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(Continued on nextpage)

(54) Title: A COMPOSITION COMPRISING PEDV ANTIGENS AND METHODS FOR MAKING AND USING THE COMPOSITION

(57) Abstract: Disclosed herein are embodiments of an immunogenic composition for porcine epidemic diarrhea virus, and a method for making the immunogenic composition. Also disclosed is a method for administering the immunogenic composition to a subject in need thereof. The immunogenic composition comprises PEDV proteins and/or antigens from one or more strains of PEDV, and may additionally comprise proteins and/or antigens from one or more additional porcine pathogens, such as PRRSV. Additionally disclosed is a combination comprising a PEDV immunogenic composition as disclosed herein, and an immunogenic composition or other therapeutic composition directed toward an additional porcine pathogen.

FIG. 2

<table>
<thead>
<tr>
<th>Lane</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prestained Protein Standard</td>
</tr>
<tr>
<td>2</td>
<td>PEDV Culture broth</td>
</tr>
<tr>
<td>3</td>
<td>PEDV Extract - 2X dilution</td>
</tr>
<tr>
<td>4</td>
<td>PEDV Extract - 3X dilution</td>
</tr>
<tr>
<td>5</td>
<td>PEDV Extract - 4X dilution</td>
</tr>
<tr>
<td>6</td>
<td>PEDV Extract - 5X dilution</td>
</tr>
</tbody>
</table>

Evaluation of PEDV Antigen Extract

Blot with mAb 6C8

Blot with mAb 3F12

kDa

240

140

100

70

50

35

25

Declarations under Rule 4.17:
— of inventorship (Rule 4.17(iv))

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— with sequence listing part of description (Rule 5.2(a))
A COMPOSITION COMPRISING PEDV ANTIGENS AND METHODS FOR MAKING
AND USING THE COMPOSITION

CROSS REFERENCE TO RELATED APPLICATIONS
This application claims the benefit of the earlier filing dates of U.S. Provisional Patent Application Nos. 62/113,976 and 62/113,979, both filed on February 9, 2015, both of which are incorporated herein by reference in their entirety.

FIELD
This disclosure relates to a composition comprising porcine epidemic diarrhea virus (PEDV) antigens, a method of making the composition, and a method of using the composition, such as by administering to a porcine.

BACKGROUND
Porcine epidemic diarrhea virus (PEDV) is a highly infectious coronavirus that infects the intestinal system of a pig, typically causing diarrhea and/or dehydration. While adult pigs mostly become sick and lose weight after becoming infected, the virus is often fatal to newborn piglets. Infected herds can suffer a loss of about 75-100% of piglets for a four- to five-week period. It has been estimated that between June 2013 and March 2014 over 4 million piglets were lost to PEDV.

SUMMARY
Embodiments of a method for making a porcine epidemic diarrhea virus (PEDV) immunogenic composition are disclosed. The immunogenic composition may be a vaccine. Certain embodiments of the method may comprise infecting cells in a culture medium with PEDV. The infected cells are incubated for a period of time effective to result in one or more replicated PEDV viral particles being released into the culture medium. Cells infected with PEDV in a population of cells comprising infected cells are isolated away from cell-free PEDV virus particles to form cells containing cell-associated PEDV proteins and antigens. PEDV proteins and antigens are separated, such as extracted or eluted, from the isolated cells, such as by using a biological buffer with or without detergent and/or metal chelating agents, and/or a freeze-thaw process, to form a first solution comprising isolated PEDV proteins and antigens. Viral particles may be
inactivated in the solution to produce a second solution. The method may further comprise combining the second solution with an adjuvant. In some embodiments, the adjuvant is an adjuvant suitable for intranasal, oral, intravaginal, intramuscular, topical or subcutaneous administration. The adjuvant may be selected to stimulate a mucosal antibody response, and may adhere to the mucous membranes. In certain examples, the adjuvant comprises a polyacrylate and/or a cross-linked polyacrylic acid polymer.

Certain additional disclosed embodiments concern an immunogenic composition comprising isolating PEDV proteins and antigens that may be made according to the disclosed method. The immunogenic composition may be a vaccine. For example, certain immunogenic composition embodiments comprise at least a spike, or S protein, or a portion thereof, in an amount sufficient to produce an immune response in a subject, and/or confer protective immunity to the subject, that is administered the immunogenic composition. The spike protein may be an intact spike protein. Certain additional immunogenic composition embodiments comprise a first antigenic component comprising isolated PEDV proteins and/or antigens from a first PEDV strain, and at least a second antigenic component. For example, the second antigenic component may comprise isolated PEDV proteins and/or antigens from a second PEDV strain. As another example, the second antigenic component may comprise isolated proteins and/or antigens from a second pathogen other than PEDV, such as an immunogenic composition where the second pathogen is porcine reproductive and respiratory syndrome virus (PRRSV), Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Clostridium tetani, Clostridium perfringens, porcine parvovirus, Erysipelothrix rhusiopathiae, Leptospira pomona, Leptospira grippotyphosa, Leptospira hardjo, Leptospira canicola, Leptospira icterohaemorrhagiae, Leptospira bratislava, porcine circovirus, Lawsonia intracellularis, Escherchia coli, Actinobacillus pleuropneumoniae, Haemophilus parasuis, Salmonella choleraesuis, Salmonella typhimurium, Streptococcus suis, Pasteurella multocida, Bordetella bronchisepitica, Actinobacillus pleuropneumoniae, Serpulina hyodysenteriae, encephalomyocarditis virus, swine influenza virus, transmissible gastroenteritis (TGE) virus, swine delta coronavirus, rotavirus diarrhea, foot and mouth disease virus, classical swine fever virus, pseudorabies virus, Japanese encephalitis virus (JEV), encephalomyocarditis virus, or any and all combinations thereof. In certain embodiments, the second pathogen is not Mycoplasma hyopneumoniae. Yet additional disclosed embodiments concern an immunogenic composition comprising a first antigenic component comprising isolated PEDV proteins and/or antigens from a first PEDV strain, isolated PEDV proteins and/or antigens from a second PEDV
strain, and at least one additional antigenic component comprising isolated proteins and/or antigens from a second pathogen other than PEDV, such as porcine reproductive and respiratory syndrome virus.

The immunogenic composition may comprise an adjuvant. The adjuvant may be selected to stimulate a mucosal antibody response and/or may adhere to the mucous membranes or may be selected to be administered parenterally, such as intramuscularly or subcutaneously.

In other embodiments, the adjuvant comprises an emulsified oil-in-water adjuvant, and optionally may comprise an ammonium salt, such as a tetraalkylammonium salt. In particular embodiments, the ammonium salt is dimethyldioctadecylammonium bromide. In other embodiments, the adjuvant comprises an acrylic acid polymer.

A method of administering a disclosed immunogenic composition to a subject, particularly a porcine subject, also is disclosed. In some embodiments the porcine subject is a piglet, and the administration method comprises administering to a piglet < 7 days old, and in some embodiments administering to a piglet ≤ 5 days old, such as a 2-day old or less piglet, an effective amount of a disclosed PEDV immunogenic composition. The immunogenic composition may be administered by any effective means, such as intranasally, orally, intramuscularly, subcutaneously, or combinations thereof, with certain particular embodiments realizing substantially beneficial results when the immunogenic composition is administered to mucosal membranes, such as by intranasal, oral, intravaginal or rectal administration. This may advantageously result in the production of IgA antibodies. In certain disclosed embodiments, the immunogenic composition is administered intranasally, particularly to piglets, such as piglets 14 days old or less, 7 days old or less, 5 days old or less, or 2 days old or less. In some embodiments, a sow is administered a first disclosed immunogenic composition, such as by intramuscular or subcutaneous administration, and the piglets farrowed from that sow are intranasally administered a second disclosed immunogenic composition. The sow may be already pregnant with the piglets, or the sow may be expected to become pregnant subsequent to the administration of the first immunogenic composition. The sow may be administered the first immunogenic composition at a time point prior to becoming pregnant such that the sow is receiving the benefit of the immunogenic composition when she becomes pregnant and/or gives birth. In the event that some degree of immunity is passed from sow to piglets, the sow may be administered the first immunogenic composition at a time point prior such that a degree of immunity is passed to piglets farrowed from the sow. In some embodiments, the sow is administered the first immunogenic composition at a time point from immediately before
conception to at least six months before conception, such as about four months before conception, about two months before conception, about one month before conception, 1 week before conception, or less than one week before conception. In certain embodiments, the first disclosed immunogenic composition comprises an oil-in-water emulsion adjuvant, such as an EMULSIGEN®-based adjuvant, and the second immunogenic composition comprises an adjuvant selected to stimulate a mucosal immune response, such as an adjuvant comprising an unsaturated carboxylic acid polymer, including, but not limited to, an adjuvant comprising a polyacrylate or polyacrylic acid, an adjuvant comprising a carbomer, an adjuvant comprising carbopel, or CARBIGEN™. The administered immunogenic composition may be any of the immunogenic compositions disclosed herein. In some embodiments, administering the first immunogenic composition to the sow and administering the second composition to the at least one piglet may be performed by the same individual and/or implemented under the guidance or instruction of a single entity.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 is a Western blot illustrating the detection of antibodies against 180-kDa to 350-kDa of PEDV spike protein.

FIG. 2 is a Western blot of PEDV proteins from cultures of infected cells, contacted with two monoclonal antibodies illustrating that the two antibodies appear to detect proteins with the same molecular weight.

FIG. 3 is a Western blot of PEDV proteins from cultures of infected cells over time, with a mixture of the two monoclonal antibodies used in FIG. 2, illustrating that an exemplary embodiment of the disclosed method successfully extracted proteins from cells infected with PEDV.

FIG. 4 is a Western blot of three MARC-cell based detergent extracts of PEDV isolates, illustrating the proteins present in the extracts.

FIG. 5 is a Western blot of the three detergent extracts of FIG. 4 and mixtures of the extracts before and after viral inactivation, illustrating the proteins present before and after inactivation.
FIG. 6 is a table illustrating the sequence homology between SEQ ID Nos: 1-9.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on February 9, 2016, 415 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is the nucleotide sequence of North American PEDV strain Colorado 2013 (GenBank Accession No. KF272920).

SEQ ID NO: 2 is the nucleotide sequence of North American PEDV strain Iowa/18984/2013 (GenBank Accession No. KF804028).

SEQ ID NO: 3 is the nucleotide sequence of North American PEDV strain North Carolina USA/NC/2013/35140.

SEQ ID NO: 4 is the nucleotide sequence of North American PEDV strain Indiana/2.83/2013 (GenBank Accession No. KJ645635).

SEQ ID NO: 5 is the nucleotide sequence of North American PEDV strain Iowa/2013 (GenBank Accession No. KJ645649).

SEQ ID NO: 6 is the nucleotide sequence of North American PEDV strain 125 1-125-10.

SEQ ID NO: 7 is the nucleotide sequence of Korean PEDV strain SM98 (GenBank Accession No. GU937797).

SEQ ID NO: 8 is the nucleotide sequence of Korean attenuated PEDV strain KR-DR13-att (GenBank Accession No. JQ023162).

SEQ ID NO: 9 is the nucleotide sequence of Chinese PEDV strain AH2012 (GenBank Accession No. KC210145).

SEQ ID NO: 10 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 1 North American PEDV strain Colorado 2013.

SEQ ID NO: 11 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 2 North American PEDV strain Iowa/18984/2013.

SEQ ID NO: 12 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 3 North American PEDV strain North Carolina USA/NC/2013/35140.
SEQ ID NO: 13 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 4 North American PEDV strain USA/Indiana12.83/2013.

SEQ ID NO: 14 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 5 North American PEDV strain USA/Iowa/2013.

SEQ ID NO: 15 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 6 North American PEDV strain 1251-125-10.

SEQ ID NO: 16 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 7 Korean PEDV strain SM98.

SEQ ID NO: 17 is the deduced amino acid sequence of the S protein encoded by SEQ ID NO: 8 Korean attenuated PEDV strain KR-DR13-att.

DETAILED DESCRIPTION

I. Definitions

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. The singular forms "a," "an," and "the" refer to one or more than one, unless the context clearly dictates otherwise. The term "or" refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. As used herein, "comprises" means "includes." Thus, "comprising A or B," means "including A, B, or A and B," without excluding additional elements. All references, including patents and patent applications cited herein, are incorporated by reference, unless expressly stated otherwise.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, percentages, temperatures, times, and so forth, as used in the specification or claims are to be understood as being modified by the term "about." Accordingly, unless otherwise indicated, implicitly or explicitly, the numerical parameters set forth are approximations that may depend on the desired properties sought and/or limits of detection under standard test conditions/methods. When directly and explicitly distinguishing embodiments from discussed prior art, the embodiment numbers are not approximates unless the word "about" is recited.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are
described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

The terms "administer," "administering," "administration," and the like, as used herein, refer to methods that may be used to enable delivery of compositions to the desired site of biological action. These methods include, but are not limited to, intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, intravaginally, orally, topically, intrathecally, inhalationally, intranasally, transdermally, rectally, and the like. Administration techniques that can be employed with the agents and methods described herein are found in e.g., Goodman and Gilman, *The Pharmacological Basis of Therapeutics*, current ed.; Pergamon; and Remington's *Pharmaceutical Sciences* (current edition), Mack Publishing Co., Easton, Pa., which are incorporated herein by reference.

Certain methods of administration deliver the immunogenic composition to mucosal membranes. These include, but are not limited to, intranasal, oral, intravaginal, and rectal. In some embodiments, an adjuvant is selected to facilitate administration to mucosal membranes, and/or stimulate a mucosal immune response. The adjuvant may adhere to the mucosal membrane. Mucosal immune responses typically comprise the production of IgA antibodies but may also stimulate IgG responses, which may be advantageous in certain disclosed embodiments.

Intranasal formulations may include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspension, solutions, emulsions, syrups or elixirs, or may be presented dry in tablet form or a product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservative.

As used herein, the terms "co-administration," "administered in combination with," and their grammatical equivalents, are meant to encompass administration of two or more therapeutic agents to a single subject, and are intended to include treatment regimens in which the agents are administered by the same or different routes of administration or at the same or different times. In some embodiments the one or more compositions described herein will be co-administered with
other agents, including, but not limited to, therapeutics such as other vaccines, antibiotics, or combinations thereof. These terms encompass administration of two or more agents to the subject so that both agents and/or their metabolites are present in the subject at the same time. They include simultaneous administration in separate compositions, administration at different times in separate compositions, and/or administration in a composition in which both agents are present. Thus, in some embodiments, the compositions described herein and the other agent(s) are administered in a single composition. In some embodiments, the compositions described herein and the other agent(s) are admixed in the composition.

The term "effective amount" or "therapeutically effective amount" refers to the amount of an active agent (such as one or more compounds provided herein alone, in combination, or potentially in combination with other therapeutic agent(s)) sufficient to induce a desired biological result. That result may be amelioration or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. The term "therapeutically effective amount" is used herein to denote any amount of a therapeutic and/or preventative that causes an improvement in a disease condition. The amount can vary with the condition being treated, the stage of advancement of the condition, and the type and concentration of formulation applied. Appropriate amounts in any given instance will be readily apparent to those of ordinary skill in the art or capable of determination by routine experimentation such as vaccination and observation of an antibody response or vaccination followed by a challenge wherein the vaccinated animals perform better than non-vaccinated animals that are challenged similarly.

The compositions provided herein, alone or in combination with other suitable components, can be made into aerosol formulations (e.g., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

The disclosed compositions can be formulated for parenteral administration, such as, for example, by intravenous, intraarterial, intramuscular, intradermal, intraperitoneal, and subcutaneous routes. Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, and emulsions. In addition, controlled-release parenteral dosage forms can be prepared. Suitable materials for such administration include sterile water; saline solution; glucose solution; aqueous vehicles, such as sodium chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose, Sodium Chloride Injection, Lactated Ringer's
Injection; ethyl alcohol, polyethylene glycol, and propylene glycol; non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate; aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this disclosure, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. In an independent embodiment, parenteral administration, oral administration, and/or intravenous administration are the methods of administration. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules, bottles, and vials.

PEDV protein refers to any polypeptide product encoded by the PEDV genome and/or produced as only as a result of PEDV infection or the PEDV lifecycle. Thus PEDV specific polypeptides not encoded by or expressed by a PEDV infected cell are within the scope of the term. Endogenous polypeptides encoded by a PEDV infected cell, but not expressed in the absence of PEDV infection and/or lifecycle, are not intended. However, endogenous polypeptides expressed only as a consequence of PEDV infection and/or lifecycle are within the scope of the term. The term also includes alternative forms of the polypeptides due to changes in secondary and/or tertiary structure, such as those resulting from partial or substantial protein denaturation as a non-limiting example. Thus denatured forms of the polypeptides are within the scope of the term.

The term "antigen" refers to a compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity. For example, PEDV antigen refers to any portion or fragment of a PEDV polypeptide that is recognized by an anti-PEDV antibody. In some cases, the portion or fragment may be a peptide with an attached moiety, such as, but not limited to, a sugar moiety, a phosphate moiety, or a lipid moiety. Alternatively, the portion or fragment may be a peptide without any attached non-peptide moieties.

The term "excipient," as used in this disclosure, is an additive that is used in combination with the PEDV antigens. An excipient can be used, for example, to dilute an active agent, such as the PEDV antigens, and/or to modify properties of a pharmaceutical composition. Examples of excipients include, but are not limited to, magnesium stearate, stearic acid, vegetable stearin,
sucrose, lactose, starches, hydroxypropyl cellulose, hydroxypropyl methylcellulose, xylitol, sorbitol, maltitol, gelatin, polyethylene glycol (PEG), phosphate buffered saline (PBS), carboxy methyl cellulose, vitamin A, vitamin E, vitamin C, retinyl palmitate, selenium, cysteine, methionine, citric acid, methyl paraben, propyl paraben, sugar, silica, talc, magnesium carbonate, sodium starch glycolate, tartrazine, aspartame, benzalkonium chloride, sesame oil, propyl gallate, sodium metabisulphite, lanolin, polyvinylpyrrolidone (PVP), tocopheryl polyethylene glycol 1000 succinate (also known as vitamin E TPGS, or TPGS), dipalmitoyl phosphatidyl choline (DPPC), trehalose, sodium bicarbonate, glycine, sodium citrate, lactose, saline, phosphate buffered saline or organic buffers including but not limited to Tris(hydroxymethyl)aminomethane, and metal chelating agents. Metal chelating agents include, but are not limited to, organic compounds such as the amino acids glutamic acid and histidine, organic diacids such as malate, and polypeptides such as phytochelatin, biomolecules such as pyochelin, pyoverdine, enterobactin and Dopa, and synthetic chelates such as ethylenediaminetetraacetic acid (EDTA).

An adjuvant is an excipient that modifies the effect of another agent, typically the active ingredient. As used herein an adjuvant may be added to an immunogenic composition, such as a vaccine, to modify the immune response to increase the amount of antibodies produced and/or increase the length of protection conferred by the vaccine. An adjuvant may also be added to stimulate a certain class of antibodies such as stimulation of mucosal antibodies like IgA. An adjuvant also may be added to a composition to help stabilize a formulation of proteins and/or antigens in a vaccine composition. In some embodiments, the adjuvant is a non-naturally occurring chemical. Examples of adjuvants include, but are not limited to, inorganic compounds, such as metal salts such as alum, aluminum hydroxide, aluminum phosphate, aluminum sulfate, or calcium phosphate hydroxide; mineral oil, such as paraffin oil; organic esters such as aryl or aliphatic esters, particularly alkyl esters such as linear alkyl esters having up to at least 25 carbons, preferably up to 10 carboxy esters of acids or of alcohols containing a linear alkyl group, such as 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; oil-in-water emulsions, such as MF59 (U.S. Patent no. 6,299,884) (containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85, optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model HOY microfluidizer (Microfluidics, Newton, MA), and SAF (containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer LI 21, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size
emulsion), EMULSIGEN®-based adjuvants including EMULSIGEN®, EMULSIGEN®-D (containing dimethyldioctadecylammonium bromide (DDA)), EMULSIGEN®-BCL (containing a block copolymer immunostimulant) and EMULSIGEN®-P (containing with a proprietary immunostimulant), and EMULSIGEN®-75 (a double adjuvant comprising an oil-in-water adjuvant with a cross-linked polymer) (Phibro Animal Health Corporation, Omaha, NE, USA); saponins, such as Stimulon™ QS- 21 (Antigenics, Framingham, MA.), described in U.S. Patent no. 5,057,540, ISCOMATRIX (CSL Limited, Parkville, Australia), described in U.S. Patent no. 5,254,339, and immunostimulating complexes (ISCOMS); surfactants, e.g., hexadecyl amine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N'-N'-bis(2-hydroxyethyl-propane di-amine), methoxyhexadecyl-glycerol, and pluronic polyols; copolymers, including low molecular weight copolymers such as Polygen™ (available from Phibro Animal Health Corporation, Omaha, NE, USA); polanions, e.g., pyran, dextran sulfate, and poly IC; peptides, e.g., muramyl dipeptide, MPL, aminomethylglycine, tuftsin, oil emulsions, alum, and mixtures thereof.

Unsaturated carboxylic acid polymers also may be useful as adjuvants for the disclosed compositions. Such polymers include polymers of acrylic or methacrylic acid, copolymers of maleic anhydride and alkenyl derivative and cross-linked acrylic acid polymers, such as polyacrylate or polyacrylic acid polymers, optionally cross-linked with polyalkenyl ethers of sugars or polyalcohols (carbomers). In certain embodiments, polymers comprising moieties having from 2 to 10 carbon atoms, more particularly 2 to 4 carbon atoms are preferred, e.g. vinyls, allyls and other ethenically unsaturated groups. The unsaturated radicals may be optionally substituted, such as with one or more alkyl moieties including methyl. Such polymers include polymers are sold under the name carbolpol (cross-linked with an allyl sucrose or with allyl pentaerythritol) including carbolpol 934P, carbolpol 971P and carbolpol 974P (available from Lubrizol Corporation, Wickliffe, Ohio)) and the polymer sold under the name CARBIGEN™ (available from Phibro Animal Health Corporation, Omaha, NE, USA). Copolymers of maleic anhydride and alkenyl derivative include the copolymers EMA (Monsanto) that are copolymers of maleic anhydride and ethylene. In certain embodiments, an adjuvant comprising an unsaturated carboxylic acid polymer, such as an adjuvant comprising carbolpol, or CARBIGEN™, are advantageous for administration to mucus membranes, such as via intranasal, oral, vaginal and rectal routes.

Other exemplary adjuvants include bacterial lipopolysaccharides; synthetic polynucleotides such as oligonucleotides containing a CpG motif (e.g., U.S. Patent no. 6,207,646); IC-31 (Intercell
AG, Vienna, Austria), described in European Patent Nos. 1 296 713 and 1 326 634; a pertussis toxin (PT) or mutant thereof, a cholera toxin or mutant thereof (e.g., U.S. Patent Nos. 7,285,281, 7,332,174, 7,361,355 and 7,384,640); or an E. coli heat-labile toxin (LT) or mutant thereof, particularly LT-K63, LT-R72 (e.g., U.S. Patent Nos. 6,149,919, 7,115,730 and 7,291,588); bacterial products, such as killed bacteria Bordetella pertussis, Mycobacterium bovis, Mycobacterium tuberculosis or toxoids; B peptide subunits of E. coli heat labile toxin or cholera toxin (McGhee, J.R., et al., "On vaccine development," Sem. Hematol., 30:3-15 (1993)); nonbacterial organics such as squalene or thimerosal; delivery systems, such as detergents (Quil A); cytokines and/or lymphokines, such as interleukins 1-a, 1-β, 2, 4, 5, 6, 7, 8 and 10, 12 (see, e.g., U.S. Patent no. 5,723,127), 13, 14, 15, 16, 17 and 18 (and its mutant forms); the interferons-α, β and γ; granulocyte-macrophage colony stimulating factor (GM-CSF) (see, e.g., U.S. Patent no. 5,078,996 and ATCC Accession Number 39900); macrophage colony stimulating factor (M-CSF); granulocyte colony stimulating factor (G-CSF); and the tumor necrosis factors α and β; chemokines, such as MCP-1, MIP-1α, MIP-1β, and RANTES; adhesion molecules, such as a selectin, e.g., L-selectin, P-selectin and E-selectin; mucin-like molecules, such as CD34, GlyCAM-1 and MadCAM-1; a member of the integrin family, such as LFA-1, VLA-1, Mac-1 and pl50.95; co-stimulatory molecules, such as CD40 and CD40L; immunoglobulin superfamily members, such as PECAM, ICAMs, e.g., ICAM-1, ICAM-2 and ICAM-3, CD2 and LFA-3; growth factors including vascular growth factor, nerve growth factor, fibroblast growth factor, epidermal growth factor, B7.2, PDGF, BL-1, and vascular endothelial growth factor; receptor molecules including Fas, TNF receptor, Fit, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, and DR6; Caspase (ICE); muramyl peptides, such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanine-2-(l'-2' dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE); the B peptide subunits of E. coli heat labile toxin or of the cholera toxin; the RIBI adjuvant system (Ribi Inc.); pluronic polyols; Amphigen; Avridine; L121/squalene; D-lactide- polylactide/glycoside; MPL™ (3-0-deacylated monophosphoryl lipid A, Corixa, Hamilton, MT; described in U.S. Patent no. 4,912,094); synthetic lipid A analogs; aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof (Corixa, Hamilton, MT; described in U.S. Patent no. 6,113,918), including 2-[(R)-3-tetradecanoyloxytetradecanoylamino]ethyl 2-deoxy-4-0-phosphono-3-0-[(R)-3-tetradecanoyloxytetradecanoyl]-2-[(R)-3-tetradecanoyloxytetradecanoylamino]-β-D-glucopyranoside (529, RC529, optionally formulated as an aqueous form (AF) or as a stable
emulsion (SE)); and combinations, such as Freund's complete adjuvant or Freund's incomplete adjuvant. Alternatively, or additionally, the proteins and antigens may be the incorporated into liposomes for use in an immunogenic composition, such as a vaccine, or may be conjugated to proteins, such as keyhole limpet hemocyanin (KLH) or human serum albumin (HSA) and/or other polymers.

The term "pharmaceutically acceptable carrier" refers to an excipient that is a carrier or vehicle, such as a suspension aid, solubilizing aid, or aerosolization aid. *Remington: The Science and Practice of Pharmacy*, The University of the Sciences in Philadelphia, Editor, Lippincott, Williams, & Wilkins, Philadelphia, PA, 21st Edition (2005), incorporated herein by reference, describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compositions and/or pharmaceutical agents.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. In some examples, the pharmaceutically acceptable carrier may be sterile to be suitable for administration to a subject (for example, by parenteral, intramuscular, or subcutaneous injection). In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

The term "immune response" refers to a response of a cell of the immune system, such as a B-cell, T-cell, macrophage or polymorphonucleocyte, to a stimulus such as an antigen. An immune response can include any cell of the body involved in a host defense response, including for example, an epithelial cell that secretes an interferon or a cytokine. An immune response includes, but is not limited to, an innate immune response or inflammation. As used herein, a protective immune response refers to an immune response that protects a subject from infection (prevents infection or prevents the development of disease associated with infection).

The terms "isolated PEDV proteins and antigens" and "isolated proteins and antigens," as used herein, refers to PEDV proteins and antigens separated from the culture medium or supernatant. The isolated proteins and antigens typically comprise cell material, such as cell wall fragments, and proteins and antigens released from the infected cells, such as by a detergent or freeze-thawing. The isolated proteins and antigens may be in a buffer solution.
The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, sequence identity to a reference sequence over a specified comparison window. Optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

The term "vaccine" refers to a preparation of immunogenic material capable of stimulating an immune response, administered for the prevention, amelioration, or treatment of disease, such as an infectious disease. The immunogenic material may include, for example, antigenic proteins, peptides or DNA derived from them. Vaccines may elicit prophylactic (preventative) and/or therapeutic responses. Methods of administration vary according to the vaccine, but may include inoculation, ingestion, inhalation or other forms of administration as discussed herein. Inoculations can be delivered by any of a number of routes, including parenteral, such as intravenous, subcutaneous, intramuscular, intranasal, oral, vaginal or rectal. Vaccines may be administered with an adjuvant to boost the immune response.

II. Overview

PEDV is a member of the subfamily Coronavirinae of the genus Alphacoronavirus. It is an enveloped virus possessing approximately a 28 kb, positive-sense, single stranded RNA genome. Although first identified in 1971 in England, variant strains of PEDV emerging since 2010 in China, and since 2013 in North America, have been associated with large-scale outbreaks of diarrhea have been more acute and severe than those associated with the early European outbreaks. These recent Chinese and North American strains have been identified as belonging to Genogroup 2. Typically, the homology between strains in this genotype is very similar. For example, North American strains typically have about 99% homology. However, the North American strains and recent China strains have less similarity to strains from Europe and Asia that are Genogroup 1. For example, FIG. 6 shows a comparison of the homologies of seven Genogroup 2 strains from North America (SEQ ID NOS: 1-6) and China in 2012 (SEQ ID NO: 9). DR13, an attenuated Korean strain of Genogroup 1 (SEQ ID NO: 8), the sequence of which would be known to a person of
ordinary skill in the art based on the disclosure provided by WO 2015/120378, incorporated herein by reference, as the sequence was known; and SM98, a Korean strain of Genogroup 1 (SEQ ID NO: 7). SM98 has 96.6 to 96.9% homology with the Genogroup 2 strains, but the Genogroup 2 strains are at least 99%, such as 99.1-99.9%, homologous with each other.

Disclosed herein are embodiments of a method for making a porcine epidemic diarrhea virus (PEDV) immunogenic composition, comprising incubating PEDV infected cells for an effective period of time to result in one or more replicated PEDV viral particles being released, such as from 24 to 60 hours or from 24 to 48 hours, isolating cells infected with PEDV away from cell-free PEDV virus particles to form cells containing cell-associated PEDV proteins and antigens, separating the PEDV proteins and antigens from the isolated cells to form a first solution comprising isolated PEDV proteins and antigens, and optionally inactivating viral particles in the first solution to produce a second solution. Any embodiments of the method may further comprise adding an adjuvant to the second solution. The adjuvant may be selected to stimulate a mucosal antibody response and/or may be selected for intranasal administration and/or intravaginal administration. The adjuvant may adhere to the mucous membranes, and/or comprise polyacrylic acid. In any of the disclosed embodiments, the immunogenic composition may be a vaccine and/or may be formulated for intranasal administration. Any embodiments of the method may comprise extracting PEDV proteins and antigens, eluting PEDV proteins and antigens, a freeze-thaw cycle, or a combination thereof.

Also disclosed are embodiments of an immunogenic composition comprising a first antigenic component comprising isolated PEDV proteins and/or antigens from a first PEDV strain. The immunogenic composition may comprise an amount of S protein, such as an amount sufficient to produce an immune response in a subject receiving the immunogenic composition. The S protein may be an intact S protein. In any of the above embodiments, the immunogenic composition may comprise a second antigenic component. The second antigenic component may comprise isolated PEDV proteins and/or antigens from a second PEDV strain or isolated proteins and/or antigens from a second pathogen other than PEDV. In some embodiments, second pathogen is porcine reproductive and respiratory syndrome virus, and in other embodiments, the second pathogen is not *Mycoplasma hyopneumoniae*.

In any of the above embodiments, the immunogenic composition may be a vaccine and/or may comprise an adjuvant. The adjuvant may be selected to stimulate a mucosal antibody response and/or adhere to the mucous membranes. The adjuvant may comprise a polyacrylic acid adjuvant
and/or an emulsified oil-in-water adjuvant. The adjuvant may comprise an ammonium salt, such as a tetraalkylammonium salt, and in certain embodiments, the adjuvant comprises dimethyldioctadecylammonium bromide.

Also disclosed are embodiments of a method of administering to a pig an effective amount of any embodiment of the immunogenic composition disclosed herein. In some embodiments, the pig is less than 7 days old, such as 5 days old or less, or 2 days old or less. In any of the above embodiments, administering may comprise administering orally, intramuscularly, or subcutaneously, or it may comprise administering intranasally.

In any of the above embodiments, the method may comprise administering a first immunogenic composition to a sow, and administering a second immunogenic composition to at least one piglet farrowed from the sow, the second immunogenic composition, and optionally the first immunogenic composition, independently being any embodiment of the immunogenic composition disclosed herein. In any embodiments, the second immunogenic composition may be administered intranasally and/or the first immunogenic composition may be administered intramuscularly.

In any of the above embodiments, the sow may be a pregnant sow, or a sow expected to become pregnant subsequent to administration of the first immunogenic composition. The first immunogenic composition may be administered at a time point prior to the sow becoming pregnant such that, when pregnant, the sow has a greater immunity to PEDV compared to a pig not administered the immunogenic composition.

Further disclosed are embodiments of a use of any embodiments of the immunogenic composition disclosed herein in the manufacture of a medicament for administration to a pig.

III. **Method of Separating PEDV Proteins and/or Antigens**

Disclosed herein are embodiments of a method of making an immunogenic composition comprising PEDV proteins and antigens. The disclosure may be practiced by use of any suitable cell line susceptible to PEDV infection and intracellular replication *in vitro*, such as PEDV having at least a 90% sequence identity (i.e., 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100%) to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9. In certain embodiments, the suitable cell line has at least 90% sequence identity (i.e., 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%,...
99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100%) to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. Thus the infected cell may be any that is capable of being productively infected by PEDV. Non-limiting examples include porcine cells, either in vitro or in vivo. One non-limiting example of cells in vitro is primary cells from a porcine subject that is infected with PEDV. Other non-limiting examples are with the use of a simian cell line, such as MA-104; VERO cells; BGM cells; MDCK cells; MARC cells, and ST cells. In certain embodiments, MARC cells are used.

Infection of cells with PEDV may be at any suitable multiplicity of infection (m.o.I), such as 0.1, 0.5 or 1 infectious particles per cell, and infection of all cells in a culture is not required. In some cases, initial infection of some cells in a culture may be followed by subsequent release of infectious PEDV that infects non-infected cells in the culture.

After infection, the PEDV is allowed to intracellularly reproduce its proteins and antigens, and so replicate, for a suitable period of time. The suitable period of time may vary between different PEDV isolates, strains, and/or subtypes. In some embodiments, post-infection times ranging from 1 hour to at least 3 days, such as from 6 hours to 3 days, from 12 hours to 60 hours, from 24 hours to 60 hours, or from 24 hours to 48 hours. PEDV culture typically does not go for more than four days, and thus the harvest may be a late term harvest, such as after 24 hours. This is in contrast to PRRSV, where the proteins and antigens are typically harvested at an early term harvest at 28-60 hours post infection. A person of ordinary skill in the art will understand that 'early term' and 'late term' are virus dependent. For example a PRRSV culture will typically take 5 days to finish, whereas a PEDV culture will typically finish after 3 days. In addition to the disclosed methods, the disclosure includes a method of assessing PEDV protein production over time, after infection, to determine possible time points for isolation of infected cells and collection of viral proteins and/or antigens from the cells. This "time course" assessment after infection may be used to select a post infection time point for the preparation of viral proteins and/or antigens. The assessment is optionally performed for each PEDV isolate, strain, and/or subtype. The protein and/or antigen yields may also be compared using different PEDV isolates and different days after virus inoculation, and optimal conditions for the highest antigenic yields may be determined by comparative testing.

PEDV produces several proteins, including spike protein having a molecular weight of about 152 kDa based on deduced amino acid sequences. After post-translational modifications the protein may have a molecular weight of from 180 kDa to at least 350 kDa, depending, for example,
on the amount of post-translational glycosylation. The molecular weight may be determined by any suitable technique, such as, for example Western blot. Spike protein has been predicted to comprise two portions - SI at the N-terminus and S2 at the C-terminus. The spike protein may be a spike protein encoded by a PEDV having least 90% sequence identity (i.e., 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100%) to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9. In certain embodiments, the spike protein is encoded by a PEDV having least 90% identity (i.e., 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100%) to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. The spike protein may have a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, sequence identity to SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In certain embodiments, the spike protein may have a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, sequence identity to SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

Without being bound to a particular theory, the PEDV proteins and antigens produced by the disclosed method include spike proteins in multiple glycosylation states. This is due, in part, to breaking open the infected cells to separate and release the PEDV proteins and antigens substantially before viral particles are released into the culture medium. As a result, spike protein glycosylation is in progress, rather than being substantially completed, and therefore, the separated spike proteins have a range of molecular weights. By administering to a subject an immunogenic composition comprising such spike proteins, the subject is exposed to spike proteins having different glycosylation states, and thus produces antibodies to these proteins. This may be advantageous for the subject, compared to a subject that is administered either a composition that only includes non-glycosylated spike protein, such as a recombinant protein generally from
prokaryote, or a composition that has substantially only fully glycosylated spike protein, such as a killed vaccine.

In some embodiments, post-infection times are selected to provide an amount of the spike protein in the composition, such as an extract or eluant, sufficient to provide an immune response, such as a protective immune response, in a subject. In certain embodiments, the post-infection time is selected such that PEDV viral particles are present in the culture medium prior to the collecting, extracting and/or eluting process. In some embodiments, the proteins and/or antigens, such as the spike protein, harvested from PEDV infected cells may be in greater amounts than those available from PEDV particles in the culture medium, such as at least 2x, 3x, 4x, 5x or more than 5x the amount available from PEDV particles in the culture medium.

In some embodiments, at some time points after infection, the majority, or entirety, of the replicated PEDV proteins may remain associated with the infected cells or are otherwise part of a cell associated viral component (CAVC). Thus the majority or entirety of PEDV proteins and/or antigens are either within the infected cells or associated with the cell membrane of the infected cells. Under such conditions, relatively few, if any, PEDV particles are present in the extracellular environment. The preparation of CAVC from an early time point, such as before the production of PEDV particles and/or the release thereof into the extracellular environment also has the benefit of increased safety in that no infectious viral particles are present as a contaminant.

However, in other embodiments, it was surprisingly found that harvesting the proteins and antigens, such as the spike protein, at a time point after the infected cells had started to release replicated PEDV viral particles resulted in improved results. This was in contrast to results obtained with certain other viruses, such as PRRSV, as disclosed in U.S. Patent Nos. 7,241,582, 7,449,296, 7,776,537, and 8,142,788.

In some embodiments, the method of preparing PEDV proteins and antigens from PEDV infected cells comprises providing a population of cells infected with PEDV; isolating the infected cells away from cell-free PEDV to form cells containing cell associated PEDV proteins and antigens; and separating the PEDV proteins and antigens to form a composition of proteins and antigens. The composition may be a solution. As used herein, the terms "separating" and "separation" refer to, by way of unlimited examples, breaking open, extracting, eluting, rupturing, lysing, centrifuging, filtering, or a combination thereof, to release the proteins and antigens from within the isolated cells. The composition may also comprise cell fragments. In cases wherein there is no cell-free virus present, then isolating the infected cells away from cell-free PEDV may
comprise isolating the infected cells from other materials that may interfere with the method, such as the culture medium used with the cells. The isolation step may be performed by any means known in the art, such as by simply pouring off the medium and leaving the cells to be extracted by detergents or freeze/thaw, or use of centrifugation to generate a cell pellet and supernatant. The supernatant can then be removed and/or discarded, such as by use of a membrane filtration, to leave the cells. The separation of viral proteins and antigens may be performed by any suitable method, such as extraction, elution and/or freeze-thawing. The extraction step is optionally performed by re-suspending the cells in a buffer. In some embodiments, the isolation step may be performed by simply pouring off the medium and leaving the cells on culture devises such as flasks, roller bottles or cell culture carriers to be extracted by detergents or freeze/thawing with buffer. The extraction or elution is performed with a detergent-containing solution, thus the buffer used to re-suspend cells may contain detergent. In other embodiments, the extraction may be performed by freeze-thaw method. Optionally, the viral proteins and/or antigens produced by the method include PEDV envelope proteins.

In some embodiments, the method comprises using a population of cells that has been infected with PEDV for a sufficient time to produce little to undetectable amounts of infectious units per ml of supernatant, such as the culture media used with the cells. In some embodiments, the time is sufficient to produce tissue culture infective doses/ml (TCID50/ml) of from $10^1$ to $10^{10}$, such as from $10^1$ to $10^7$, or from $10^1$ to $10^{5.5}$. Non-limiting examples include using less than $10^{5.5}$, such as $10^4$ or less, or $10^3$ or less TCID50/ml.

The detergent-containing solution may be any that is suitable for separating, such as by extracting and/or eluting, the PEDV proteins and antigens from the infected cells. One non-limiting example is the use of a non-ionic detergent. Suitable detergents include, but are not limited to poly(ethylene glycol) p-isooctyl-phenyl ether, octylphenoxy polyethoxyethanol (Nonidet P-40), or Triton X-100. The detergent is used at a concentration effective for extracting or eluting PEDV proteins and/or antigens, such as a concentration of from greater than zero to 5% in solution, such as from greater than zero to 2%, 0.25% to 1%, or 0.5% in solution. In certain embodiments, the detergent is a solution of 0.5% Triton X-100. The collecting, extracting and/or eluting may be performed for from greater than zero to at least about 24 hours, such as from 0.1 hours to 24 hours, 0.1 hours to 15 hours, 0.2 hours to 10 hours or 0.2 hours to 5 hours, or in certain embodiments from 0.5 hours to 2 hours. The collecting, and optional extracting and/or eluting is performed at a suitable temperature. In some embodiments, the temperature is from -20 °C to less than 30 °C,
such as from -20 °C to 25 °C, from 1 °C to 25 °C, from 5 °C to 25 °C or from 10 °C to 25 °C. In certain exemplary embodiments, the temperature is 4 °C, or room temperature, such as from 20 to 25 °C.

For a freeze-thaw process a buffer may be added to the cells, typically after the culture medium is removed. The buffer can be any suitable buffer, such as Tris or phosphate buffered saline (PBS) with or without EDTA. The cells are coated with the buffer, such as by agitation, for example, swirling or stirring. The cells are then placed in a freezer at a temperature suitable to freeze the cells and buffer, such as -10°C or below. After the cells are frozen, they are allowed to thaw. The thawing causes cells to break, thereby releasing the proteins and antigens. Additional freeze-thaw cycles may be performed to release additional proteins and antigens.

After the release of the proteins and antigens, an inactivating agent may be added. Suitable inactivating agents include any agent that will inactivate viral particles present in the culture medium, such as binary ethylenimine (BEI), formalin, or beta propiolactone (BPL). The concentration of activating agent may be from 0.01 mol/L to at least 2 mol/L, such as from 0.01 mol/L to 1 mol/L. The culture medium is mixed with the inactivating agent until inactivation is complete, such as for from 5 minutes to 2 hours or more, such as 15 minutes to 2 hours, or from 30 minutes to 1 hour. The inactivation process can be stopped by the addition of a sufficient amount of thiosulfate solution, such as sodium thiosulfate, to neutralize the excess inactivating agent. After inactivation, the culture may be optionally diluted before a suitable adjuvant is added to the culture to produce the immunogenic composition.

The immunogenic composition will contain an effective amount of PEDV antigens in the solution, the effective amount being readily determined by a person of ordinary skill in the art. The effective amount may be sufficient to produce a desired immune response in a subject, such as a substantially protective immune response. The amount of PEDV antigens may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends upon factors such as the age, weight and physical condition of the animal considered for vaccination. The quantity also depends upon the capacity of the animal’s immune system to synthesize antibodies, and the degree of protection desired. Effective dosages can be readily established by a person of ordinary skill in the art through routine trials establishing dose response curves. In some embodiments, the concentration of viral antigens in the solution of PEDV protein and antigens is from 0.01 ng/ml to 10,000 ng/ml, such as from 0.1 ng/ml to 5,000 ng/ml, from 0.5 ng/ml to 1,000 ng/ml or from 1 ng/ml to 100 ng/ml. The volume of the dosage is
from greater than 0 mL to at least 10 mL, such as from 0.1 mL to 10 mL, from 0.5 mL to 5 mL, or from 0.5 mL to 2 mL.

The PEDV proteins and antigens may be prepared from PEDV infected cells. In certain embodiments, the method comprises preparing the proteins and antigens from a population of the cells prepared by in vitro and in vivo methods. For the in vitro method, VERO cells or MARC cells are cultured, and the cells are harvested following an infection of PEDV.

IV. Composition and Applications

Disclosed herein are embodiments of a composition comprising the isolated PEDV proteins and/or antigens prepared according to embodiments of the disclosed method. The composition is suitable for use for any purpose for which PEDV proteins and/or antigens are used. Non-limiting examples of applications of the proteins and/or antigens include the preparation of antibodies against the proteins/antigens; using the proteins and/or antigens as reference markers for PEDV proteins; and using the proteins and/or antigens in an immunogenic composition, such as in a vaccine formulation, optionally with a suitable excipient, carrier, adjuvant, etc., and combination thereof. The immunogenic composition may be administered to an animal to generate an immune response. Additional non-limiting examples of the compositions include those where the protein(s)/antigen(s) is/are in soluble or lyophilized (freeze dried) form.

The disclosed immunogenic composition has a different composition to that of a conventional PEDV vaccine, such as a killed or attenuated vaccine. Embodiments of the disclosed method isolate infected cells containing the PEDV proteins and antigens away from the supernatant, which contains the culture medium, at a time point substantially before viral particles have been released into the culture medium. Any viral particle that have been released into the culture medium are removed with the supernatant. The PEDV proteins and antigens are then released from the infected cells by a suitable separation technique, such as extracting and/or eluting, freeze/thawing, or other techniques. In some embodiments, the infected cells may be lysed to release the PEDV proteins and antigens. Any viral particles also released from the infected cells are inactivated, such as by a detergent and/or other inactivating agent. In some embodiments, the detergent is a detergent that is also used to extract and/or elute the proteins and antigens from the infected cells. In other embodiments, the inactivating agent is affirmatively added to the proteins and antigens. Thus, the composition made by the method comprises a high concentration of
proteins produced by the infected cells and/or by the virus while within an infected cell, but a low concentration of actual viral particles.

This is in contrast to a killed vaccine, which typically is prepared by allowing the virus to carry the cell infection through the cytopathic effect (CPE) to substantial release of viral particles. Without being bound to a particular theory, the proteins produced by the infected cells and/or by the virus while within an infected cell may be useful for production of the viral particles, but are not necessarily properly exposed to the animal's immune system.

Therefore, the composition and concentration of the proteins and antigens included in the present immunogenic composition is very different from those found in killed or attenuated PEDV vaccines.

In some embodiments, the immunogenic composition comprises, consists essentially of, or consists of, PEDV proteins and antigens prepared by the disclosed method, a buffer solution, an adjuvant, an inactivating agent and a neutralizing agent. In certain embodiments, the buffer is PBS with EDTA, the inactivating agent is BEI, and/or the neutralizing agent comprises thiosulfate, such as sodium thiosulfate. In particular embodiments, the adjuvant is an oil-in-water adjuvant, such as an EMULSIGEN®-based adjuvant, or an adjuvant that adheres to the mucosal membranes, such as an adjuvant comprising polyacrylic acid, typically an adjuvant comprising carbopol such as CARBIGENTM.

Strains suitable for use in the composition include any strain of PED virus, such as strains from North America, Europe and Asia. In particular embodiments, the PEDV strain is a Genogroup 2 strain, and may be a North American strain. In some embodiments, the disclosed immunogenic composition comprises PEDV proteins and antigens encoded by a PEDV strain having at least 90% identity (i.e., 90%, 91%, 92%, 93%, or 94%, or 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9%, or 100%) to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. In certain embodiments, the PEDV has at least 99%, 99.9% or 99.99% identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6. Exemplary strains include, but are not limited to, the original US PEDV strain, Colorado 2013 (SEQ ID NO: 1), Iowa/18984/2013 (SEQ ID NO: 2), North Carolina USA/NC/2013/35140 (SEQ ID NO: 3), Indiana2.83/2013 (SEQ ID NO: 4), Iowa/2013 (SEQ ID NO: 5), 1251-125-10 (SEQ ID NO: 6), SM98 (SEQ ID NO: 7), KR-DR13-att (SEQ ID NO: 8), the INDEL strain, the S2aa-del strain, CV777, Chinese PEDV strains such as Chinese CH/ZMDZY/11, and AH2012 (SEQ ID NO: 9).
In certain embodiments, the composition comprises proteins and/or antigens from more than one strain of PEDV. The composition may comprise proteins and/or antigens from a first PEDV strain and a second PEDV strain, and optionally may further comprise proteins and/or antigens from at least a third PEDV strain.

The composition may comprise proteins and/or antigens from at least one additional pathogen. The additional pathogen may be any pathogen that causes illness and/or an infection in a porcine subject. Exemplary pathogens include, but are not limited to, porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae*, *Mycoplasma rhinitis*, *Clostridium tetani*, *Clostridium perfringens*, porcine parovirus, *Erysipelothrix rhusiopathiae*, *Leptospira pomona*, *Leptospira grippotyphosa*, *Leptospira hardjo*, *Leptospira canicola*, *Leptospira icterohaemorrhagiae*, *Leptospira bratislava*, porcine circovirus, *Lawsonia intracellularis*, *Escherchia coli*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Salmonella choleraesuis*, *Salmonella typhimurium*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Actinobacillus pleuropneumoniae*, *Serpulina hyodysenteriae*, encephalomyocarditis virus, swine influenza virus, transmissible gastroenteritis virus (TGE), swine delta coronavirus, rotavirus diarrhea, foot and mouth disease virus, classical swine fever virus, pseudorabies virus, Japanese encephalitis virus (JEV), encephalomyocarditis virus, or a combination thereof. In certain embodiments, the additional pathogen is not *Mycoplasma hyopneumoniae*. In other embodiments, the additional pathogen is *Clostridium tetani*, *Clostridium perfringens*, porcine parovirus, *Erysipelothrix rhusiopathiae*, *Leptospira pomona*, *Leptospira grippotyphosa*, *Leptospira hardjo*, *Leptospira canicola*, *Leptospira icterohaemorrhagiae*, *Leptospira bratislava*, porcine circovirus, *Lawsonia intracellularis*, *Escherchia coli*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Salmonella choleraesuis*, *Salmonella typhimurium*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Actinobacillus pleuropneumoniae*, *Serpulina hyodysenteriae*, encephalomyocarditis virus, swine influenza virus, transmissible gastroenteritis virus (TGE), swine delta coronavirus, rotavirus diarrhea, foot and mouth disease virus, classical swine fever virus, pseudorabies virus, Japanese encephalitis virus (JEV), encephalomyocarditis virus, or a combination thereof.

In particular embodiments, the second pathogen is, or comprises, PRRSV. The PRRSV may comprise one or more North American strains, one or more European strains or combinations thereof. PRRSV strains may include, but are not limited to, Lelystad, VR2332 or HP-PRRSV. In
Certain embodiments, the PRRSV proteins and/or antigens are extracted or eluted by a method according to one or more of U.S. Patent Nos. 7,241,582, 7,449,296, 7,776,537 or 8,142,788.

In some embodiments, an immunogenic composition as disclosed herein may comprise PEDV proteins and/or antigens from one or more PEDV strains, and proteins and/or antigens from one or more additional pathogens. In certain embodiments, the additional pathogen comprises PRRSV.

Also disclosed herein are combinations of immunogenic compositions, comprising at least one PEDV immunogenic composition and at least one immunogenic composition directed toward a non-PEDV porcine pathogen. In certain embodiments, the additional pathogen is or comprises PRRSV. In particular embodiments, the PRRSV immunogenic composition is an immunogenic composition prepared according to the methods disclosed in one or more of U.S. Patent Nos. 7,241,582, 7,449,296, 7,776,537 or 8,142,788.

The immunogenic compositions in the combination of immunogenic compositions may be administered sequentially in any order, or at substantially the same time. In some embodiments, the immunogenic compositions may be mixed to form a single administrable composition. In other embodiments, the immunogenic compositions are administered as separate formulations.

The immunogenic compositions disclosed herein may also be administered in combination with other therapeutic agents suitable for administration to the subject, such as antibiotics, antiviral agents, antifungal agents, antiparasitic agents, or combinations thereof.

V. Detection of Protective Antibodies Against PEDV

Disclosed herein are embodiments of a method of detecting protective antibodies against PEDV. Antibodies against PEDV spike proteins having a molecular weight between 180-kDa to 350-kDa provide immunological protection in livestock such as swine. Accordingly, certain disclosed embodiments provide an agent that binds the antibodies against 180-kDa to 350-kDa of porcine epidemic diarrhea virus (PEDV), or PEDV. The agent may be used in embodiments of a method, device, and/or kit for detecting the presence of the antibodies against 180-kDa to 350-kDa of PEDV spike protein in a biological fluid.

Thus, disclosed herein are embodiments of a method of detecting antibodies against 180-kDa to 350-kDa post translationally modified PEDV spike protein in a sample of a biological fluid from a subject, particularly but not necessarily a porcine subject, suspected of being infected with PEDV. The method comprises contacting the sample, or a diluted form thereof, with a binding
agent that binds the antibodies against 180-kDa to 350-kDa of PEDV spike protein. The binding of
the agent, or agents, to the antibodies against 180-kDa to 350-kDa of PEDV spike protein forms a
complex that may be detected to indicate the presence of the antibodies against 180-kDa to 350-
kDa of PEDV spike protein post translational variants, and thus the presence of a PEDV infection
in the subject from which the sample was obtained.

The biological fluid may be any fluid in which antibodies against 180-kDa to 350-kDa of
PEDV spike protein post translational variants may be present in detectable amounts. Non-limiting
examples include bodily secretions, such as saliva, tears, mucous, nasal discharge, and vaginal
secretions as well as other bodily fluids such as blood, serum, plasma, semen, seminal fluid, milk,
and urine as well as any fluid component of feces or a fluid extract of feces.

The binding agent which binds the antibodies against 180-kDa to 350-kDa of PEDV spike
protein may be the spike protein, a 180-kDa to 350-kDa of PEDV spike protein post translational
variants, or derivative thereof. In particular, binding agents may also be used to immobilize the
antibodies against 180-kDa to 350-kDa of PEDV spike protein, or a macromolecular complex
containing it, to facilitate its detection.

Also contemplated are labeled forms of the binding agent to facilitate its detection when
bound to antibodies against 180-kDa to 350-kDa of PEDV spike protein. The binding agent may
be labeled to permit direct detection, such as by conjugation to a label which is visible to the eye
upon sufficient aggregation. Alternatively, the binding agent may be labeled for indirect detection,
such as by conjugation to an enzyme, which is detected based upon its activity on a detectable
substrate or to produce a detectable product. The binding agent may also be unlabeled and then
detected based upon use of a detectable reagent, which binds the binding agent after formation of
the complex. As a non-limiting example with the use of an antibody as the binding agent, the
antibody complex comprising antibodies against 180-kDa to 350-kDa of PEDV spike protein post
translational variants may be detected by a detectably labeled secondary antibody which binds the
antibody bound to the antibodies against 180-kDa to 350-kDa of PEDV spike protein.

In another embodiment, detecting the binding agent complex is facilitated by
immobilization of the complex. In some embodiments, the complex is immobilized to a solid
substrate comprising an immobilized second binding agent which binds and immobilizes the
complex. A first non-limiting example of such an embodiment comprises using a second binding
agent to localize the complexes in discrete areas of the substrate to improve detection. In another
non-limiting embodiment, immobilization forms a "sandwich" wherein antibodies against 180-kDa
to 350-kDa of PEDV spike protein are "sandwiched" between the binding agent and a second agent immobilized on a solid substrate which also binds antibodies against 180-kDa to 350-kDa of PEDV spike protein. As a non-limiting example, the complex may be immobilized by binding to a second binding agent immobilized to a solid substrate, such as a surface of a well, plate, dish or tube. The complex may then by detected based on localization on the surface. Alternatively the solid substrate may be a bead or chromatographic media which permits detection based on localization on the bead or media. The second binding agent preferably binds the antibodies against 180-kDa to 350-kDa of PEDV spike protein and the binding agent as described herein. Alternatively, the second binding agent is the same as the binding agent.

Also disclosed are embodiments of a device for practicing the above described method. Generally, such devices are useful for detecting the presence of antibodies against 180-kDa to 350-kDa of PEDV spike protein in a sample of a biological fluid as an indicator of PEDV infection in the subject from which the sample was taken. Thus the devices may be used as a rapid means of diagnosing the presence of PEDV infection.

The test strip may be uniform in composition, such as by being a unitary membrane strip comprising the first and second portions as described herein. Non-limiting examples include a strip of nitrocellulose membrane of appropriate pore size. Non-limiting examples of pore sizes include those in the range of 1-250 microns. Other non-bibulous materials may also be used, along with one or more mobilization agent as described herein to improve the mobilization of a dried first binding agent (the detector agent or preferably the detector antibody). Non-limiting examples of a mobilization reagent include glazes comprising sugar and/or BSA (bovine serum albumin).

Alternatively, the test strip is non-unitary in construction but the different components are functionally linked to permit fluid communication therebetween. In some embodiments, the first portion of the test strip as defined herein is composed of a porous or bibulous material. Non-limiting examples include cellulose or glass wool.

Placement of the first binding agent in a mobilizable form on the first portion of a device of the invention is preferably by drying a solution containing the agent thereon. In some embodiments, the solution is sprayed on and then dried prior to use. A non-limiting representative example of such a solution is one containing a detector reagent of the invention. Preferably, the first binding agent is labeled as described herein, such as with colloidal gold as a non-limiting example. In other embodiments, the test strip is within a housing or casing comprising liquid impermeable material to facilitate the manipulation and use of the test strip.
The test strip may be designed to operate solely based on the liquid available from a sample applied thereto (see for example U.S. Patent no. 5,591,645 for analogous test strip embodiments). Alternatively, the test strip may be designed to operate in connection with a solvent or developing solution which increases the volume of the sample applied to the test strip (see for example U.S. Patent no. 4,235,601 for analogous embodiments). In other embodiments, the test strip is embodied in a housing or casing, preferably composed of a plastic, polyacrylate or other liquid resistant material, to form a device of the invention. The test strip may include a backing composed of similar materials.

A test strip or other device of the invention may also comprise a control site or control region as described herein. The control site or region may comprise a reagent that produces a color upon being wetted. Non-limiting examples include cobalt chloride, copper chloride, and the like. Alternatively, the reagent may be a pH indicator which exhibits a color at the pH of the traversing fluid different from the color in the dry state. In a further alternative, the reagent is one that binds, and thus permits the detection of, a labeled first binding agent regardless of whether it has bound antibodies against 180-kDa to 350-kDa of PEDV spike protein.

The device may comprise both a first binding agent which binds antibodies against 180-kDa to 350-kDa of PEDV spike protein to form a complex and a second binding agent which immobilizes the complex. The first binding agent may thus be viewed as a "detector agent" and is as described herein. Where the first binding agent is 180-kDa to 350-kDa of PEDV spike protein, it may be viewed as a "detector antigen." The first binding agent may be located in a mobilizable form on a first portion of the device. A non-limiting example of how to make such a mobilizable first binding agent comprises drying the agent on a first portion of a device. Upon hydration with a liquid, such as a sample of a biological fluid, the agent is mobilized within the sample and thus may move with the liquid. Where the liquid, such as a sample of a biological fluid, contains antibodies against 200-kDa to 350-kDa of PEDV spike protein, the first binding agent binds the antibodies against 180-kDa to 350-kDa of PEDV spike protein to form a complex which moves with the liquid.

A second binding agent is immobilized on a second portion of a device to bind and immobilize a complex comprising the first binding agent and antibodies against 180-kDa to 350-kDa of PEDV spike protein when such a complex contacts the second binding agent. The second binding agent may thus be viewed as the "capture agent," or in the case of an antigen as the second binding agent, a "capture antigen." Contact between the second binding agent and the complex
occurs via the movement of a liquid containing the complex, such as a sample of a biological fluid that contains a complex of antibodies against 180-kDa to 350-kDa of PEDV spike protein and mobilized first binding agent as described above, into contact with the second binding agent. Such movement is readily accomplished by the first and second portions of the device being in fluid communication with each other such that fluid in the first portion will move into and through the second portion. Such fluid communication may be direct, with no intervening material between the first and second portions, or indirect, with an intervening material between the first and second portions that permits liquid to pass from the first to second portions.

Detection of immobilized complex in the device, preferably by detection of a detectably labeled first binding agent immobilized in the second portion as permitted by the device, may be used to indicate the presence of antibodies against 180-kDa to 350-kDa of PEDV spike protein in a sample of biological fluid. The presence of antibodies against 180-kDa to 350-kDa of PEDV spike protein may be used as an indication of PEDV infection in the subject from which the sample was obtained. The sample is preferably from a porcine subject, or other subject suspected of being infected with PEDV, but any subject which may be infected by PEDV carrier may be used in the devices of the invention.

Biological fluids that may be used in the device include any fluid in which antibodies against 180-kDa to 350-kDa of PEDV spike protein may be detectably present. Non-limiting examples have been provided above and below, and dilutions of such fluids may also be used as the sample.

The present disclosure provides a binding agent capable of binding antibodies against 180-kDa to 350-kDa of PEDV spike protein in a sample of a biological fluid from a subject. Preferably, the binding agent specifically binds 180-kDa to 350-kDa of PEDV spike protein antibodies to the exclusion of other molecules present in the biological fluid. In many embodiments of the disclosure, the subject is a pig, and thus the sample may be of a bodily fluid or secretion from a pig. Non-limiting examples of pigs from which samples may be obtained for use with the present invention include boar, sow, fattener, gilt, nursery pigs, finishing pigs, and weaned pigs. The pigs may range in age from 1 day to at least 60 days, such as from 1 day to about 30 days, 30 days to about 40 days, 41 days to about 50 days, or 51 days to about 60 days or older.

The binding agent preferably, or substantially selectively, binds an antibody against 180-kDa to 350-kDa of PEDV spike protein as found in multiple PEDV strains and isolates. In other embodiments, the binding agent does not cross react with other porcine viruses, such as circovirus,
porcine parvovirus (PPV), Japanese encephalitis virus (JEV), rotavirus, pseudorabies,
encephalomyocarditis virus, swine influenza virus, PRRSV or transmissible gastroenteritis (TGE) virus.

The binding agent is preferably a 180-kDa to 350-kDa of PEDV spike protein, or a fragment thereof, which binds 180-kDa to 350-kDa of PEDV spike protein antibodies. Accordingly, the disclosure provides an immunochromatographic-based method for detecting PEDV. FIG. 1 provides a Western blot illustrating detection of antibodies against 180-kDa to 350-kDa of PEDV spike protein. With reference to FIG. 1, pigs in cases 1 and 2 maintained healthy status without diarrhea, but pigs in cases 3 and 4 had severe diarrhea caused by PEDV.

The 180-kDa to 350-kDa of PEDV spike protein may be generated by any appropriate method known in the art. Suitable methods include, but are not limited to recombinant, extraction, and/or synthetic methods.

As explained herein, the binding agent may be labeled to facilitate its detection, such as, for example, by attachment to another moiety. The moiety is preferably a detectable label, including a directly detectable label, such as a radioactive isotope, a fluorescent label (Cy3 and Cy5 as non-limiting examples) or a particulate label. Non-limiting examples of particulate labels include latex particles, metal sols, and colloidal gold particles. Alternatively, the label may be for indirect detection. Non-limiting examples of labels suitable for indirect detection include an enzyme, such as, but not limited to, luciferase, alkaline phosphatase, and horse radish peroxidase. Other non-limiting examples include a molecule bound by another molecule, such as, but not limited to, biotin, an affinity peptide, or a purification tag. Preferably, the label is covalently attached.

The binding agent may be used to detect antibodies against 180-kDa to 350-kDa of PEDV spike protein in a sample of a biological fluid from a subject as described herein. The sample is preferably from an individual suspected of being infected with PEDV due to the presence of symptoms indicative of an infection. Alternatively, embodiments of the disclosed method may be used as part of routine screening of animals, such as those of a farm to permit rapid identification and isolation of infected individuals. Certain embodiments also may be used in specific instances, such as prior to transport or transfer of an animal from one location to another to permit identification of infection and prevent spread of infection.
Also disclosed herein are embodiments of a kit comprising a binding agent, or a composition and/or device comprising the binding agent, for use in one or more embodiments of the method disclosed herein. Such kits optionally further comprise an identifying description or label or instructions relating to their use in the methods of the present invention. Such a kit may comprise containers, each with one or more of the various reagents (typically in concentrated form) or devices utilized in the methods. A set of instructions will also typically be included.

Embodiments of a kit comprising a device may further comprise one or more additional reagents or pieces of equipment for use with the device. Non-limiting examples of additional materials for inclusion are sample diluent solution, diluent vial, and a dropper for transfer of sample.

VI. Examples

Example 1

PEDV proteins may be prepared from a PEDV strain by infecting susceptible cells in vitro or in vivo, and harvesting the infected cells at an optimal time, typically from 24 to 60 hours post infection, to prepare cell-associated viral components. For in vitro methods, the antigen(s) may be prepared by a cell culture system or by using recombinant technologies.

In an exemplary embodiment, the cells were harvested by pouring off the culture medium. The infected cells layer may be optionally washed with PBS. PEDV proteins and antigens were extracted from the cell layer by suspending the cells in a 0.05M tris (hydroxymethyl) aminomethane 0.025M EDTA buffer containing 0.5% Triton X-100 at a volume of one half to one tenth of culture medium. The mixture was stirred for 0.5-10 hours at 4-25 °C and filtered to remove cell debris. The resulting filtrate comprised the PEDV proteins and antigens. Optionally, before filtering, the antigen-containing solution may then undergo one or more freeze-thawing cycles, one or more of each, followed by an additional extraction cycle, to further break up intact cells and increase the efficiency of the extraction process. The freeze-thawing process also may facilitate ensuring that the antigen solution is non-infectious and allowing its use without a risk of spreading the virus.

FIG. 2 provides a photograph of a Western blot of PEDV proteins. The proteins were obtained from infected cells by an exemplary embodiment of the disclosed method and were mixed with one of two monoclonal antibodies, 6C8 or 3F12, which were selected to detect certain PEDV
proteins. Lanes identified as 'S' contained pre-stained protein molecular weight markers. The other lanes contained samples from a PEDV infected cell culture medium at the end of culture (lanes 1), an extract of isolated PEDV infected cells diluted 2x (lanes 2), an extract of isolated PEDV infected cells diluted 3x (lanes 3), an extract of isolated PEDV infected cells diluted 4x (lanes 4), and an extract of isolated PEDV infected cells diluted 5x (lanes 5). Surprisingly, in this example, both of the monoclonal antibodies used appeared to detect proteins with the same molecular weight.

FIG. 3 provides a photograph of a Western blot of PEDV proteins from cultures of infected cells over time with a mixture of the two monoclonal antibodies used in FIG. 2. Lane S contained pre-stained protein molecular weight markers. The other lanes contained extracted samples from isolated PEDV infected cells 24 hours post infection (lane 1), 30 hours post infection (lane 2), 35 hours post infection (lane 3), 47 hours post infection (lane 4), and 52 hours post infection (lane 5).

The results from these two experiments demonstrated that the disclosed method successfully extracted proteins from cells infected with PEDV. Moreover, the Western blots illustrate that the extraction method results in a concentrated mixture of proteins compared to a culture medium, such as the culture medium of a killed virus vaccine. The concentration of proteins in the extracts or eluents may be more than twice the concentration of proteins in the culture medium, such as 4 times, 6 times, 8 times, 10 times or more than 10 times the concentration of proteins in the culture medium.

**Example 2**

PEDV proteins may be prepared from a PEDV strain by infecting susceptible cells *in vitro* or *in vivo*, and harvesting the infected cells at an optimal time, typically from 24 to 60 hours post infection, to prepare cell-associated viral components. For *in vitro* methods, the antigen(s) may be prepared by a cell culture system or by using recombinant technologies.

In an exemplary embodiment, the supernatant containing whole virus particles was poured off from the cells and removed or discarded. The cells may optionally be washed. PEDV proteins and antigens were extracted from the cell pellets by suspending the cell pellets in a 0.05M tris (hydroxymethyl) aminomethane 0.025M EDTA buffer containing 0.5% Triton X-100 at a volume of 5-10 times that of the cells. The cells were incubated with the buffer for 30 minutes to 15 hours at 4 °C and then optionally frozen. The resulting extract comprised the PEDV proteins and antigens. Optionally, the antigen-containing solution may then undergo one or more freeze-
thawing cycles, one or more of each, followed by an additional extraction cycle, to further break up intact cells and increase the efficiency of the extraction process. The freeze-thawing process also may facilitate ensuring that the antigen solution is non-infectious and allowing its use without a risk of spreading the virus. After this extraction process it was found that the proteins and antigens were inactivated. Optionally, an inactivating agent such as binary ethyleneimine is added to insure inactivation.

**Example 3**

**Vaccine Preparation**

Detergent extracts (DE samples) were prepared from MARC (monkey kidney) cells infected with Colorado (CO), Iowa (IA), and North Carolina (NC), SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, respectively (FIGS. 7-9). FIG. 4 is a Western blot of the detergent extracts. For each extract, 2x, 3x, and 4x diluted samples were run (lanes 1-3, respectively). A sample of each full grown virus culture also was run (lane 4).

The three DE samples were mixed in equal volume to make 1,000 mL to form a DE mixture. The DE mixture was inactivated with binary ethyleneimine (BEI) and neutralized with thiosulfate. The resulting solution had a volume of about 1,200 mL. Samples taken before and after the inactivation process were tested to confirm that there was no live virus in the DE mixture after the inactivation process. FIG. 5 is a Western blot of the three DE extracts (lanes 1-3), the DE mixture before inactivation (lane 4), the DE mixture after inactivation (lane 5), and a mixture of the three viral cultures in equal volume.

The inactivated DE mixture was split into two parts - 850 mL (Part A) and 350 mL (Part B). Part A was diluted with PBS (650 mL) and mixed with 300 mL of Emulsigen-D adjuvant (Phibro Animal Health Corporation, Omaha, NE, USA) to form an intramuscular (IM) vaccine (2 mL/dose). Part B was not diluted and was mixed with 70 mL of CARBIGEN™ adjuvant (Phibro Animal Health Corporation, Omaha, NE, USA) to form an intranasal (IN) vaccine (1 mL/dose). Each mixture was blended with an industrial grade blender (Commercial Blender 7011, Model 31BL92, Dynamic Corporation of America, New Hartford, CT). The blended solution was aliquoted into 50 mL batches for IM vaccine or 20 mL batches for IN vaccine, and stored until use. In trials, the IN vaccine was also used as a subcutaneous (SQ) vaccine.
**Example 4**

*Pre-Wean Mortality in Vaccinated and Unvaccinated Pigs*

Table 1 shows multiple farm examples of pre-wean mortality based on days post initial whole herd PEDV exposure. The data was used as a baseline to compare vaccine performance.

<table>
<thead>
<tr>
<th>Days post exposure</th>
<th>Farm 1</th>
<th>Farm 2</th>
<th>Farm 3</th>
<th>Farm 4</th>
<th>Farm 5</th>
<th>Farm 6</th>
<th>All farm average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-7</td>
<td>100</td>
<td>99</td>
<td>97</td>
<td>100</td>
<td>97</td>
<td>100</td>
<td>98%</td>
</tr>
<tr>
<td>8-15</td>
<td>98</td>
<td>94</td>
<td>98</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>98%</td>
</tr>
<tr>
<td>16-23</td>
<td>51</td>
<td>47</td>
<td>78</td>
<td>89</td>
<td>90</td>
<td>100</td>
<td>76%</td>
</tr>
<tr>
<td>24-31</td>
<td>23</td>
<td>24</td>
<td>34</td>
<td>54</td>
<td>45</td>
<td>100</td>
<td>46%</td>
</tr>
<tr>
<td>32-39</td>
<td>19</td>
<td>14</td>
<td>27</td>
<td>43</td>
<td>34</td>
<td>100</td>
<td>39%</td>
</tr>
<tr>
<td>40-47</td>
<td>19</td>
<td>25</td>
<td>17</td>
<td>30</td>
<td>16</td>
<td>22</td>
<td>21%</td>
</tr>
<tr>
<td>48-55</td>
<td>24</td>
<td>12</td>
<td>18</td>
<td>27</td>
<td>20</td>
<td>37</td>
<td>23%</td>
</tr>
<tr>
<td>56-63</td>
<td>19</td>
<td>11</td>
<td>16</td>
<td>25</td>
<td>19</td>
<td>32</td>
<td>20%</td>
</tr>
<tr>
<td>Individual farm average prior to PEDV</td>
<td>15%</td>
<td>14%</td>
<td>15%</td>
<td>20%</td>
<td>17%</td>
<td>12%</td>
<td></td>
</tr>
</tbody>
</table>

In one trial sows received the PEDV vaccine 3-5 days pre-farrow (endemic infection, 150 days post clinical break). Treated and non-treated litters were in the same room. Table 2 summarizes the results.

<table>
<thead>
<tr>
<th>Vaccinated sows - 10 head, 2 cc dose</th>
<th>Born alive</th>
<th>Pigs remaining at weaning</th>
<th>Piglet mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>133 (13.3 avg.)</td>
<td>113 (11.3 avg.)</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>Non-vaccinated sows, 33 head</td>
<td>405 (12.2 avg.)</td>
<td>291 (8.8 avg.)</td>
<td>28%</td>
</tr>
</tbody>
</table>
In another trial, sows received the vaccine at 3-5 days pre-farrow (1 cc dose) in an endemic herd (150 days post clinical break) where all litters within a room were vaccinated or non-vaccinated. The results are shown in Table 3.

**Table 3**

<table>
<thead>
<tr>
<th></th>
<th># of litters</th>
<th>Born alive</th>
<th>Pigs weaned</th>
<th>Pre-wean mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire room vaccinated</td>
<td>128</td>
<td>1466 (11.4 avg.)</td>
<td>1324 (10.3 avg.)</td>
<td>9.6%</td>
</tr>
<tr>
<td>No vaccine used in room</td>
<td>88</td>
<td>1059 (12.0 avg.)</td>
<td>872 (9.9 avg.)</td>
<td>17.6%</td>
</tr>
</tbody>
</table>

In another trial, nursing piglets were vaccinated intranasally between 2-4 days of age (endemic farm, 150 days post clinical break). Table 4 summarizes the results.

**Table 4**

<table>
<thead>
<tr>
<th></th>
<th>Vaccinated nursing piglets (1 cc dose)</th>
<th>Non-vaccinated nursing piglets</th>
</tr>
</thead>
<tbody>
<tr>
<td># of litters exhibiting clinical scour</td>
<td>5 of 15 litters (33%)</td>
<td>8 of 27 litters (30%)</td>
</tr>
<tr>
<td># of litters where all pigs died</td>
<td>0 of 15 litters (0%)</td>
<td>5 of 27 litters (18%)</td>
</tr>
<tr>
<td>Piglet mortality</td>
<td>22%</td>
<td>44%</td>
</tr>
<tr>
<td>Piglet morbidity</td>
<td>12%</td>
<td>13%</td>
</tr>
</tbody>
</table>

In another trial, PEDV vaccine was administered intramuscularly to nursing piglets in an endemic herd (150 days post clinical break). The results are shown in Table 5.

**Table 5**

<table>
<thead>
<tr>
<th></th>
<th>Born alive</th>
<th>Pigs remaining at weaning</th>
<th>Pre-wean mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nursing piglets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 cc IM, 2-3 days old, 7 litters</td>
<td>79 (11.2 avg.)</td>
<td>60 (8.6 avg.)</td>
<td>24%</td>
</tr>
<tr>
<td><strong>Nursing piglets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 cc IM, 2-3 days old, 7 litters</td>
<td>81 (11.5 avg.)</td>
<td>72 (10.3 avg.)</td>
<td>11%</td>
</tr>
</tbody>
</table>
In another trial, PEDV vaccine was administered to PEDV naive isowean pigs at weaning on Day 0. Vaccines were administered subcutaneously (SubQ), intramuscularly (IM), or intranasally (IN). The pigs were weighed and given a booster vaccine on Day 21. The pigs were challenged and weighed on Day 45, and weighed again on Day 56. The results are shown in Table 6.

### Table 6

<table>
<thead>
<tr>
<th>Group</th>
<th>Wean weight and 1&lt;sup&gt;st&lt;/sup&gt; vaccination (Day 0)</th>
<th>Booster vaccination weight (Day 21)</th>
<th>Day of challenge weight (Day 45)</th>
<th>End weight (Day 56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (red tags)</td>
<td>12.9</td>
<td>24.9</td>
<td>44.6</td>
<td>54.1</td>
</tr>
<tr>
<td>SubQ vaccination (green tags)</td>
<td>13.6</td>
<td>23.5</td>
<td>50.8</td>
<td>61.3</td>
</tr>
<tr>
<td>IN vaccination (pink tags)</td>
<td>13.3</td>
<td>27.8</td>
<td>51.0</td>
<td>66.6</td>
</tr>
<tr>
<td>IM vaccination (purple tags)</td>
<td>13.4</td>
<td>27.4</td>
<td>48.8</td>
<td>68.0</td>
</tr>
</tbody>
</table>

Table 7 shows the average daily gain for days 1-45 post vaccination and days 1-11 post challenge.

### Table 7

<table>
<thead>
<tr>
<th>Group</th>
<th>ADG Days 1-45 post vaccination</th>
<th>ADG Days 1-11 post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (red tags)</td>
<td>0.71</td>
<td>0.85</td>
</tr>
<tr>
<td>SubQ vaccination (green tags)</td>
<td>0.83</td>
<td>0.95</td>
</tr>
</tbody>
</table>
Fecal shedding was evaluated 11 days post challenge in naive isowean pigs. The values shown in Table 8 are PCR cycle times (CT) values. The lower the number, the higher the level of viral material in the sample. The negative cut-off is 35.

Table 8

<table>
<thead>
<tr>
<th>Group</th>
<th>Average CT value</th>
<th>Range in CT values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>28.3</td>
<td>21.9-33.0</td>
</tr>
<tr>
<td>SubQ vaccination</td>
<td>32.8</td>
<td>30.0-33.1</td>
</tr>
<tr>
<td>IN vaccination</td>
<td>26.2</td>
<td>21.3-30.0</td>
</tr>
<tr>
<td>IM vaccination</td>
<td>32.4</td>
<td>31.2-34.7</td>
</tr>
</tbody>
</table>

In another trial, the disclosed vaccine (MJ PEDV) was evaluated against a commercial PEDV vaccine in an endemic farm 150 days post clinical break. The vaccines were administered 3-5 days pre-farrow. The results are summarized in Table 9. The data is a composite of three farrowings in which all three groups were scattered throughout rooms.

Table 9

<table>
<thead>
<tr>
<th></th>
<th># of litters</th>
<th>Born alive</th>
<th>Pigs remaining</th>
<th>Pre-wean mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ PEDV</td>
<td>36</td>
<td>412 (11.4 avg.)</td>
<td>357 (9.9 avg.)</td>
<td>13.3%</td>
</tr>
<tr>
<td>1 cc IM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commercial PEDV</td>
<td>50</td>
<td>598 (11.9 avg.)</td>
<td>458 (9.1 avg.)</td>
<td>23.4%</td>
</tr>
<tr>
<td>1 cc IM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-vaccinates</td>
<td>33</td>
<td>422 (12.7 avg.)</td>
<td>311 (9.4 avg.)</td>
<td>26.3%</td>
</tr>
</tbody>
</table>
In another trial, the disclosed vaccine (MJ PEDV) was evaluated against two commercial PEDV vaccines during an acute outbreak. Sows farrowed 9 to 22 days post PEDV whole herd feedback. The results are shown in Table 10.

<table>
<thead>
<tr>
<th>Group treatment</th>
<th>Sow dosage of vaccine</th>
<th>Days farrowed post feedback exposure</th>
<th>Number of litters in group</th>
<th>Average born alive</th>
<th>Viable pigs remaining at 16 days of age</th>
<th>Pre-wean mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>No vaccine</td>
<td>9-21 days</td>
<td>6 litters</td>
<td>13.1</td>
<td>6.1</td>
<td>53.4%</td>
</tr>
<tr>
<td>Commercial vaccine 1</td>
<td>2 cc / 2 cc</td>
<td>10-22 days</td>
<td>6 litters</td>
<td>7.8</td>
<td>4.8</td>
<td>38.4%</td>
</tr>
<tr>
<td>Commercial vaccine 2</td>
<td>2 cc / 2 cc</td>
<td>9-21 days</td>
<td>7 litters</td>
<td>12.4</td>
<td>7.2</td>
<td>41.9%</td>
</tr>
<tr>
<td>MJ</td>
<td>2 cc / 2 cc</td>
<td>12-22 days</td>
<td>10 litters</td>
<td>9.3</td>
<td>6.9</td>
<td>25.8%</td>
</tr>
</tbody>
</table>

**Example 5**

**PEDV Active Farm Trials**

A farm experienced an outbreak of PEDV. After feeding back, the herd had stabilized. A few months after the initial outbreak, bringing in naive gilts acclimated for PEDV caused a second PEDV event on the farm. The farm was farrowing 145-150 sows/week. Each farrowing room included 44 crates; each farrowing group occupied 3+ farrowing rooms.

Five litters in Room #1 may be selected at 5 days old (day zero). Each piglet may be given 1 mL of IN vaccine, and the immunized piglets may be marked. Mortality may be compared between vaccinated and unvaccinated litters at weaning during days 14-19.

Rooms 2-4 may include sows bred at the same time. Sows in Room #2 and half the sows in Room #3 may not be vaccinated. The remaining sows in Room #3 and the sows in Room #4 may be vaccinated with IM vaccine (2 cc/sow) on day zero. The sows may farrow on days 14-19. Five litters at 5 days old (days 21-26 post sow vaccine) may be selected from Room #4; the selected piglets may be immunized with 1 mL of IN vaccine and marked. On days 37-44 post sow vaccination, mortality may be compared at weaning among litters that receive no vaccine, litters in which the sows receive IM vaccine and the piglets are unvaccinated, and litters in which the sows receive IM vaccine and the piglets receive IN vaccine.
Rooms 5-7 may include sows bred at the same time. Sows in Room #5 and half the sows in Room #6 may not be vaccinated. The remaining sows in Room #6 and the sows in Room #7 may be vaccinated with IM vaccine (2 cc/sow) on day zero. The vaccinated sows may receive a booster vaccination 14-19 days post initial vaccination. The sows may farrow on days 28-34 post initial vaccination. Five litters at 5 days old may be selected from Room #7 on days 33-39; the piglets may be vaccinated with 1 mL of IN vaccine and marked. On days 47-53, mortality may be compared at weaning among litters that receive no vaccine, litters in which the sows receive IM vaccine and the piglets are unvaccinated, and litters in which the sows receive IM vaccine and the piglets receive IN vaccine.

**Example 6**

*Comparison of Vaccination Protocols*

Sows and/or piglets were immunized intramuscularly with 1-4 cc of PEDV vaccine. The piglets were monitored to determine the effect on mortality.

**Table 11 - Room 3**

<table>
<thead>
<tr>
<th>Dose/pig IM</th>
<th># litters vaccinated</th>
<th>Total pigs vaccinated</th>
<th>Born alive per litter</th>
<th>Pigs wean vaccinated</th>
<th>Pigs weaned per litter</th>
<th>Piglet mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cc</td>
<td>7</td>
<td>79</td>
<td>11.2</td>
<td>60</td>
<td>8.6</td>
<td>24%</td>
</tr>
<tr>
<td>2 cc</td>
<td>7</td>
<td>81</td>
<td>11.5</td>
<td>72</td>
<td>10.3</td>
<td>11.1%</td>
</tr>
<tr>
<td>--</td>
<td>24</td>
<td>265</td>
<td>11.0</td>
<td>191</td>
<td>7.9</td>
<td>28%</td>
</tr>
</tbody>
</table>

One litter in the 1 cc group of pigs had severe scours. Pigs were vaccinated at 1-2 days of age. Pigs were weaned at 17-19 days of age.

**Table 12 - Room 4**

<table>
<thead>
<tr>
<th>Dose/pig IM</th>
<th># sows vaccinated</th>
<th>Born alive per litter</th>
<th>Pig inventory from vaccinated sows</th>
<th>Pig inventory at 15 days of age</th>
<th>Piglet mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 cc</td>
<td>10</td>
<td>11.2</td>
<td>112</td>
<td>99</td>
<td>11.6%</td>
</tr>
<tr>
<td>--</td>
<td>33</td>
<td>12.1</td>
<td>399</td>
<td>333</td>
<td>16.7%</td>
</tr>
</tbody>
</table>

Sows were given vaccine between day of farrowing out to day 6 pre-farrow. Average timing was 2.2 days pre-farrow for vaccinated sows.
Forty PEDV naive isowean pigs (20 days old) may be divided into 4 groups randomly and tagged (Groups A, B, C, and D). Four days later, each pig may be weighted and a blood sample obtained before vaccination. On day zero, Group A may receive 2 ml of a control vaccine, Group B may receive 2 mL of IM vaccine, Group C may receive 1 mL of IN vaccine (0.5 mL x 2 spots), and Group D may receive 1 mL of SQ vaccine (0.5 mL x 2 spots). On day 21, blood samples may be obtained and each pig may receive a booster vaccination. On day 34, the pigs may be moved to a new location. On day 35, a third blood sample may be obtained, and the pigs may be challenged by giving each pig 1 mL of "gut-homogenizer" by mouth. Each pig's behavior may be observed daily for two weeks. On day 41 (6 days post challenge), a fourth blood sample may be obtained. On day 45 (10 days post challenge), a fifth blood sample may be obtained.

**Example 8**

**Vaccination of Sows Previously Exposed to PEDV**

A PED stabilized farm may be selected. Sero-converting gilts may be kept by the PEDV-feedback method. Pigs may be identified in 4 groups - 8, 6, 4, and 2 prefarrowing groups, 30 gilts per group (15 for control, and 15 for vaccination). Blood samples may be obtained before vaccination. Pigs may be vaccinated with 2 mL of IM vaccine; controls may receive PBS + adjuvant. Blood samples may be obtained before a booster vaccination (2 mL of IM vaccine) at 2, 3, or 4 weeks later.
Example 9

PEDV proteins usable in vaccines may be prepared from a PEDV strain by infecting susceptible cells in vitro or in vivo, and harvesting the infected cells at an optimal time to prepare cell-associated viral components. For in vitro methods, the antigen(s) may be prepared by a cell culture system or by using recombinant technologies.

For instance, MARC cells can be grown in cell culture and infected with PEDV either with or without the addition of trypsin. The trypsin is added at a concentration that will help the virus infect the cells sheet without destroying the cells. For instance, at a concentration of 1-10 μg/mL.

Once the cells show evidence of infection by the PEDV, the culture medium is removed and discarded. The cells may be optionally washed. A buffer such as Tris or phosphate buffered saline (PBS) with EDTA is added, swirling to coat the cell sheet and then the PEDV proteins and antigens are extracted by placing the culture vessels (roller bottles, flasks, beads or other types of matrix) with the buffer into a freezer at a temperature at or below -10°C. The vessels are allowed to freeze and then are thawed. Thawing breaks open the cells and releases the PEDV proteins and antigens useful for preparation of a vaccine. Optionally, the thawed culture may be refrozen and rethawed to release more proteins and antigens. After release of the proteins/antigens, an inactivating agent such as binary ethyleneimine (BEI), formalin, beta propiolactone (BPL) or any other effective inactivating agent is added while mixing. Mixing of the inactivating agent with the culture is
continued until inactivation is complete, usually at least 30 minutes. After inactivation, the culture may be diluted and adjuvanted or adjuvanted without further dilution. Acceptable adjuvants include oil-in-water adjuvants such as those containing EMULSIGEN®, adjuvants comprising polymers such as those comprising acrylic acids or carbomers such as CARBIGEN™, or other types of polymers such as POLYGEN™. Once the antigens are inactivated and adjuvanted they may be administered to animals, preferably pigs, via intramuscular, subcutaneous, intranasal or oral routes.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.
We claim:

1. A method, comprising:
   incubating porcine epidemic diarrhea virus (PEDV) infected cells for an effective period of time to result in one or more replicated PEDV viral particles being released from the infected cells;
   isolating cells infected with PEDV away from cell-free PEDV virus particles to obtain isolated cells containing cell-associated PEDV proteins and antigens;
   separating the PEDV proteins and antigens from the isolated cells, to form an immunogenic composition comprising isolated PEDV proteins and antigens.

2. The method of claim 1, wherein separating inactivates viral particles released from the infected cells.

3. The method of claim 1, further comprising adding an adjuvant to the composition.

4. The method of claim 3, wherein the adjuvant is selected to stimulate a mucosal antibody response.

5. The method of claim 4, wherein the adjuvant is selected for intranasal administration.

6. The method of claim 4, wherein the adjuvant is selected for intravaginal administration.

7. The method of claim 4, wherein the adjuvant adheres to the mucous membranes.

8. The method of claim 5, wherein the adjuvant comprises polyacrylic acid.

9. The method of claim 1, wherein separating comprises extracting PEDV proteins and antigens.
10. The method of claim 1, wherein separating comprises eluting PEDV proteins and antigens.

11. The method of claim 1, wherein separating comprises a freeze-thaw cycle.

12. The method of claim 1, wherein separating PEDV proteins and antigens comprises breaking open, extracting, rupturing, freezing and thawing, lysing, centrifuging, filtering the cells, or any combination thereof to release the PEDV proteins and antigens from the cells.

13. The method of claim 1, further comprising adding an inactivating agent.

14. The method of claim 13, wherein the inactivating agent comprises binary ethyleneimine.

15. The method of claim 1, wherein separating the PEDV proteins and antigens from the isolated cells comprises contacting the isolated cells with a detergent.

16. The method of claim 15, wherein contacting forms inactivated viral particles.

17. The method of claim 15, wherein the detergent is Triton X-100.

18. The method of claim 15, wherein separating further comprising adding an inactivating agent.

19. The method of claim 18, wherein the inactivating agent comprises binary ethyleneimine.

20. The method of claim 1, wherein the immunogenic composition is a vaccine.

21. The method of claim 1, comprising:
   incubating PEDV infected cells for a period of from 24 to 60 hours;
isolating cells infected with PEDV away from cell-free PEDV virus particles to obtain isolated cells containing cell-associated PEDV proteins and antigens;
lysing the isolated cells to form a first composition comprising isolated PEDV proteins and antigens;
inactivating viral particles in the first composition to produce a second composition; and adding an adjuvant comprising polyacrylic acid to the second composition to form an immunogenic composition formulated for intranasal administration.

22. The method of claim 20, comprising incubating the PEDV infected cells for from 24 to 48 hours.

23. The method of claim 1, wherein incubating PEDV infected cells comprises incubating cells infected with PEDV having at least 90% sequence identity to at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 or SEQ ID NO: 9.

24. The method of claim 1, wherein incubating PEDV infected cells comprises incubating cells infected with PEDV having at least 99% sequence identity to at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

25. The method of claim 1, wherein incubating PEDV infected cells comprises incubating cells infected with PEDV having at least 99.5% sequence identity to at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

26. The method of claim 1, wherein incubating PEDV infected cells comprises incubating cells infected with PEDV having a sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

27. The method of claim 1, wherein the isolated PEDV proteins and antigens comprise an amount of S protein.
28. The method of claim 27, wherein the amount of S protein is sufficient to produce an immune response in a subject receiving the immunogenic composition.

29. The method of claim 27, wherein the S protein is an intact S protein.

30. The method of claim 27, wherein the S protein has at least 90% sequence identity to at least one of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17.

31. The method of claim 27, wherein the S protein has at least 99% sequence identity to at least one of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15.

32. The method of claim 27, wherein the S protein has a sequence selected from SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15.

33. The method of claim 1, comprising:

- incubating PEDV infected cells for a period of from 24 to 60 hours sufficient to produce to tissue culture infective doses/ml (TCIDso/ml) of from $10^1$ to $10^{10}$, the cells infected with a PEDV strain that has at least 99% sequence identity to at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6;

- isolating cells infected with PEDV away from cell-free PEDV virus particles to obtain isolated cells containing cell-associated PEDV proteins and antigens;

- lysing the isolated cells to form a first composition comprising isolated PEDV proteins and antigens comprising S protein having a molecular weight of about 152 kDa based on deduced amino acid sequences, and a molecular weight of from 180 kDa to at least 350 kDa after post-translational modifications;

- adding an inactivating agent comprising binary ethyleneimine to the first composition to produce a second composition; and

- adding an adjuvant comprising polyacrylic acid to the second composition to form an immunogenic composition formulated for intranasal administration.
34. An immunogenic composition, comprising a first antigenic component comprising isolated PEDV proteins and/or antigens from a first PEDV strain.

35. The immunogenic composition of claim 34, comprising an S protein.

36. The immunogenic composition of claim 35, comprising an amount of S protein sufficient to produce an immune response in a subject receiving the immunogenic composition.

37. The immunogenic composition of claim 35, wherein the S protein is an intact S protein.

38. The immunogenic composition of claim 35, wherein the S protein has at least 90% sequence identity to at least one of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17.

39. The immunogenic composition of claim 35, wherein the S protein has at least 99% sequence identity to at least one of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15.

40. The immunogenic composition of claim 39, wherein the S protein has a sequence selected from SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15.

41. The immunogenic composition of claim 34, wherein the first PEDV strain has at least 90% sequence identity to at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 or SEQ ID NO: 9.

42. The immunogenic composition of claim 34, wherein the first PEDV strain has at least 99% sequence identity to at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.
43. The immunogenic composition of claim 34, wherein the first PEDV strain has at least 99.5% sequence identity to at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

44. The immunogenic composition of claim 34, wherein the first PEDV strain has a sequence identity selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

45. The immunogenic composition of claim 34, comprising a second antigenic component.

46. The immunogenic composition of claim 45, wherein the second antigenic component comprises isolated PEDV proteins and/or antigens from a second PEDV strain different from the first PEDV strain.

47. The immunogenic compositions of claim 45, wherein the second antigenic component comprises isolated proteins and/or antigens from a second pathogen other than PEDV.

49. The immunogenic composition of claim 48, wherein the second pathogen is porcine reproductive and respiratory syndrome virus.

50. The immunogenic composition of claim 48, wherein the second pathogen is not *Mycoplasma hyopneumoniae*.

51. The immunogenic composition of claim 34, wherein the immunogenic composition is a vaccine.

52. The immunogenic composition of any one of claims 34-51, further comprising an adjuvant.

53. The immunogenic composition of claim 52, wherein the adjuvant is selected to stimulate a mucosal antibody response.

54. The immunogenic composition of claim 52, wherein the adjuvant adheres to the mucous membranes.

55. The immunogenic composition of claim 52, wherein the adjuvant comprises emulsified oil-in-water adjuvant.

56. The immunogenic composition of claim 55, wherein the adjuvant comprises an ammonium salt.

57. The immunogenic composition of claim 55, wherein the ammonium salt is a tetraalkylammonium salt.

58. The immunogenic composition of claim 55, wherein the tetraalkylammonium salt is dimethyldioctadecylammonium bromide.

59. The immunogenic composition of claim 52, wherein the adjuvant comprises polyacrylic acid.
60. A method, comprising administering to a first pig an effective amount of a first immunogenic composition according to claim 34.

61. The method of claim 60, wherein the first immunogenic composition comprises an adjuvant.

62. The method of claim 61, wherein the adjuvant is selected to stimulate a mucosal antibody response.

63. The method of claim 62, wherein the adjuvant adheres to the mucous membranes.

64. The method of claim 61, wherein the adjuvant comprises an emulsified oil-in-water adjuvant.

65. The method of claim 64, wherein the adjuvant comprises an ammonium salt.

66. The method of claim 65, wherein the ammonium salt is a tetralkylammonium salt.

67. The method of claim 66, wherein the tetralkylammonium salt is dimethyldioctadecylammonium bromide.

68. The method of claim 61, wherein the adjuvant comprises a polyacrylic acid.

69. The method of claim 60, wherein the first pig is less than 7 days old.

70. The method of claim 60, wherein the first pig is 5 days old or less.

71. The method of claim 60, wherein the first pig is 2 days old or less.

72. The method of any one of claims 60-71, wherein administering comprises administering orally, intramuscularly, or subcutaneously.
73. The method of any one of claims 60-71, wherein administering comprises administering intranasally.

74. The method of claim 60, wherein the immunogenic composition further comprises a second antigenic component different from the first antigenic component.

75. The method of claim 74, wherein the second antigenic component comprises isolated PEDV proteins and/or antigens from a second PEDV strain.

76. The method of claim 74, wherein the second antigenic component comprises isolated proteins and/or antigens from a second pathogen other than PEDV.

77. The method of claim 76, wherein the second pathogen is porcine reproductive and respiratory syndrome virus, *Mycoplasma hyopneumoniae, Mycoplasma hyosynoviae, Mycoplasma hyorhinis, Clostridium tetani, Clostridium perfringens, porcine parvovirus, Erysipelothrix rhusiopathiae, Leptospira pomona, Leptospira grippotyphosa, Leptospira hardjo, Leptospira canicola, Leptospira icterohaemorrhagiae, Leptospira bratislava, porcine circovirus, Lawsonia intracellulars, Escherchia coli, Actinobacillus pleuropneumoniae, Haemophilus parasuis, Salmonella choleraesuis, Salmonella typhimurium, Streptococcus suis, Pasteurella multocida, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Serpulina hyodysenteriae, encephalomyocarditis virus, swine influenza virus, transmissible gastroenteritis virus (TGE), swine delta coronavirus, rotavirus diarrhea, foot and mouth disease virus, classical swine fever virus, pseudorabies virus, Japanese encephalitis virus (JEV), encephalomyocarditis virus or a combination thereof.

78. The method of claim 76, wherein the second pathogen is porcine reproductive and respiratory syndrome virus.

79. The method of claim 76, wherein the second pathogen is not *Mycoplasma hyopneumoniae.*
80. The method of claim 60, comprising administering a second immunogenic composition to a second pig, the second pig being a sow and the first pig being a piglet farrowed from the sow.

81. The method of claim 80, wherein the first immunogenic composition is administered intranasally.

82. The method of claim 80, wherein the second immunogenic composition is administered intramuscularly.

83. The method of claim 80, wherein the second immunogenic composition comprises an immunogenic composition according to claim 34.

84. The method of claim 80, wherein the sow is a pregnant sow.

85. The method of claim 80, wherein the sow is a sow expected to become pregnant subsequent to administration of the second immunogenic composition.

86. The method of claim 80, wherein administering the second immunogenic composition comprises administering the second immunogenic composition at a time point prior to the sow becoming pregnant such that, when the sow becomes pregnant, the sow has a greater immunity to PEDV compared to a pig not administered the immunogenic composition.

87. A use of an immunogenic composition made by the method of any one of claims 1-33 in the manufacture of a medicament for administration to a swine.
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Std, Protein Molecular Weight Standard
S, Sow serum samples
P, Piglet serum samples

FIG. 1
FIG. 2
Time Course Study
mAB 6C8 + 3F12

kDa
240
140
100
70
50
35
25

S 1 2 3 4 5

FIG. 3
1. 2x diluted sample
2. 3x diluted sample
3. 4x diluted sample
4. Fully grown virus culture itself
S. SeeBlue Protein MW Standard
M. Magic Protein MW Standard
CO. PEDv isolate from sample taken in Colorado
IA. PEDv isolate from sample taken in Iowa
NC. PEDv isolate from sample taken in North Carolina

FIG. 4

1. DE sample from CO-virus culture
2. DE sample from IA-virus culture
3. DE sample from NC-virus culture
4. Mix of 3 DE samples before inactivation process
5. Mix of 3 DE samples after inactivation process
6. Mix of 3 virus cultures in equal volume
S. SeeBlue Protein MW Standard
M. Magic Protein MW Standard

FIG. 5
### FIG. 6

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- **SEG-ID-1**: Whole gene
- **SEG-ID-2**: Whole gene
- **SEG-ID-3**: Whole gene
- **SEG-ID-4**: Whole gene
- **SEG-ID-5**: Whole gene
- **SEG-ID-6**: Whole gene
- **SEG-ID-7**: Whole gene
- **SEG-ID-8**: Whole gene
- **SEG-ID-9**: Whole gene
INTERNATIONAL SEARCH REPORT

A CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/12
ADD.

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

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A61K C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>X</td>
<td>OH JIN SI K ET AL: &quot;Compari son of an enzyme- 1 inked immunosorobent assay wi t h serum neutral izati on test for serodi agnosti s of porc e epidemi c diarrhea vi r us infecti on. &quot;, JOURNAL OF VETERINARY SCI ENCE DEC 2005, vol . 6, no . 4, December 2005 (2005-12) , pages 349-352 , XP002757049, ISSN : 1229-845X</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

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

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

Date of the actual completion of the international search: 27 April 2016
Date of mailing of the international search report: 26/07/2016

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer
Heder, Andreas
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<td>Wo 02/074940 AL (ONCOLYTICS BIOTECH INC [CA]; COFFEY MATTHEW C [CA]; THOMPSON BRADLEY G) 26 September 2002 (2002-09-26) claims 1, 2</td>
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### INTERNATIONAL SEARCH REPORT

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

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

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

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

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

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

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

   see additional sheet

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

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

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

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

   1, 2, 9-12, 15-17, 20-26, 33(al partial ly)

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☑ No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 9-12, 15-17, 20-26, 33 (all 1 partially)
   Method comprising incubating PEDV infected cells, isolating infected cells from cell-free PEDV, and separating PEDV proteins and antigens from the cells to form an immunogenic composition, wherein PEDV has SEQ ID NO: 1

2-9. claims: 1, 2, 9-12, 15-17, 20-26, 33 (all 1 partially)
   Like invention 1, wherein PEDV has one of SEQ ID NO: 2-9

10. claims: 1, 2, 9-12, 15-17, 20-26, 33 (all 1 partially)
    Multiple inventions, like inventions 1-9, wherein PEDV has a sequence different from SEQ ID NO: 1-9

11. claims: 3-8 (completely); 33 (partially)
    Multiple inventions, like inventions 1-10, further comprising adding an adjuvant to the composition

12. claims: 13, 14, 18, 19
    Multiple inventions, like inventions 1-10, further comprising adding an inactivating agent

13. claims: 27-32
    Multiple inventions, like inventions 1-10, wherein an S protein having one of SEQ ID NO: 11-17, or a different sequence, is composed

14. claims: 34-59
    Multiple inventions, relating to immunogenic compositions comprising PEDV proteins / antigens

15. claims: 60-87
    Multiple inventions, relating to medical uses of immunogenic compositions comprising PEDV proteins / antigens
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