, which received only one does of the same vaccine. Groups 3,

5, 6, 7, which received decreasing levels of rORF2 – Carbopol, of 16, 4, 1, and 0.25 ug respectively, demonstrated correspondingly decreasing antibody titers on Days 25 and 32.

Table 19. Summary of Group PCV2 IFA Titers

Group	Treatment	Day -3	Day 14**	Day 25***	Day 32	Day 50****
1	rORF2 - 16 µg – IMS 1314 2 doses	50	64	646	3326	4314
2	vORF2 – 16 µg – Carbopol 2 doses	50	110	2507	5627	4005
3	rORF2 – 16 µg – Carbopol 2 doses	50	80	1503	5120	6720
4	vORF2 - 16 µg – Carbopol 1 dose	50	113	1920	3720	1257
5	rORF2 - 4 µg – Carbopol 1 dose	50	61	1867	3933	4533
6	rORF2 - 1 µg – Carbopol 2 doses	50	70	490	2360	5740
7	rORF2 – 0.25 µg – Carbopol 2 doses	50	73	63	382	5819
8	KV > 8.0 log – Carbopol 2 doses	50	97	4617	7619	10817
9	Challenge Controls	50	53	50	129	4288
10	Strict Negative Controls	50	50	50	78	11205

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

*For calculation purposes, a ≤100 IFA titer was designated as a titer of "50"; a ≥6400 IFA titer was designated as a titer of "12,800".

**Day of Challenge

***Day of Necropsy

The results from the post-challenge clinical observations are presented below. Table

20 includes observations for Abnormal Behavior, Abnormal Respiration, Cough and Diarrhea. Table 21 includes the results from the Summary of Group Overall Incidence of Clinical Symptoms and Table 22 includes results from the Summary of Group Mortality Rates Post-challenge. The incidence of abnormal behavior, respiration and cough post-challenge were low in pigs receiving 16 ug rORF2–IMS 1314 (Group 1), 16 ug rORF2–Carbopol (Group 3), 1 ug rORF2–Carbopol (Group 6), 0.25 ug rORF2–Carbopol (Group 7), and in pigs in the Challenge Control Group (Group 9). The incidence of abnormal behavior, respiration, and cough post-challenge was zero in pigs receiving 16 ug vORF2–Carbopol (Group 2), a single dose of 16ug vORF2–Carbopol (Group 4), 4 ug rORF2–Carbopol (Group 5), >8 log KV–Carbopol (Group 8), and in pigs in the strict negative control group (Group 10).

The overall incidence of clinical symptoms varied between groups. Pigs receiving 16 ug vORF2–Carbopol (Group 2), a single dose of 16 ug vORF2–Carbopol (Group 4), and pigs in the Strict negative control group (Group 10) had incidence rates of 0%; pigs receiving 16 ug rORF2–Carbopol (Group 3), and 1 ug rORF2–Carbopol (Group 6) had incidence rates of 6.7%; pigs receiving 16 ug rORF2–IMS 1314 (Group 1) had an overall incidence rate of 7.1%; pigs receiving 4 ug rORF2–Carbopol (Group 5), 0.25 ug rORF2–Carbopol (Group 7), and >8 log KV vaccine had incidence rates of 13.3%; and pigs in the Challenge Control Group (Group 9) had an incidence rate of 14.3%.

Overall mortality rates between groups varied as well. Group 8, which received 2 doses of KV vaccine had the highest mortality rate of 20.0%; followed by Group 9, the challenge control group, and Group 7, which received 0.25 ug rORF2–Carbopol and had mortality rates of 14.3% and 13.3% respectively. Group 4, which received one dose of 16 ug vORF2–Carbopol had a 6.7% mortality rate. All of the other Groups, 1, 2, 3, 5, 6, and 10,

had a 0% mortality rate.

Table 20. Summary of Group Observations for Abnormal Behavior, Abnormal Respiration, and Cough Post-Challenge

Group	Treatment	N	Abnormal Behavior[1]	Abnormal Behavior[2]	Cough[3]
1	rORF2 - 16 µg – IMS 1314 2 doses	14	0/14 (0%)	0/14 (0%)	1/14 (7.1%)
2	vORF2 – 16 µg – Carbopol 2 doses	15	0/15 (0%)	0/15 (0%)	0/15 (0%)
3	rORF2 – 16 µg – Carbopol 2 doses	15	0/15 (0%)	0/15 (0%)	1/15 (6.7%)
4	vORF2 - 16 µg – Carbopol 1 dose	15	0/15 (0%)	0/15 (0%)	0/15 (0%)
5	rORF2 - 4 µg – Carbopol 1 dose	15	1/15 (6.7%)	1/15 (6.7%)	0/15 (0%)
6	rORF2 - 1 µg – Carbopol 2 doses	15	0/15 (0%)	0/15 (0%)	1/15 (6.7%)
7	rORF2 – 0.25 µg – Carbopol 2 doses	15	0/15 (0%)	1/15 (6.7%)	1/15 (06.7%)
8	KV > 8.0 log – Carbopol 2 doses	15	1/15 (6.7%)	1/15 (6.7%)	0/15 (0%)
9	Challenge Controls	14	1/14 (7.1%)	1/14 (7.1%)	2/14 (14/3%)
10	Strict Negative Controls	15	0/15 (0%)	0/15 (0%)	0/15 (0%)

[1]Total number of pigs in each group that demonstrated any abnormal behavior for at least one day

[2]Total number of pigs in each group that demonstrated any abnormal respiration for at least one day

[3]Total number of pigs in each group that demonstrated a cough for at least one day

Table 21. Summary of Group Overall Incidence of Clinical Symptoms Post-Challenge

Group	Treatment	N	Incidence of pigs with Clinical Symptoms[1]	Incidence Rate
1	rORF2 - 16 µg – IMS 1314 2 doses	14	1	7.1%
2	vORF2 – 16 µg – Carbopol 2 doses	15	0	0.0%
3	rORF2 – 16 µg – Carbopol 2 doses	15	1	6.7%
4	vORF2 - 16 µg – Carbopol 1 dose	15	0	0.0%
5	rORF2 - 4 µg – Carbopol 1 dose	15	2	13.3%
6	rORF2 - 1 µg – Carbopol 2 doses	15	1	6.7%
7	rORF2 – 0.25 µg – Carbopol 2 doses	15	2	13.3%
8	KV > 8.0 log – Carbopol 2 doses	15	2	13.3%
9	Challenge Controls	14	2	14.3%
10	Strict Negative Controls	15	0	0.0%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

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[1]Total number of pigs in each group that demonstrated any clinical symptom for at least one day

Table 22. Summary of Group Mortality Rates Post-Challenge

Group	Treatment	N	Dead Post-challenge	Mortality Rate
1	rORF2 - 16 µg – IMS 1314 2 doses	14	0	0.0%

2	vORF2 – 16 μg – Carbopol 2 doses	15	0	0.0%
3	rORF2 – 16 μg – Carbopol 2 doses	15	0	0.0%
4	vORF2 - 16 μg – Carbopol 1 dose	15	1	6.7%
5	rORF2 - 4 μg – Carbopol 1 dose	15	0	0.0%
6	rORF2 - 1 μg – Carbopol 2 doses	15	0	0.0%
7	rORF2 – 0.25 μg – Carbopol 2 doses	15	2	13.3%
8	KV > 8.0 log – Carbopol 2 doses	15	3	20.0%
9	Challenge Controls	14	2	14.3%
10	Strict Negative Controls	15	0	0.0%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

The Summary of Group Mean Percentage Lung Lesions and Tentative Diagnosis is given below in Table 23. Group 9, the challenge control group, had the highest percentage lung lesions with a mean of 10.81 ± 23.27%, followed by Group 7, which received 0.25 ug rORF2–Carbopol and had a mean of 6.57 ± 24.74%, Group 5, which received 4 ug rORF2–Carbopol and had a mean of 2.88 ± 8.88%, and Group 8, which received the KV vaccine and had a mean of 2.01 ± 4.98%. The remaining six (6) groups had lower mean percentage lung lesions that ranged from 0.11 ± 0.38% to 0.90 ± 0.15%.

Tentative diagnosis of pneumonia varied among the groups. Group 3, which received two doses of 16 ug rORF2–Carbopol, had the lowest tentative diagnosis of pneumonia, with 13.3%. Group 9, the challenge control group, had 50% of the group tentatively diagnosed with pneumonia, followed by Group 10, the strict negative control group and Group 2, which received two doses of 16 ug vORF2–Carbopol, with 46.7% and 40% respectively, tentatively

diagnosed with pneumonia.

Groups 1, 2, 3, 5, 9, and 10 had 0% of the group tentatively diagnosed as PCV2 infected; while Group 8, which received two doses if KV vaccine, had the highest group rate of tentative diagnosis of PCV2 infection, with 20%. Group 7, which received two doses of 0.25 ug rORF2–Carbopol, and Group 4, which received one dose of 16 ug vORF2–Carbopol had tentative group diagnoses of PCV2 infection in 13.3% and 6.7% of each group, respectively.

Gastric ulcers were only diagnosed in one pig in Group 7 (6.7%); while the other 9 groups remained free of gastric ulcers.

Table 23. Summary of Group Mean % Lung Lesion and Tentative Diagnosis

Group	Treatment	N	No. Of pigs that shed for at least one day	Incidence Rate
1	rORF2 - 16 μg – IMS 1314 2 doses	15	0	0%
2	vORF2 – 16 μg – Carbopol 2 doses	15	1	6.7%
3	rORF2 – 16 μg – Carbopol 2 doses	15	3	20.0%
4	vORF2 - 16 μg – Carbopol 1 dose	15	2	13.3%
5	rORF2 - 4 μg – Carbopol 1 dose	15	3	20.0%
6	rORF2 - 1 μg – Carbopol 2 doses	15	6	40.0%
7	rORF2 – 0.25 μg – Carbopol 2 doses	15	7	46.7%
8	KV > 8.0 log – Carbopol 2 doses	15	12	80%

9	Challenge Controls	14	14	100.0%
10	Strict Negative Controls	15	14	93.3%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

The Summary of Group IHC Positive Incidence Results is shown below in Table 24. Group 1 (16 ug rORF2 – IMS 1314) had the lowest group rate of IHC positive results with 0% of the pigs positive for PCV2, followed by Group 2 (16 ug vORF2 – Carbopol) and Group 4 (single dose 16 ug vORF2 – Carbopol), which had group IHC rates of 6.7% and 13.3% respectively. Group 9, the challenge control group, had the highest IHC positive incidence rate with 100% of the pigs positive for PCV2, followed by Group 10, the strict negative control group, and Group 8 (KV vaccine), with 93.3% and 80% of the pigs positive for PCV2, respectively.

Table 24. Summary of Group IHC Positive Incidence Rate

Group	Treatment	N	No. Of pigs that shed for at least one day	Incidence Rate
1	rORF2 - 16 μg – IMS 1314 2 doses	15	0	0%
2	vORF2 – 16 μg – Carbopol 2 doses	15	1	6.7%
3	rORF2 – 16 μg – Carbopol 2 doses	15	3	20.0%
4	vORF2 - 16 μg – Carbopol 1 dose	15	2	13.3%
5	rORF2 - 4 μg – Carbopol 1 dose	15	3	20.0%
6	rORF2 - 1 μg – Carbopol 2 doses	15	6	40.0%

7	rORF2 – 0.25 µg – Carbopol 2 doses	15	7	46.7%
8	KV > 8.0 log – Carbopol 2 doses	15	12	80%
9	Challenge Controls	14	14	100.0%
10	Strict Negative Controls	15	14	93.3%

vORF2 = isolated viral ORF2; rORF2 = recombinant baculovirus expressed ORF2; KV or killed whole cell virus = PCV2 virus grown in suitable cell culture

Discussion

Seven PCV2 vaccines were evaluated in this example, which included a high dose (16 µg) of rORF2 antigen adjuvanted with IMS 1314 administered twice, a high dose (16 µg) of vORF2 antigen adjuvanted with Carbopol administered once to one group of pigs and twice to a second group of pigs, a high dose (16 µg) of rORF2 antigen adjuvanted with Carbopol administered twice, a 4 µg dose of rORF2 antigen adjuvanted with Carbopol administered twice, a 1 µg dose of rORF2 antigen adjuvanted with Carbopol administered twice, a low dose (0.25 µg) of rORF2 antigen adjuvanted with Carbopol administered twice, and a high dose (> 8 log) of killed whole cell PCV2 vaccine adjuvanted with Carbopol. Overall, Group 1, which received two doses of 16 µg rORF2 – IMS 1314, performed slightly better than Groups 2 through 7, which received vaccines containing various levels of either vORF2 or rORF2 antigen adjuvanted with Carbopol and much better than Group 8, which received two doses of killed whole cell PCV2 vaccine. Group 1 had the third highest ADWG (1.80 ± 0.30 lbs/day), the lowest incidence of abnormal behavior (0%), the lowest incidence of abnormal respiration (0%), a low incidence of cough (7.1%), a low incidence of overall clinical symptoms (7.1%), was tied with three other groups for the lowest mortality rate (0%), the second lowest rate for mean % lung lesions (0.15 ± 0.34%), the second lowest rate for pneumonia (21.4%) and the lowest incidence rate for positive IHC tissues (0%). Group 1

was, however, the only group in which injection site reactions were noted, which included 50% of the vaccinates 1 day after the second vaccination. The other vaccines administered to Groups 2 through 7 performed better than the killed vaccine and nearly as well as the vaccine administered to Group 1.

Group 8, which received two doses of killed PCV2 vaccine adjuvanted with Carbopol, had the worst set of results for any vaccine group. Group 8 had the lowest ADWG (0.93 ± 0.33 lbs/day), the second highest rate of abnormal behavior (6.7%), the highest rate of abnormal respiration (6.7%), was tied with three other groups for the highest overall incidence rate of clinical symptoms (13.3%), had the highest mortality rate of all groups (20%), and had the highest positive IHC rate (80%) of any vaccine group. There was concern that the killed whole cell PCV2 vaccine may not have been fully inactivated prior to administration to Group 8, which may explain this group's poor results. Unfortunately, definitive data was not available to confirm this concern. Overall, in the context of this example, a Conventional Killed PCV2 vaccine did not aid in the reduction of PCV2 associated disease.

As previously mentioned, no adverse events were associated with the test vaccines with exception of the vaccine adjuvanted with IMS 1314. Injection site reactions were noted in 50.0% of the pigs 1 day after the second vaccination with the vaccine formulated with IMS 1314 and in 28.6% of the pigs 2 days after the second vaccination. No reactions were noted in any pigs receiving Carbopol adjuvanted vaccines. Any further studies that include pigs vaccinated with IMS 1314 adjuvanted vaccines should continue to closely monitor pigs for injection site reactions.

All pigs were sero-negative for PCV2 on Day -3 and only Group 2 had a titer above 100 on Day 14. On Day 25 (day of challenge), Group 8 had the highest PCV2 antibody titer

(4619), followed by Group 2 (2507). With the exception of Groups 7, 9 and 10, all groups demonstrated a strong antibody response by Day 32. By Day 50, all groups including Groups 7, 9 and 10 demonstrated a strong antibody response.

One of the hallmarks of late stage PCV2 infection and subsequent PMWS development is growth retardation in weaned pigs, and in severe cases, weight loss is noted. Average daily weight gain of groups is a quantitative method of demonstrating growth retardation or weight loss. In this example, there was not a large difference in ADWG between groups. Group 8 had the lowest ADWG of 0.88 ± 0.29 lbs/day, while Group 4 had the highest ADWG of 1.16 ± 0.26 lb/day. Within the context of this study there was not a sufficient difference between groups to base future vaccine efficacy on ADWG.

In addition to weight loss – dyspnea, leghargy, pallor of the skin and sometimes icterus are clinical symptoms associated with PMWS. In this example, abnormal behavior and abnormal respiration and cough were noted infrequently for each group. As evidenced in this study, this challenge model and challenge strain do not result in overwhelming clinical symptoms and this is not a strong parameter on which to base vaccine efficacy.

Overall, mortality rates were not high in this example and the lack of a high mortality rate in the challenge control group limits this parameter on which to base vaccine efficacy. Prior to Day 46, Groups 4 and 7 each had one out of fifteen pigs die, Group 9 had two out of fourteen pigs die and Group 8 had three out of fifteen pigs die. Due to the fact that Group 9, the challenge control group was not demonstrating PCV2 clinical symptoms and only two deaths had occurred in this group by Day 46, Porcine Respiratory and Reproductive Syndrome Virus (PRRSV) MLV vaccine was administered to all pigs on Day 46. Earlier studies had utilized INGELVAC® PRRS MLV as an immunostimulant to exasperate PCV2-associated PMWS disease and mortality rates were higher in these earlier studies. Two

deaths occurred shortly after administering the PRRS vaccine on Day 46 – Group 4 had one death on Day 46 and Group 7 had one death on Day 47 – which were probably not associated with the administration of the PRRS vaccine. By Day 50, Group 8, which received two doses of killed vaccine, had the highest mortality rate (20%), followed by Group 9 (challenge control) and Group 7 (0.25 ug rORF2 – Carbopol), with mortality rates of 14.3% and 13.3% respectively. Overall, administration of the PRRS vaccine to the challenge model late in the post-challenge observation phase of this example did not significantly increase mortality rates.

Gross lesions in pigs with PMWS secondary to PCV2 infection typically consist of generalized lymphadenopathy in combination with one or more of the following: (1) interstitial pneumonia with interlobular edema, (2) cutaneous pallor or icterus, (3) mottled atrophic livers, (4) gastric ulcers , (5) nephritis and (6) reproductive disorders, e.g. abortion, stillbirths, mummies, etc. At necropsy (Day 50), icterus, hepatitis, and nephritis were not noted in any groups. A gastric ulcer was noted in one Group 7 pig, but lymphadenopathy was not specifically examined for. Based on the presence of lesions that were consistent with PCV2 infection, three groups had at least one pig tentatively diagnosed with PCV2 (PMWS). Group 8, which received two doses of killed vaccine, had 20% tentatively diagnosed with PCV2, while Group 7 and Group 4 had 13.3% and 6.7%, respectively, tentatively diagnosed with PCV2. The mean % lung lesion scores varied between groups at necropsy. Groups 1, 2, 3, 4, 6 and 10 had low % lung lesion scores that ranged from 0.11 ± 0.38% to 0.90 ± 0.15%. As expected, Group 9, the challenge control group, had the highest mean % lung lesion score (10.81 ± 23.27%). In four groups, the mean % lung lesion scores were elevated due to one to three pigs in each of these groups having very high lung lesion scores. The lung lesions were red/purple and consolidated. Typically, lung lesions associated with PMWS are described as

tan, non-collapsible, and with interlobular edema. The lung lesions noted in this study were either not associated with PCV2 infection or a second pulmonary infectious agent may have been present. Within the context of this study, the % lung lesion scores probably do no reflect a true measure of the amount of lung infection due to PCV2. Likewise, tentative diagnosis of pneumonia may have been over-utilized as well. Any pigs with lung lesions, some as small as 0.10% were listed with a tentative diagnosis of pneumonia. In this example, there was no sufficient difference between groups with respect to gross lesions and % lung lesions on which to base vaccine efficacy.

IHC results showed the largest differences between groups. Group 1 (16 μg rORF2 – IMS 1314) had the lowest positive IHC results for PCV2 antigen (0%); while Groups 9 and 10 had the highest positive IHC results with incidence rates of 100% and 93.3% respectively. Groups 3, 5, 6 and 7, which received 16, 4, 1 or 0.25 μg of rORF2 antigen, respectively, adjuvanted with Carbopol, had IHC positive rates of 20%, 20%, 40% and 46.7%, respectively. Group 2, which received two doses of 16 μg vORF2 adjuvanted with Carbopol had an IHC positive rate of 6.7%, while Group 4 which received only one dose of the same vaccine, had an IHC positive rate of 13.3%. Due to the objective nature of this test and the fact that IHC results correlated with expected results, IHC testing is probably one of the best parameters on which to base vaccine efficacy.

Thus in one aspect of the present invention, the Minimum Protective Dosage (MPD) of PCV2 rORF2 antigen adjuvanted with Carbopol in the CDCD pig model in the face of a PCV2 challenge is determined. Groups 3, 5, 6 and 7 each received two doses of rORF2 antigen adjuvanted with Carbopol, but the level of rORF2 antigen varied for each group. Groups 3, 5, 6 and 7 each received 16, 4, 1 or 0.25 μg of rORF2 antigen respectively. In general, decreasing the level of rORF2 antigen decreased PCV2 antibody titers, and increased

the mortality rate, mean % lung lesions, and the incidence of IHC positive tissues. Of the four groups receiving varying levels of rORF2 – Carbopol, Groups 3 and 5, which received two doses of 16 or 4 μg of rORF2 antigen, respectively, each had an IHC positive rate of only 20%, and each had similar antibody titers. Overall, based on IHC positive results, the minimum protective dosage of rORF2 antigen administered twice is approximately 4 μg.

In another aspect of the present invention, the antigenicity of recombinant (rORF2) and VIDO R-1 (vORF2) PCV2 antigens were assessed. Group 2 received two doses of 16 μg vORF2 and Group 3 received two doses of 16 μg rORF2. Both vaccines were adjuvanted with Carbopol. Both vaccines were found to be safe and both had 0% mortality rate. Group 2 had a PCV2 antibody titer of 2507 on Day 25, while Group 3 had a PCV2 antibody titer of 1503. Group 3 had a lower mean % lung lesion score than Group 2 (0.11 ± 0.38% vs. 0.90 ± 0.15%), but Group 2 had a lower IHC positive incidence rate that Group 3 (6.7% vs. 20%). Overall, both vaccines had similar antigenicity, but vORF2 was associated with slightly better IHC results.

In yet another aspect of the present invention, the suitability of two different adjuvants (Carbopol and IMS 1314) was determined. Groups 1 and 3 both received two doses of vaccine containing 16 ug of rORF2 antigen, but Group 1 received the antigen adjuvanted with IMS 1314 while Group 3 received the antigen adjuvanted with Carbopol. Both groups had essentially the same ADWG, essentially the same incidence of clinical signs post-challenge, the same mortality rate, and essentially the same mean % lung lesions; but Group 1 had an IHC positive rate of 0% while Group 3 had an IHC positive rate of 20%. However, Group 3, which received the vaccine adjuvanted with Carbopol, had higher IFAT PCV2 titers on Days 25, 32, and 50 than Group 1, which received the vaccine adjuvanted with IMS 1314. Overall, although the PCV2 vaccine adjuvanted with IMS 1314 did provide better IHC results, it did

not provide overwhelmingly better protection from PCV2 infection and did induce injection site reaction. Whereas the PCV2 vaccine adjuvanted with Carbopol performed nearly as well as the IMS 1314 adjuvanted vaccine, but was not associated with any adverse events.

In still another aspect of the present invention, the feasibility of PCV2 ORF2 as a 1ml, 1 dose product was determined. Groups 2 and 4 both received 16 μg of vORF2 vaccine adjuvanted with Carbopol on Day 0, but Group 2 received a second dose on Day 14. Group 4 had a slightly higher ADWG and a lower mean % lung lesions than Group 2, but Group 2 had higher IFAT PCV2 titers on Day 25, 32 and 50, and a slightly lower incidence rate of IHC positive tissues. All other results for these two groups were similar. Overall, one dose of vORF2 adjuvanted with Carbopol performed similar to two doses of the same vaccine.

CLAIMS

1. Use of an immunogenic composition capable of eliciting or enhancing an immune response against porcine circovirus type 2 (PCV) and comprising recombinantly produced PCV2 ORF2 protein in the manufacture of a medicament for preventing one or more of the following symptoms associated with PCV2 infection:
nasal shedding, increased mortality rate, decreased average daily weight gain,
wherein the PCV2 ORF2 protein is to be administered once to a pig.

2. The use according to claim 1, wherein the recombinantly produced PCV2 ORF2 protein is baculovirus expressed PCV2 ORF2 protein.

3. The use according to claim 1 or 2, wherein the recombinantly produced PCV2 ORF2 protein has been obtained by a method, wherein
- susceptible cells are infected with a recombinant baculovirus vector containing PCV2 ORF2 DNA coding sequences,
- PCV2 ORF2 polypeptide is expressed by the recombinant baculovirus, and
- the expressed PCV2 ORF2 polypeptide is recovered from the supernate by filtration and inactivated.

4. The use according to any one of claims 1 to 3, wherein the recombinantly produced PCV2 ORF2 protein has been recovered from the supernate of *in vitro* cultured cells, wherein said cells were infected with a recombinant baculovirus vector containing PCV2 ORF2 DNA and expressing PCV2 ORF2 protein, and wherein said cell cultire was treated with 2 to 8 mM binary ethylenimine (BEI) to

inactivate the baculovirus vector, and an equivalent concentration of a neutralization agent.

5. The use according to any one of claims 1 to 4, the composition further comprising at least a portion of recombinant baculovirus vector expressing said PCV2 ORF2 protein.

6. The use according to claim 5, the composition further comprising a portion of cell culture supernate.

7. The use according to any one of claims 1 to 6, wherein the composition is a vaccine.

8. The use according to any one of claims 1 to 7, the composition comprising from 0.3 to 200 μg of the recombinantly produced PCV2 ORF2 protein per dose.

9. The use according to any one of claims 1 to 8, wherein the pigs are pigs of 2 weeks of age or older, but not older than 15 weeks of age.

10. The use according to any one of claims 1 to 9, wherein the composition further comprises an adjuvant.

11. The use according to claim 10. wherein the adjuvant can include aluminum hydroxide and aluminum phosphate, saponins, water-in-oil emulsion, oil-in-water emulsion, water-in-oil-in-water emulsion, or wherein the adjuvant is a compound

chose from the polymers of acrylic or methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative.

12. The use according to claim 10 or 11, the composition comprising 100 µg to 10 mg of adjuvant per dose.

13. The use according to any one of claims 1 to 12, wherein the immunogenic composition is substantially free of antigens or polypeptides from PCV2 other than PCV2 ORF2 protein.

14. The use according to claim 4, wherein the immunogenic composition further comprises an adjuvant listed in claim 11 and is stable over a period of 24 months.

15. The use according to any one of claims 1 to 14, wherein the medicament is to be administered intramuscularly.