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(71) Applicant *(for all designated States except US)*: **BOEHRINGER INGELHEIM VETMEDICA, INC.** [US/US]; 2621 North Belt Highway, Saint Joseph, MO 64506 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **ROOF, Michael** [US/US]; 2501 North Loop Drive, Suite 1000, Ames, IA 50014 (US). **EICHMEYER, Marc** [US/US]; 2501 North Loop Drive, Suite 1000, Ames, IA 50014 (US).

(74) Agent: **MORRIS, Michael, P.**; Boehringer Ingelheim Corporation, 900 Ridgebury Road, P.O.Box 368, Ridgefield, CT 06877-0368 (US).

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(54) Title: PCV2 MYCOPLASMA HYOPNEUMONIAE IMMUNOGENIC COMPOSITIONS AND METHODS OF PRODUCING SUCH COMPOSITIONS

(57) Abstract: Multivalent combination vaccines are provided which include an immunological agent effective for reducing the incidence of or lessening the severity of M. *hyo* infection, preferably M. *hyo* bacterin, or an immunogenic composition comprising M. *hyo* bacterin, and at least one immunogenic active component of another disease-causing organism in swine, preferably PCV2 wherein the preferred PCV2 antigen for such a multivalent vaccine is PCV2 ORF 2 protein.

PCV2 MYCOPLASMA HYOPNEUMONIAE IMMUNOGENIC COMPOSITIONS AND METHODS OF PRODUCING SUCH COMPOSITIONS

RELATED APPLICATIONS

This application claims the priority benefit of U.S. provisional application serial number 61/023,086, filed on January 23, 2008 and U.S. provisional serial number 61/025,293, filed on January 31, 2008. The teachings and content of both of these applications are hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

Field of the Invention

In one aspect of the present invention, an immunogenic composition effective for inducing an immune response against infection by *Mycoplasma hyopneumoniae* (*M. hyo*) is provided. More particularly, the present invention is concerned with an immunogenic composition effective for inducing, eliciting, or providing an immune response that protects an animal receiving such a composition and reduces the incidence of, or lessens the severity of, the clinical symptoms associated with *M. hyo* infection. Still more particularly, the present invention is concerned with an immunogenic composition that includes therein cell-based antigen, preferably a bacterin, which confers a protective immune response against infection by *M. hyo*. Even more particularly, the present invention is concerned with an immunogenic composition comprising *M. hyo* bacterin, wherein administration of the bacterin results in a protective immune response against infection by *M. hyo*. Most particularly, the present invention is concerned with an immunogenic composition effective for conferring a protective immune response to a swine receiving the immunogenic composition, and wherein a dose of the composition comprises *M. hyo* bacterin with a relative potency (RP) of at least 1.22. Additionally, methods for producing and administering such immunogenic compositions are provided.

In another aspect of the present invention, an immunogenic composition effective for inducing an immune response against infection by porcine circovirus 2 (PCV2) is provided. More particularly, the present invention is concerned with an immunogenic composition

effective for providing an immune response that protects an animal receiving the composition by reducing the incidence of, or lessening the severity of the clinical symptoms associated with PCV2 infection. Still more particularly, the present invention is concerned with a protein-based immunogenic composition that confers a protective immune response against infection by PCV2. Even more particularly, the present invention is concerned with an immunogenic composition comprising ORF2 of PCV2, wherein administration of PCV2-ORF2 results in a protective immune response against infection by PCV2. Most particularly, the present invention is concerned with an immunogenic composition effective for conferring a protective immune response to a swine receiving the immunogenic composition, and wherein the composition comprises the protein expressed by ORF2 of PCV2 and has an RP value of at least 1.38. Additionally, the present invention provides methods for producing and administering such immunogenic compositions.

In another aspect of the present invention, a combination immunogenic composition, combination vaccine, or multivalent immunogenic composition or vaccine is provided. More particularly, the present invention provides immunogenic compositions effective at inducing an immune response against infection by *M. hyo* and at least one other disease-causing organism for swine. Even more particularly, the present invention provides a composition effective at inducing an immune response against infection by *M. hyo* and inducing an immune response against infection by PCV2. Preferably, the immune response against *M. hyo* and PCV2 is sufficient to reduce the severity of or incidence of clinical signs or symptoms associated with each respective pathogen up to and including preventing the same. In preferred forms of such an immunogenic composition, the *M. hyo* portion thereof will have an RP value of at least 1.22 and the PCV2 portion thereof will also have an RP value of at least 1.38. The present invention further provides methods of producing and administering such compositions.

Description of the Prior Art

Mycoplasma hyopneumoniae is a small bacterium (400-1200 nm) classified in the mycoplasmataceae family. *M. hyo* is associated with Enzootic Pneumonia, a swine respiratory disease commonly seen in growing and finishing pigs. *M. hyo* attacks the cilia of epithelial cells of the windpipe and lungs, causing the cilia to stop beating (ciliostasis) and eventually causing areas of the lungs to collapse. Depending on the extent of the disease,

daily live weight gain of infected swine can be reduced by up to 17%. Enzootic Pneumonia is widespread in swine populations and present in almost every swine herd. *M. hyo* is considered to be a primary pathogen that facilitates entry of PRRSV and other respiratory pathogens into the lungs. Three separate strains, 232, J, & 7448, have had their genomes sequenced (Minion et al., J. Bacteriol. 186: 7123-33, 2004; Vasconcelos et al., J. Bacteriol. 187: 5568-77, 2005). Clinical signs of *M. hyo* infection include a dry cough, impaired performance, and lung lesions.

Porcine circovirus type 2 (PCV2) is a small (17 -22 nm in diameter), icosahedral, non-enveloped DNA virus, which contains a single-stranded circular genome. PCV2 shares approximately 80% sequence identity with porcine circovirus type 1 (PCV1). However, in contrast with PCV1, which is generally non-virulent, swine infected with PCV2 exhibit a syndrome commonly referred to as Post-weaning Multisystemic Wasting Syndrome (PMWS). PMWS is clinically characterized by wasting, paleness of the skin, unthriftiness, respiratory distress, diarrhea, icterus, and jaundice. In some affected swine, a combination of all symptoms will be apparent while other swine will only have one or two of these symptoms. During necropsy, microscopic and macroscopic lesions also appear on multiple tissues and organs, with lymphoid organs being the most common site for lesions. A strong correlation has been observed between the amount of PCV2 nucleic acid or antigen and the severity of microscopic lymphoid lesions. Mortality rates for swine infected with PCV2 can approach 80%. In addition to PMWS, PCV2 has been associated with several other infections including pseudorabies, porcine reproductive and respiratory syndrome (PRRS), Glasser's disease, streptococcal meningitis, salmonellosis, postweaning colibacillosis, dietetic hepatosis, and suppurative bronchopneumonia.

Open reading frame 2 (ORF2) protein of PCV2, having an approximate molecular weight of 30 kDa when run on SDS-PAGE gel, has been utilized in the past as an antigenic component in vaccines for PCV2. Typical methods of obtaining ORF2 for use in such vaccines generally consist of amplifying the PCV2 DNA coding for ORF2, transfecting a viral vector with the ORF2 DNA, infecting cells with the viral vector containing the ORF2 DNA, permitting the virus to express ORF2 protein within the cell, and extracting the ORF2 protein from the cell via cell lysis. These procedures generally take up to about four days after infection of the cells by the viral vector. However, these procedures have a disadvantage in that the extraction procedures are both costly and time-consuming.

Additionally, the amount of ORF2 recovered from the cells is not very high; consequently, a large number of cells need to be infected by a large number of viral vectors in order to obtain sufficient quantities of the recombinant expressed protein for use in vaccines and the like.

Accordingly, what is needed in the art is an immunogenic composition that confers a protective immune response against, reduces the incidence of, and/or lessens the severity of or prevents the clinical signs associated with PCV2 infection and *M. hyo* infection. In particular, what is needed in the art is a combination vaccine comprising *M. hyo* antigen and PCV-2 antigen in sufficient amounts to confer a protective immune response against, reduces the incidence of, and/or lessens the severity of or prevents the clinical signs associated with PCV2 infection and *M. hyo* infection after a single administration of such vaccine. Such a vaccine would improve the compliance of swine vaccines.

SUMMARY OF THE INVENTION

The present invention overcomes the problems inherent in the prior art and provides a distinct advance in the state of the art. In one aspect of the present invention, an immunogenic composition for eliciting a protective immune response in a pig against *M. hyo* is provided. Preferably, the immunogenic composition comprises porcine circovirus type 2 antigen and *M. hyo* antigen. Even more preferably, the amount of the *M. hyo* antigen in each dose has a relative potency (RP) value of at least 1.22, wherein a relative potency value of 1.22 means that at least 95% and preferably 100% of mice receiving an administration of one-fortieth (1/40) of such amount of *M. hyo* antigen develop a detectable amount of antibodies within or at 21 days post treatment in a *M. hyo* specific antibody detection assay. Thus, the 40-fold amount of *M. hyo* antigen that is needed to induce a detectable *M. hyo* specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment is sufficient to confer a protective immune response against, reduces the incidence of, and/or lessens the severity of or prevents the clinical signs associated with a *M. hyo* infection, when administered together with a porcine circovirus type 2 antigen. In other words the amount of *M. hyo* antigen as described above has been shown to be able to overcome any negative interference with the PCV2 antigen, when mixed and administered as a combination vaccine. Consequently, the present invention also relates to an immunogenic composition comprising porcine circovirus type 2 antigen and *M. hyo* antigen, wherein the amount of the *M. hyo* antigen per dose has a relative potency (RP) value of at least 1.22,

wherein said RP value of 1.22 corresponds to the 40-fold amount of *M. hyo* antigen, that is needed to induce a detectable *M. hyo* specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment. In some preferred forms, the composition further includes or comprises an adjuvant. A variety of adjuvants will be useful with the present invention and can be selected by those of skill in the art, but carbomer and even more preferably Carbopol are particularly preferred. Advantageously, the immunogenic composition of the present invention confer a protective immune response against, reduces the incidence of, and/or lessens the severity of or prevents the clinical signs associated with a *M. hyo* infection when administered to a pig as a single dose administration. Such a single dose elicits a duration of immunity of at least 100, more preferably at least 110, even more preferably at least 120, still more preferably at least 130, even more preferably at least 140, still more preferably at least 150, even more preferably at least 160, still more preferably at least 170, even more preferably at least 180, and most preferably at least 184 days when administered to a pig. In other words, one dose of the immunogenic composition of the present invention, without boosters or subsequent doses, provides an animal or group of animals with a reduced incidence of or lessened severity of clinical signs of infection from *M. hyo* for at least 100 (110, 120, 130, 140, 150, 160, 170, 180, etc) and most preferably at least 184 days. With respect to the antibody detection assay, those of skill in the art will be able to identify and utilize appropriate products. ELISA assays and especially the IDEXX Herdchek *M. hyo* Test Kit (IDEXX Laboratories, Inc., Westbrook, ME) are preferred. In particular, the IDEXX Herdchek *M. hyo* Test Kit (IDEXX Laboratories, Inc., Westbrook, ME) was used as a reference assay according to the present invention.

In another aspect of the present invention, an immunogenic composition for eliciting a protective immune response in a pig against PCV2 is provided. Preferably, the immunogenic composition comprises porcine circovirus type 2 antigen and *M. hyo* antigen. Even more preferably, the amount of the PCV2 antigen in each dose has a relative potency (RP) value of at least 1.38, wherein a relative potency value of 1.38 means that at least 85% of mice receiving an administration of one-twentieth (1/20) of such amount of PCV2 antigen develop a detectable amount of antibodies within or at 21 days post treatment in a PCV2 specific antibody detection assay. Preferably, a relative potency value of 1.38 means that at least 95% and preferably 100% of mice receiving an administration of one-tenth (1/10) of such amount of PCV2 antigen develop a detectable amount of antibodies within or at 21 days

post treatment in a PCV2 specific antibody detection assay. Thus the 20-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 85% of the mice within or at 21 days post treatment or preferably the 10-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 95% and preferably 100% of the mice within or at 21 days post treatment has been shown to be sufficient to confer a protective immune response against, reduce the incidence of, and/or lessen the severity of or prevent the clinical signs associated with a PCV2 infection, when administered together with a *M. hyo* antigen. In other words the amount of PCV2 antigen as described above has been shown to be able to overcome any negative interference with the *M. hyo* antigen, when mixed and administered as a combination vaccine. Consequently, the present invention also relates to an immunogenic composition comprising porcine circovirus type 2 antigen and *M. hyo* antigen, wherein the amount of the PCV2 antigen per dose has a relative potency (RP) value of at least 1.38, wherein said RP value of 1.38 corresponds to the 20-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 85% of mice within or at 21 days post treatment. According to another aspect, the present invention also relates to an immunogenic composition comprises porcine circovirus type 2 antigen and *M. hyo* antigen, wherein the amount of the PCV2 antigen per dose has a relative potency (RP) value of at least 1.38, wherein said RP value of 1.38 corresponds to the 10-fold amount of PCV2 antigen, that is needed to induce a detectable PCV2 specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment.

In some preferred forms of this embodiment, the composition further includes or comprises an adjuvant. A variety of adjuvants will be useful with the present invention and can be selected by those of skill in the art, but carbomer and even more preferably Carbopol are particularly preferred. Advantageously, the immunogenic compositions of the present invention confer a protective immune response against, reduce the incidence of, and/or lessen the severity of or prevent the clinical signs associated with a PCV2 infection when administered to a pig as a single dose administration. Such a single dose elicits a duration of immunity of at least 100, more preferably at least 110, even more preferably at least 120, still more preferably at least 130, even more preferably at least 140, still more preferably at least 150, even more preferably at least 160, still more preferably at least 170, even more preferably at least 180, and most preferably at least 184 days when administered to a pig. In

other words, one dose of the immunogenic composition of the present invention, without boosters or subsequent doses, provides an animal or group of animals with a reduced incidence of or lessened severity of clinical signs of infection from PCV2 for at least 100 (110, 120, 130, 140, 150, 160, 170, 180, etc) and most preferably for at least 184 days. With respect to the antibody detection assay, those of skill in the art will be able to identify and utilize appropriate methods and products. ELISA assays and the procedures described by Nawagitgul, P., et al. (the teachings and content of which are hereby incorporated by reference) in *Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV* Clin. Diagn. Lab. Immunol. **9:**33-40 (2002), are particularly preferred.

In other aspect of the present invention, an immunogenic composition comprising an effective amount of *M. hyo* antigen and an effective amount of PCV2 antigen is provided. Preferably, both the *M. hyo* antigen and the PCV2 antigen will have an RP value of at least 1.22 and 1.38, respectively, as described above wherein an RP value of 1.22 for *M. hyo* and 1.38 for PCV2 means that 95% and preferably 100% of mice receiving an administration of one-fortieth of such amount of *M. hyo* and one-tenth of such amount of PCV2 antigen develop a detectable amount of antibodies within or at 21 days post treatment in an *M. hyo* and PCV2 specific antibody detection assay. Thus the 40-fold amount of *M. hyo* antigen that is needed to induce a detectable *M. hyo* specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment and the 10-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment have been shown to be sufficient to confer a protective immune response against, reduce the incidence of, and/or lessen the severity of or prevent the clinical signs associated with a PCV2 infection and/or *M. hyo* infection, when administered together with a *M. hyo* antigen. In other words the amounts of PCV2 antigen and *M. hyo* antigen as described above have been shown to be able to overcome any negative interference between both antigens, when mixed and administered as a combination vaccine. Consequently, the present invention also relates to an immunogenic composition comprising porcine circovirus type 2 antigen and *M. hyo* antigen, wherein the amount of the *M. hyo* and PCV2 antigen per dose has a relative potency value of at least 1.22 and 1.38 respectively, wherein said RP value of 1.22 for the *M. hyo* antigen corresponds to the 40-fold amount of *M. hyo* antigen that is needed to induce a detectable *M. hyo* specific

antibody response in at least 95% and preferably 100% of mice within or 21 days post treatment and wherein said RP value of 1.38 for the PCV2 antigen corresponds to the 20-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 85% of mice within or 21 days post treatment. Preferably, said RP value of 1.38 for the PCV2 antigen corresponds to the 10-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment. In some preferred forms of this embodiment, the composition further includes or comprises an adjuvant. A variety of adjuvants will be useful with the present invention and can be selected by those of skill in the art, but carbomer and especially Carbopol are particularly preferred. Advantageously, the immunogenic composition of the present invention elicits a protective immune response when administered to a pig as a single dose administration. Such a single dose elicits a duration of immunity of at least 100, more preferably at least 110, even more preferably at least 120, still more preferably at least 130, even more preferably at least 140, still more preferably at least 150, even more preferably at least 160, still more preferably at least 170, even more preferably at least 180, and most preferably at least 184 days when administered to a pig. In other words, one dose of the immunogenic composition of the present invention, without boosters or subsequent doses, provides an animal or group of animals with a reduced incidence of or lessened severity of clinical signs of infection from *M. hyo* and PCV2 for at least 100 (110, 120, 130, 140, 150, 160, 170, 180, etc) and most preferably for at least 184 days. With respect to the antibody detection assay, those of skill in the art will be able to identify and utilize appropriate methods and products. ELISA assays and the procedures described by Nawagitgul, P., et al. (the teachings and content of which are hereby incorporated by reference) in *Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV* Clin. Diagn. Lab. Immunol. **9:**33-40 (2002), are particularly preferred for a PCV2 antibody detection assay and the IDEXX Herdchek M hyo Test Kit is particularly preferred for the *M. hyo* antibody detection assay.

In another aspect of the present invention, a method for eliciting a protective immune response in a pig against *M. hyo* is provided. Generally, the method comprises the step of administering an immunogenic composition in accordance with the present invention to an animal in need thereof, preferably a pig. Preferably, the immunogenic composition

comprises porcine circovirus type 2 antigen and *M. hyo* antigen. Even more preferably, the amount of the *M. hyo* antigen in each dose has a relative potency (RP) value of at least 1.22, wherein an RP value of 1.22 for *M. hyo* means that 95% and preferably 100% of mice receiving an administration of one-fortieth of such amount of *M. hyo* antigen develop a detectable amount of antibodies within or at 21 days post treatment in an *M. hyo* specific antibody detection assay. Consequently, the present invention also relates to method for eliciting a protective immune response in a pig against *Mycoplasma hyopneumoniae* (*M. hyo*) comprising administering to said pig an immunogenic composition which comprises porcine circovirus type 2 antigen and *M. hyo* antigen, wherein the amount of the *M. hyo* antigen per dose has a relative potency value of at least 1.22, wherein said RP value of 1.22 corresponds to the 40-fold amount of *M. hyo* antigen that is needed to induce a detectable *M. hyo* specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment. In some preferred forms, the composition further includes or comprises an adjuvant. A variety of adjuvants will be useful with the present invention and can be selected by those of skill in the art, but carbomer, and even more preferably Carbopol, are particularly preferred. Advantageously, the immunogenic composition of the present invention elicits a protective immune response when administered to a pig as a single dose administration. Such a single dose elicits a duration of immunity of at least 100, more preferably at least 110, even more preferably at least 120, still more preferably at least 130, even more preferably at least 140, still more preferably at least 150, even more preferably at least 160, still more preferably at least 170, even more preferably at least 180, and most preferably at least 184 days when administered to a pig. In other words, one dose of the immunogenic composition of the present invention, without boosters or subsequent doses, provides an animal or group of animals with a reduced incidence of or lessened severity of clinical signs of infection from *M. hyo* for at least 100 (110, 120, 130, 140, 150, 160, 170, 180, etc) and most preferably at least 184 days. With respect to the antibody detection assay, those of skill in the art will be able to identify and utilize appropriate products. ELISA assays and especially the IDEXX Herdchek *M. hyo* Test Kit (IDEXX Laboratories, Inc., Westbrook, ME) are preferred.

In another aspect of the present invention, a method for eliciting a protective immune response against PCV2 in a pig is provided. Generally, the method comprises the step of administering an immunogenic composition in accordance with the present invention to an animal in need thereof, preferably a pig. Preferably, the immunogenic composition

comprises porcine circovirus type 2 antigen and *M. hyo* antigen. Even more preferably, the amount of the PCV2 antigen in each dose has a relative potency (RP) value of at least 1.38, wherein an RP value of 1.38 for PCV2 means that at least 85% of mice receiving an administration of one-twentieth of such amount of PCV2 antigen develop a detectable amount of antibodies within or at 21 days post treatment in a PCV2 specific antibody detection assay. Preferably, an RP value of 1.38 for PCV2 means that at least 95% and preferably 100% of mice receiving an administration of one-tenth of such amount of PCV2 antigen develop a detectable amount of antibodies within or at 21 days post treatment in a PCV2 specific antibody detection assay. Consequently, the present invention also relates to method for eliciting a protective immune response in a pig against PCV2 comprising administering to said pig an immunogenic composition which comprises PCV2 antigen and *M. hyo* antigen, wherein the amount of the PCV2 antigen per dose has a relative potency value of at least 1.38, wherein said RP value of 1.38 corresponds to the 20-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 85% of mice within or at 21 days post treatment. Preferably, said RP value of 1.38 corresponds to the 10-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 95% and preferably 100% of mice within or at 21 days post treatment. In some preferred forms of this embodiment, the composition further includes or comprises an adjuvant. A variety of adjuvants will be useful with the present invention and can be selected by those of skill in the art, but carbomer and even more preferably Carbopol are particularly preferred. Advantageously, the immunogenic composition of the present invention elicits a protective immune response when administered to a pig as a single dose administration. Such a single dose elicits a duration of immunity of at least 100, more preferably at least 110, even more preferably at least 120, still more preferably at least 130, even more preferably at least 140, still more preferably at least 150, even more preferably at least 160, still more preferably at least 170, even more preferably at least 180, and most preferably at least 184 days when administered to a pig. In other words, one dose of the immunogenic composition of the present invention, without boosters or subsequent doses, provides an animal or group of animals with a reduced incidence of or lessened severity of clinical signs of infection from PCV2 for at least 100(110, 120, 130, 140, 150, 160, 170, 180, etc) and most preferably for at least 184 days. With respect to the antibody detection assay, those of skill in the art will be able to identify and utilize

appropriate methods and products. ELISA assays and the procedures described by Nawagitgul, P., et al. (the teachings and content of which are hereby incorporated by reference) in *Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV* Clin. Diagn. Lab. Immunol. **9:**33-40 (2002), are particularly preferred.

In yet another aspect of the present invention, a method for inducing or eliciting a protective immune response against *M. hyo* infection and PCV2 infection is provided. In preferred forms, this aspect includes an immunogenic composition comprising an effective amount of *M. hyo* antigen and an effective amount of PCV2 antigen. Preferably, both the *M. hyo* antigen and the PCV2 antigen will have an RP value of at least 1.22 and 1.38, respectively, as described above wherein an RP value of 1.22 for *M. hyo* and 1.38 for PCV2 means that 95% and preferably 100% of mice receiving an administration of one-fortieth of such amount of *M. hyo* and one-tenth of such amount of PCV2 antigen develop a detectable amount of antibodies within or at 21 days post treatment in an *M. hyo* and PCV2 specific antibody detection assay. Consequently, the present invention also relates to method for eliciting a protective immune response in a pig against *M. hyo* and PCV2 comprising administering to said pig an immunogenic composition which comprises PCV2 antigen and *M. hyo* antigen, wherein the amount of the *M. hyo* and PCV2 antigen per dose has a relative potency value of at least 1.22 and 1.38 respectively, wherein said RP value of 1.22 for the *M. hyo* antigen corresponds to the 40-fold amount of *M. hyo* antigen that is needed to induce a detectable *M. hyo* specific antibody response in at least 95% and preferably 100% of mice within or 21 days post treatment and wherein said RP value of 1.38 for the PCV2 antigen corresponds to the 20-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 85% of mice within or 21 days post treatment. Preferably, said RP value of 1.38 for the PCV2 antigen corresponds to the 10-fold amount of PCV2 antigen that is needed to induce a detectable PCV2 specific antibody response in at least 95% and preferably 100% of mice within or 21 days post treatment. In some preferred forms of this embodiment, the composition further includes or comprises an adjuvant. A variety of adjuvants will be useful with the present invention and can be selected by those of skill in the art, but carbomer and especially Carbopol are particularly preferred. Advantageously, the immunogenic composition of the present invention elicits a protective immune response when administered to a pig as a single dose administration. Such a single

dose elicits a duration of immunity of at least 100, more preferably at least 110, even more preferably at least 120, still more preferably at least 130, even more preferably at least 140, still more preferably at least 150, even more preferably at least 160, still more preferably at least 170, even more preferably at least 180, and most preferably at least 184 days when administered to a pig. In other words, one dose of the immunogenic composition of the present invention, without boosters or subsequent doses, provides an animal or group of animals with a reduced incidence of or lessened severity of clinical signs of infection from *M. hyo* and PCV2 for at least 100 (110, 120, 130, 140, 150, 160, 170, 180, etc) and most preferably at least 184 days. With respect to the antibody detection assay, those of skill in the art will be able to identify and utilize appropriate methods and products. ELISA assays and the procedures described by Nawagitgul, P., et al. (the teachings and content of which are hereby incorporated by reference) in *Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV* Clin. Diagn. Lab. Immunol. **9:**33-40 (2002), are particularly preferred for a PCV2 antibody detection assay and the IDEXX Herdchek M hyo Test Kit is particularly preferred for the *M. hyo* antibody detection assay.

As used herein, a "protective immune response" refers to a reduced incidence of or reduced severity of clinical, pathological, or histopathological signs of infection from *M. hyo* or PCV2 infection up to and including the complete prevention of such signs.

In another aspect of the present invention, a method for preparing a composition, preferably an antigenic or immunogenic composition, such as for example a vaccine, for invoking an immune response against PCV2 and *M. hyo* is provided. Generally, this method includes the steps of transfecting a construct into a virus, wherein the construct comprises **i)** recombinant DNA from ORF2 of PCV2, **ii)** infecting cells in growth media with the transfected virus, **iii)** causing the virus to express the recombinant protein from PCV2 ORF2, **iv)** recovering the expressed ORF2 protein from the supernate, **v)** and preparing the composition by combining the recovered protein with a suitable adjuvant and/or other pharmaceutically acceptable carrier, together with *M. hyo* bacterin and a suitable adjuvant. Due to the long-term stability of the components of the immunogenic composition, the composition can be administered to animals up to 3 years after being mixed together. Preferred forms of the composition described herein will have an *M. hyo* portion with an RP value greater than about 1.22, more preferably between about 1.22 to about 4.5, even more

preferably between about 1.22 and 3.5, and most preferably between about 1.22 and 2.8. An RP value for *M. hyo* is greater than about 1.22 when 95% and more preferably 100% of mice treated with one-fortieth of the amount of *M. hyo* antigen associated with the RP value of 1.22 develop a detectable amount of antibodies against *M. hyo* using an *M. hyo*-specific antibody detection assay within or at 21 days post treatment or post administration of the *M. hyo* antigen.

"Adjuvants" as used herein, can include aluminum hydroxide and aluminum phosphate, saponins e.g., Quil A, QS-21 (Cambridge Biotech Inc., Cambridge MA), GPI-0100 (Galenica Pharmaceuticals, Inc., Birmingham, AL), water-in-oil emulsion, oil-in-water emulsion, water-in-oil-in-water emulsion. The emulsion can be based in particular on light liquid paraffin oil (European Pharmacopea type); isoprenoid oil such as squalane or squalene oil resulting from the oligomerization of alkenes, in particular of isobutene or decene; esters of acids or of alcohols containing a linear alkyl group, more particularly plant oils, ethyl oleate, propylene glycol di-(caprylate/caprate), glyceryl tri-(caprylate/caprate) or propylene glycol dioleate; esters of branched fatty acids or alcohols, in particular isostearic acid esters. The oil is used in combination with emulsifiers to form the emulsion. The emulsifiers are preferably nonionic surfactants, in particular esters of sorbitan, of mannide (e.g. anhydromannitol oleate), of glycol, of polyglycerol, of propylene glycol and of oleic, isostearic, ricinoleic or hydroxystearic acid, which are optionally ethoxylated, and polyoxypropylene-polyoxyethylene copolymer blocks, in particular the Pluronic products, especially L121. See Hunter et al., The Theory and Practical Application of Adjuvants (Ed.Stewart-Tull, D. E. S.). John Wiley and Sons, NY, pp51-94 (1995) and Todd et al., Vaccine 15:564-570 (1997).

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

A further instance of an adjuvant is a compound chosen from the polymers of acrylic or methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative. Advantageous adjuvant compounds are the polymers of acrylic or methacrylic acid, which are cross-linked, especially with polyalkenyl ethers of sugars or polyalcohols. These compounds are known by the term carbomer (Phameuropa Vol. 8, No. 2, June 1996). Persons

skilled in the art can also refer to U. S. Patent No. 2,909,462 which describes such acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than 8, the hydrogen atoms of at least three hydroxyls being replaced by unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents, such as methyl. The products sold under the name Carbopol; (BF Goodrich, Ohio, USA) are particularly appropriate. They are cross-linked with an allyl sucrose or with allyl pentaerythritol. Among then, there may be mentioned Carbopol 974P, 934P and 971P. Most preferred is the use of Carbopol 971P. Among the copolymers of maleic anhydride and alkenyl derivative, the copolymers EMA (Monsanto), which are copolymers of maleic anhydride and ethylene, are also contemplated. The dissolution of these polymers in water leads to an acid solution that will be neutralized, preferably to physiological pH, in order to give the adjuvant solution into which the immunogenic, immunological or vaccine composition itself will be incorporated. Further suitable adjuvants include, but are not limited to, the RIBI adjuvant system (Ribi Inc.), Block co-polymer (CytRx, Atlanta GA), SAF-M (Chiron, Emeryville CA), monophosphoryl lipid A, Avridine lipid-amine adjuvant, heat-labile enterotoxin from E. coli (recombinant or otherwise), cholera toxin, IMS 1314 or muramyl dipeptide among many others.

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

Thus, according to a further aspect, the method for preparing an antigenic composition, such as for example a vaccine, for invoking an immune response against PCV2 and *M. hyo* comprises **i)** admixing PCV2 ORF2 protein with *M. hyo* bacterin and a suitable adjuvant. Preferably, the adjuvant is a carbomer, and more preferably is Carbopol 971P. Even more preferably, Carbopol 971P is added in an amount of about 500 μg to about 5 mg per dose, even more preferably in an amount of about 750 μg to about 2.5 mg per dose and most preferably in an amount of about 1 mg per dose.

Additionally, the vaccine composition can include one or more pharmaceutical-acceptable carriers. As used herein, "a pharmaceutical-acceptable carrier" includes any and all solvents, dispersion media, coatings, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like.

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

A “PCV2 antigen” refers to any composition of matter that comprises at least one antigen that can induce, stimulate or enhance the immune response against PCV2 infection, when administered to a pig. Preferably, said PCV2 antigen is the whole PCV2 virus, preferably in an inactivated form, a life modified or attenuated PCV2 virus, a chimeric virus that comprises at least an immunogenic amino acid sequence of PCV2, any other polypeptide or component that comprises at least an immunogenic amino acid sequence of PCV2. The terms "immunogenic protein”, “immunogenic polypeptide” or “immunogenic amino acid sequence” as used herein refer to any amino acid sequence which elicits an immune response in a host against a pathogen comprising said immunogenic protein, immunogenic polypeptide or immunogenic amino acid sequence. An “immunogenic protein”, “immunogenic polypeptide” or “immunogenic amino acid sequence” as used herein, includes the full-length sequence of any proteins, analogs thereof, or immunogenic fragments thereof. By "immunogenic fragment" is meant a fragment of a protein, which includes one or more epitopes and thus elicits the immunological response against the relevant pathogen. Such fragments can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the

peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) Proc. Natl. Acad. Sci. USA 81:3998-4002; Geysen et al. (1986) Molec. Immunol. 23:709-715. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols, supra. Synthetic antigens are also included within the definition, for example, polyepitopes, flanking epitopes, and other recombinant or synthetically derived antigens. See, e.g., Bergmann et al. (1993) Eur. J. Immunol. 23:2777-2781; Bergmann et al. (1996), J. Immunol. 157:3242-3249; Suhrbier, A. (1997), Immunol. and Cell Biol. 75:402-408; Gardner et al., (1998) 12th World AIDS Conference, Geneva, Switzerland, June 28-July 3, 1998.

Most preferably, the vaccine composition that can be used according to the invention contains PCV2 ORF2 protein, preferably expressed in and recovered from *in vitro* cultured cells. Preferred examples of PCV2 ORF2 proteins are described in the international patent application WO2006-072065, the teaching and content of which is entirely incorporated herein by reference.

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

composition. The term "immunogenic portion" as used herein refers to truncated and/or substituted forms, or fragments of PCV2 ORF2 protein and/or polynucleotide, respectively. Preferably, such truncated and/or substituted forms, or fragments will comprise at least 6 contiguous amino acids from the full-length ORF2 polypeptide. More preferably, the truncated or substituted forms, or fragments will have at least 10, more preferably at least 15, and still more preferably at least 19 contiguous amino acids from the full-length ORF2 polypeptide. Two preferred sequences in this respect are provided herein as SEQ ID NOs. 9 and 10. It is further understood that such sequences may be a part of larger fragments or truncated forms. A further preferred PCV2 ORF2 polypeptide provided herein is encoded by the nucleotide sequences of SEQ ID NO: 3 or SEQ ID NO: 4. However, it is understood by those of skill in the art that this sequence could vary by as much as 6-20% in sequence homology and still retain the antigenic characteristics that render it useful in immunogenic compositions. In some forms, a truncated or substituted form, or fragment of ORF2 is used as the antigenic component in the composition. Preferably, such truncated or substituted forms, or fragments will comprise at least 18 contiguous nucleotides from the full-length ORF2 nucleotide sequence, e.g. of SEQ ID NO: 3 or SEQ ID NO: 4. More preferably, the truncated or substituted forms, or fragments will have at least 30, more preferably at least 45, and still more preferably at least 57 contiguous nucleotides the full-length ORF2 nucleotide sequence, e.g. of SEQ ID NO: 3 or SEQ ID NO: 4.

"Sequence Identity" as it is known in the art refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences are "identical" at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by known methods, including but not limited to, those described in Computational Molecular Biology, Lesk, A. N., ed., Oxford University Press, New York (1988), Biocomputing: Informatics and Genome Projects, Smith, D.W., ed.,

Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinge, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988), the teachings of which are incorporated herein by reference. Preferred methods to determine the sequence identity are designed to give the largest match between the sequences tested. Methods to determine sequence identity are codified in publicly available computer programs that determine sequence identity between given sequences. Examples of such programs include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research, 12(1):387 (1984)), BLASTP, BLASTN and FASTA (Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al., NCVI NLM NIH Bethesda, MD 20894, Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990), the teachings of which are incorporated herein by reference). These programs optimally align sequences using default gap weights in order to produce the highest level of sequence identity between the given and reference sequences. As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 85%, preferably 90%, even more preferably 95% "sequence identity" to a reference nucleotide sequence, it is intended that the nucleotide sequence of the given polynucleotide is identical to the reference sequence except that the given polynucleotide sequence may include up to 15, preferably up to 10, even more preferably up to 5 point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, in a polynucleotide having a nucleotide sequence having at least 85%, preferably 90%, even more preferably 95% identity relative to the reference nucleotide sequence, up to 15%, preferably 10%, even more preferably 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 15%, preferably 10%, even more preferably 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having a given amino acid sequence having at least, for

example, 85%, preferably 90%, even more preferably 95% sequence identity to a reference amino acid sequence, it is intended that the given amino acid sequence of the polypeptide is identical to the reference sequence except that the given polypeptide sequence may include up to 15, preferably up to 10, even more preferably up to 5 amino acid alterations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a given polypeptide sequence having at least 85%, preferably 90%, even more preferably 95% sequence identity with a reference amino acid sequence, up to 15%, preferably up to 10%, even more preferably up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 15%, preferably up to 10%, even more preferably up to 5% of the total number of amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or the carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in the one or more contiguous groups within the reference sequence. Preferably, residue positions that are not identical differ by conservative amino acid substitutions. However, conservative substitutions are not included as a match when determining sequence identity.

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

A "conservative substitution" refers to the substitution of an amino acid residue or nucleotide with another amino acid residue or nucleotide having similar characteristics or

properties including size, hydrophobicity, etc., such that the overall functionality does not change significantly.

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

Preferably, said PCV2 ORF2 protein is

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

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

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

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

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

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

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

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

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

As mentioned above, the relative potency (RP) of the PCV2 ORF2 portion of the composition is at least 1.38, more preferably between about 1.38 and 4.5, still more preferably between about 1.38 and 3.5, even more preferably between about 1.38 and 3, and most preferably between about 1.38 and 2.75. Similar to the determination of an RP value of at least 1.22 with respect to *M.hyo*, an RP value for PCV2 is greater than about 1.38 when

85% of mice treated with one-twentieth of the amount of PCV2 antigen associated with an RP value of 1.38 develop a detectable amount of antibodies against PCV2 using a PCV2-specific antibody detection assay within or at 21 days post treatment or post administration of the PCV2 antigen. Preferably, an RP value for PCV2 is greater than about 1.38 when 95% and preferably 100% of mice treated with one-tenth of the amount of PCV2 antigen associated with an RP value of 1.38 develop a detectable amount of antibodies against PCV2 using a PCV2-specific antibody detection assay within or at 21 days post treatment or post administration of the PCV2 antigen.

The PCV2 ORF2 protein used in an immunogenic composition in accordance with the present invention can be derived in any fashion including isolation and purification of PCV2 ORF2, standard protein synthesis, and recombinant methodology. Preferred methods for obtaining PCV2 ORF2 polypeptide are also provided in the international patent application WO 2006-072065, which corresponds to the U.S. Patent Application Serial No. 11/034,797, the teachings and content of which are hereby incorporated by reference.

According to further embodiment, said PCV2 antigen is Ingelvac®CircoFLEX, (Boehringer Ingelheim Vetmedica Inc, St Joseph, MO, USA), CircoVac® (Merial SAS, Lyon, France), CircoVent (Intervet Inc., Millsboro, DE, USA), or Suvaxyn PCV-2 One Dose® (Fort Dodge Animal Health, Kansas City, KA, USA), or any of the PCV2 antigens included in any of the vaccines mentioned above. Most preferably, the PCV2 antigen is that which is included in Ingelvac®CircoFLEX or is Ingelvac®CircoFLEX.

A "*M. hyo* antigen" refers to any composition of matter that comprises at least one antigen that can induce, stimulate or enhance the immune response against *M. hyo* infection, when administered to a pig. Preferably, said *M. hyo* antigen is the whole *M. hyo* bacterin, preferably in an inactivated form, a live modified or attenuated *M. hyo* bacterium, a chimeric virus that comprises at least an immunogenic amino acid sequence of *M. hyo*, or any other polypeptide or component that comprises at least an immunogenic amino acid sequence of *M. hyo*. Preferably the *M. hyo* antigen is an inactivated *M. hyo* bacterin. More preferably the *M. hyo* antigen is derived from the *M. hyo* J-strain. Most preferably the *M. hyo* bacterin is the inactivated *M. hyo* bacterin that is included in Ingelvac®MycoFlex vaccine (Boehringer Ingelheim Vetmedica Inc, St Joseph, MO, USA) or is Ingelvac®MycoFlex. However, the *M. hyo* antigen that can be used according to the invention can also selected from any one which is included in the following vaccine compositions: Porcilis M. hyo, Myco Silencer® BPM,

Myco Silencer® BPME, Myco Silencer® ME, Myco Silencer® M, Myco Silencer® Once, Myco Silencer® MEH (all of Intervet Inc., Millsboro, DE, USA) Stellamune Mycoplasma (Pfizer Inc., New York, NY, USA), Suvaxyn Mycoplasma, Suvaxyn *M. hyo*, Suvaxyn MH-One (all of Fort Dodge Animal Health, Overland Park, KS, USA (Wyeth).

Injection timing is flexible. Compositions as described herein can be used as early as three weeks of age through the time when pigs leave the nursery with the objective of vaccinating at least 2 weeks prior to exposure to *Mycoplasma hyopneumoniae*. The composition according to the invention may be applied in any conventional manner including intradermally, intratracheally, or intravaginally. The composition preferably may be applied intramuscularly or intranasally. In an animal body, it can prove advantageous to apply the pharmaceutical compositions as described above via an intravenous injection or by direct injection into target tissues. For systemic application, the intravenous, intravascular, intramuscular, intranasal, intra-arterial, intraperitoneal, oral, or intrathecal routes are preferred. A more local application can be effected subcutaneously, intradermally, intracutaneously, intracardially, intralobally, intramedullarly, intrapulmonarily or directly in or near the tissue to be treated (connective-, bone-, muscle-, nerve-, epithelial tissue). Depending on the desired duration and effectiveness of the treatment, the compositions according to the invention may be administered once or several times, also intermittently, for instance on a daily basis for several days, weeks or months, and in different dosages. However, as detailed herein, the combination of *M. hyo* bacterin and PCV2 ORF 2 provides an immunogenic composition that provides effective immunity and/or reduces the severity of or incidence of clinical signs associated with *M. hyo* and/or PCV2 infection in pigs receiving such a composition after just one dose.

"Decrease" or "reduction in the incidence of or severity of clinical, pathological, and/or histopathological signs" shall mean that any of such signs are reduced in incidence or severity in animals receiving an administration of the vaccine in comparison with a "control group" of animals when both have been infected with or challenged by the pathogen from which the immunological active component(s) in the vaccine are derived and wherein the control group has not received an administration of the vaccine or immunogenic composition. In this context, the term "decrease" or "reduction" means a reduction of at least 10%, preferably 25%, even more preferably 50%, and most preferably of 100% in the vaccinated group as compared to the control group as defined above.

"Clinical signs" shall refer to signs of infection from a pathogen that are directly observable from a live animal such as symptoms. Representative examples will depend on the pathogen selected but can include things such as nasal discharge, lethargy, coughing, elevated fever, weight gain or loss, dehydration, diarrhea, swelling, lameness, and the like. PCV2 clinical signs can include wasting, paleness of the skin, unthriftiness, respiratory distress, diarrhea, icterus, and jaundice. Clinical signs of *M. hyo* infection include a dry cough, impaired performance, and lung lesions.

"Pathological" signs shall refer to signs of infection that are observable at the microscopic or molecular level, through biochemical testing, or with the naked eye upon necropsy. For PCV2, pathological signs will include microscopic and macroscopic lesions on multiple tissues and organs, with lymphoid organs being the most common site for lesions.

"Histopathological" signs shall refer to signs of tissue changes resulting from infection.

In another embodiment of the present invention *M.hyo* antigen, preferably *M.hyo* bacterin, is combined with antigen from another disease-causing organism in swine. In such cases, the RP value of the *M.hyo* portion is as described above. In another embodiment of the present invention, PCV2 antigen is combined with antigen from another disease-causing organism in swine. In such cases, the RP value of the PCV2 portion is as described above. Furthermore, in another embodiment of the present invention, PCV2 antigen and *M. hyo* antigen, preferably *M. hyo* bacterin, are combined with antigen from another disease-causing organism in swine. In such cases, the RP value of the *M. hyo* and PCV2 portion are as described above.

Preferably the other disease-causing organism in swine is selected from the group consisting of: Actinobacillus pleuropneumonia **(1)**; Adenovirus **(2)**; Alphavirus such as Eastern equine encephalomyelitis viruses **(3)**; Bordetella bronchiseptica **(4)**; Brachyspira spp. **(5)**, preferably B. hyodyentheriae **(6)**; B. piosicoli **(7)**, Brucella suis, preferably biovars 1, 2, and 3 **(8)**; Classical swine fever virus **(9)**; Clostridium spp. **(10)**, preferably Cl. difficile **(11),** Cl. perfringens types A, B, and C **(12)**, Cl. novyi **(13)**, Cl. septicum **(14)**, Cl. tetani **(15)**; Coronavirus **(16)**, preferably Porcine Respiratory Corona virus **(17)**; Eperythrozoonosis suis **(18)**; Erysipelothrix rhusiopathiae **(19)** Escherichia coli **(20)**; Haemophilus parasuis, preferably subtypes 1, 7 and 14 **(21)** Hemagglutinating encephalomyelitis virus **(22)**;

Japanese Encephalitis Virus **(23)**; Lawsonia intracellularis**(24)** Leptospira spp. **(25)**, preferably Leptospira australis **(26)**; Leptospira canicola **(27)**; Leptospira grippotyphosa **(28)**; Leptospira icterohaemorrhagicae **(29)**; and Leptospira interrogans **(30)**; Leptospira pomona **(31)**; Leptospira tarassovi **(32)**; Mycobacterium spp. **(33)** preferably M. avium **(34),** M. intracellulare **(35)** and M.bovis **(36);** Pasteurella multocida **(37)**; Porcine cytomegalovirus **(38)**; Porcine Parvovirus **(39)**; Porcine Reproductive and Respiratory Syndrome (PRRS) Virus **(40)** Pseudorabies virus **(41)**; Rotavirus **(42)**; Salmonella spp. **(43)**, preferably S. thyphimurium **(44)** and S. choleraesuis **(45);** Staph. hyicus **(46)**; Staphylococcus spp. **(47)** preferably Streptococcus spp. **(48)**, preferably Strep. suis **(49)**; Swine herpes virus **(50)**; Swine Influenza Virus **(51)**; Swine pox virus **(52)**; Swine pox virus **(53);** Vesicular stomatitis virus **(54)**; Virus of vesicular exanthema of swine **(55);** Leptospira Hardjo **(56)**; Porcine circovirus **(57)**; and/or Mycoplasma hyosynoviae **(58)**.

Any reference made in connection with a swine pathogen in the following can be made by naming the pathogen, for example Pasteurella multocida, or by making reference to the number in ( ) behind the pathogen that is found above. For example reference to Pasteurella multocida can be made by Pasteurella multocida or by (37).

Thus, the present invention relates to a combination vaccine for the treatment and /or prophylaxis of swine, that includes an immunological agent effective for reducing the incidence of or lessening the severity of *M. hyo* and/or PCV2 infection(s), and further an immunological active component effective for the treatment and/or prophylaxis of infections caused by any of the swine pathogens (1), (2), (3), (4), (5), (6), (7), (8), (9), (10), (11), (12), (13), (14), (15), (16), (17), (18), (19), (20), (21), (22), (23), (24), (25), (26), (27), (28), (29), (30), (31), (32), (33), (34), (35), (36), (37), (38), (39), (40), (41), (42), (43), (44), (45), (46), (47), (48), (49), (50), (51), (52), (53), (54), (55), (56), (57) and/or (58), or is an immunological active component of said swine pathogen(s).

An "immunological active component" as used herein means a component that induces or stimulates the immune response in an animal to which said component is administered. According to a preferred embodiment, said immune response is directed to said component or to a microorganism comprising said component. According to a further preferred embodiment, the immunological active component is an attenuated microorganism, including modified live virus (MLV), a killed-microorganism or at least an immunological active part of a microorganism.

"Immunological active part of a microorganism" as used herein means a protein-, sugar-, and or glycoprotein containing fraction of a microorganism that comprises at least one antigen that induces or stimulates the immune response in an animal to which said component is administered. According to a preferred embodiment, said immune response is directed to said immunological active part of a microorganism or to a microorganism comprising said immunological active part.

According to further aspect, the further immunological active component of the combination vaccine is selected from the group consisting Enterisol® Ileitis, Enterisol® Ileitis FF, Enterisol® SC-54, Enterisol® SC-54 FF, Enterisol® ERY-ALC, Ingelvac® APP ALC, Ingelvac® AR4, Ingelvac® HP-1, Ingelvac® HPE-1, Ingelvac® PRRS MLV, Ingelvac® PRRS ATP, Ingelvac® PRV-G1, Reprocyc® PRRS PLE, Reprocyc® PLE, Tetguard™, Toxivac® AD+E, Toxivac® Plus Parasuis, (all of **Boehringer Ingelheim, St. Joseph, MO, USA**); Circovent, Porcilis Coli, Porcilis ERY + PARVO, Porcilis Ery, Porcilis Glasser, Porcilis Parvo, Porcilis Porcoli DF, Porcilis APP, Porcilis AR-T, Porcilis AR-T DF, Porcilis Porcoli, Porcilis Porcoli Diluvac forte, Porcilis PRRS, Porcilis Porcol 5, Porcilis Aujeszky, Porcilis Begonia Diluvac, Porcilis Begonia I.D.A.L., Porcilis Begonia Unisole, Porcilis M. hyo, Porcilis Atrinord, Rhinogen® BPE, Rhinogen® CTE 5000, Rhinogen® CTSE, Score, Sow Bac® E II, Sow Bac® CE II, Sow Bac® TREC, ProSystem® CE, ProSystem® RCE, ProSystem® TREC, ProSystem® Pillmune, ProSystem® Rotamune® with Imugan® II, ProSystem® Rota, ProSystem® Rotamune KV, ProSystem® TG-Emune® Rota with Imugan® II, ProSystem® TGE/Rota, ProSystem® TG-Emune® with Imugen®, ProSystem® TGE, MaGESTIC 7, MaGESTIC 8, MaGESTic™ with Spur®, MaGESTic® 7 with Spur®, MaGESTic® 8 with Spur®, End-FLUence® with Imugen® Il, End-FLUence® 2, PRRomiSE®, PRV-Begonia with Dlluvac Forte®, Argus® SC/ST, Strep Bac, Strep Bac® with Imugen® II, Colisorb, Heptavac, Lambivac, Porcovac plus, Erysorb Parvo all of **Intervet Inc., Millsboro, DE, USA**); Hyoresp, Circovac, Neocolipor, Parvoruvac, Parvosuin, Progressis, Viraflu, Akipor 6.3, Jespur gl-, Jesflu gl- **(**all of **Merial LTD, Duluth, GA);** ER BAC® PLUS, ER BAC®, ER BAC® PLUS/LEPTOFERM-5®, ER BAC® Leptoferm-5®, Farrowsure®, Farrowsure® B, FARROWSURE® PLUS B, FARROWSURE® PLUS, FARROWSURE® PRV, FARROWSURE B-PRV, FLUSURE™, FLUSURE™ RTU, FLUSURE™/ER BAC® PLUS, FLUSURE™/ER BAC PLus®, FLUSURE™/RESPISURE®, FLUSURE™/RESPISURE® RTU, FLUSURE™/RESPISURE-ONE®/ER BAC® PLUS,

FLUSURE□/RespiSure 1 ONE®/ER BAC Plus®, FLUSURE™/RESPISURE ONE®, FLUSURE□/RESPISURE 1 ONE®, FLUSURE/Farrowsure Plus, FLUSURE/Farrowsure Plus B, LITTERGUARD® LT-C, LITTERGUARD® LT, PleuroGuard® 4, Pneumosuis III, Stellamune One, Stellamune Uno, Stellamune Once, Stellamune Mono, Respisure One, Respisure®, Respisure 1 ONE®, Respisure 1 One®/ER Bac Plus®, Enduracell T, Zylexis (formerly known as Baypamune), Atrobac® 3, BratiVac®, BratiVac®-B, Leptoferm-5°°Parvo-Vac®/Leptoferm-5®, PR-Vac®-Killed, PR-Vac®, PR-Vac Plus™ (all of **Pfizer Inc., New York, NY, USA);** Suvaxyn MH One, Suvaxyn RespiFend® MH, Suvaxyn Aujeszky Bartha + Diluent, Suvaxyn Aujeszky Bartha + o/w, Suvaxyn Aujeszky-Flu, Suvaxyn Aujeszky 783 + o/w, Suvaxyn Ery, Suvaxyn Flu, *M. hyo* Suvaxyn Parvo ST, Suvaxyn Parvo/E, Suvaxyn RespiFend® APP, Suvaxyn RespiFend® HPS, Suvaxyn RespiFend® MH/HPS, Suvaxyn RespiFend® MH, Suvaxyn® AR/T/E, Suvaxyn® EC-4, Suvaxyn® E, Suvaxyn®-E, Suvaxyn® E-oral, Suvaxyn® PLE, Suvaxyn® PLE/PrV gpl-, Suvaxyn® LE+B, Suvaxyn® PLE + B, Suvaxyn® PLE+B/PrV gpl-, Suvaxyn® SIV, Suvaxyn® SIV/Mh-one, Suvaxyn® P, Suvaxyn® PrV gpl-, Suvaxyn® PCV-2 One Shot (all of **Fort Dodge Animal Health, Overland Park, KS, USA (Wyeth);** SCOURMUNE®, SCOURMUNE®-C, SCOURMUNE®-CR, AR-PAC®-PD+ER, AR-PARAPAC®+ER, M+ Rhusigen®, M+PAC®, MaxiVac Excell®3, MaxiVac® H1N1, MaxiVac® H3N2, MaxiVac®-FLU, MaxiVac®-M+, MaxiVac Excell®, MaxiVac Excell 3, PARAPAC®, PNEU PAC®, PNEU PAC®-ER, PNEU PAC®+ER, PRV/Marker Gold®, PRV/Marker Gold®, PRV/Marker Gold®-MaxiVac® FLU, Rhusigen™, Gletvax 6, Covexin 8, M + PAC, Gletvax plus, M-Parapac™SS PAC® (all of **Schering-Plough Animal Health Corporation, Kenilworth, NJ, USA**); AMERVAC-PRRS, AUSKIPRA-BK, AUSKIPRA-GN, COLISUIN-CL, COLISUIN-TP, ERYSIPRAVAC, GRIPORK, HIPRASUIS-GLÄSSER, MYPRAVAC SUIS, NEUMOSUIN, PARVOSUIN, PARVOSUIN-MR, PARVOSUIN-MR/AD, RINIPRAVAC-DT, SUIPRAVAC-PRRS, SUIPRAVAC-RC, TOXIPRA PLUS (all of **Laboratorios Hipra S.A., Amer, Girona, Spain**); Clostricol, Coliporc Plus, Haeppovac, Per-C-Porc, Porciparvac, RESPIPORC ART + EP, RESPIPORC FLU, Respiporc M. HYO 1 SHOT, Rhusiovac, Rotlauf-Lebendimpfstoff, Salmoporc, Suisaloral, AK-vac MK35 (all of **IDT Impfstoffwerk DessaTornau, Tornau, Germany**); Mypravac Suis, (**Albrecht GmbH, Germany**); Haemo Shield® P, Parapleuro Shield® P, Parapleuro Shield® P+BE, Rhinicell® FD, Rhini Shield™ TX4, Prefarrow Shield® 9, Prefarrow Strep Shield®, Clostratox® BCD,

Clostratox® C, Clostratox® Ultra C 1300, Porcine Ecolizer® 3 + C, Porcine Pili Shield ™+C, Porcine Pili Shield ™Porcine Ecolizer® 3, Ery Serum™Ery Shield™Ery Vac Oral, Ery Shield™ +L5, PanSTAR™ Ery, Erycell™Parvo Shield® E, Parvo Shield® L5E, Parvo Shield® L5, Parvo Shield®, Para Shield®, PneumoSTAR SIV, PneumoSTAR™ Myco, Lepto Shield™ 5, Myco Shield™Salmo Shield® 2, Salmo Shield® Live, Amitox Tet™C. Perfringens Type A Toxoid (all of **Novartis Animal Health, Basel, Switzerland);** Nitro-Sal (**Akro**); or any antigen included in the compositions described above.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following examples set forth preferred materials and procedures in accordance with the present invention. It is to be understood, however, that these examples are provided by way of illustration only, and nothing therein should be deemed a limitation upon the overall scope of the invention.

EXAMPLE 1

The study design was set up to evaluate the combination efficacy of PCV2-*M.hyo* at full and 2 x 1/2 dose, at two different inclusion levels of *M. hyo* for the combination product and also to demonstrate any interference of the combination product compared to the monovalent *M. hyo*.

Materials and Methods:

Pigs were divided into 6 groups. All of the challenge groups (Groups 1-5) had around 19 pigs in each group. The control group containing pigs that were not given treatment and were not challenged (Group 6) had 5 pigs in the group. All pigs were necropsied on Day 61 of the study. The Schedule of the Investigation is shown below in Table 1

Table 1

Study Day (approximately)	Study Event
Before Day 0 (D0)	ID pigs; Health examination
Before D0	Randomize pigs to one of five groups
D-2	All pigs: Collect blood samples; Clinical Assessments
D0	All pigs: Clinical Assessments
D0	Administer Investigational Veterinary Product 1 (IVP1) to Group 1; Administer Investigational Veterinary Product 2 (IVP2) to Group 2; Administer Investigational Veterinary Product 3 (IVP3) to Group 3; Administer Investigational Veterinary Product 3 (IVP3) to Group 4; Administer Control Product (CP) to Group 5.
D1 to D33	All pigs: Clinical Assessments
D12	Collect blood samples from all animals. Administer IVP-3 to Group 3; CP administered to Group 5.
D32	Move at least the strict control group (Group 6) to a separate room
D33	All pigs: Collect blood samples All pigs: Anesthetize; Challenge with virulent *M. hyopneumoniae* strain 232
D32 to D63	All pigs: Daily clinical observations
D47	All pigs: Collect blood samples
D61	All pigs: Collect blood samples All pigs: Euthanize; Necropsy; Score lungs; Collect lung samples

This is a vaccination-challenge efficacy study conducted in a minimum of 100 piglets, approximately 21 ± 6 days of age on Day 0 (D0).

Prior to the start of the study, piglets' sera was drawn and screened for serological status to *M. hyopneumoniae* and Porcine Reproductive and Respiratory Syndrome virus (PRRSV). Only piglets that were negative for *M. hyopneumoniae* and PRRSV antibodies in serum were considered for the study. Table 2 gives a summary of the study.

Table 2

Group	Number	Treatment on D0	Treatment on D12	Challenge with *M. hyopneumoniae* On D33	Day of Necropsy
1	≥ 19	1.0 mL of **IVP-1** (50 mL of *M. hyo* Bacterin) IM in the neck region	N/A	Yes	D61
2	≥ 19	2.0 mL of **IVP-2** (100 mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	N/A	Yes	D61
3	≥ 19	1.0 mL of **IVP-3** (100 mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	1.0 mL of **IVP-3** (100mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	Yes	D61

4	≥ 19	2.0 mL of **IVP-3** (100 mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	N/A	Yes	D61
5	≥ 19	2.0 mL of **CP** (50 mL saline) IM in the neck region	1.0 mL of **CP** (50 mL saline) IM in the neck region	Yes	D61
6	≥ 5	N/A	N/A	N/A	D61

IVP-1 (monovalent *M. hyo*) was serial 273-011B that had a release RP of 1.44 and an RP of 1.22 at 18 months. IVP-2 (PCV2-*M. hyo*) utilized *M. hyo* serial 273-011B (RP of 1.22 at 18 months) and PCV2 serial 309-035 (release RP of 1.38) IVP-3 (PCV2- *M. hyo*) was composed of *M. hyo* serial 273-010B (release RP 2.8) and PCV2 serial 309-035 (release RP of 1.38)

Prior to D0, all pigs were assigned completely at random to one of six groups. Group 1 consisted of ≥ 19 pigs and was assigned to the *M. hyo* vaccine treatment group (Investigational Veterinary Product-1 (IVP-1)) treated group. Group 2 consisted of ≥ 19 pigs and was assigned to a *M. hyo*-PCV2 vaccine (Investigational Veterinary Product-2 (IVP-2)) treatment group and was administered a 1 x 2.0 mL dose on day 0. Group 3 consisted of ≥ 19 pigs and was assigned to a *M. hyo*-PCV2 vaccine treatment group (IVP-3) and was administered a 1.0 mL dose on days 0 & 12. Group 4 consisted of ≥ 19 pigs and received a *M. hyo*-PCV2 vaccine (IVP-3) 1 x 2.0 mL on Day 0. Group 5 served as challenge controls and received 2.0 mL of control product on Day 0 and 1.0 mL of control product on Day 12. Group 6 served as strict controls and did not receive any product. Prior to D0, each pig was examined for overall health and had their blood sampled.

On D0, 1) ≥ 19 piglets assigned to Group 1 received 1.0 mL of IVP-1 IM; 2) ≥ 19 piglets assigned to Group 2 received 2.0 mL of IVP-2 IM; 3) ≥ 19 piglets assigned to Group 3 received 1.0 mL of IVP-3 IM on days 0 &12; 4) ≥ 19 piglets assigned to Group 4 received 2.0 mL of IVP-3 IM on Day 0; 5) ≥ 19 piglets assigned to Group 5 received 2.0 mL of CP IM on Study Day 0 and 1.0 mL IM on Study Day 12; and, 6) ≥ 5 piglets assigned to Group 6 did not receive product and served as strict controls.

On Study Day 0, treatments were administered in the right neck region, midway between the base of the ear and the point of the shoulder. On Study Day 12, treatment was administered in the left neck region, midway between the base of the ear and the point of the shoulder. All piglets were observed daily from D1 to D33 for any adverse events and overall

health. All piglets were blood sampled on D33. On D33, each pig was challenged intratracheally with 10 ml of virulent *M. hyopneumoniae.* All pigs were observed daily for any clinical signs of disease from D32 to D61. Blood samples were collected from all pigs on D47 and D61. Blood samples collected prior to IVP administration were tested for *M. hyopneumoniae* and PRRS serology. Blood samples collected on D12, D33, D47 and D61 were tested for *M. hyopneumoniae* serology only. On D61, all pigs were euthanized and necropsied. The lungs and trachea were removed and the lungs scored. A sample was removed from each lung and tested by PCR for *M. hyopneumoniae* DNA. To determine if the study objective was achieved, lung lesion score data from Group 1, 2, 3 and 4 was compared to controls (Group 5) for statistically significant differences. Data from Group 6 was not included in any statistical analysis and was for information purposes only. Other *M. hyopneumoniae* parameters that were analyzed between Group 1, 2, 3 and 4 and controls (Group 5) included *M. hyopneumoniae* serology, post-vaccination clinical assessments and post-challenge clinical signs. PCR testing for the presence of *M. hyopneumoniae* DNA was completed on samples taken from the lungs to demonstrate an effective challenge.

Results and Discussion:

Upon necropsy, lungs were scored. One animal in Group 1 had consolidation and multiple abscesses and one animal in Group two had consolidation plus pleuritis. Statistical analysis was performed both with and without these two animals. As shown below, Group 3 had the lowest lung score of the vaccinated groups, with a score of 2.20. Group 4 had the highest lung score of the vaccinated groups, with a score of 4.19. Both Groups 1 and 2 had a lower lung score when animals 729 and 712, respectively, had been removed, with lung score for Group 1 being 2.13 and 2.56 for Group 2 with the animals removed. Lung scores for all of the vaccinated groups were significantly lower than the lung score of Group 5 (14.27) in which the pigs were challenged and no vaccine was administered.

Lung Scores:

Group	Average Lung Scores
1	5.54
1 (with 729 removed)	2.13

2	3.90
2 (with 712 removed)	2.56
3	2.20
4	4.19
5	14.27
6	0.00

Below is the statistical pairwise comparison summary. This includes analysis both with and without the two animals with conflicting factors.

Results of Pairwise Test - Scores.

Comparison (Group v. Group)	Wilcoxon Two-Sample Test	P Value Median Two-Sample Test	P Value Two-Sample T Test
1 vs. 5	<0.0001	<0.0001	<0.0001
1 vs. 5 (729 Removed)	<0.0001	<0.0001	<0.0001
2 vs. 5	<0.0001	<0.0001	<0.0001
2 vs. 5 (712 Removed)	<0.0001	<0.0001	<0.0001
3 vs. 5	<0.0001	<0.0001	<0.0001
4 vs. 5	<0.0001	<0.0001	<0.0001
1 vs. 2	0.3707	0.3872	0.3012
1 vs. 2 (712 and 729 Removed)	0.1636	0.2931	0.1139
3 vs. 4	0.0006	0.0005	0.0165
2 vs. 4	0.2196	0.2706	.7767

2 vs. 4 (712 Removed)	0.1045	0.1511	0.5296
1 vs. 3	0.1752	0.1365	0.0247
1 vs. 3 (729 Removed)	0.6212	0.3629	0.9196
1 vs. 4	0.0356	0.0515	0.3785
1 vs. 4 (729 Removed)	0.0022	0.0135	0.0150

All vaccinated groups showed statistically significant results when compared to controls. There were differences between the 2x1/2 dose and the 1x2ml dose (Groups 3 and 4). These groups had lung scores of 2.2 and 4.19 respectively. There were also differences between the monovalent and the high *M. hyo* antigen inclusion combo group (Group 1 versus 4). The results of this study provide surprising results that *M. hyo* in combination with PCV2 can provide an immunogenic effect when administered to pigs, as well as preserve the efficacy of the *M. hyo* component. Additionally, the results show that a single dose of the composition provides quick, long-lasting immunity. A single dose of this kind requires less time and manual labor and puts less stress on the pigs.

EXAMPLE 2

This study design was set up to determine the duration of immunity of the combination vaccine.

Materials and Methods:

Pigs were divided into 6 groups. All of the challenge groups (Groups 1-5) had around 19 pigs in each group. The control group containing pigs that were not given treatment and were not challenged (Group 6) had 5 pigs in the group. The Schedule of the Investigation is shown below in Table 3.

Table 3

Group	Number	Treatment on D0	Treatment on D12	Challenge with

				***M. hyopneumoniae* On D33**
1	≥ 19	1.0 mL of **IVP-1** (50 mL of *M. hyo* Bacterin) IM in the neck region	N/A	Yes
2	≥ 19	2.0 mL of **IVP-2** (100 mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	N/A	Yes
3	≥ 19	1.0 mL of **IVP-3** (100 mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	1.0 mL of **IVP-3** (100mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	Yes
4	≥ 19	2.0 mL of **IVP-3** (100 mL: 50 mL of *M. hyo* Bacterin and 50 mL of PCV2 vaccine) IM in the neck region	N/A	Yes
5	≥ 19	2.0 mL of **CP** (50 mL saline) IM in the neck region	1.0 mL of **CP** (50 mL saline) IM in the neck region	Yes
6	≥ 5	N/A	N/A	N/A

The pigs in the challenged groups were challenged on D184 of the study. All piglets were observed daily from D1 to D33 for any adverse events and overall health. All piglets were blood sampled on D33. On D184, each pig was challenged intratracheally with 10 ml of virulent *M. hyopneumoniae*. All pigs were observed daily for any clinical signs of disease from D32 to D184. Blood samples were collected from all pigs on D47 and D61. Blood samples collected prior to IVP administration were tested for *M. hyopneumoniae* and PRRS serology. On D184, all pigs were euthanized and necropsied. The lungs and trachea were removed and the lungs scored.

Results and Discussion

After the pigs were necropsied their lungs were removed, and the lungs were observed for lung lesions. The vaccinated groups (Groups 1-4) had an average gross lung pathology of 6.2% ($P>0.0023$), the challenge control group (Group 5) had an average gross lung pathology of 14.9%, and the strict controls group (Group 6) had an average gross lung pathology of 1.6%. These results are summarized in Table 4 below.

Table 4

Efficacy of Ingelvac MycoFLEX 26 weeks post-vaccination

Treatment Group	Average Gross Lung Pathology (% of lung)
Ingelvac MycoFLEX	6.2% (P<0.0023)
Challenge Controls	14.9%
Strict Controls	1.6%

The results of this study indicate that the vaccine combination of *M. hyo* and PCV2 has a long-lasting effect in pigs that is at least 184 days or 26 weeks post vaccination. This is a surprising result given that the combination of *M. hyo* and PCV2 antigens is a novel combination.

EXAMPLE 3

This investigation was carried out to determine the amount of interference observed when mixing an antigen of *M. hyo* with an antigen of PCV2. The study demonstrated that the *M. hyo* component of the vaccine was still effective in the presence of PCV2.

Materials and Methods

Pigs were 3 weeks ± 5 days of age at vaccination. Group 1 was vaccinated with a single 2 ml dose of *M. hyo* antigen in the form of a bacterin. Group 2 was vaccinated with a single 2 ml dose of equal amounts of *M. hyo* antigen and PCV2 antigen. Group 3 was a challenge control group and Group 4 was a strict control group. Groups 1-3 were subsequently challenged on D33 with a virulent *M. hyo* isolate. All animals in the study were necropsied at D61.

Results and Discussion

Upon necropsy, lungs were scored. Group 1, vaccinated with *M. hyo* antigen, only had average gross lung pathology of 5.5% (P>0.001). Group 2, vaccinated with *M. hyo* antigen and PCV2 antigen, had average gross lung pathology of 3.9% (P>0.001). Group 3, the challenge controls, had average gross lung pathology of 14.3% and Group 4, strict controls, had an average gross lung pathology of 0. The results are summarized below in Table 5.

Table 5

Efficacy of Ingelvac MycoFLEX as a monovalent and after mixing with CircoFLEX

Treatment Group	Average Gross Lung Pathology (% of lung)
Ingelvac MycoFLEX	5.5 % (P<0.001)
MycoFLEX mixed with CircoFLEX	3.9% (P<0.001)
Challenge Controls	14.3%
Strict Controls	0

The results of this study show that the difference in lung pathology, between those pigs vaccinated with *M. hyo* antigen alone versus those pigs vaccinated with a combination of *M. hyo* and PCV2 antigens, was not statistically significant. Therefore, this study shows that surprisingly, PCV2 antigen does not interfere with the immunogenic effect of the *M. hyo* antigen component. This makes the combination vaccine comprising *M. hyo* antigen and PCV2 antigen efficacious for infection by *M. hyo*. Additionally, it shows that this novel combination of antigens can be mixed and still provide the necessary protection against these pathogens after a single dose.

EXAMPLE 4

This investigation was carried out to determine the efficacy of the composition, comprised of *M. hyo* antigen and PCV2 antigen, when challenged with PCV2.

Materials and Methods

The PCV2 efficacy evaluation was performed in sixty caesarian-derived, colostrum-deprived piglets. At approximately three weeks of age, piglets were vaccinated with Ingelvac® MycoFLEX™, Ingelvac® PRRS MLV™ and Ingelvac® CircoFLEX™ in a single 2 ml dose. On day 31 post vaccination, vaccinate and control animals were administered a virulent PCV2 challenge virus. Twenty-two days following the administration of the challenge material, all animals were euthanized and the select tissues were removed and submitted for histology and immunohistochemistry (IHC) for PCV2. All procedures for handling and housing the piglets were observed as described in Example 1.

Results and Discussion

The criteria for the PCV2 efficacy evaluation were lymphoid depletion, lymphoid inflammation, and lymphoid IHC. The vaccinated group had 0% lymphoid depletion, 4.2% (1/24) lymphoid inflammation, and 8.3% (2/24) lymphoid IHC. The control group had 83.3% (20/24) lymphoid depletion, 87.5% (21/24) lymphoid inflammation, and 91.7% (22/24) lymphoid IHC. The results are summarized below in Table 6.

Table 6

Summary of PCV2 Primary Challenge Results

Treatment	Lymphoid depletion +/ total (%)	Lymphoid inflammation +/total (%)	Lymphoid IHC +/total (%)
Vaccine	0/24 (0.0%)	1/24 (4.2%)	2/24 (8.3%)
Controls	20/24 (83.3%)	21/24 (87.5%)	22/24 (91.7%)
P-value between groups	<0.0001	<0.0001	<0.0001

There were no injection site or other adverse reactions that could be attributed to the vaccine mixture.

The results, together with the results of the *M. hyo* challenge study described in Example 1, demonstrate that the mixture of *M. hyo* antigen and PCV2 antigen delivered in a single 2 ml dose is efficacious against infections by *M. hyo* and PCV2. The ability to mix these antigens provides needed protection against the pathogens *M. hyo* and PCV2 as well as decreasing the number of injection sites and subsequent stress on the animal while decreasing the labor required to administer two separate vaccines without sacrificing the safety of the individual vaccines. Further this demonstrates that the vaccine combination is efficacious for infection against PCV2.

EXAMPLE 5

This example determined the number of mice producing detectable levels of antibodies to *M. hyo* or PCV2 after receiving an administration of an immunogenic composition comprising *M. hyo* antigen or PCV2 antigen in accordance with the present invention.

Materials and Methods

Antibody production was measured in mice from 5 different treatment groups, each having 20 mice therein. The first group received a dose of *M. hyo* antigen, the second and third groups received a dose of PCV2 antigen, and the last two groups served as a control group and received a dose of physiological saline and Carbopol. Each mouse in Group 1 received a 0.1 ml administration of IVP-1 (monovalent *M. hyo* antigen) from serial 273-011B that had a release RP of 1.44 and an RP of 1.22 at 18 months (see Example 1 above). Prior to administration, the *M. hyo* antigen for each dose in Group 1 was diluted 1:4. Thus, the amount of antigen administered to the mice was 40-fold less than the amount of antigen administered to pigs in Example 1. Each mouse in Group 2 received a 0.2ml administration of PCV2 antigen serial 309-035 (release RP of 1.38) (See Example 1 above). Similar to the *M. hyo* antigen for Group 1, the PCV2 antigen for each dose of Group 2 was diluted 1:2 with physiological saline such that it contained one-tenth of the PCV2 antigen administered to the pigs in Example 1 above. Each mouse in Group 3 received a 0.2ml administration of PCV2 antigen serial 309-035 (release RP of 1.38) that had been diluted 1:4 with physiological saline such that it contained one-twentieth of the PCV2 antigen administered to the pigs in Example 1 above. Groups 4 and 5 each received a 0.1ml (Group 4) or 0.2ml (Group 5) administration of physiological saline/Carbopol. After 21 days, antibody production was measured using a specific *M Hyo* antibody detection assay (ELISA) for Groups 1 and 4, and using a specific PCV2 antibody detection assay (ELISA) for groups 2, 3, and 5. If detectable amounts of antibodies were found using the assay, the result was termed "positive". If detectable amounts of antibodies were not found using the assay, the result was termed "negative."

Results

All mice in Group 1 were found to have detectable levels of *M. hyo* antibodies and all mice in Group 2 were found to have detectable levels of PCV2 antibodies 21 days after administration of the antigen-containing composition. In contrast, no detectable levels of *M. hyo* antibodies were found in Group 4 and no detectable levels of PCV2 antibodies were found in Group 5 21 days after administration of the physiological saline/Carbopol composition. Thus, one-fortieth of the antigen effective at inducing a protective immune response in pigs was able to produce detectable amounts of antibodies to *M. hyo* in 100% of

mice receiving an administration of a composition in accordance with the present invention. This amount was defined as an RP of 1.22 for *M. hyo*. Moreover, one-tenth of the antigen effective at inducing a protective immune response in pigs was able to produce detectable amounts of antibodies to PCV2 in 100% of mice receiving an administration of a composition in accordance with the present invention. This amount was defined as an RP of 1.38 for PCV2.

Claims

1. An immunogenic composition for eliciting a protective immune response in a pig against *Mycoplasma hyopneumoniae* (*M. hyo*) wherein said immunogenic composition comprises porcine circovirus type 2 antigen and *M. hyo* antigen, wherein the amount of the *M. hyo* antigen per dose has a relative potency (RP) value of at least 1.22, wherein an RP value of 1.22 for *M. hyo* means that 95% and preferably 100% of mice receiving an administration of one-fortieth (1/40) of such amount of *M. hyo* antigen develop a detectable amount of antibodies within or at 21 days post treatment in an *M. hyo* specific antibody detection assay.

2. The immunogenic composition according to claim 1, wherein the amount of porcine circovirus type 2 antigen per dose has a relative potency (RP) value of at least 1.38, wherein an RP value of 1.38 for porcine circovirus type 2 antigen means that 95% and preferably 100% of mice receiving an administration of one-tenth (1/10) of such amount of porcine circovirus type 2 antigen develop a detectable amount of antibodies within 21 days post treatment in an porcine circovirus type 2 specific antibody detection assay.

3. The immunogenic composition according to claim 1 or 2, wherein said composition further comprises an adjuvant.

4. The immunogenic composition according to claim 3, wherein the adjuvant is a carbomer.

5. The immunogenic composition according to claim 4, wherein said carbomer is Carbopol.

6. The immunogenic composition according to any one of claims 1 to 5, wherein said immunogenic composition elicits a protective immune response against *M. hyo* when administered to a pig as a single dose administration.

7. The immunogenic composition according to any one of claims 2 to 5, wherein said immunogenic composition elicits a protective immune response against *M. hyo* and porcine circovirus type 2 when administered to a pig as a single dose administration.

8. The immunogenic composition according to any one of claims 1 to 7, wherein the immunogenic composition elicits a duration of immunity against *M. hyo* and/or porcine circovirus type 2 of at least 130 days when administered to a pig.

9. The immunogenic composition according to claim 8, wherein the immunogenic composition elicits a duration of immunity against *M. hyo* and/or porcine circovirus type 2 of at least 150 days when administered to a pig.

10. The immunogenic composition according to claim 8, wherein the immunogenic composition elicits a duration of immunity against *M. hyo* and/or porcine circovirus type 2 of at least 184 days when administered to a pig.

11. The immunogenic composition according to any one of claims 1 to 10, wherein the specific antibody detection assays are an ELISA.

12. The immunogenic composition according to any one of claims 1 to 11, wherein the specific antibody detection assay for detecting the *M. hyo* specific antibodies is the IDEXX Herdchek *M. hyo* Test Kit.

13. The immunogenic composition according to any one of claims 2 to 12, wherein the specific antibody detection assay for detecting the porcine circovirus type 2 specific antibodies is the *modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV* as exemplarily described in Clin. Diagn. Lab. Immunol. **9:**33-40 (2002).

14. A method for eliciting a protective immune response in a pig against *Mycoplasma hyopneumoniae* (*M. hyo*) comprising administering to said pig an immunogenic composition which comprises porcine circovirus type 2 antigen and *M. hyo* antigen, wherein the amount of the *M. hyo* antigen per dose has a relative potency value of at least 1.22, wherein an RP value of 1.22 for *M. hyo* means that 95% and preferably 100% of mice receiving an administration of one-fortieth (1/40) of such amount of *M. hyo* antigen develop a detectable amount of antibodies within or at 21 days post treatment in an *M. hyo* specific antibody

detection assay.

15. The method of claim 14, wherein the amount of porcine circovirus type 2 antigen per dose has a relative potency (RP) value of at least 1.38, wherein an RP value of 1.38 for porcine circovirus type 2 antigen means that 95% and preferably 100% of mice receiving an administration of one-tenth (1/10) of such amount of porcine circovirus type 2 antigen develop a detectable amount of antibodies within 21 days post treatment in an porcine circovirus type 2 specific antibody detection assay.

16. The method according to claim 14 or 15, wherein said composition further comprises an adjuvant.

17. The method composition according to claim 16, wherein the adjuvant is a carbomer.

18. The method according to claim 17, wherein said carbomer is Carbopol.

19. The method according to any one of claims 14 to 18, wherein said immunogenic composition elicits a protective immune response against *M. hyo* when administered to said pig as a single dose administration.

20. The method according to any one of claims 14 to 18, wherein said immunogenic composition elicits a protective immune response against *M. hyo* and porcine circovirus type 2 when administered to said pig as a single dose administration.

21. The method according to any one of claims 14 to 20, wherein the immunogenic composition elicits a duration of immunity against *M. hyo* and/or porcine circovirus type 2 of at least 130 days when administered to said pig.

22. The method according to claim 21, wherein the immunogenic composition elicits a duration of immunity against *M. hyo* and/or porcine circovirus type 2 of at least 150 days when administered to said pig.

23. The method according to claim 21, wherein the immunogenic composition elicits a duration of immunity against *M. hyo* and/or porcine circovirus type 2 of at least 184 days when administered to said pig.

24. The method according to any one of claims 14 to 23, wherein the specific antibody detection assays are an ELISA.

25. The method according to any one of claims 14 to 24, wherein the specific antibody detection assay is the IDEXX Herdchek *M. hyo* Test Kit.

26. The method according to any one of claims 15 to 25, wherein the specific antibody detection assay for detecting the porcine circovirus type 2 specific antibodies is the *modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV* as exemplarily described in Clin. Diagn. Lab. Immunol. **9:**33-40 (2002).