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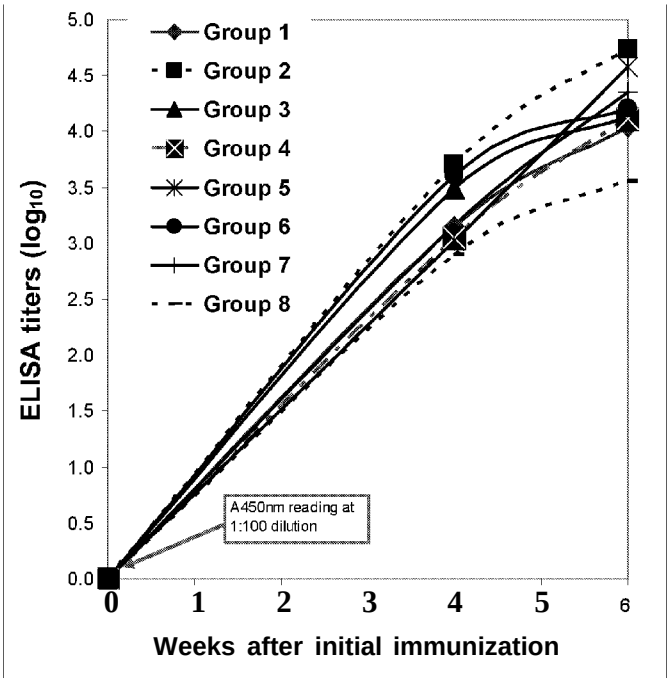
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(54) Title: DESIGNER PEPTIDE-BASED PCV2 VACCINE

Figure 2



(57) Abstract: Porcine circovirus (PCV2) vaccine compositions comprising a peptide antigen derived from a PCV2 capsid protein are described. In various embodiments, the peptide antigen contains amino acids of the capsid protein from about amino acid 47 to about amino acid 202. In some embodiments, the peptide antigen is optionally linked to an artificial T helper epitope and/or mixed with T helper epitopes derived from the ORF1 and ORF3 proteins of PCV2. Methods of using PCV2 vaccine compositions are also described. In various embodiments, a vaccine composition is used in animals for the prevention of PCV2 infection. In other embodiments, a PCV2 vaccine composition is used as an antigen for diagnosing PCV2 infection.

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DESIGNER PEPTIDE-BASED PCV2 VACCINE

FIELD OF THE INVENTION

This disclosure relates to a peptide-based vaccine against porcine circovirus type 2 (PCV2) and a method for the manufacture of such a vaccine for protecting piglets against PCV2 infection even in the face of short-lived but relatively high titers of maternally derived antibodies against PCV2.

Vaccine formulations according to various embodiments of the invention contain a peptide derived from open reading frame 2 (ORF2)-encoded capsid protein, which is optionally linked to an artificial T helper epitope to enhance its B cell immunogenicity, which can be optionally supplemented with a mixture of peptides derived from PCV2 ORF1 and ORF3 containing clusters of PCV2 T helper epitopes, prepared in a veterinarily acceptable delivery system as vaccine formulations.

BACKGROUND OF THE INVENTION

Porcine circovirus (PCV) was identified in 1974 as a picornavirus-like contaminant of a pig kidney tissue culture cell line (PK15) (1). In 1998, an antigenically and genetically distinct PCV was isolated from pig tissue and named *Porcine circovirus* type 2 (PCV2); PCV2 is associated with clinical disease in pigs (2).

Postweaning multisystemic wasting syndrome (PMWS), an emerging and economically important disease in swine, is caused by PCV2. However, evidence suggests that manifestation of the signs of PMWS requires co-infection with a pathogen such as *Porcine parvovirus* (PPV) or a similar immune stimulant, stress, or cofactor. This syndrome debilitates swine 7 to 15 weeks of age, with wasting, respiratory distress, enlarged lymph nodes, diarrhea, pallor, and jaundice. Also, gross and histologic lesions can affect multiple organ systems and are associated with interstitial pneumonia, lymphadenopathy, hepatitis, nephritis, myocarditis, enteritis, dermatitis, and pancreatitis (1,3).

Antibodies specific for PCV2 have been retrospectively detected in swine serum dating back to 1973 (4). Diagnosis of PMWS relies on the detection of either PCV2-specific nucleic acid or antigen associated with lesions in affected tissues. The virus has been isolated from heart, lung, liver, kidney, spleen, salivary gland, lymph node, thyroid, thymus, gastrointestinal tract, feces, pancreas, testes, and brain (1,5).

The primary route of transmission is unknown, but evidence suggests that PCV2 can

be transmitted both horizontally and vertically. It has been detected in ocular, nasal, and fecal samples from naturally infected swine. Isolation of PCV2 from aborted pig fetal tissue suggests vertical transmission. Detection of PCV2 nucleic acid in the semen of naturally and experimentally infected boars suggests transmission from boars to PCV2-narve gilts and their litters (5).

Porcine circovirus type 2 (PCV2) is a small nonenveloped virus with a circular single-stranded DNA (ssDNA) genome. The genome comprises six open reading frames (ORFs) encoding putative proteins (2) with four ORFs sharing significant homology with analogous ORFs from the nonpathogenic porcine circovirus type 1 (PCVI) (6). Only proteins encoded by ORF1, ORF2, and ORF3 have been detected in PCV2- infected cells. ORF1 encodes the 312 amino acid replicase protein and also gives rise to a 168 amino acid spliced variant, both of which are essential for PCV2 replication (7). The structural capsid protein is encoded by ORF2 (8); and ORF3 encodes a highly conserved protein that is not essential for replication but plays an important role in PCV2-induced apoptosis and pathogenicity (9).

Only the surface-exposed PCV2 ORF2-encoded capsid protein is capable of eliciting a protective antibody response by the B cells of the host's immune system (10). Consistent with that observation, PEPSCAN mapping studies localized clusters of immunodominant B cell epitopes with specificity to PCV2 primarily to areas on the PCV2 capsid protein, at residues 65-87, 113-139, 169-183, and 193-207 (11). In addition to B cell epitopes, immunodominant T cell epitopes, including T helper cell epitopes, are another important factor affecting the antigenicity of a virus. T helper cell epitopes elicit protective T helper cell (Th) responses that signal B cells to produce antibody. In contrast to the localization of B cell epitopes to the capsid protein (11), T cell epitope mapping of PCV2 with 20mer peptides and lymphocyte proliferation assays indicated that immunodominant Th responses are most consistently localized to epitopes on the nonstructural proteins of ORF1 and ORF3, whereas no linear Th epitopes encoded by ORF2 displayed immunodominance (12).

Presentation of Th determinants to the immune system by a synthetic peptide is a critical factor controlling the immunogenicity of a synthetic peptide. Th epitopes that are immunodominant and promiscuous are highly and broadly reactive in populations of divergent MHC types (13). Thus, the scarcity of immunodominant Th sites on the capsid protein helps to account for the limited immunogenicity of subunit capsid vaccines for PCV2. The immunogenicity of a peptide subunit immunogen can be strengthened by covalent

linkage of a targeted B cell epitope to selected foreign promiscuous Th sites, including a promiscuous Th epitope whose genetic responsiveness is enlarged through combinatorial chemistry (14, 15, 16). In addition, the immunopotency of a vaccine in swine and protection of swine from viral infection afforded by the vaccine can be influenced by multiple Th epitopes of the virus, and these can be cross-protective in combination with B cell epitopes from different proteins (17).

There is a need for a low cost highly specific vaccine that protects piglets against PCV2-related diseases such as PMWS. Conventional vaccines for pigs are based on inactivated whole PCV2 virus. However, PCV2 does not replicate to high titers in cell culture, making conventional vaccines costly and of low potency. Alternative vaccines are based on recombinant PCV2 ORF2 antigens. PCV2 ORF2 capsid subunits have been expressed in various expression systems including the baculovirus expression system in insect cells (3,10). In most applications, insect cells which produce the recombinant PCV2 capsid proteins are lysed and formulated into vaccines that are used to vaccinate piglets. Such recombinant antigens frequently contain host cell and vector-encoded antigens thus rendering the vaccine nonspecific and can cause severe untoward immune responses including anaphylaxis.

Another problem with inactivated virus PCV2 vaccines, and with subunit capsid vaccines either as recombinant virus lysates or as purified protein, is their relatively low potencies when used in normal field piglets. They are commonly tested in specific pathogen-free (SPF) piglets or cesarean-derived colostrum deprived (CDCD) piglets because those in the field often have maternally-derived antibodies (MDA). These maternal antibodies, while typically of titers too low and of too short duration to provide protection, are still high enough to interfere with vaccination. This interference by MDA can be partially overcome by administration of a high dose of recombinant ORF2 capsid antigen (18). A recombinant full-length ORF2 antigen was secreted into the medium by the baculovirus expression system disclosed in US2009/0022749A1 (19). This system partially alleviates problems of low yield and purity; however, this system and the other biological expression systems suffer from the inherently limited immunogenicity of full-length PVC2 antigen preparations, and they endure costly problems stemming from non-reproducibility, low yields, and complicated quality control protocols. Thus, it is desirable to develop a PCV2 vaccine through a process of rational design, through which immunogenic peptides are designed and synthesized. Such peptide-based vaccines can be designed to contain capsid-specific B cell epitopes, potent foreign Th epitopes and PCV2 Th epitopes derived from other PCV2 proteins, and

used in combination as the key ingredient of a vaccine formulation, to elicit anti-capsid antibody responses that precisely confront PCV2 infection in piglets.

The rational design of immunogenic peptides for a virus vaccine begins with the identification of immunodominant epitopes by epitope mapping. Epitope mapping employs a series of overlapping peptides corresponding to regions of interest on a targeted virus protein to identify sites which participate as immunogenic determinants in interactions with the immune system. Most commonly, epitope mapping employs peptides of relatively short length to precisely detect linear determinants. A fast method of epitope mapping known as "PEPSCAN" is based on the simultaneous synthesis of hundreds of overlapping peptides, coupled to solid supports. The coupled peptides are tested for their abilities to bind antibodies or to stimulate T cell proliferation. This approach is effective in localizing linear B and T cell determinants; however, the immunodominant B cell epitopes of a targeted virus or protein that are essential for vaccine development are usually long high affinity discontinuous epitopes that are difficult to define by the PEPSCAN method (20).

There is a need for the identification of immunogenic PCV2 peptides bearing long discontinuous epitopes which can be chemically synthesized in milligram to kilogram quantities by controlled and reproducible solid-phase peptide synthesis. This controlled commercial scale process for the synthesis of PCV2 capsid immunogens, together with the straightforward means to characterize such peptide products, would provide a framework for the low cost commercial scale manufacture and quality control of PCV vaccine formulations (21).

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BRIEF DESCRIPTION OF THE INVENTION

This disclosure relates to porcine circovirus type 2 (PCV2) vaccines comprising a peptide or peptide composition wherein the peptide or peptide composition is optimized by screening overlapping short and long PCV2 capsid peptides for serological reactivities. Such empirical findings detected a cluster of immunodominant B cell epitopes within the PCV2 capsid protein as exemplified by GenBank Accession Number AAN62766. Specifically, the cluster of immunodominant B cell epitopes was found on a 154 amino acid long peptide of the PCV2 capsid protein (peptide Capl, SEQ ID NO:l, also shown in Table 1). This peptide was incorporated into a peptide-based vaccine as a key immunogen to elicit protective antibody and cell-mediated immune responses against PCV2 infection and was augmented by additional foreign and PCV2-derived T cell epitopes. Said peptide immunogens are useful for the formulation of vaccines against PCV2.

Various embodiments of the invention relate to methods for the manufacture of such peptide, peptide composition and pharmaceutical formulation as vaccines for elicitation of immune responses that can protect piglets against PCV2 infection, and for the manufacture of diagnostic kits for detection of PCV2 infection.

Various embodiments of the invention are directed to vaccine formulations which contain the designed PCV2 Capl peptide (SEQ ID NO: 1), and homologues and analogues thereof, derived from ORF2 capsid protein. The PCV2 Capl peptide in these formulations is preferentially, but optionally, linked to an artificial combinatorial T helper epitope (SEQ ID NO: 2, also shown in Table 1) to enhance its B cell immunogenicity. The PCV2 Capl peptide can also be optionally mixed with a mixture of PCV2 ORF1 (SEQ ID NOS: 3 and 4) and ORF3 (SEQ ID NO: 5) peptides containing clusters of PCV2 Th epitopes (also shown in Table 1). Such peptide-based PCV2 vaccine formulations elicit PCV2-specific antibody responses that protect against PCV2 infection in piglets, including those that may have maternally derived antibodies (MDA) that may interfere with vaccination. Such designed peptide-based vaccines contain peptide compositions as immunogens that can be chemically synthesized at milligram to kilogram scales for industrial application, and be readily quality controlled.

Also provided by various embodiments of the invention are adjuvants and/or delivery vehicles and other ingredients routinely incorporated with vaccine formulations.

BRIEF DESCRIPTIONS OF THE FIGURES

Figure 1A is an illustration showing the mechanism for detecting antibodies to PCV2 capsid protein bound to capsid inside the nucleus of co-transfected HTK cell line cells by immunofluorescence according to an embodiment of the invention. The illustration shows HTK cells infected with T7 polymerase recombinant vaccinia virus (T7/vac) (22) and co-transfected with the pCR-orf2 plasmid by lipofectamine™ (Invitrogen). The recombinant capsid protein transports to the nucleus after it is translated (23). Anti-capsid antibodies, according to one embodiment of the invention, become bound to the nucleus of the transfected HTK host cells where they are detected by the immunofluorescence of a labeled secondary antibody. This method affords for detection of antibodies to authentic PCV2 capsid protein with high specificity through transient eukaryotic expression in the HTK cells of full length PCV2 capsid protein, mediated by the prokaryotic T7 promotor.

Figure IB is an Immunofluorescence Assay (IFA) carried out according to the mechanism described in Figure 1A. Co-transfected HTK cell line cells with antibodies to PCV2 capsid protein bound to capsid inside the nucleus are detected.

Figure 2 is a graph that reports the immunogenicity of piglets raised on a regular farm with endemic PCV2 infection that were immunized with eight Capl peptide-based PCV2 vaccine formulations and doses as described in Table 5. Immunogenicity was evaluated by PCV2 Capl peptide-based ELISA (titer in Log_{10}).

Figure 3A is a photograph of a kidney of an unvaccinated pig infected by PCV2. Lesions are bracketed (*).

Figure 3B is a photograph of the kidneys from an uninfected vaccinated pig, one sectioned (left), one intact (right). Light reflection from the flash is bracketed (**).

DETAILED DESCRIPTION OF THE INVENTION

Peptide antigens can detect immunological responses and certain peptide antigens may also stimulate immunological responses. Many peptide antigens can be used for the sensitive and specific detection of immune responses but most often they do not by themselves act as immunogens. Peptide immunogens are a special class of peptide antigens that can be used to stimulate immune responses as well as detect them. According to one embodiment of the invention, the peptide antigens in the PCV2 vaccine are peptide immunogens that have both B cell and T helper cell (Th) epitopes that together act to stimulate the generation of protective immune responses, in addition to being capable of

detecting immune responses to PCV2 infection.

One method for identification of B cell epitopes relies on a set of nested and overlapping peptides of multiple lengths, typically ranging from 20 to 60 residues or longer in length. These longer peptides are synthesized by a laborious series of independent solid-phase peptide syntheses, rather than by the rapid and simultaneous PEPSCAN syntheses. The resulting sets of nested and overlapping peptides can then be used in antibody binding studies and experimental immunizations to identify long peptides which best present immunodominant determinants, including discontinuous conformational B cell epitopes. This method has been utilized for the selection of a non-linear determinant on CD4 that is used in a vaccine to block binding by the gpl20 glycoprotein of HIV (24), and for the design of a potent peptide antigen from the G-H loop domain of the VPl protein of foot-and-mouth disease virus (25).

One embodiment of the invention provides for PCV2 ORF2-encoded capsid peptides that comprise a 154mer amino acid sequence (SEQ ID NO: 1, also shown in Table 1) having a cluster of B cell epitopes for optimal antibody recognition. These antigenic peptides were empirically identified and optimized using serum samples from PCV2-infected piglets and an ELISA immunoassay format. Any immunoassay format that can be adapted to an antibody capture phase comprising peptide antigens, e.g., ELISA, can be used to detect and quantitate antibodies that bind to a particular fragment of a PCV2 capsid protein in a blood, serum, or plasma sample from a PCV2-infected pig.

In a specific embodiment, an optimized PCV2 antigenic peptide of about 154 amino acids (SEQ ID NO: 1), which corresponds to amino acid residues 47-202 of a full-length PCV2 protein, presents immunogenic and antigenic sites of PCV2 capsid that are most useful both for the generation of protective antibodies by vaccination and for the detection of antibodies for diagnosis by ELISA. This highly antigenic and immunogenic capsid peptide was identified from among a collection of more than 50 overlapping peptides with lengths from 20 to 170 residues that were designed, synthesized and tested for reactivities to a panel of PCV2 positive sera from infected piglets. Among the more than 50 candidate peptide antigens tested, the PCV2 Capl antigenic peptide (SEQ ID NO. 1), was found to have a cluster of immunodominant B cell epitopes and had the most significant and consistent antigenicity for the PCV2 positive serum panel. The Capl peptide also was proven to be of high immunogenicity when tested in a trial of vaccines comprising this peptide sequence. The production and use of vaccines comprising a PCV2 Capl antigenic/immunogenic peptide

(e.g., SEQ ID NO: 1) are within the scope of various exemplary embodiments of the invention.

Specific embodiments of the PCV2 Capl immunogenic/antigenic peptide invention are further defined as being immunologically functional homologues of SEQ ID NO: 1 that have corresponding sequences and conformational elements from mutant and variant strains of PCV2. Homologous PCV2 Capl antigenic peptides have amino acid residues that correlate approximately with capsid protein positions 47-202 of the originating variant PCV2 strains. Such homologues are readily demonstrated through sequence alignment programs such as ClustalW (produced by Julie D. Thompson, Toby Gibson of European Molecular Biology Laboratory, Germany and Desmond Higgins of European Bioinformatics Institute, Cambridge, UK. Algorithmic). Table 2 shows the alignment by ClustalW of eleven Capl antigen sequences taken from diverse strains of PCV2 identified by GenBank Accession numbers. The originating PCV strains of the Capl homologues aligned in Table 2 include viruses of genotypes 2a, 2b, 2d, l/2a, and these diverse strains were isolated from animals in Taiwan, China, U.S.A, Canada, Brazil, Spain, Germany and Denmark. Table 2 also exemplifies a homologue as being a consensus Capl sequence wherein the amino acids assigned to the variable positions are those most frequently applied for those positions. In one embodiment, the homologue has an amino acid sequence from about amino acid position 47 to about amino acid position 202 of a PCV2 capsid protein.

Homologues of the invention are further defined as having at least 80% identity to SEQ ID NO: 1. In one embodiment, the variant strain homologue has at least 85% identity to SEQ ID NO: 1. In another embodiment, the variant strain homologue has at least 90% identity to SEQ ID NO: 1. In yet another embodiment, the variant strain homologue has at least 95% identity to SEQ ID NO: 1.

Other embodiments of the invention provide for immunologically functional analogues of the PCV2 Capl antigenic peptide. An immunologically functional analogue of the Capl peptide includes variants of SEQ ID NO: 1 and homologues which retain substantially the same antigenicity and immunogenicity as the original antigenic peptide. For example, variants that are functional analogues of SEQ ID NO: 1 or of a homologue can have a conservative substitution in an amino acid position; a change in overall charge; a covalent attachment to another moiety; or small additions, insertions, deletions or conservative substitutions and/or any combination thereof. Thus, antibodies that bind to a PCV2 Capl antigenic peptide (e.g., SEQ ID NO: 1) will also bind to the immunologically

functional analogues of that PCV2 Cap1 antigenic peptide with substantially similar efficacy. In one embodiment, the functional analogue has at least 50% identity to SEQ ID NO: 1 or homologue. In another embodiment, the functional analogue has at least 80% identity to SEQ ID NO: 1 or homologue. In yet another embodiment, the functional analogue has at least 85% homology to SEQ ID NO: 1 or homologue. In still another embodiment, the functional analogue has at least 90% homology to SEQ ID NO: 1 or homologue.

In one embodiment, immunologically functional analogues of the PCV2 Capl antigenic peptide encompasses versions of PCV2 Capl antigenic peptide that have been modified by conservative substitutions, and by insertions or deletions. In this embodiment, immunologically functional analogues can be modified from SEQ ID NO: 1 or from a homologue of SEQ ID NO: 1 by substitutions that are conservative.

Conservative substitutions are when one amino acid residue is substituted for another amino acid residue with similar chemical properties. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine; the polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; the positively charged (basic) amino acids include arginine, lysine and histidine; and the negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In another embodiment, immunologically functional analogues can be modified by amino acid additions to the N-terminus, C-terminus, and/or by insertions into the middle of the peptide. In various embodiments of the invention, additions are to the N-terminus or C-terminus of the peptide. Additions can be of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues. Such additions may constitute amino acid sequences which are not present in PCV2 capsid and which do not alter the immunogenicity of the PCV2 capsid portion of the peptide. Additions which are not present in PCV2 capsid include, but are not limited to, small charged sequences (e.g., lysine-lysine-lysine), amino acids that enable the formation of branched structures (e.g., εN-lysine) or enable the formation of cyclized structures (e.g., cysteine). In an embodiment of the invention, additions of amino acid sequences that are not present in PCV2 Capl are of 5 amino acids or less. Amino acid additions can be either classical or non-classical amino acids or a mixture thereof.

In another specific embodiment, immunologically functional analogues can be modified by amino acid deletions to the N-terminus, C-terminus, and/or middle of the peptide.

In various embodiments, deletions are to the N-terminus or C-terminus of the peptide. Deletions can be of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues. In a specific embodiment, deletions of amino acid sequences are of 10 amino acids or less.

In another embodiment, immunologically functional analogues of PCV2 Capl antigenic peptide encompass PCV2 cap antigenic peptides that have been modified by an alteration in charge. Such alteration in charge may be the result of amino acid substitutions, additions, or deletions, or the covalent attachment of a charged molecule. The alteration in charge may have the result of making the peptide more basic, more acidic, or more neutral as compared to the unmodified peptide. In a specific embodiment, the peptide is made more basic by the addition of 1-5 lysine residues to the N-terminus or C-terminus. In a more specific embodiment, the peptide is made more basic by the addition of 3 lysine residues to the N-terminus.

By way of a non-limiting example, immunologically functional analogues of the peptide of the invention can have from 1 to about 5 additional amino acids (classical and non-classical) added to the terminal amino acids. For example, the sequence Lys-Lys-Lys can be added to the amino terminus of this PCV2 Capl peptide for a change in charge.

The peptides can be readily synthesized using standard techniques, such as the Merrifield solid phase method of synthesis and the myriad of available improvements on that process. The peptides can also be made using recombinant DNA technology. As such, nucleic acid molecules encoding the PCV2 Capl antigenic peptide and immunologically functional analogues of the PCV2 Capl antigenic peptide and compliments thereof are encompassed by various exemplary embodiments of the invention. Vectors, especially expression vectors, comprising the nucleic acid molecules encoding PCV2 Capl antigenic peptides and immunologically functional analogues are also encompassed by various exemplary embodiments of the invention. Host cells containing the vectors are also encompassed by various exemplary embodiments of the invention.

Various exemplary embodiments of the invention also encompass methods of producing the PCV2 Capl antigenic peptide and immunologically functional analogues of the PCV2 Capl antigenic peptide. For example, the method can comprise incubating a host cell containing an expression vector comprising a nucleic acid molecule encoding a PCV2 Capl antigenic peptide and/or immunologically functional analogue of an PCV2 Capl antigenic peptide under such conditions that the PCV2 Capl peptide and/or immunologically

functional analogue of a PCV2 Capl peptide is expressed. This embodiment can use controlled and well-defined immunogens derived from the lysates or secretions of such cells.

One embodiment of the invention provides peptide compositions produced by solid-phase synthesis. The quality of antigens produced by the chemical process of this embodiment are controlled and defined and, as a result, reproducibility of antigenicity, immunogenicity and yield can be assured. Also, no biohazardous materials are used in the manufacture of peptide antigens, reducing risks and eliminating the need for expensive biological containment. As site-specific immunogens presenting high molar concentrations of selected epitopes, both the safety and immunopotency of the vaccine employing PCV2 Capl antigenic peptide compositions are assured.

In one embodiment, the peptides of the invention are synthesized. The use of defined Capl synthetic peptides minimizes the false-positive results when used as antigen for antibody detection and diagnosis in piglets. The use of defined synthetic peptides, having known B cell and Th epitopes, as immunogens eliminates the undesired non-PCV2-specific immune responses caused by the presence of antigenic materials originating from PCV2-infected or recombinant virus-infected host cells and from recombinant protein expression systems that may be co-purified with PCV2 virus and/or recombinant proteins, when used as the immunogenic ingredients of a vaccine. For example, sera from pigs may have antibodies to host cells, or to recombinant *Escherichia coli,* yeast or baculovirus which are then cross-reactive with the antigenic materials used in diagnostic tests based on the biologically-derived antigens, and such immune responses generated by vaccines having these extraneous immunogens as ingredients will be non-protective. In contrast, pigs receiving a PCV2 Capl peptide vaccine of the invention will generate focused immune responses devoid of untoward antibodies and other immune responses to proteins originating from host cells or expression vectors, e.g., proteins from recombinant *Escherichia coli,* yeast or baculovirus that had been co-purified with the biologically-derived PCV2 antigens.

This embodiment of synthetic peptides also minimizes interference from impurities that are generated during production. With long syntheses, despite the rigorous control of coupling efficiency, peptide analogues are also produced due to events during elongation cycles, including amino acid insertion, deletion, substitution, and premature termination, thus yielding to the generation of multiple peptide analogues along with the targeted peptide syntheses. Nonetheless, such peptide analogues are still suitable in peptide preparations as contributors to antigenicity and immunogenicity when used in immunological application

either as solid phase antigen for purpose of immunodiagnosis or as immunogen for purpose of vaccination.

During 25 years of experience in immunological applications of synthetic peptides, we have found that the range in structural variability that allows for retention of an intended immunological activity is far more accommodating than the range in structural variability allowed for retention of a specific drug activity by a small molecule drug or the desired activities and undesired toxicities found in large molecules that are co-produced with biologically-derived drugs. This is why peptide analogues, either intentionally designed or inevitably produced by errors of the synthetic process as a mixture of deletion sequence byproducts that have chromatographic and immunologic properties similar to the intended peptide, are frequently as effective as a purified preparation of the desired peptide. Designed analogues and unintended analogue mixtures are effective as long as a discerning QC procedure is developed to monitor both the manufacturing process and the product evaluation process so as to guarantee the reproducibility and efficacy of the final products employing these peptides.

In other embodiments of the invention, Th peptides are included in the vaccine compositions. The presence of Th peptides can improve immunogenicity of the PCV2 Capl peptide vaccine. PCV2 Capl peptides (including the homologues and analogues described above) can be covalently linked to and/or mixed with Th epitopes.

In one embodiment, Th peptides having clusters of immunodominant PCV2 Th epitopes from ORF 1 and ORF 3, described as SEQ ID NOS: 3-5 (also shown in Table 1) and unlinked to the Capl immunogen, can be used to supplement the immunogenicity of PCV2 Capl peptide vaccines. Including SEQ ID NOS: 3-5 as free peptides, without covalent linkages, can improve immunogenicity of the vaccine formulations.

In another embodiment, PCV2 Capl peptides (including homologues and analogues described above) can be covalently linked, with or without a spacer, to a peptide containing a sequence known to contain a Th epitope. This embodiment can offer enhanced immunogenicity over the equivalent Capl immunogens without the covalently linked Th epitope. In a specific embodiment, the peptide containing the Th epitope is covalently linked to the N-terminus and/or C-terminus of the PCV2 peptide. In another specific embodiment, the spacer has the sequence Lys-Lys-Lys-eNLys (SEQ ID NO: 7), also shown in Table 1. In an embodiment, the peptide containing the Th epitope is covalently linked to the amino terminus of the PCV2 Capl peptide. In a specific embodiment, the peptide

containing the Th epitope is the artificial combinatorial Th peptide SEQ ID NO:2 (as shown in Table 1) linked to the amino terminus through a Lys-Lys-Lys-eNLys spacer, and presented as SEQ ID NO: 6 (also shown in Table 1).

Various embodiments of the invention relate to vaccine compositions for protecting pigs against PCV2. In exemplary embodiments, the vaccine comprises an immunogenic peptide antigen and an acceptable delivery vehicle or adjuvant. In various embodiments, the PCV2 vaccine composition, comprises a peptide antigen and a veterinarily acceptable delivery vehicle or adjuvant, wherein the peptide antigen comprises an amino acid sequence selected from the group consisting of:

a) from about amino acid position 47 to about amino acid position 202 of a PCV2 capsid protein;
b) SEQ ID NO: 1;
c) a homologue of (b);
d) an antigenically and immunologically functional analogue of (a), (b), or (c);
e) (a), (b), (c), or (d) having at least one conservative amino acid substitution, amino acid addition, and/or amino acid deletion; and
f) any combination of (a)-(e).

In an embodiment of the PCV2 vaccine, the charge of the peptide antigen is altered by adding or deleting 1 to 5 amino acids. In another embodiment of the PCV2 vaccine, the antigenically and immunologically functional homologue or analogue has at least 80% identity to the antigen of the amino acid sequence that is approximately positions 47-202 of a PCV2 capsid protein. In a particular embodiment, the peptide antigen has an amino acid sequence selected from the group consisting of SEQ ID NOs 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20.

In another embodiment of the PCV2 vaccine the peptide antigen further comprises a T helper epitope covalently linked to the N-terminus or C-terminus of the peptide antigen. In a specific embodiment, the T helper epitope is covalently linked to the amino terminus of the peptide antigen. In another specific embodiment, the T helper epitope is covalently linked to the peptide antigen through a spacer having at least one amino acid. In a particular embodiment, the T helper epitope is SEQ ID NO: 2. In yet another particular embodiment, the spacer is SEQ ID NO: 7. In a specific embodiment, the peptide antigen is SEQ ID NO: 1 or SEQ ID NO: 6.

In various exemplary embodiments, any amount of immunogenic peptide antigen can

be used to elicit immune responses in the animal. In a particular embodiment, the amount of peptide antigen is between about 0.1 μg to about 100 mg. In another particular embodiment, the amount of peptide antigen is between about 1 μg to about 10 mg. In yet another embodiment, the amount of peptide antigen is between about 10 μg to about 1 mg.

In various embodiments of the PCV2 vaccine composition the composition further comprises an equimolar mixture of three PCV2 T helper epitope peptides of SEQ ID NOS: 3, 4, and 5. In a specific embodiment, the amount of the equimolar mixture of SEQ ID NOS: 3, 4, and 5 is between about 0.1 μg to about 1 mg. In a more specific embodiment, the amount of the equimolar mixture of SEQ ID NOS: 3, 4, and 5 is between about 1 μg to about 100 μg.

In various exemplary embodiments, any type or amount of delivery vehicle or adjuvant can be used. In a particular embodiment, the delivery vehicle and adjuvant is Montanide™ ISA 50V2 (an oil vaccine adjuvant composition comprised of mineral oil and mannide oleate for production of water-in-oil emulsions), Tween® 80 (also known as: Polysorbate 80 or Polyoxyethylene (20) sorbitan monooleate), a CpG oligonucleotide, and/or any combination thereof.

In a specific embodiment, the PCV2 vaccine composition, comprises a peptide antigen of SEQ ID NO: 6 and a veterinarily acceptable delivery vehicle or adjuvant, wherein the amount of peptide antigen is between about 10 μg to about 1 mg.

Another embodiment of the invention relates to a method for protecting piglets that are or are not PCV2 MDA positive against PCV2 infection, comprising administering a vaccine encompassed by any of the exemplary embodiments as described above.

A PCV2 Capl peptide prepared in accordance with the present disclosure can also be used to detect PCV2 antibodies by using the peptide in an antigenically effective amount in the capture phase of an immunoassay, e.g., in the solid phase immunosorbent of ELISA test kits. In accordance with an embodiment of the present invention, any compatible immunoassay format can be used with the subject peptides. Such formats are well known to the ordinarily skilled artisan and have been described in many standard immunology manuals and texts, see for example Harlow et al.1988 (26). These include, among other well-known immunoassay formats, an enzyme-linked immunoadsorbent assay (ELISA), an enzyme immunodot assay, an agglutination assay, an antibody-peptide-antibody sandwich assay, a peptide-antibody-peptide sandwich assay. In an embodiment, the immunoassay is an ELISA using a solid phase coated with a PCV2 Capl peptide composition.

According to one embodiment of the invention, the peptide is capable of testing sera

from sows and gilts, boars and barrows, and piglets for PCV2 infection by a screening ELISA, for the evaluation of sera from pre-vaccinated piglets for levels of maternally derived anti-PCV2 antibodies, and for determining the levels of immune responses in vaccinated piglets towards a vaccine employing PCV2 Capl antigenic peptide, full-length capsid protein , or inactivated PCV2 virions.

In a specific embodiment, an ELISA immunoassay can be used to test swine blood, serum or plasma samples for the presence of anti-PCV2 antibodies comprising the steps of:

i. attaching a PCV2 Capl peptide to a solid support,

ii. exposing said peptide attached to said solid support to a swine blood, serum or plasma sample containing antibodies, under conditions conducive to binding of the antibody to the peptide, and

iii. detecting the presence of antibodies bound to said peptide attached to said solid support.

In an exemplified use of the subject ELISA kit, a pig serum sample to be tested is diluted in sample diluent and then contacted with one or more of the PCV2 Capl peptides described above for a time and under conditions for any antibodies, if present, to bind to the peptide-sensitized solid phase. After removal of unbound material (e.g., by washing with phosphate-buffered-saline), the secondary complex is contacted with labeled antibodies to pig-specific IgG or labeled protein A, protein G, or protein A/G. These antibodies or proteins A, G or A/G bind to the secondary complex to form a tertiary complex and, since the second antibodies or proteins A, or G or A/G are labeled with a reporter molecule, when subjected to a detecting means, the tertiary complex is detected. The reporter molecule can be an enzyme, radioisotope, fluorophore, bioluminescent molecule, chemiluminescent molecule, biotin, avidin, streptavidin or the like. For ELISA the reporter molecule is preferably an enzyme.

The following examples serve to illustrate the present invention and are not to be used to limit the scope of the invention.

Table 1

Amino acid sequences of PCV2 immunogenic peptides derived from PCV2 ORF1 , ORF2 and ORF3 proteins, and an artificial combinatorial Th peptide for enhancement of ORF2 Cap1 peptide immunogenicity. Combinatorial sequences are shaded.

B cell epitope cluster peptide derived from PCV2 ORF2 capsid protein (Cap1 peptide)	
TIU-SRTFGYTVKATWRTPSWAVDl^I^NISDFVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVT KATALTYDPYVNYSSRHTIPQPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSANVDHVGLGT	(SEQ ID No. 1)
Artificial combinatorial Th peptide (UBITh3)	
ISISEIKGVIVHKIETILF *T RT TR*	*(SEQ ID No. 2)*
T helper epitope cluster peptides derived from PCV2 ORF 1 Rep protein	
CHIEKAKGTDQQNKEYCSKE	(SEQ ID No.3)
KWWDGYHGEEVWIDDFYGW	(SEQ ID No. 4)
T helper epitope cluster peptide derived from PCV2 ORF3 Apo protein	
PRWPHNDVYIGLPITLLHFP	(SEQ ID No. 5)
B cell epitope cluster peptide derived from PCV2 ORF2 capsid protein (Cap1 peptide) linked through spacer to artificial combinatorial Th peptide (UBITh3)	(SEQ ID NO. 6)
ISISEIKGVIVHK**JETILF**-KKKeK-TRLSRTFGYTVKATTVRTPSWAVDMMRFNISDFVPPGGGTNKISIPFEYYRIRKVKVE *T RT TR* **FTOCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYWYSSR^ TIPQPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWLRLQT SANVDHVGLGT**	

TABLE 2

Alignments for homologous Cap1 sequences from various PCV2 strains and a consensus. Variable positions are shaded.

Access No.	Country*	47	130
ACC59783	US	TRL	3RTFGYT1KRTTVRTPSWAVDMMRFNINDFLPPGGGSNPRS1PFEYYRIRKVKVEFWPCSPITQGDRGVGS1AVILDDNFV
ACQ99546	DK	TRL	BRTFGYTIKRTTVITPSWAVDMMRFNINDFLPPGGGSNPRSLPFEYYRIRKVKVEFWPCSPITQGDRGVGSLAVILDDNFV
ACX47360	DE	TRL	3RTFGYT|IKRTTVITPSWAVDMMRFNINDFLPPGGGSNPRSIPFEYYRIRKVKVEFWPCSPITQGDRGVGS|AVILDDNFV
ACV53396	ES	----	-RTFGYTVKRTPVRTPSWAVDMMRFNINDFLPPGGGSNHRS||PFEYYRIRKVKVEFWPCSPITQGDRGVGS|AVILDDNFV
ADI44325	CN	TRL	SRTIGYTVKKTRVRTPSWIVDMMRFNINDFLPPGGGSNPLTIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFV
ADD25772	CN	TRL	SRTIGYTVKKTRVRTPSW||VDMMRFNINDFLPPGGGSNPLT|PFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFV
ADB97547	CN	|RL	SRTFGYTVKRTRVITPSWAVDMMRF|I|DFIPPGGGTNKIS|PFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFV
ABW76695	BR	TRL	SRTFGYTVKRTRVITPSWAVDMMRF|I|DFIPPGGGTNKIS|PFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFV
ABI2 9888	US	SRL	SRTFGYTVKATTviTPSWAVDMMRFN|iDF||pPGGGTNKIslPFEYYRIRKVKVEFWPCSPITQGDRGVGSlA|lLDDNFV
YP_003422531	CA	TRL	SRTFGYTVKATTVRTPSWAVDMMRFNI|DF||PPGGGTNKIS1PFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFV
AAN62766	TW	TRL	SRTFGYTVKATRVRTPSWAVDMMRFNINDF|PPGGGTNKIS|PFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFV
consensus		TRL	SRTFGYTVKRTPVRTPSWAVDMMRFNINDFLPPGGGSNHRSIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFV

		131	200	SEQ ID NO
ACC59783	US	TKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTlGNVDHVGLGT		9
ACQ99546	DK	TKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTIGNVDHVGLGT		10
ACX47360	DE	TKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLW!RLQTSRNVDHVGLGT		11
ACV53396	ES	TKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWLRLQT|GNVDHVGLGT		12
ADI44325	CN	TKA||ALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLD|TIDYFQPNNKRNQLWLRLQT|GNVDHVGLGT		13
ADD25772	CN	TKA||ALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLD1TIDYFQPNNKRNQLWLRLQT|GNVDHVGLGT		14
ADB97547	CN	|KA||ALTYDPYVNYS|RHTI|QPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLW|R|QTSRNVDHVGLGT		15
ABW76695	BR	IKAIALTYDPYVNYSSRHTIIQPFSYHSRYFTPKPVLDSTIDYFQPNNKINQLWLRLQTSRNVDHVGLGT		16
ABI2 9888	US	|KATAiTYDPYVNYSSRHTl|QPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWiRLQTSRNVDHVGLGT		17
YP_003422531	CA	TKATALTYDPYVNYSSRHTI|QPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLW|RLQTSRNVDHVGLGT		18
AAN62766	TW	TKATALTYDPYVNYSSRHTI!QPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSANVDHVGLGT		19
Consensus		TKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSANVDHVGLGT		20

*US- United States of America, DK- Denmark, DE- Germany, ES- Spain, CN- China, BR- Brazil, CA- Canada, TW- Taiwan

EXAMPLE 1

IMMUNOFLUORESCENCE ASSAY USING CAPSID PROTEIN INSIDE THE NUCLEUS OF CO-TRANSFECTED HTK CELLS FOR DETECTION OF ANTIBODIES TO PCV2

Antibodies directed against PCV2 can be detected by immunofluoresence using HTK cells co-transfected with PCV2 capsid protein. This method provides for transient expression of full length PCV2 capsid antigen in the HTK cells. This immunoassay method also provides for capsid protein expression through recombinant virus infection, while ensuring preservation of native capsid protein conformation during synthesis and maturation stages, providing conditions for antibody detection of high specificity and sensitivity.

HTK cell line cells were infected with T7 polymerase recombinant vaccinia virus (T7/vac) (22), and co-transected with the pCR-orf2 plasmid by lipofectamine™ (Invitrogen) (23) according to the mechanism illustrated in Figure 1A. Specifically, construction of the pCR-orf2 plasmid for expression of the native PCV2 capsid protein mediated by the T7 polymerase promoter was accomplished as follows: A full length of PCV2 ORF2 gene (from Taiwan PCV2 strain Cyc08; Accession No. AAN62766 with an N→S mutation at position 77) was amplified by polymerase chain reaction (PCR) and cloned into the pCR®2.1-TOPO® plasmid vector (Invitrogen). The expression ability of the pCR-orf2 plasmid was confirmed by sequencing which shows the full nucleotide sequence for ORF2 from PCV2 strain Cyc08 (23).

HTK cells were grown to 80% confluence in 96-well plates, infected with T7 polymerase recombinant vaccinia virus (T7/vac) (22), and then co-transected with the pCR-orf2 plasmid by lipofectamine™ (Invitrogen). The pCR-orf2 plasmid co-transfected cells were found to reliably express PCV capsid antigen within the nucleus of HSK cells and thus were suitable to be used for the capture and detection of antibodies to PCV2 capsid protein through immunofluorescence assay (IFA).

Titration of PCV2 antibody by immunofluorescence assay (IFA).

Plates with native PCV2 capsid protein as antigen were prepared in batch by coating with the transfected HTK cells, and stored at -80°C. Antigen coated plates were prepared in batches of 100 each time to ensure quality control. Serum samples were initially diluted 50-fold in PBS followed by a 2-fold serial dilution series. For each test run, a positive

control serum from a PCV2-infected SPF pig and negative control serum from an uninfected SPF pig were both included to validate the expression of the capsid protein by the pCR-orf2 plasmid within the nuclei of the HSK cells. Serum samples giving fluorescence signals localized to the nuclei (as shown in Figure IB) at dilutions higher than 1:50 were scored as having IFA titers >50; and these titers were indicative of animals infected by PCV2 or of animals having developed anti-PCV antibodies as a result of vaccination. Observation by IFA can also facilitate the differentiation of true positive antibody signals from false positive signals by distinguishing specific nucleus-localized anti-capsid antibody binding from non-specific protein binding.

All testing of serum samples collected from either pigs with natural infection or from pigs given PCV2 Capl peptide-based vaccine were performed under code.

EXAMPLE 2

ELISA METHOD FOR DETECTION OF SERUM ANTIBODIES FOR IDENTIFICATION OF NATURALLY PCV2-INFECTED PIGS AND PIGS GIVEN CAP1 PEPTIDE-BASED PCV2 VACCINES

The wells of 96-well plates were coated separately for 1 hour at 37°C with 100 μL of individual PCV2 capsid peptides, e.g., peptide of SEQ ID NO.:l (PCV2 Capl peptide), at 2 μg/mL in lOmM $NaHC0_3$ buffer, pH 9.5 unless noted otherwise.

The peptide-coated wells were incubated with 250 μL of 3% by weight of gelatin in PBS in 37°C for 1 hour to block non-specific protein binding sites, followed by three washes with PBS containing 0.05% by volume of TWEEN® 20 and dried. Pig serum positive for PCV2 antibody by IFA and negative control sera were diluted 1:20, unless otherwise noted, with PBS containing 20% by volume normal goat serum, 1% by weight gelatin and 0.05% by volume TWEEN® 20. One hundred microliters of the diluted specimens were added to each of the wells and allowed to react for 60 minutes at 37°C.

The wells were then washed six times with 0.05% by volume TWEEN® 20 in PBS in order to remove unbound antibodies. Horseradish peroxidase-conjugated goat anti-swine IgG was used as a labeled tracer to bind with the antibody/peptide antigen complex formed in positive wells. One hundred microliters of the peroxidase-labeled goat anti-swine IgG at a pre-titered optimal dilution and in 1% by volume normal goat serum with 0.05% by volume

TWEEN® 20 in PBS, was added to each well and incubated at 37°C for another 30 minutes. The wells were washed six times with 0.05% by volume TWEEN® 20 in PBS to remove unbound antibody and reacted with 100 μE of the substrate mixture containing 0.04% by weight 3',3',5',5'-Tetramethylbenzidine (TMB) and 0.12% by volume hydrogen peroxide in sodium citrate buffer for another 15 minutes. This substrate mixture was used to detect the peroxidase label by forming a colored product. Reactions were stopped by the addition of 100 μE of 1.0M H_2SO_4 and absorbance at 450 nm (A_{450}) determined.

Serum dilutions were done in accordance with the purpose for detecting PCV2 antibodies in the animal sera: (a) For identification of potential natural infection, a dilution of 1:20 was used, the A_{450} reading was recorded, and a built-in intrinsic negative control for cutoff calculation was used; or (b) For the determination of antibody titers of pigs that received peptide-based PCV2 vaccine formulations, 10-fold serial dilutions of sera from 1:10 to 1:10,000 were tested, and the titer of a tested serum, expressed as Log_{10}, was calculated by linear regression analysis of the A_{450}.

The serological validity of the Cap 1-based ELISA for detection of PCV2 infection was confirmed by the high correlation coefficient (0.782 shown in Table 3) between the results for the ELISA and for the highly specific native capsid-based IFA (Example 1).

EXAMPLE 3

SITE-SPECIFIC SEROLOGY FOR IDENTIFICATION OF OPTIMAL PCV2 CAPSID PEPTIDES FOR USE IN BOTH VACCINE AND DIAGNOSTIC APPLICATIONS

The genomic sequences of PCV2 from the previously published sequence of PCV2 isolate 1010-Stoon (2) and from a Taiwan strain Cyc08 were used to deduce the protein sequences from open reading frames, and the data obtained from the two ORF2 sequences were used to design candidate peptide antigens for immunological testing. Over 70 capsid peptides with overlapping sequences of lengths from about 20 to over 170 amino acids were designed and synthesized for serological validation, with an aim to identify antigenic peptides that are targets for B and T cell recognition associated with PCV2 infection. Capsid peptides having optimal B cell epitopes were then identified by a process of serological validation. This method for epitope mapping with long and short peptides identifies discontinuous conformational epitopes as well as linear epitopes (24, 25). A panel of 24

sera characterized with respect to anti-PCV2 capsid protein reactivities by the IFA test described in Example 1 was used for the serological validation of the peptides. The sera were used to screen the overlapping PCV2 capsid peptides for strong and consistent antigenicities that could be useful for diagnosis and for prediction of antibody-mediated protective immunogenicities. The sera used to construct this PCV2 reactivity panel were collected from pigs in the field found to have natural infection or from normal and SPF pigs known to be free of PCV2 infection, each having had their respective anti-PCV2 capsid protein reactivity titered by IFA as shown in Table 3.

TABLE 3

Sera from piglets with or without PCV2 infection used for the serological validation of Capl peptide ELISA by correlation to capsid IFA titers

Swine Serum Code	Host description	IFA titer	Cap1 Peptide ELISA (A_{450})
RS 3	SFP	< 50	0.251
RS 4	SFP	< 50	0.204
RS 6	SFP	< 50	0.134
RS 8	SFP	< 50	0.284
RS 9	SFP	< 50	0.285
RS 10	SFP	< 50	0.192
RS 12	SFP	< 50	0.179
RS 13	SFP	< 50	0.171
RS 15	SFP	< 50	0.233
NS 5	Normal serum	< 50	0.123
NS 6	Normal serum	< 50	0.140
NS 9	Normal serum	< 50	0.245
RS 28	Field	200	0.259
RS 24	Field	400	0.354
RS 25	Field	800	0.528
RS 26	Field	800	0.479
RS 1	SFP	1600	0.794
RS 39	Field	1600	0.492
RS 7	SFP	3200	0.633
RS 16	SFP	3200	1.129
RS 42	PCV2-infected	3200	3.000
RS 43	PCV2-infected	3200	2.782
RS 41	PCV2-infected	6400	3.000
RS 45	PCV2-infected	12800	2.657
Correlation coefficient		**0.782**	

A panel including 12 PCV2 IFA positive (IFA titer >1:50) swine sera and 12 PCV2 IFA negative (titer < 1:50) swine sera was used for serological validation of the PCV2 Capl (SEQ ID NO: 1) peptide-based ELISA. Sera were diluted 1:21 for ELISA testing. A correlation coefficient of 0.782 was found between IFA titers and the A_{450} readings from the peptide-based ELISA.

Among the overlapping peptides, short peptides comprising about 20 amino acids were synthesized by an Applied BioSystems Inc. automated peptide synthesizer Model 433 (Lexington, KY); and longer peptides comprising from about 25 up to about 170 amino acids were synthesized using Applied BioSystems Peptide Synthesizer Models 430A, 431 and 433, using Fmoc chemistry.

Each peptide was produced by an independent synthesis on a solid-phase support, with Fmoc protection for the terminus and side chain protecting groups of trifunctional amino acids. Completed peptides were cleaved from the solid support and side chain protecting groups removed by 90% trifluoroacetic acid. Synthetic peptide preparations were characterized for correct composition by Matrix-Assisted Laser Desorption Time-Of-Flight (MALDTOF) Mass Spectrometry, and for content including synthesis profile, content and concentration by Reverse Phase HPLC. With long syntheses, despite the rigorous control of coupling efficiency, peptide analogues were also produced due to events during elongation cycles, including amino acid insertion, deletion, substitution, and premature termination, thus yielding to the generation of multiple peptide analogues along with the targeted peptide syntheses. Nonetheless, such peptide analogues were still suitable in peptide preparations as contributors to antigenicity and immunogenicity when used in immunological application either as solid phase antigen for purpose of immunodiagnosis or as immunogen for purpose of vaccination. Typically, such peptide analogues, either intentionally designed or generated through synthetic process as a mixture of byproducts, are frequently as effective as a purified preparation of the desired peptide, as long as a discerning QC procedure is developed to monitor both the manufacturing process and the product evaluation process for guaranteeing the reproducibility and efficacy of the final products employing these peptides.

Antigenicities of the synthesized peptides of varied lengths were tested for initial reactivity profiles with the serum panel. The antibodies present in the serum panel from the naturally infected pigs followed a trend of increasing reactivities to PCV2 capsid peptides of increasing lengths. As a consequence of extensive serological screening data acquired with the sera in the lower panel of Table 3, a long capsid peptide designated as "Capl" (SEQ ID NO: 1) of length 154 amino acids, provided for an unusually sensitive ELISA. The Capl peptide also imparted high specificity to this ELISA as shown in the upper panel of Table 3 by low reactivities with the IFA-validated negative pig sera. In fact, the predominant serological reactivities of the PCV-infected pigs were associated only with the Capl 154mer peptide (SEQ ID NO. 1). It is therefore reasonable to assume that a majority of the

serological reactivities present in the PCV2-infected pigs are due to antibodies recognizing clusters of B cell epitopes that were present only on the long peptide. Furthermore, the unexpected immunodominant antigenicity of the 154mer is consistent with the long peptide representing a large exposed surface on the capsid protein that presents processions of long continuous and discontinuous epitopes. These epitopes comprise B cell epitopes that are linked to T helper cell (Th) epitopes requisite for the stimulation of the B cell antibody responses.

EXAMPLE 4

IMMUNIZATIONS OF GUINEA PIGS WTH VACCINES FORMULATED WITH PCV2 CAP1 PEPTIDE FOR INITIAL IMMUNOGENICITY EVALUATION AND CORRELATION OF ANTIBODY TITERS IN VACCINEES BY CAP1 PEPTIDE-BASED ELISA AND NATIVE CAPSID-BASED IFA

Guinea pig immunization.

In an exemplified use of vaccines of the invention, vaccines having as immunogens peptides comprising the Capl antigenic peptide without linkage to a foreign Th epitope (for example SEQ ID NO: 1) or with covalent linkage through the amino terminus and a Lys-Lys-Lys-eNLys (SEQ ID NO: 7) spacer to a foreign T helper epitope such as UBITh®3 (SEQ ID NO: 2), for example SEQ ID NO: 6, were formulated into water-in-oil emulsions using a commercially available oil vaccine delivery vehicle, Montanide ISA 50V2. Two groups of guinea pigs were immunized with the peptide-based vaccines as shown in Table 4.

This was an initial study conducted in small animals to evaluate the immunogenicity of the Capl peptide (SEQ ID NO: 1) with and without covalent linkage to a foreign T helper site, UBITh®3 (SEQ ID NO: 2), in a commonly used water-in-oil emulsion formulation to obtain a serum panel with a range of antibody responses. The unlinked Capl peptide is designated as SEQ ID NO: 1 (Table 1). The Capl peptide linked on the amino terminus to the foreign T helper site through a spacer is designated as SEQ ID NO: 6 (Table 1). Two experimental groups of three Duncan Hartley guinea pigs (female, 9 weeks old, 450 gm, virus-free) each were used in this immunogenicity study of PCV2 Capl vaccine formulations, as shown in Table 4.

TABLE 4

Immunogenicity evaluation of Cap1 PCV2 vaccines in hosts by comparison of anti-capsid antibody titers determined by Capl peptide ELISA (Titer Logio) and anti-capsid protein IFA

				ELISA		IFA titer
				0 wpi	4 wpi	
Grp	Immunogen	Dose	Animal ID #	A_{450} at 1/100	Titer Log_{10}	4 wpi
A	PCV2 Cap1 (SEQ ID NO: 1)	Prime: 25µg/0.25 ml/IM Boost: 25µg/0.25 ml/IM (in ISA 50V2 1:1 v/v)	3963	0.050	4.108	100
			3964	0.055	3.280	
			3965	0.051	3.347	
			Avg	**0.052**	**3.578**	
			SD	**0.002**	**0.376**	
B	UBITh3-KKK-εK-PCV2 Cap1 (SEQ ID NO: 6)	Prime: 25µg/0.25 ml/IM Boost: 25µg/0.25 ml/IM (in ISA 50V2 1:1 v/v)	3957	0.053	4.349	400
			3958	0.056	4.404	
			3959	0.053	4.051	
			Avg	**0.054**	**4.268**	
			SD	**0.001**	**0.155**	

Each animal was immunized intramuscularly. For group A, peptide PCV2 Capl (SEQ ID NO: 1) was used as the immunogen which was formulated in a water-in-oil emulsion having Montanide™ ISA 50V2 (Seppic, Paris France), an oily adjuvant composition of mannide oleate and mineral oil commonly used for swine vaccines, emulsified with equal volumes of the aqueous phase peptide solution in PBS. For the vaccine given to group B, the Capl peptide was covalently linked at the amino terminus to the artificial combinatorial UBITh®3 T helper epitope (SEQ ID NO: 2) through a spacer of sequence Lys-Lys-Lys-eNLys (SEQ ID NO: 7) to bridge the targeted PCV Capl B cell epitopes and the UBITh®3 T cell epitope, resulting in the peptide immunogen designated as SEQ ID NO: 6 (Table 1), and formulated into the water-in-oil emulsion as was used for group A. Guinea pigs of groups A and B were administered 25 μg doses by the intramuscular route on weeks 0 (prime) and 2 (boost). The guinea pigs were bled on 0 and 4 weeks post-initial immunization (wpi) for immunogenicity testing of sera by both ELISA and IFA. As discussed below, a vaccine having the peptide immunogen without the covalently linked spacer and artificial UBITh®3 (SEQ ID No:l) elicited anti-Capl antibody responses; and, the use of the linked UBITh®3 T helper epitope, as in SEQ ID No: 6, resulted in more impressive anti-capsid titers for evidence of improved immunogenicity, as evaluated by either assay method (Table 4).

Immunogenicity evaluation.

Immunogenicity testing of the designed PCV2 Capl peptide vaccine formulations was accomplished by target peptide-based ELISA using PCV2 peptide Capl (SEQ ID NO: 1) as solid phase antigen that had been coated on the wells at 2 μg/mL in 100 μE per well. Experimental animal sera serially diluted from 1:10 to 1:10,000 were tested and positive titers were expressed as Logio of the reciprocal dilution. Seropositive samples were pooled by group and immunogenicity results for the pools were determined by the Capl ELISA and by the IFA method (immunofluorescense assay). By this means, ELISA immunogenicities evaluated by reactivity to the Capl peptide were compared to the IFA titers evaluated by reactivity to full-length naturally configured PCV2 capsid protein expressed inside HTK cell line cells (Figures 1A and IB).

Results

The results for group A show that the PCV2 Capl peptide (SEQ ID NO: 1) in the water-in-oil emulsion was immunogenic on its own without linkage to the artificial Th epitope (SEQ ID NO: 2) at the 25 μg dose. Addition of the artificial combinatorial Th epitope through the multi-lysine spacer enhanced immunogenicity by an estimated five-fold (4.268-3.578= 0.69 Logio in titer) as detected by ELISA. In a positive correlation with the Capl -based ELISA results which serve to validate the Capl ELISA, the native capsid-based IFA method for determining immunogenicity for groups A and B also indicated an improved response to the artificial Th epitope-linked PCV2 peptide (SEQ ID NO: 6) over the Capl peptide by itself (SEQ ID NO: 1), although the less precise immunofluorescence assay indicated only a four-fold improvement, 100 vs. 400, for the chimerical SEQ ID NO: 6 immunogen. Similarly, piglets immunized with either the Capl peptide without (SEQ ID NO: 1) or with (SEQ ID NO: 6) linkage to the UBITh®3 T helper epitope (SEQ ID NO: 2) also display strong anti-Capl antibody responses with responses significantly improved by linkage to the SEQ ID NO: 2 T helper epitope (Example 5, Table 5).

The more immunogenic SEQ ID NO: 6 chimerical peptide was then tested in guinea pigs for its upper range of immunogenicity by administrations at a higher dosage with an additional adjuvant. A priming dose of 400 μg, in which the peptide immunogen was complexed with a CpG oligonucleotide adjuvant (SEQ ID No: 8) (27) in the amount described in Table 5, was injected followed by a booster dose of 100 μg of the CpG-augmented immunogen at 2 wpi. Immunogenicity exerted by this CpG-augmented higher dosage vaccination protocol yielded an unexpected ten-fold enhancement in immunogenicity by ELISA, and, consistent with that increase in ELISA titer, there was a 16-fold enhancement by IFA. That is, in comparison to the pooled group B IFA titer of 400 shown in Table 4, the guinea pig group immunized by the higher dosage CpG-augmented vaccine protocol reached an impressive IFA titer of 1600.

EXAMPLE 5

IMMUNOGENICITY EVALUATION OF PVC2 CAP1 PEPTIDE VACCINE FORMULATIONS AT VARYING DOSES IN PIGLETS RAISED ON A REGULAR FARM

Piglet immunizations.

As shown in Table 5, 80 piglets at 4 weeks of age, on a regular not specific pathogen-free (SPF) farm, were divided into eight groups (ten piglets/group). These groups were immunized intramuscularly at weeks 0 and 4 with vaccines homogenized into Montanide ISA 50V2 as 1:1 (v/v) water-in-oil emulsions containing either 50 μg of peptide Capl (SEQ ID NO:l) and Tween® 80; or 50 to 400 μg of peptide Capl linked to the UBITh®3 artificial Th epitope (SEQ ID NO: 6). The varying doses of the SEQ ID NO: 6 immunogen were complexed (27) or not complexed with the CpG oligonucleotide (SEQ ID NO: 7), before being formulated into the water-in-oil emulsions, in the presence or absence of Tween® 80 (Table 5).

Blood samples for ELISA analysis were collected at the time of first vaccination, and at 4 and 6 wpi. Individual sera were prepared and examined for PCV2 antibodies by ELISA as described in Examples 2 and 3. Sera from each group were pooled and subjected to further ELISA testing.

Results

Immunogenicity results by ELISA for the serum pools are shown by group in Table 5 and Figure 2. All vaccine formulations were immunogenic even at the low 50 μg dose. Every piglet in each of the groups gave individual immune responses with ELISA titers of 1:1,000 or higher by weeks 4 and 6, and at over 1:10,000 by week 6 for the group pools, with the exception of the group 8 piglets that were given the unenhanced immunogen Capl peptide (SEQ ID NO: 1) at low 50 μg doses for both prime and boost.

TABLE 5

Immunogenicity evaluation of PCV2 Capl peptide vaccines. Vaccines varied by T helper epitope, peptide dose, and adjuvant formulation, administered to piglets raised on a regular farm, evaluation by Capl ELISA

Grp No.[#]	Immunogen Description	Immunogen amount (µg/mL)	Additional Adjuvant[§]	0 wpi (A_{450nm} at 1/100 dil)	ELISA titers (Log_{10})	
					4 wpi	6 wpi
1	UBITh3-KKK-εK- Cap1*	Prime: 400µg/1.0 mL Boost: 100µg/0.25mL	CpG1 at 1.2:1[‡]	0.168	3.154	4.036
2	UBITh3-KKK-εK- Cap1*	Prime: 200µg/2mL Boost: 200µg/2mL	0.1% Tween 80	0.104	3.705	4.734
3	UBITh3-KKK-εK- Cap1*	Prime: 200µg/2mL Boost: 200µg/2mL	none	0.134	3.488	4.128
4	UBITh3-KKK-εK- Cap1*	Prime: 100µg/1mL Boost: 100µg/1mL	CpG1 at 1.2:1[‡] and 0.1% Tween 80	0.096	3.045	4.116
5	UBITh3-KKK-εK- Cap1*	Prime: 100µg/1mL Boost: 100ug/1mL	0.1% Tween 80	0.120	3.027	4.576
6	UBITh3-KKK-εK- Cap1*	Prime: 50µg/0.5mL Boost: 50µg/0.5mL	0.1% Tween 80	0.090	3.603	4.203
7	UBITh3-KKK-εK- Cap1*	Prime: 50µg /0.5mL Boost: 50µg/0.5mL	none	0.153	3.157	4.350
8	Cap1[†]	Prime: 50µg /0.5mL Boost: 50µg /0.5mL	0.1% Tween 80	0.104	2.901	3.554

[#] Pooled sera used for each group

* SEQ ID NO: 6

[†] SEQ ID NO: 1

§ All vaccines were 1:1 v/v water-in-oil emulsions with peptide dissolved in PBS or peptide/CpG complexes suspended in PBS as aqueous phase, Montanide™ ISA 50V2 as oil phase and primary adjuvant, and indicated additional adjuvants.

*Charge ratio of cationic peptide: anionic oligonucleotide.

Sera from individual pigs from weeks 0, 4, 6, and 10 were also evaluated for anti-capsid antibodies by IFA as described in Example 1. The IFA titers were found to be in the range of 400 to 1600 in most of the animals by week 10 with a few sera from group 3 having IFA titers as unexpectedly high as 6,400 after receiving two shots of the vaccine. No injection site reactogenicity was found in any of these pigs.

Among the 80 immunized piglets, eight were found to have maternally derived antibodies (MDA) as detected by IFA prior to the first immunization at week 0, as shown in Table 6. MDA are typically present in piglets raised on a regular farm in Taiwan and they typically act to inhibit anti-PCV capsid responses. In all eight piglets with MDA, anti-Cap 1 antibodies of increasing titers were detected by ELISA between weeks 4 and 6 regardless of the doses and formulations given (Table 6). Thus, all eight animals with MDA had successful vaccine takes to the PCV2 Capl vaccine formulations despite interference by the presence of maternally-derived antibodies. This serves as evidence for the focused nature of the response to the vaccine of the invention.

IFA titers for immune sera collected 10 weeks after initial immunization from MDA-positive animals 26, 27, 86, and 87 had unprecedented IFA titers of 6400, 3200, 3200 and 3200, respectively. Comparable piglets having MDA were included for PCV2 vaccine studies with the Ft. Dodge/Wyeth (now Pfizer Animal Health) Suvaxyn® vaccine for PCV2, an inactivated virus vaccine, and with the Boehringer Ingleheim Inglevac® CircoFLEX® vaccine, a recombinant full length PCV2 ORF2 capsid protein vaccine. The piglets responded to those vaccines with IFA titers that did not develop beyond a usual range of 50 - 100, with only 5 - 10% reaching IFA titers up to 400. The "focused" nature of the responses to designed PCV2 Capl peptide immunogen thus differentiates the peptide-based vaccines from the already available types of PCV2 vaccines.

TABLE 6

Vaccine takes in piglets having maternally derived antibodies (MDA) prior to immunization by PCV2 Capl peptide-based vaccines at various doses.

Individual Animal Ear Tag No.	IFA Titer*	Doses (µg per dose)		Vaccine Take ELISA Titer (Log_{10})	
	0 wpi	Prime	Boost	4 wpi	6 wpi
7	200	400	100	3.039	3.750
26	100	200	200	2.948	3.812
27	200	200	200	2.988	3.982
29	50	200	200	3.749	4.081
68	400	50	50	2.446	3.939
85	50	100	100	3.690	4.608
86	50	100	100	2.720	3.939
87	100	100	100	3.090	4.499

* MDA

Throughout the study period, the survival rate among the 80 pigs vaccinated with two shots of the various peptide CapI PCV2 vaccines was 93% while the survival rate for the 400 unvaccinated pigs housed in the same barn in this regular farm was 82%. The 93% survival rate for the vaccinees is a large improvement over the average survival rate of 80% for the 5000 pigs raised in the other housing facilities on this regular farm. This remarkable reduction in expected loss, an unintended result for this immunogenicity study, has further demonstrated the efficacy of CapI peptide-based PCV2 vaccines.

In summary, the CapI peptide PCV2 vaccines have shown significant immunogenicities from just a single dose as shown by ELISA titers at week 4, and have shown excellent immunogenicities by week 6 after two shots, with IFA titers reaching as high as 6400 in animals from group 3. All eight animals found to have prior maternally derived antibodies had immunogenic responses to the CapI vaccines regardless of the dose and formulation they received. The designed peptide-based PCV2 vaccines provided for a survival rate among vaccinated regular (non-SPF) piglets of 93% in comparison to the survival rate of 82% for the 400 unvaccinated piglets housed in the same building and in comparison to the 80% survival for another 5000 pigs housed in the other facilities of this regular farm, thus providing evidence for significant economic benefit.

EXAMPLE 6

CAP1 PEPTIDE PCV2 VACCINE IMPROVED FOR ONE SHOT PROTECTION BY ADDITION TO THE FORMULATION OF CLUSTERED T HELPER CELL PEPTIDES FROM THE ORF1 AND ORF3 PROTEINS OF PCV2

Immunogenicity of the CapI peptide PCV2 vaccine was further improved by incorporating the PCV2 T cell epitope peptides encoded by ORF 1 and ORF 3 and shown in Table 1. These epitopes were initially tested by Stevenson et al (12) for their induction of T cell proliferation in swine. PCV2 CapI peptide (for example SEQ ID NOS: 1 or 6) mixed with an equimolar combination of the three short PCV2 Th peptides (SEQ ID NOS.: 3, 4 and 5) at molar ratios from 1:1 down to 1:0.1 to 1:0.05 were formulated in the water phase prior to emulsion preparation. A PCV2 CapI vaccine formulation supplemented by these immunodominant PCV2 Th epitopes was tested on piglets to determine the effect on the immune response through the stimulation of PCV2-related T cell responses, including cytokine production, after a single shot of the vaccine.

Results

As shown in Table 7, significantly enhanced IFA titers were observed in piglets given a lower dose PCV2 Capl vaccine formulation with the supplemental PCV2 Th peptides. IFA titers of 50 to 400 were developed by week 4 in the group 10 piglets given a single dose of vaccine having 50 μg of Capl peptide immunogen with linked UBITh®3 (SEQ ID NO: 6) mixed with 20 μg of the three PCV2 T helper peptide immunogens (SEQ ID NOS: 3, 4, 5), in comparison to the IFA titers of <50 to 50 seen in the group 9 piglets at week 4 who received a single dose of a vaccine having 200 μg of the SEQ ID NO: 6 Capl immunogen without the three additional PCV2 Th peptide immunogens.

Therefore, a vaccine formulation containing a predominantly B cell epitope-clustered PCV2 capsid peptide (SEQ ID NO: 6) in combination with the equimolar mixture of immunodominant Th peptides from other PCV2 proteins (SEQ ID NOS.: 3, 4 and 5) provided for immunogen sparing by allowing for a lower total dose of immunogen content while maintaining balanced B cell antibody responses and T cell responses, and thereby exerting effective protective immune responses against infection with lower doses of peptide immunogen. Significant immunogen-sparing results by the three supplemental PCV Th peptides were also obtained when a Capl immunogen without linked UBITh®3 (SEQ ID NO: 1) was the target B cell immunogenic peptide in comparable vaccine formulations with and without the supplemental Th immunogens (Table 8). In this outcome, the dose having 50 μg of SEQ ID NO: 1 with 10 μg of the supplemental Th peptides was more effective than 100 and 200 μg doses of SEQ ID NO: 1 without the three supplemental Th peptides. Such combination Capl vaccine formulations should be particularly effective when supplied as an inexpensive low dose one shot vaccine intended for emergency use.

TABLE 7

Induction of IFA antibody titers against PCV2 capsid after single immunization of piglets with Capl peptide vaccines without or with PCV2 ORFl and ORF3 Th epitope peptides

			IFA Titer		
Grp No.	**Vaccine Formulation**	**Animal ID**	**0 wpi**	**2 wpi**	**4 wpi**
9	200 µg SEQ ID NO: 6 in 2 mL (1:1 v/v emulsion with ISA 50V2)	154-03	<50	<50	**<50**
		154-07	<50	50	**50**
		152-03	<50	<50	**<50**
		152-10	<50	<50	**<50**
		152-04	<50	<50	**<50**
10	50 µg SEQ ID NO: 6 + 20 µg SEQ ID NOS: 3, 4 and 5 at equal weights in 1mL (1:1 v/v emulsion with ISA 50V2)	139-01	<50	<50	**200**
		139-02	<50	<50	**50**
		139-05	<50	<50	**200**
		139-13	<50	<50	**200**
		139-15	<50	<50	**200**
		139-03	<50	<50	**400**
		139-04	<50	50	**200**
		139-10	<50	<50	**400**
		139-12	<50	<50	**200**
		139-14	<50	<50	**400**

EXAMPLE 7

CAP1 PEPTIDE PCV2 VACCINE TESTED FOR ABILITY TO PROVIDE PROTECTION TO PIGLETS FROM VIRUS TRANSMISSION AND PATHOGENICITY CAUSED BY EXPOSURE TO PCV2-INFECTED PIGS

A field trial was designed to evaluate the ability of Cap1 peptide PCV2 vaccines to elicit protective immunity in a variety of doses and formulations. SPF (specific pathogen-free) piglets that were exposed to PCV2 by being housed together with otherwise pathogen-free piglets carrying PCV2 infection were vaccinated with Capl vaccines. These conditions, designed for testing the vulnerability of specially housed transgenic pigs to infection by the natural transmission of PCV2, are ideally suited for assessing the protective immunity provided by a PCV2 vaccine.

Housin2 conditions for PCV2-infected and 2erm-free animals

The facility used for the field trial is the "Transgenic Animal Testing Station" (TATS) at Xiang Hill Ranch in northern Taiwan. TATS is used for the safety testing of transgenic pigs being kept under quarantine prior to intentional release to the field. It is managed by the Department of Animal Medicine of the Animal Technology Institute of Taiwan (ATIT) (23). The laboratories, animal housing, and animal management practices are in compliance with the guidelines of ISO/IEC 17025:2005 and ISO9001 (SGS TW07/03565). The transgenic pigs of the farm are routinely monitored for SPF status by observation and routine tests for Classical Swine Fever, pseudorabies virus, porcine reproductive and respiratory syndrome virus, hog cholera virus, atrophic rhinitis, Mycoplasma hyopneumoniae, foot-and-mouth disease virus, Swine Dysentery, Scabies, toxoplasma and Actinobacillus pleuropneumoniae. Routine monitoring found no evidence for the presence of these infectious diseases and agents.

In addition, testing for PCV2 infection by the recently developed IFA test described in Example 1 has been used for the past two years, and this pig population was found to have widespread PCV2 infection. The PCV2 positive rate of this farm, as determined by serological surveillance, has reached as high as 89.4% (47/52). These otherwise SPF animals have PCV2 as the only circulating pathogen.

Conditions for PCV2 vaccination and exposure

The selection of germ-free piglets from this "PCV2 only" environment began by identifying expectant sows that were negative for PCV2 antibodies first in serum and later in colostrum, by PCV2 IFA titer. Piglets from sows with serum and colostrum both negative were enrolled in the trial at about four weeks of age. At this time, the germ-free piglets were randomly divided into five groups, five piglets per group for vaccine groups 11-13, 15 piglets for vaccine group 14, and six piglets for control group 15. The vaccine groups were then administered the PCV2 Capl vaccine priming doses described in Table 8 (100 or 200 μg of SEQ ID NO: 1, or either 50 μg of SEQ ID NO: 1 + 50 μg of SEQ ID NOS: 3, 4, and 5 in equal weights or 50 μg of SEQ ID NO: 1 + 10 μg of SEQ ID NOS: 3, 4, and 5 in equal weights) by intramuscular injection (i.m.). At 4 weeks post-initial immunization (wpi), the groups were given booster doses of the same vaccines. Antibody responses were monitored by IFA at 2, 6, and 10 wpi. The IFA titers, seen in Table 8, were at baseline level for all groups at 2 wpi. High antibody responses were achieved for all vaccinated groups by week 6 although immunogenicity varied for the groups at 6 and 10 wpi. Titers for the control group remained at baseline throughout the period of serological testing.

The germ-free piglets, in both the vaccinated and control groups, were housed together in a barn with the general population of "PCV2 only" transgenic pigs for about two months, putting them at risk for transmission of PCV2. At the end of this period, when the weights of all the experimental pigs had reached approximately 120 kg liveweight (at 5 - 6 months age), the animals were sacrificed and examined for evidence of PCV2 infection by necropsy.

Signs of PCV2 infection were subtle and many pathological lesions that appeared during PCV2 infection were not found after recovery during the two month exposure period. Therefore, a necropsy procedure, one that provides for more sensitive diagnosis than the usual more direct methods used to examine the vaccinated animals of a vaccine/challenge study or field trial for signs of infection by the transmitted pathogen, was used as the method for detecting infection in the vaccinated/exposed animals. Necropsy examinations included observations of lymph nodes, lung and kidney. Little evidence for infection was found except for signs of lesions on the kidneys of control animals. White spots and streaks were retained as signs of fibrosis on the surfaces of kidneys of unvaccinated, infected animals, as shown in Figure 3A (*), while such signs marking a history of lesions were absent from the kidneys of vaccinated pigs scored as having no lesions as shown in Figure 3B (the dull white

streak seen in Figure 3B is reflected light (**)).

The pathology results scored from the necropsies on the experimental pigs, shown in Table 9, were obtained with white spots on the kidney capsule being the predominant indication of prior infection. This trial was conducted with PCV2 peptide vaccine formulations under code.

Results and conclusions

The results of PCV2 antibody titers by PCV 2 IFA for both of control and vaccine testing groups, Table 8, show increasing antibody responses over time in the vaccinated animals with higher responses by 10 wpi. The immunogenicities of the Capl peptide-based PCV2 vaccine formulations varied and the most immunogenic was the formulation given to the largest group, group 14.

Three out of six pigs in the control group (50%) were found to have white spots on the kidney capsule as the typical lesions marking a history of PCV2 infection (Figure 3A), with one of the control pigs having slight lesions and two having moderate lesions. In contrast, only two out of 30 vaccinated pigs (6.7%) displayed pathological changes, and these were both scored for having slight evidence of kidney pathology (Table 9). These two were found only among the largest group of vaccinated pigs, group 14 with 15 pigs. No pathological changes at all were noted for lymph nodes and lungs in the vaccinated pigs, with only slight kidney lesions noted for two of the vaccinees. Thus, protective efficacy is demonstrated for multiple formulations and doses of the peptide-based PCV2 vaccine of this disclosure.

In the specification, the inventors have not attempted to exhaustively enumerate all possible variations. That alternate embodiments may not have been presented for a specific portion of the invention, and may result from a different combination of described portions, or that other undescribed alternate embodiments may be available for a portion, is not to be considered a disclaimer of those alternate embodiments. It will be appreciated that many of those undescribed embodiments are within the literal scope of the following claims, and others are equivalent. Furthermore, all references, publications, U.S. Patents, and U.S. Patent Application Publications cited throughout this specification are hereby incorporated by reference as if fully set forth in this specification.

TABLE 8

Geometric mean antibody titers by IFA for each group of pigs after immunizations with Capl peptide vaccines with or without PCV2 ORFl and ORF2 T epitope peptides

Grp No.	Vaccine Description*†	No. of Animals	IFA Titer		
			2 wpi	6 wpi	10 wpi
11	SEQ ID NO:1 200 µg/2 mL	5	10	48	66
12	SEQ ID NO:1 100 µg/1 mL	5	10	60	105
13	50µg SEQ ID No: 1 + 50µg SEQ ID NOS: 3, 4, 5 in equal ratio /1mL	5	10	100	100
14	50µg SEQ ID No: 1 + 10µg SEQ ID NOS: 3, 4, 5 in equal ratio /1mL	15	10	127	174
15	Control	6	10	10	10

* All formulations were 1:1 v/v water-in-oil emulsions with Montanide™ ISA 50V2 as adjuvant.

† Vaccine given i.m. in two shots with priming dose given at 0 wpi and booster dose at 4 wpi.

TABLE 9

Observations of PCV2 pathology in the experimental pigs

Grp No.	Vaccine Description*	No. of Animals	Animal No.	Score†
11	SEQ ID NO:1 (200 µg/2 mL)	5	154-03	0
			154-07	0
			152-03	0
			152-10	0
			152-04	0
12	SEQ ID NO:1 (100 µg/1 mL)	5	135-01	0
			135-02	0
			135-03	0
			135-10	0
			135-14	0
13	50µg SEQ ID No: 1 + 50µg SEQ ID NOS: 3, 4, 5 in equal ratio /1mL	5	135-04	0
			135-11	0
			135-12	0
			135-13	0
			135-15	0
14	50µg SEQ ID No: 1 + 10µg SEQ ID NOS: 3, 4, 5 in equal ratio /1mL	15	141-04	0
			140-11	0
			140-01	0
			140-02	1
			140-10	0
			140-12	0
			141-01	0
			141-05	0
			140-14	0
			140-03	0
			154-02	0
			154-06	0
			154-05	0
			152-02	0
			152-01	1
1-4 total		30	**2/30=6.7% infected**	
15	Control	6	140-04	0
			139-11	2
			146-03	2
			146-11	1
			146-12	0
			146-10	0
5 total		6	**3/6=50% infected**	

*Same two dose vaccinations as described in Table 8.

†Score: 0 = no lesions, 1 = slight, 2 = moderate, 3 = severe

CLAIMS:

1. A porcine circovirus type 2 (PCV2) vaccine composition, comprising a peptide antigen and a veterinarily acceptable delivery vehicle or adjuvant,

wherein the peptide antigen comprises an amino acid sequence selected from the group consisting of:

a) from about amino acid position 47 to about amino acid position 202 of a PCV2 capsid protein;

b) SEQ ID NO: 1;

c) a homologue of (b); and

d) any combination of (a)-(c).

2. The PCV2 vaccine according to claim 1, wherein the peptide antigen comprises the amino acid sequence of SEQ ID NO:l.

3. The PCV2 vaccine according to claim 1, wherein the homologue comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 9-19 and 20.

4. The PCV2 vaccine according to any of claims 1 to 3, wherein the charge of the peptide antigen is altered by adding or deleting 1 to 5 amino acids.

5. The PCV2 vaccine according to any of claims 1 to 4, further comprising a T helper epitope covalently linked to the amino terminus of the peptide antigen.

6. The PCV2 vaccine according to claim 5, wherein the T helper epitope is covalently linked to the amino terminus of the peptide antigen through a spacer having at least one amino acid.

7. The PCV2 vaccine according to any of claims 5 to 6, wherein the T helper epitope is SEQ ID NO:2.

8. The PCV2 vaccine according to any of claims 6 or 7, wherein the spacer is SEQ ID NO:7.

9. The PCV2 vaccine according to any of claims 1 to 8, further comprising an equimolar mixture of three PCV2 T helper epitope peptides of SEQ ID NOS: 3, 4, and 5.

10. The PCV2 vaccine according to any of claims 1 to 9, wherein the total amount of peptide antigen is between about 10 μg to about 1 mg.

11. The PCV2 vaccine according to any of claims 9 to 10, wherein the total amount of the equimolar mix of SEQ ID NOS: 3, 4, and 5 is between about 1 μg to about 100 μg.

12. The PCV2 vaccine according to any of claims 1 to 11, wherein the delivery vehicle and adjuvant is selected from a group consisting of Montanide ISA 50V2, Polyoxyethylene (20) sorbitan monooleate, and a CpG oligonucleotide.

13. The PCV2 vaccine according to any of claims 1 to 12, where in the peptide antigen is SEQ ID NO: 1 or SEQ ID NO: 6.

14. A PCV2 vaccine composition, comprising a peptide antigen of SEQ ID NO: 6 and a veterinarily acceptable delivery vehicle or adjuvant, wherein the amount of peptide antigen is between about 10 μg to about 1 mg.

15. A method for protecting piglets that are or are not PCV2 MDA positive against PCV2 infection, comprising administering a vaccine according to any of claims 1 to 14.

16. A method for diagnosing PCV2 infection comprising the steps of

a) attaching a peptide antigen to a solid support, wherein the peptide antigen comprises an amino acid sequence selected from the group consisting of:
   i) from about amino acid position 47 to about amino acid position 202 of a PCV2 capsid protein;
   ii) SEQ ID NO: 1;
   iii) a homologue of (ii); and
   iv) any combination of (i)-(iii);
b) exposing said peptide attached to said solid support to a swine blood, serum or plasma sample containing antibodies, under conditions conducive to binding

of the antibody to the peptide, and

c) detecting the presence of antibodies bound to said peptide attached to said solid support.

Figure 1

Fig 1A

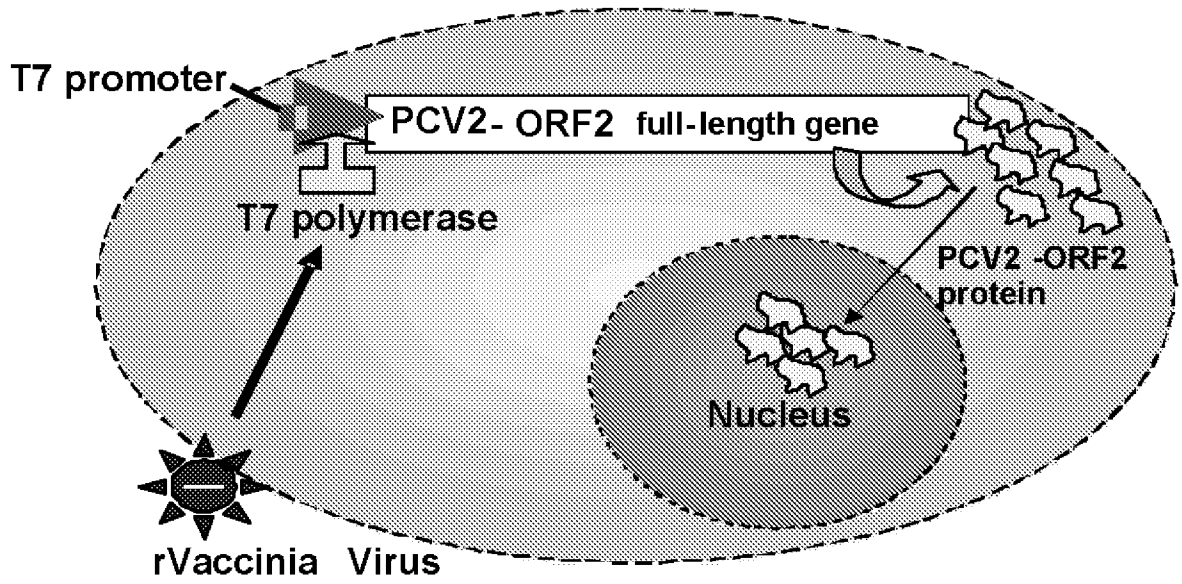


Fig 1B

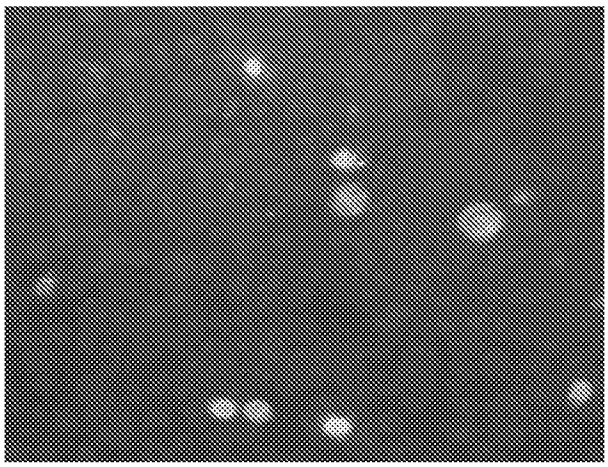


Figure 2

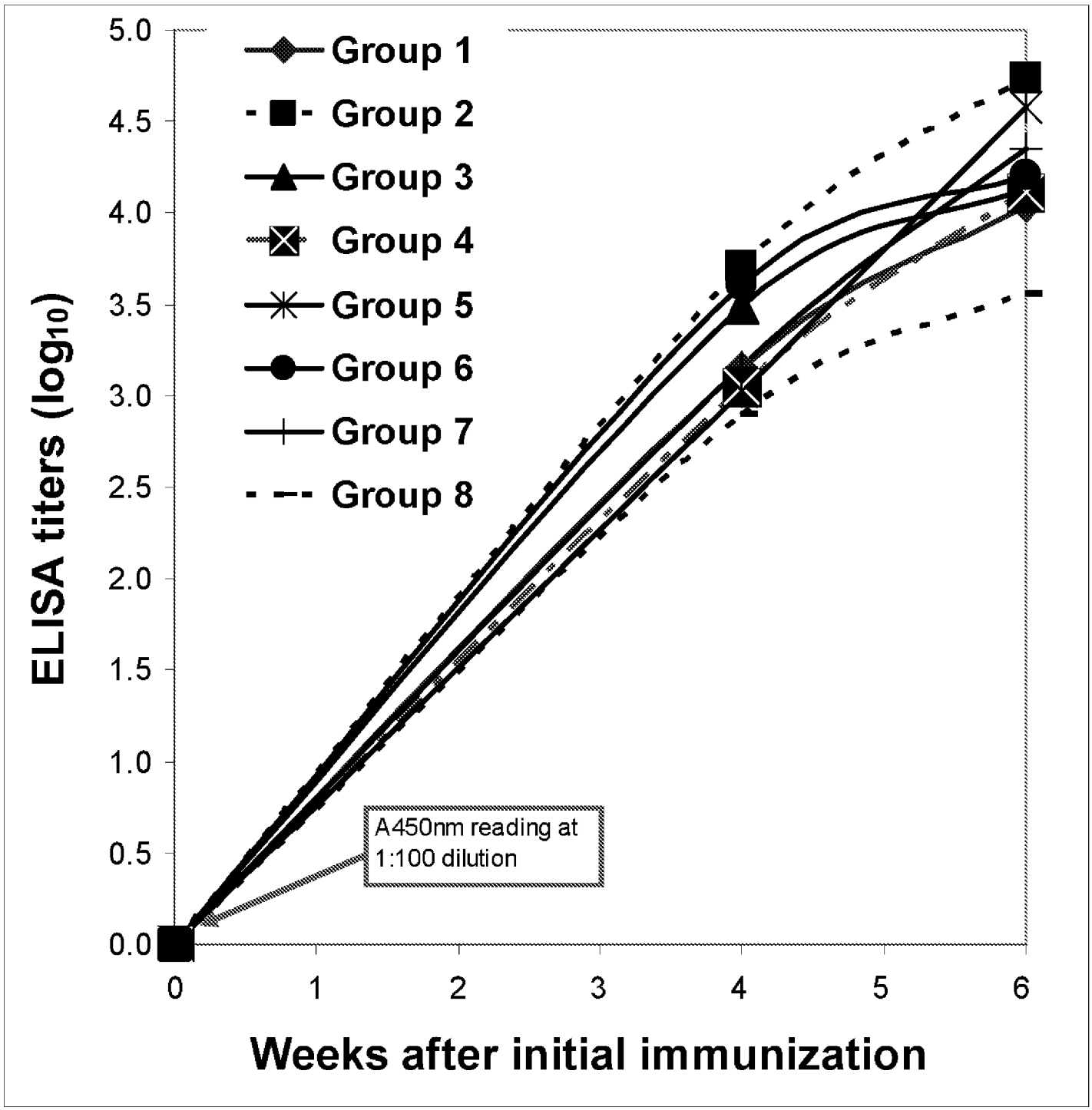

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ELISA titers (log_{10})
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
A450nm reading at
1:100 dilution
0
1
2
3
4
5
6
Weeks after initial immunization

Figure 3

Fig 3A

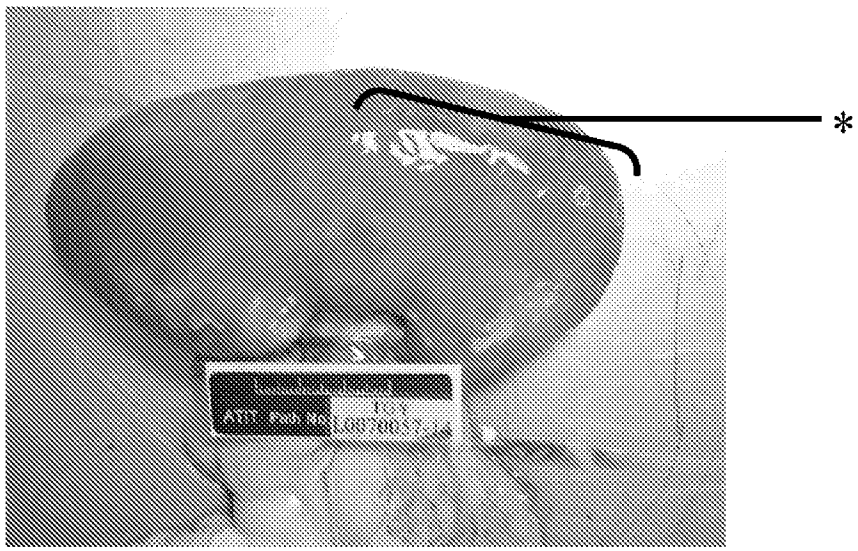
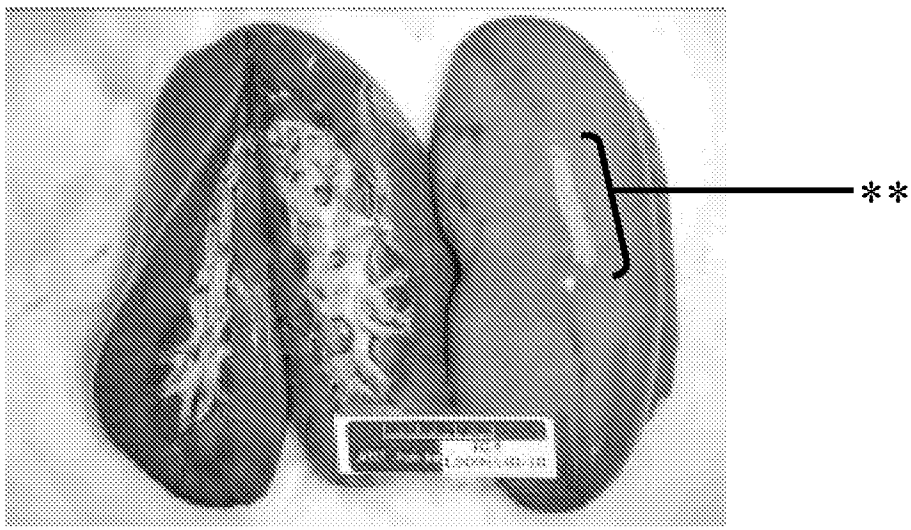


Fig 3B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/414406

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/12 (2010.01)
USPC - 424/204.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 424/201.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST -- PGPB,USPT,USOC,EPAB,JPAB; Dialog Classic Files - 654, 652, 349, 6, 35, 65, 155; USPTO Web Page; Google Scholar;
Search terms - porcine circovirus 2, PCV2, vaccine, antigen, delivery vehicle, peptide sequence homologs, altered charge

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y -- A	US 2009/0017064 A1 (WU et al.) 15 January 2009 (15.01.2009) para [0011], [0054], [0075], [0076], SEQ ID NO: 14	1 ------ 4/(1) ------ 2, 4/(2)
Y	US 2006/0029615 A1 (REN et al.) 09 February 2006 (09.02.2006) para [0083], [0089], [0105]	4/(1)
A	US 2010/0129397 A1 (FACHINGER et al.) 27 May 2010 (27.05.2010) para [0016], [0017], [0019], [0026], SEQ ID NO: 11	2, 4/(2)
X -- Y -- A	US 2009/0162398 A1 (WU) 25 June 2009 (25.06.2009) para [0015], [0020], [0037], [0092], [0093], SEQ ID NO: 3	1 ------ 4/(1) ------ 2, 4/(2)

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying tne invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
28 December 2010 (28.12.2010)

Date of mailing of the international search report
19 JAN 2011

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Authorized officer
Lee W. Young

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PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/41406

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item l.c of the first sheet)

1. Witli regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/41406

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-13 and 15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Group l+: Claims 1-4 and 14, drawn to a PCV2 vaccine, where the first named invention will be searched and is limited to a peptide antigen comprising SEQ ID NO: 1 (claims 1, 2, and 4). Due to the number of sequences in this application, the inventions of Groups l+ will be defined as necessary depending on Applicant's ultimate payment of additional fees. The additional sequences will be searched if applicant pays for each additional sequence or shows that the sequences share a special technical feature, i.e. a common structure or feature that defines a contribution over the prior art. Note that each additional sequence to be searched must be specified by the Applicant in the response to this invitation and must either (1) have an additional invention fee paid or (2) have a showing that the sequences share a common structure or feature that defines a contribution over the prior art.

Group II: Claim 16, drawn to a method for diagnosing PCV2 infection.

SEE CONTINUATION SHEET.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2 and 4.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

No protest accompanied the payment of additional search fees.

Continuation of Box III

The groups listed above do not relate to a single general inventive concept under PCT Rule 13.1 because under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The special technical feature of Group II is a diagnosing PCV2 infection which is not present in the inventions of Group I+. The inventions of Group I+ share the technical feature of the PCV2 vaccine of claim 1, comprising SEQ ID NO: 1 or a homologue thereof (SEQ ID NOS: 6, 9-19 and 20). However, this does not represent an improvement over the prior art. US 2009/0017064 (Jan 2009) to Wu et al. teaches a porcine circovirus type 2 (PCV2) vaccine composition, comprising a peptide antigen and a veterinarily acceptable delivery vehicle or adjuvant (para [0024]; method of immunizing a pig), wherein the peptide antigen comprises:
c) a homologue of SEQ ID NO: 1 (para [0024]; SEQ ID NO: 14, which is identical to Applicant's SEQ ID NO: 9).

Accordingly, unity of invention is lacking.