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(54) Title: PORCINE PESTVIRUS, VACCINES, AND ASSAYS

(57) Abstract: Porcine pestivirus designated herein as atypical porcine pestivirus ("APPV") (Genbank accession no. KR011347.1). Immunogenic compositions to induce an immune response against porcine pestivirus infection in a pig are described, which APPV antigenic agents (e.g., isolated whole virus, derivatives thereof, functional fragments thereof, and combinations of the foregoing). Methods of vaccinating against porcine pestivirus infection using the immunogenic compositions are also described. The methods can be also applied for clinical research and/or study, including diagnostic methods for detecting pestivirus infection using monoclonal antibodies specifically binding to APPV epitopes.

PORCINE PESTVIRUS, VACCINES, AND ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the priority benefit of U.S. Provisional Patent Application Serial No. 62/155,004, filed April 30, 2015, entitled PORCINE PESTVIRUS, VACCINES, AND ASSAYS, incorporated by reference in its entirety herein.

SEQUENCE LISTING

The following application contains a sequence listing in computer readable format (CRF), submitted as a text file in ASCII format entitled "47343-PCT Sequence Listing," created on April 28, 2016, as 51 KB. The content of the CRF is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to atypical porcine pestivirus isolates, and methods of use thereof.

Description of Related Art

Species in the genus Pestivirus: bovine viral diarrhea virus type 1 (BVDV-1), bovine viral diarrhea virus type 2 (BVDV-2), classical swine fever virus (CSFV) and border disease virus (BDV), are some of the most significant pathogens affecting ruminants and swine. Clinical disease can lead to high morbidity and mortality. Host immunosuppression and persistent viremia are hallmarks of pestivirus infections. Pestiviruses cause economically significant disease in ruminants and swine.

In 2003, a divergent pestivirus, Bungowannah virus (BWV), was isolated from a farm in Australia exhibiting an outbreak of sudden death in three to four week-old pigs and increase in stillborn fetuses. Pathologically, multifocal non-suppurative myocarditis with myonecrosis was observed. Phylogenetic analysis found Bungowannah virus to be the most divergent pestivirus and antigenically it failed to react with pan-reactive pestivirus monoclonal antibodies. More recently, two novel pestiviruses were identified by next generation sequencing (NGS). Analysis of the virome from the bat species *Rhinolophus affinis* in China identified a 5 kb contig with 32% amino acid sequence identity to known pestiviruses. Metagenomic sequencing of Norway rats in New York City also identified a highly divergent pestivirus (Norway rat pestivirus,

NRPV) which shared a maximum of 60% amino acid identity with known pestivirus polyproteins. These discoveries have challenged our understanding of the diversity and ecology of pestiviruses. These two newly described pestiviruses are the first identified in species outside the order *Artiodactyla* and suggest a wider pestivirus host range. Nothing is known on the ability for these viruses to cause disease.

SUMMARY OF THE INVENTION

The present invention is broadly concerned with immunogenic compositions to induce an immune response against porcine pestivirus infection in a pig. The compositions generally comprise a therapeutically-effective amount of atypical porcine pestivirus (APPV) antigenic agents dispersed in a pharmaceutically-acceptable carrier. Exemplary APPV antigenic agents are selected from the group consisting of isolated whole virus having an mRNA complementary coding sequence according to Genbank accession no. KR011347.1 (SEQ ID NO:1), derivatives thereof, functional fragments thereof, and combinations of the foregoing.

Also described herein are methods of vaccinating a pig to induce an immune response against porcine pestivirus infection. The methods generally comprise administering an immunogenic composition according to any one of the embodiments described herein to the pig.

Kits for inducing an immune response against porcine pestivirus infection in a pig are also described herein. The kit generally comprise an immunogenic composition according to any one of the embodiments described herein, and instructions for administering the composition to the pig.

The disclosure also concerns the use of an immunogenic composition according to any one of the embodiments described herein for inducing an immune response against porcine pestivirus infection in a pig.

Also described herein are methods of detecting pestivirus antigen in a biological sample. The methods generally comprise contacting a biological sample from a pig with a monoclonal antibody against the atypical porcine pestivirus (APPV) described herein. The monoclonal antibody specifically binds to an antigen of the pestivirus if present in the sample. The antibody-antigen binding, if present, can then be detected to confirm or refute pestivirus infection.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office

upon request and payment of the necessary fee.

Figure (Fig.) 1A shows phylogenetic trees of non-structural pestivirus proteins Npro, NS2, NS4a, and NS4b for various pestiviruses;

Fig. 1B shows phylogenetic trees of non-structural pestivirus proteins NS3, NS5a, NS5b, and P7 for various pestiviruses;

Fig. 1C shows phylogenetic trees of structural pestivirus proteins C, Erns, E1, and E2 for various pestiviruses;

Fig. 2A shows SDS-PAGE analysis of protein purity for the APPV Erns fragments (Erns-A: 20-120aa; Erns-B: 45-150aa) expressed in *E. coli* N-terminal 6xHis fusion proteins and purified using affinity chromatography;

Fig. 2B shows Western blot analysis using a monoclonal anti-6xHis antibody of protein purity for the APPV Erns fragments (Erns-A: 20-120aa; Erns-B: 45-150aa) expressed in *E. coli* N-terminal 6xHis fusion proteins and purified using affinity chromatography;

Fig. 3A shows the detection of APPV antigen by indirect immunofluorescence assay. The top two panels show MARC-145 cells transfected with a plasmid DNA expressing pestivirus Erns protein or mock-transfected (control), as indicated, and stained with DAPI for visualizing the cell nucleus. The bottom two panels show immunofluorescent staining with DAPI in of lymph node section from formalin-fixed paraffin-embedded tissues. Tissue sections are shown from samples identified as APPV positive by qRT-PCR ("LN-APPV infection"), and from a healthy pig, identified as APPV negative by qRT-PCR ("LN-control"), as indicated. Images were taken by a confocal microscope (LSM 880, Zeiss);

Fig. 3B shows an image from Western blot analysis of HEK293T cells mock-transfected (lane 2) or transfected with a plasmid DNA expressing pestivirus Erns protein (lane 3), and analyzed using anti-Erns mAb. The image was obtained on a digital Odyssey infrared imaging system. The molecular weight marker (Line 1) is shown with red fluorescence; and

Fig. 4 shows images from detection of APPV antigen by immunohistochemistry test. Immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissues. Tissue sections were from samples identified as APPV positive by qRT-PCR, as compared to tissue sections from a healthy pig, identified as APPV negative by qRT-PCR, as indicated.

DETAILED DESCRIPTION

The present invention is concerned with identification of a novel virus designated herein as atypical porcine pestivirus ("APPV") (aka porcine pestivirus 1 (PPeV1) strain 000515). The

virus and associated information is useful in diagnostic assays to detect porcine pestivirus, and vaccines to induce a protective immune response against porcine pestivirus infection.

The pestivirus genome generally consists of a single positive strand RNA approximately 12kb in length which encodes a polyprotein approximately of 3,900 amino acids flanked by untranslated regions ~400bp in length. The polyprotein is co- and post-translationally cleaved by a combination of cellular and viral-encoded proteases to generate 11-12 proteins. Genomic organization is conserved with protein order: Npro-C-Erns-E1-E2-p7-NS2/3-NS4A-NS4B-NS5A-NS5B. The pestivirus virion contains three structural glycoproteins: Erns, E1, and E2. Erns is a pestivirus envelope glycoprotein that is unique to pestiviruses.

Sequence information for the novel APPV isolate is available under Genbank accession no. KR011347.1, incorporated by reference herein. Molecular epidemiology and serology suggest that this virus is common in U.S. swine. The mRNA complementary (cDNA) coding sequence of the novel APPV isolate viral genome comprises (consists of) SEQ ID NO:1, and encodes for a polyprotein according to SEQ ID NO:2. The viral cDNA template can be used to synthesize mRNAs, which are then translated into viral proteins. In one or more embodiments, the APPV isolate comprises a gene (SEQ ID NO:3) encoding for an Erns protein. In one or more embodiments, the APPV isolate is one presenting an Erns epitope according to SEQ ID NO:4, or an epitope having at least 95% sequence identity to SEQ ID NO:4.

The APPV isolate can be used to prepare vaccines to induce a protective immune response against porcine pestivirus infection in a pig. The APPV isolate can be used as a live, attenuated (whole) virus vaccine. Various protocols for attenuating viruses are known in the art, including passaging of the virus in cell culture or in an unnatural host animal (e.g., mouse, chick embryos, etc.). The APPV isolate can also be used as an inactivated (non-replicating) whole virus vaccine. Such viruses can be prepared by heat or chemical treatment to inactivate the replicative function of the virus. Genetic modifications may also be made to yield attenuation or inactivation. For example, mutations in the catalytic active sites for the Erns protein can lead to virus attenuation (e.g., a virus that can replicate, but not infect). Synthetically generated viral constructs can also be used in such vaccines, wherein the virus is synthetically generated from genome segments constructed directly using known sequences and chemical or enzymatic synthesis and assembly of the oligonucleotides. For example, the synthetic expression construct can drive expression in a eukaryotic cell of viral segments encoded therein. The expressed viral segment RNA can be translated into a viral protein that can be incorporated into a virion. Molecular techniques can be used to synthesize an infectious clone of the virus, and

subsequently introduce targeted mutations to knock down the function of virulent gene(s) to generated attenuated clones as well.

Sequence fragments may also be used so long as they are "functional fragments" meaning that they nonetheless encode a functional protein for the virus from which the sequence was derived. Vaccines can also be made using "derivatives" of the APPV isolate, which as used herein, refers to recombinant viral particles and chimeras including the APPV isolate or a functional fragment thereof. Vaccines can also be made with functional fragments of the APPV isolate, including subunits, purified antigens, surface proteins, and recombinant or synthetically generated forms thereof. Such viral fragments are "functional" so long as they encode for a functional antigenic protein of the APPV isolate that will provoke an immune response in the subject animal. Examples include the Erns protein, E2 protein, or NS3 protein of the APPV isolate. As such, the vaccines can include recombinant proteins or vectors that express APPV E2 or Erns recombinant proteins from APPV as vaccines. In some embodiments, such functional fragments can be coupled to a carrier protein or adjuvant for delivery. Similarly, functional fragments from APPV can be used in chimeric or recombinant viral particles to generate an immune response against APPV. Examples include recombinant or chimeric pestiviruses created via reverse genetics and pestivirus backbones other than APPV (e.g., BVD backbone with BVD E2 or Erns gene replaced with E2 or Erns from APPV).

As used herein, the term "vaccine" refers to an immunogenic composition capable of provoking an immune response against a disease or condition in the subject (e.g., swine) to which it has been administered. Compositions according to the embodiments disclosed herein are useful in treating viral infection from pestivirus in a subject (e.g., swine) and/or preventing or reducing clinical symptoms of infection. Such clinical symptoms are the manifestation of the neurological disease caused by the pestivirus and include tremors, increased respiration rate, lack of coordination (e.g., unbalanced walking, inability to stand-up), and/or inability to swallow or control mouth movements. Thus, embodiments described herein have therapeutic and/or prophylactic uses, and in particular can be used for prophylactic treatment of a viral infection. In general, the compositions are administered prophylactically, that is, before the subject demonstrates detectable clinical signs of an infection, such that the subject develops an adaptive immune response to infection by the virus. As such, the methods are useful for preventing the development of observable clinical symptoms from viral infection, and/or reducing the incidence or severity of clinical symptoms, and/or effects of the infection, and/or reducing the duration of the infection/symptoms/effects, and/or reducing the amount and/or duration viral

shedding/viremia (e.g., excretion or expulsion of the virus or viral particles from an infected subject), as compared with unvaccinated control animals. Thus, the composition may only partially prevent and/or lessen the extent of morbidity due to the viral infection (i.e., reduce the severity of the symptoms and/or effects of the infection, and/or reduce the duration of the infection/symptoms/effects), as compared with unvaccinated control animals. Yet, the composition is still considered is still considered to treat or "prevent" the target infection or disease, even though it is not 100% effective. Another advantageous aspect of the invention is that protective immunity may be transmitted from vaccinated subjects to the offspring.

The vaccines comprise the APPV isolate described herein, derivative thereof, or functional fragment thereof, dispersed in a pharmaceutically-acceptable carrier. For ease of reference, the term "APPV antigenic agents" will be used to refer collectively to the APPV isolates (whole virus), derivatives thereof, or functional fragments thereof. The term carrier is used herein to refer to diluents, excipients, vehicles, and the like, in which the APPV antigenic agents may be dispersed for administration. Suitable carriers will be pharmaceutically acceptable. As used herein, the term "pharmaceutically acceptable" means not biologically or otherwise undesirable, in that it can be administered to a subject without excessive toxicity, irritation, or allergic response, and does not cause unacceptable biological effects or interact in a deleterious manner with any of the other components of the composition in which it is contained. A pharmaceutically-acceptable carrier would naturally be selected to minimize any degradation of the APPV antigenic agents or other ingredients and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art. Pharmaceutically-acceptable ingredients include those acceptable for veterinary use, and will depend on the route of administration. For example, compositions suitable for administration via injection are typically solutions in sterile isotonic aqueous buffer. Exemplary carriers include aqueous solutions such as normal (n.) saline (~0.9% NaCl), phosphate buffered saline (PBS), sterile water/distilled autoclaved water (DAW), aqueous dextrose solutions, aqueous glycerol solutions, ethanol, normal allantoic fluid, various oil-in-water or water-in-oil emulsions, as well as dimethyl sulfoxide (DMSO) or other acceptable vehicles, and the like.

The vaccine can comprise a therapeutically effective amount of APPV antigenic agents dispersed in the carrier. As used herein, a "therapeutically effective" amount refers to the amount that will elicit the biological or medical response of a tissue, system, or subject that is being sought by a researcher or clinician, and in particular elicit some desired protective effect as against the viral infection by priming or stimulating an immune response specific to the APPV

isolate. One of skill in the art recognizes that an amount may be considered therapeutically "effective" even if the condition is not totally eradicated or prevented, but it or its symptoms and/or effects are improved or alleviated partially in the subject. In some embodiments, the composition will comprise from about 5% to about 95% by weight of APPV antigenic agents described herein, and preferably from about 30% to about 90% by weight of APPV antigenic agents, based upon the total weight of the composition taken as 100% by weight. In some embodiments, combinations of more than one type of the described APPV antigenic agents can be included in the composition, in which case the total levels of all such viral particles will preferably fall within the ranges described above.

Other ingredients may be included in the composition, such as adjuvants, other active agents, preservatives, buffering agents, salts, other pharmaceutically-acceptable ingredients, including residual amounts of ingredients used in vaccine manufacturing. The term "adjuvant" is used herein to refer to substances that have immunopotentiating effects and are added to or co-formulated in the vaccine composition in order to enhance, elicit, and/or modulate the innate, humoral, and/or cell-mediated immune response against the vaccine components. Suitable adjuvants include: aluminum salts, such as aluminum hydroxide, aluminum phosphate, alum (potassium aluminum sulfate), or mixed aluminum salts, peptides, oil or hydrocarbon emulsions, or any other adjuvant deemed suitable for animal use. Other active agents that could be included in the composition include other antiviral compounds or any immunogenic active components (e.g., antigens) such as those that resemble a disease-causing microorganism or infectious agent other than the APPV, and/or are made from weakened or killed forms of the same, its toxins, subunits, particles, and/or one of its surface proteins, such that it provokes an immune response to that microorganism or infectious agent. In addition to live, modified, or attenuated vaccine components, active agents using synthetic peptides, carbohydrates, or antigens can also be used. Antibiotics can also be used as part of vaccine production and may be present in small amounts in the vaccine, such as neomycin, polymyxin B, streptomycin and gentamicin. In some embodiments, the vaccine composition is substantially free of any other active (immunogenic) agents, other than the APPV antigenic agents and optional adjuvant, dispersed in the carrier.

In use, the vaccine composition is administered to a subject. In general, the subject would be an animal susceptible to pestivirus. In some embodiments, the immunogenic composition is administered to a pregnant animal to induce immunity indirectly in her offspring through passive transfer of maternal antibodies. In some embodiments, the invention is concerned with methods of conferring immunity to piglets against pestivirus by administering to

pregnant sows an effective amount of APPV antigenic agents, wherein the resulting piglet(s) have a reduced morbidity and/or mortality as compared to piglets born by unvaccinated sows.

Various routes of administration can be used depending upon the particular carrier and other ingredients used. For example, the vaccine can be injected intramuscularly, subcutaneously, intradermally, or intravenously using a needle and syringe, or a needleless injection device. The vaccine can also be administered mucosally, such as intranasal administration. For intranasal administration, the vaccine composition is usually administered through the nasal passage as drops, large particle aerosol (greater than about 10 microns), or spray into the upper respiratory tract. Oral administration may encompass, for example, adding the compositions to the feed or drink of the animals. While stimulation of a protective immune response with a single dose is preferred, additional dosages can be administered, by the same or different route, to achieve the desired prophylactic effect. The vaccine can also be administered using a prime and boost regime if deemed necessary. In some embodiments, the methods described herein are useful for reducing the occurrence or incidence of pestivirus and/or reducing the effects of pestivirus infection, as described above.

Regardless, administration of the APPV antigenic agents elicits an immune response in the animal (or offspring, if applicable). Such an "immune response" includes, for example, the production or activation of antibodies, B cells and/or the various T cells, directed specifically to an antigen or antigenic component of the APPV isolate (e.g., E1, E2, Erns, NS3, etc.). The immune response will be demonstrated by a lack of observable clinical symptoms, or reduction of clinical symptoms normally displayed by an infected animal, faster recovery times from infection, reduced duration or amount of viral shedding, and the like. Accordingly, vaccinated animals will display resistance to new infection (or observable signs of infection) or reduced severity of infection, as compared to unvaccinated animals. The invention is particularly concerned with pigs, in all stages of development, including newborn, embryonic, and fetal stages.

"Reducing" the incidence, severity, and/or duration of clinical symptoms and/or viral shedding, means reducing the number of infected animals in a group, reducing or eliminating the number of animals exhibiting clinical signs of infection, or reducing the severity of any clinical signs that are present in the animals, in comparison to wild-type infection in unvaccinated animals. Preferably, these are reduced in animals receiving the APPV antigenic agents of the present invention by at least 10% in comparison to animals not receiving the vaccination which may become infected. More preferably, clinical symptoms of infection are reduced in animals

receiving the vaccination by at least 20%, more preferably by at least 30%, even more preferably by at least 40%, and even more preferably by at least 50%.

In some embodiments, the vaccine can be provided in unit dosage form in a suitable container. The term "unit dosage form" refers to a physically discrete unit suitable as a unitary dosage for animal use. Each unit dosage form may contain a predetermined amount of the vaccine (and/or other active agents) in the carrier calculated to produce the desired effect. In other embodiments, the vaccine can be provided separate from the carrier (e.g., in its own vial, ampule, sachet, or other suitable container) for on-site mixing before administration to a subject. A kit comprising the vaccine is also disclosed herein. The kit further comprises instructions for administering the vaccine to a subject. The APPV antigenic agents can be provided as part of a dosage unit, already dispersed in a pharmaceutically-acceptable carrier, or the APPV antigenic agents can be provided separately from the carrier. The kit can further comprise instructions for preparing the virus for administration to a subject, including for example, instructions for dispersing the APPV antigenic agents in a suitable carrier.

In one or more embodiments, vaccination against pestivirus can be combined with other vaccinations within the framework of vaccination programs, in the form of immunization or vaccination kits or methods, or in the form of multivalent immunogenic compositions and multivalent vaccines, i.e. comprising or consisting essentially of at least one vaccine component against APPV and at least one vaccine component against at least one other pathogenic agent..

The methods can be also applied for clinical research and/or study. A diagnostic method for pestivirus infection in a subject is also disclosed. The method includes contacting a biological sample from a subject with a monoclonal antibody against the APPV isolate or a fragment thereof. The term "antibody" means an immunoglobulin molecule that recognizes and specifically binds to a target, such as a protein, polypeptide, peptide, carbohydrate, polynucleotide, lipid, or combinations of the foregoing through at least one antigen recognition site within the variable region of the immunoglobulin molecule. An antibody can be of any the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses (isotypes) thereof (e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu, respectively. Antibodies can be naked or conjugated to other molecules such as toxins, radioisotopes, etc. A "monoclonal antibody" refers to a homogeneous antibody population involved in the highly specific recognition and binding of a single antigenic determinant, or epitope. This is in contrast to polyclonal antibodies that typically include different antibodies directed against different

antigenic determinants. The term encompasses both intact and full-length monoclonal antibodies as well as antibody fragments (such as Fab, Fab', F(ab')2, Fv), single chain (scFv) mutants, fusion proteins comprising an antibody portion, and any other modified immunoglobulin molecule comprising an antigen recognition site. Furthermore, "monoclonal antibody" refers to such antibodies made in any number of manners including but not limited to by hybridoma, phage selection, recombinant expression, and transgenic animals. The term "specifically binds" refers to the antibody reacting or associated more frequently, more rapidly, with greater duration, with greater affinity, or with some combination of the above to an epitope or protein than with alternative substances, including unrelated proteins.

In one or more embodiments, the monoclonal antibody is one that specifically binds to the a surface protein (e.g., E1, E2, Erns) of the described APPV isolate, if present, in the sample. In one or more embodiments, the antibody binds to an epitope having SEQ ID NO:4, or at least 95% sequence identity to SEQ ID NO:4. In one or more embodiments, functional antibody fragments are contemplated as long as they nonetheless bind specifically to the target epitope. This antibody-antigen binding can then be detected using various techniques. For example, in one or more embodiments, the antibody (or fragment) can be conjugated with a detectable label. Exemplary labels include fluorophores, biotin, radioisotopes, enzymes, and the like. Various techniques are available for detecting the antibody-antigen binding, depending upon the label used. Non-limiting examples include enzyme-linked immunosorbent assay (ELISA), immunofluorescence, immunohistochemistry, immunochromatography, flow cytometry, immunoprecipitation, and Western blot.

Additional advantages of the various embodiments of the invention will be apparent to those skilled in the art upon review of the disclosure herein and the working examples below. It will be appreciated that the various embodiments described herein are not necessarily mutually exclusive unless otherwise indicated herein. For example, a feature described or depicted in one embodiment may also be included in other embodiments, but is not necessarily included. Thus, the present invention encompasses a variety of combinations and/or integrations of the specific embodiments described herein.

As used herein, the phrase "and/or," when used in a list of two or more items, means that any one of the listed items can be employed by itself or any combination of two or more of the listed items can be employed. For example, if a composition is described as containing or excluding components A, B, and/or C, the composition can contain or exclude A alone; B alone; C alone; A and B in combination; A and C in combination; B and C in combination; or A, B, and

C in combination.

The present description also uses numerical ranges to quantify certain parameters relating to various embodiments of the invention. It should be understood that when numerical ranges are provided, such ranges are to be construed as providing literal support for claim limitations that only recite the lower value of the range as well as claim limitations that only recite the upper value of the range. For example, a disclosed numerical range of about 10 to about 100 provides literal support for a claim reciting "greater than about 10" (with no upper bounds) and a claim reciting "less than about 100" (with no lower bounds).

EXAMPLES

The following examples set forth methods in accordance with the invention. It is to be understood, however, that these examples are provided by way of illustration and nothing therein should be taken as a limitation upon the overall scope of the invention.

EXAMPLE 1

Characterization of new porcine pestivirus

In this study, a novel porcine pestivirus isolate designated as PPeV1 or alternatively as APPV ("atypical porcine pestivirus") was identified and characterized.

Materials and Methods:

Ethics Statement. Porcine serum samples used in this study were submitted to Kansas State Veterinary Diagnostic Laboratory (KSVDL), Iowa State University Veterinary Diagnostic Laboratory or the South Dakota State University Animal Disease Research and Diagnostic Laboratory for routine diagnostic testing. The samples were obtained from naturally infected animals in the field by licensed veterinarians as a part of normal veterinary care and diagnostic investigations.

Collection of Samples. Metagenomic sequencing was performed on 182 swine serum samples that were quantitative real time reverse transcription PCR (qRT-PCR) positive for PRRSV. The serum samples were submitted to KSVDL, Iowa State University Veterinary Diagnostic Laboratory or the South Dakota State University Animal Disease Research and Diagnostic Laboratory for PRRSV qRT-PCR. The samples originated from thirteen states (Iowa [n=35], Minnesota [n=39], South Dakota [n=2], Texas [n=9], North Carolina [n=18], Nebraska [n=14], Kansas [n=22], Oklahoma [n=1], Illinois [n=2], Indiana [n=1], Missouri [n=1], Arizona [n=2], and Colorado [n=4]), Mexico (n=4) and unknown (n=28). A collection of 292 PRRSV-

negative swine serum samples submitted to KSVDL for diagnostic testing were screened for the presence of porcine pestivirus 1 (PPeV1) using a qRT-PCR assay targeting the E2 region of the genome.

Metagenomic Sequencing. Sample preparation for metagenomic sequencing was performed similar to previously described (Hause BM, Collin EA, Anderson J, Hesse RA, Anderson G. 2015. Bovine rhinitis viruses are common in U.S. cattle with bovine respiratory disease. PLOS One 10:e0121998). Serum samples were treated with a cocktail of nucleases to degrade host or unprotected environmental nucleic acids at 37°C for 90 minutes. Viral nucleic acids were isolated using the MinElute Virus spin filter kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed using primers consisting of a known 20 nt sequence followed by a random hexamer at the 3' end using a Superscript III reverse transcription kit (Life Technologies, Grand Island, NY). Second strand synthesis was performed using Sequenase 2.0 (Affymetrix, Santa Clara, CA). Double stranded cDNA was purified using a Qiagen Minelute PCR spin column and subsequently amplified using primers identical to the ones used for reverse transcription but lacking the random hexamer. Amplicons were purified using a Qiagen Minelute PCR spin column and quantified using a Qubit fluorimeter (Life Technologies, Grand Island, NY). Sequencing libraries were prepared using the Nextera XT library preparation kit (Illumina, San Diego, CA) according to manufacturer's instructions. Pooled barcoded libraries were sequenced on an Illumina MiSeq instrument using paired 150bp reads.

Reads from each sequencing library were parsed into individual folders based on barcoded sequences. Reads were imported into the CLC Genomics software package (Qiagen, Valencia, CA). Reads were mapped to the host genome (*Sus scrofa*) and unmapped reads were collected. *De novo* assembly was performed on unmapped reads and assembled contigs were analyzed by BLASTN. Sequences were aligned using ClustalW and phylogenetic analyses were performed by using Mega6.06 software using the Maximum Likelihood algorithm with tree topology verified by performing 1000 bootstrap replicates.

Molecular screening for porcine pestivirus 1

A 5'-nuclease reverse transcription PCR assay was designed to detect PPeV1 targeting the E2 region of the genome: probe, 5'-FAM-TTT AGA CAC GAC CCC TCA GCC C-Iowa Black-3' (SEQ ID NO:5); Forward: 5'-CCA CTT GCC CAT TAT AGA CCG -3'(SEQ ID NO:6); Reverse: 5'-TTA TGG TGC CTG TTA CTG TCT G-3'(SEQ ID NO:7). A second 5'-

nuclease reverse transcription PCR assay targeting the Erns region of the genome was also designed: probe, 5'-FAM-ACC TCG TCT CTG GCC TGT CTC A -Iowa Black-3'(SEQ ID NO:8); Forward, 5'-AGT GTG CTG TCA TCT GTC G -3'(SEQ ID NO:9); Reverse, 5'-CTT CCT TAC ACC CTG TCA GTG -3'(SEQ ID NO:10). Viral RNA was extracted using the MagMAX-96 viral RNA isolation kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Quantitative real time reverse transcription PCR (qRT-PCR) was performed using Qiagen Quantitect RT-PCR with E2 or Erns primers and probe as follows: 50°C, 30 minutes; 95°C, 15 minutes; followed by 40 cycles of 94°C for 15 seconds and 60°C for 60 seconds. The PCR assay specificity was confirmed using PPeV1 positive samples as determined by metagenomic sequencing as well as with cultures of common swine respiratory viruses, influenza A virus and PRRSV and two different pestivirus species, BVDV-1 and BVDV-2.

Viral Isolation

Virus isolation was attempted on various primate, bovine, swine and canine cell lines. They included primate MARC-145, Vero, Vero 76, and human rectal tumor cells (HCT-8). Bovine cells included bovine turbinates (BT) and Madin-Darby bovine kidney (MDBK) cells. Swine cell lines included swine testicle cells (ST) and porcine kidney cells (PK-15). Virus isolation was also attempted on Madin-Darby canine kidney cells (MDCK). All cell lines were maintained in minimal essential media (MEM) supplemented with L-glutamine and 5% fetal bovine sera. Cell culture fluids were removed from the 12-well plates (>80% confluency) and 25-100uL of sample (depending on available sample volume) was inoculated into 1mL of viral replacement media, which consisted of MEM and penicillin-streptomycin solution. Plates were incubated 5 days before being frozen, thawed, and passaged as above to fresh monolayers. Cells were observed daily for cytopathic effects and PPeV1 growth was monitored by qRT-PCR.

Serology

The PPeV1 Erns regions (fragment A: 20-120aa; fragment B: 45-150aa) were amplified from genomic RNA by RT-PCR and the PCR products were cloned into the pET-28a (+) vector (Novagen, Madison, WI), which contains a His-tag at its N-terminus for facilitating downstream protein purification. The resulting plasmids, pET28-Erns-A and pET28-Erns-B, were transformed into *E. coli* BL21 (DE3) cells. Transformed cells were cultured in 2× yeast extract tryptone (YT) medium and the protein expression was induced using isopropyl β-d-1-

thiogalactopyranoside (IPTG). The recombinant proteins were extracted using B-PER reagent (Pierce, Rockford, IL), and further purified by the Ni-NTA agarose (Qiagen, Valencia, CA) following the manufacturer's instruction. The purified recombinant proteins Erns-A and Erns-B were suspended in 1× phosphate buffered saline (PBS), aliquoted and frozen at −80 °C.

Erns-A and Erns-B purity were assessed by polyacrylamide gel electrophoresis using denaturing conditions and Western blotting using an antibody directed against the His-tag. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by staining with Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA). To confirm the expression of target proteins, Western blotting assay was performed. The proteins in the SDS-PAGE gel were electro-transferred onto a nitrocellulose membrane (Whatman, Piscataway, NJ). The membrane was then incubated for 2 hour at room temperature with anti-Histidine monoclonal antibody (mAb; Novagen, Madison, WI). After a 3× wash with phosphate buffered saline containing 0.05% Tween 20 (PBST), IRDye 800CW-conjugated goat anti-mouse antibody (LI-COR Biosciences, Lincoln, NE) was added and the membrane was incubated at room temperature for 2 h. The Image of the membrane was finally taken under the appropriate excitation wavelength using a digital imaging system (Odyssey infrared imaging system; LI-COR Biosciences).

ELISA assays were performed similar to previous studies on CSFV. Immulon 2HB plates were coated with 100 µL of 0.1 M sodium carbonate buffer (pH 9.6) containing 2µg/mL of each recombinant Erns fragment at room temperature for 20 hours. The coating solution was removed and plates were frozen at -20°C until use. Plates were thawed and washed with PBST before being blocked 1 hour at 37°C with 100 µL/well Starting Block (Thermo Fisher Scientific, Waltham, MA). Plates were washed three times with PBST and then 100 µL of 1:100 diluted serum samples (diluted in PBST) were applied to single wells. Plates were incubated at 37°C for 1 hour before being washed three times with PBST. Horse radish peroxidase-labeled goat anti-swine IgG was diluted 1:2,000 in PBST and 100 µL was added to each well and incubated at 37°C for 1 hour. Plates were washed three times with PBST and developed with a commercial peroxidase colorimetric assay kit (ABTS ELISA HRP substrate, KPL, Gaithersburg, MD) at room temperature for 10 minutes before the reaction was stopped with stop solution. The absorbance at 405 nm was measured with a plate reader. Mean optical density values per group were analyzed using ANOVA and Tukey-Kramer honest significant difference tests as implemented in JMP software (SAS, Cary, NC). Samples were analyzed concurrently on the same day.

Nucleotide Accession Numbers. The genome sequence of PPeV1 was submitted to GenBank under accession number KR011347, incorporated by reference herein.

Results

Identification of a novel porcine pestivirus. Metagenomic sequencing was performed on a sample that was qRT-PCR positive for PRRSV as part of a PRRSV metagenomic sequencing project. Following subtraction of reads mapping to the host *Sus scrofa*, reads were assembled *de novo* into 2,167 contigs and analyzed by BLASTN. The largest contig, 1,343 base pairs (bp), was most similar to CSFV (expectation value, E=4.2x10^{-11}). Four additional contigs were identified which were most similar to pestiviruses (E=2.73x10^{-48} to BVD; E=9.9x10^{-8}, 4.04x10^{-87} and 1.50x10^{-60} to *Rhinolophus affininis* pestivirus 1 [RaPV1]). Resequencing of the sequencing library coupled with PCR targeting gaps in the assembly resulted in an 11,276 bp contig encoding a predicted 3,635 amino acid (a.a.) polyprotein with an overall 68% identity to the partial polyprotein sequence of RaPV1 and approximately 40% identity to complete polyprotein amino acid sequences of BVDV, BDV and CSFV (Table 1).

Table 1. BLASTP analysis of the twelve putative mature proteins and the polyprotein of atypical porcine pestivirus (APPV)

Region	Size (amino acids)	Best BLAST Hit (accession number)	Query Coverage (%)	*E* value	Identity (%)
Npro	180	none	none	none	none
C	111	BVDV (NP_776260)	89	1e^{-5}	37
Erns	210	CSFV (AFE56244)	97	2e^{-43}	43
E1	199	BVDV (ACV83744)	98	1e^{-27}	32
E2	241	RaPV (AFK85014)	100	4e^{-87}	54
P7	64	RaPV (AFK85014)	100	2e^{-19}	67
NS2	314	RaPV (AFK85014)	100	4e^{-125}	60
NS3	687	RaPV (AFK85014)	100	0	74
NS4a	67	RaPV (AFK85014)	100	1e^{-20}	61
NS4b	339	RaPV (AFK85014)	76	4e^{-110}	76
NS5a	472	BDV (AHM88396)	45	2e^{-6}	26

NS5b	751	CSFV (AAT85641)	89	0	50
Polyprotein	3635	RaPV (AFK85014)	46	0	68

Notably, this polyprotein is greater than 250 residues smaller than other pestiviruses. The results suggest that this virus represents a novel member of the Pestivirus genus and was provisionally named porcine pestivirus 1 (PPeV1). Putative PPeV1 mature protein sequences were identified by alignment with reference pestivirus genomes and known cleavage sites as determined for CSFV strain Alfort.

Genome Characterization

Untranslated Regions

The 5'-untranslated region (UTR) determined encompassed 123 bp which is considerably shorter than those for other pestiviruses (~370-498 bp). Attempts to verify the termini sequences by random amplification of cDNA ends (RACE) failed, likely due to insufficient virus titer (Ct~30). BLASTN analysis of the 5'-UTR failed to identify any significant (E<1x10^{-5}) hits. The 5'-UTR of the genome is the most highly conserved region of pestiviruses and is frequently the target for molecular detection and taxonomical classification. With the exception of the newly described NRPV, all pestiviruses share >70% nt identity in the 5'UTR. The PPeV1 5'-UTR has less than 20% nt identity to all known pestiviruses. Likewise, the 3'UTR failed to show any similarity to known sequences in Genbank. The 245 bp of sequence determined for the 3'UTR is consistent with the length of other pestivirus 3'UTR's (~200-500 bp) but further experimentation is needed to demonstrate its completeness.

Npro

The Npro protein is unique to the genus Pestivirus and is a non-structural autoprotease. Npro catalyzes self-cleavage from the polyprotein between Cys168 and Ser169. The predicted Npro protein of PPeV1 is 180 amino acids, slightly larger than the typical 168 amino acids of most pestiviruses with the exception of NRPV (273 a.a.), and contains a Cys180-Ser181 cleavage motif. The conserved Npro catalytic site consisting of Glu22, His49 and Cys69 were identified in PPeV1 at Glu 20, His69 and Cys89 by pairwise alignment with known pestiviruses. Despite conservation of the catalytic and cleavage sites, the Npro protein sequence had no significant similarity to any known proteins by BLASTP analysis.

Core

Pestiviruses encode a small, basic core protein (C) which possesses RNA chaperone activity and plays a role in RNA packaging into virions. At 113 a.a. in length, the C protein of PPeV1 is slightly larger than other pestivirus C proteins (97-102 a.a.) but has a predicted isoelectric point of 10.4, similar to other pestiviruses. The PPeV1 C protein has ~37% identity to those of BVDV, BDV, CSFV and BWV.

Envelope Proteins

Pestiviruses encode three envelope glycoproteins, E1, E2 and Erns. The Erns protein, found only in pestiviruses, is the only known viral structural protein with a ribonuclease T2 domain with uridinylate specificity. The ribonuclease T2 domain was identified in the PPeV1 Erns region of the genome from a.a. 319-373 using the National Center for Biotechnology Information (NCBI) Conserved Domain Database. The PPeV1 Erns length was slightly shorter than other pestiviruses (208 a.a. and 214-227 a.a., respectively) and has 39-42% identity to BVDV, BDV and CSFV.

E1 and E2 form heterodimers on the virus surface that are crucial for viral entry into cells. The E2 protein is also immunodominant and possesses neutralization epitopes and consequently is the pestivirus protein that exhibits the greatest amount of diversity. Both proteins displayed 29-33% identity to BVD and CSFV however E2 was 54% identical to the recently described RaPV1. It should be noted that only 5.1kb of the RaPV1 genome is known, encompassing the E2, P7, NS2, NS3 and NS4a regions of the genome. Interestingly, while the length of the E1 proteins were similar for all pestiviruses (195-215 a.a.), the E2 proteins for PPeV1 and RaPV1 were significantly shorter (241 and 244 a.a., respectively) than all other pestiviruses (373-378 a.a.). The deletion accounting for the smaller size of the PPeV1 E2 protein was located at the N-terminus.

Nonstructural Proteins

NS2 is a cysteine autoprotease responsible for cleavage of NS2 from NS3. The length of PPeV1 NSP2 (315 a.a.) was similar to that of RaPV1 NSP2 (318 a.a.) and significantly shorter than NSP2 from other pestiviruses (455-543 a.a.). A search of PPeV1 NSP2 failed to identify conserved protease domains. BLASTP analysis of the PPeV1 NSP2 found significant similarity only to RaPV1 NSP2 (60% identity). The active site of NSP2 has a His1447 and Cys1512 and is thought to include a glutamate residue between 1447-1512. Multi-sequence alignment identified

a conserved His1237 and Glu1253 however the conserved cysteine residue of the catalytic triad could not be identified. One candidate cysteine residue at position 1280 was identified. NS3 is a chymotrypsin-like serine protease catalyzing both *cis* and *trans*-cleavage. The PPeV1 NS3 protein shared relatively high identity to the NS3 of RaPV1 (74% identity) and 45-47% identity to other pestiviruses. Conserved domains identified include a DEAD-like helicase from 1547-1672 and a pestivirus peptidase S31 domain from 1320-1530. NS4a, NS4b, NS5a and NS5b are all involved with pestivirus replication. NS4a and NS4b were 61% and 76% identical to RaPV1 NS4a and NS4b, respectively, and 37-43% identical to BDV, BVDV and CSFV. NS5a shared only 24-28% identity to other pestiviruses. NS5b codes for the RNA dependent RNA polymerase (RdRp) and is one of the most conserved pestivirus proteins. NS5b from PPeV1 was 50% identical to CSFV and a RdRp domain was identified from 3153-3438. The P7 protein is a small hydrophobic peptide 61-72 a.a. in length that functions as a viroporin that is essential for virus production *in vitro* and virulence *in vivo*. The P7 of PPeV1 was 67% identical to the P7 of RaPV1 and lacked significant similarity to any other proteins in GenBank.

Phylogenetic Analysis

Phylogenetic trees constructed using amino acid sequences of predicted pestivirus nonstructural and structural proteins are shown in Figs. 1A-1C. Maximum-likelihood analysis in combination with 500 bootstrap replicates as implemented in MEGA 6.06 was used to derive trees based on the predicted protein sequences. A scale representing the number of amino acid changes is shown in each Figure. Abbreviations are as follows with GenBank accession numbers in parentheses: BDV, border disease virus (NC003679); RPV, reindeer pestivirus (AAF02524); CSFV, classical swine fever virus (NC002657); GPV, giraffe pestivirus (NP620053); BVDV, bovine viral diarrhea virus (NC001461); Hobi, HoBi virus (BAO04453); APV, antelope pestivirus (NC024018); Bungo, Bungowannah virus (NC023176); NRPV, Norway rat pestivirus (NC025677); RaPV, *Rhinolophus affinis* pestivirus 1 (JQ814854); PPV1, atypical porcine pestivirus (KR011347). APPV/PPeV1 and RaPV1 formed a distinct cluster for all proteins where RaPV1 sequence is available with the exception of P7. The PPV1/RaPV1 cluster represented a highly divergent lineage of pestiviruses that evolved from an ancestral pestivirus.

Virus Isolation

Virus isolation was attempted on MARC-145, Vero, Vero 76, HCT-8, BT, MDBK, ST, PK15 and MDCK cell lines. No CPE was evident in any cell line. Viral titers were monitored

by the E2 RT-PCR. Following two passages on cells all samples were qRT-PCR negative.

Molecular Epidemiology

As part of a project investigating PRRSV genetic diversity using metagenomic sequencing of swine serum samples, a total of 182 samples were sequenced using viral metagenomic methodology. Templated assembly was performed on these 182 samples using the PPeV1 genome. Reads mapping to the PPeV1 genome were identified in five samples. To confirm these results, two Taqman qRT-PCR assays were designed, targeting either the E2 or Erns region of the genome. The original sample, #146, was positive for both assays with cycle threshold (Ct) values of 25.1 and 30.5 (1:10 dilution of RNA), respectively. Sample #208 was only tested on the E2 qRT-PCR assay due to insufficient sample and was positive with Ct=19.3. Samples #51 and #98 were positive on both assays with Ct=33.2 and 36.2 for the E2 assay and Ct=33.9 and 34.1 for the Erns assay, respectively. Sample #28 was negative on the E2 assay but was positive with a Ct=32.3 for the Erns assay. All samples were positive for PRRSV as determined using a commercial PRRSV qRT-PCR assay and metagenomic sequencing. We also screened a collection of 292 PRRSV qRT-PCR negative serum samples. These samples were submitted to KSVDL for unrelated diagnostic testing. Using the E2 qRT-PCR assay all samples were negative.

For samples #208 and #28, there was sufficient read coverage for the E2 region of the genome to assemble complete and partial E2 sequences, respectively. The E2 sequence for #208 was identical to #146. For sample #28, a 197 a.a. portion of the E2 protein was assembled that was 88% identical to sample #146. The five positive samples were collected in 2014 from Nebraska, Arizona, North Carolina, Minnesota and Kansas, suggesting widespread distribution of PPeV1 in the U.S. swine herd. The finding of only 88% a.a. identity for the partial E2 sequence of sample #28 also suggests significant genetic diversity is present in PPeV1 and is likely the reason for the failure of the E2 assay to detect sample #28.

Serology

The purity of the two Erns fragments were assessed by protein electrophoresis and Western blotting (Fig. 2). Large bands approximately 20 kDa were observed (as expected) for both fragments and only very faint additional bands were evident. A collection of 78 PRRSV qRT-PCR positive serum samples collected from multiple states, 90 PRRSV antibody-positive samples collected from a single site, 30 and 48 PRRSV antibody-negative serum samples

collected from individuals at two production sites and 15 specific pathogen free pigs were assayed on the PPeV1 Erns ELISA. Mean group optical density (O.D.) values ranged from 0.48-1.25 (Table 2).

Table 2. Detection of antibodies cross reactive to atypical porcine pestivirus Erns peptides produced in *E. coli*. PRRSV qRT-PCR positive samples were collected from multiple sites representing at least seven states. Sera in other groups were collected from a single site.

Serum Samples	n[1]	Mean O.D.[2]	Standard Deviation (O.D)	Positive Samples[3] (%)
PRRSV qRT-PCR Positive	78	1.25[A]	0.33	73/78 (94)
Farm 1	90	1.06[B]	0.15	90/90 (100)
Farm 2	48	0.96[C]	0.12	46/48 (96)
Farm 3	30	0.57[D]	0.06	0/30 (0)
Specific Pathogen Free Pigs	15	0.48[D]	0.08	0/15 (0)

[1]Number of serum samples

[2]Optical Density (O.D.). Groups not connected by the same letter are significantly different ($P<0.05$)

[3]A cutoff of O.D.>0.72 was used to determine positivity

All group mean O.D. values were significantly different from one another with the exception of the PRRSV antibody negative farm 2 and the negative control SPF pig group ($P<0.05$). The value of the mean negative control plus three standard deviations has been used to determine the negative cutoff for ELISA assays without well-defined positive controls. Applying this formula to the negative controls yielded a cutoff value O.D.>0.72. For the PRRSV qRT-PCR sample set, 73/78 (94%) of samples are positive. For samples from a single farm, 90/90 (100%) pigs from the PRRSV antibody positive and 46/48 (96%) and 0/30 (0%) of the pigs in the two PRRSV antibody-negative farms were positive.

Discussion

Pestiviruses are economically one of the most significant genera of viruses affecting livestock. Prior to eradication from the U.S. in 1978, classical swine fever plagued swine production since its identification in the 1830's. Currently, CSFV is widespread in Central and South America, the Caribbean, Asia and Eastern Europe. Likewise, BVDV, first reported in the

1940's, is generally regarded as one of the most economically significant diseases affecting cattle with near worldwide distribution. The other major pestivirus, BDV, mainly affects sheep and is also widely distributed. CSFV, BVDV and BDV are closely related and interspecies transmission and infection have been well documented. With the exception of descriptions of pestivirus genotypic variants in other species such as pronghorn antelope pestivirus and giraffe pestivirus, our knowledge of the breadth of pestivirus diversity has been limited until recent times. An outbreak of stillbirths and sudden deaths in swine in Australia, 2003, was attributed to a novel, divergent pestivirus, BWV, which was proposed to represent a new species. More recently with the advent of next generation sequencing technology, yet more divergent pestiviruses were identified in rats and bats (NRPV and RaPV1, respectively), expanding the diversity and host range of pestiviruses. While little is known on the pathogenic potential of the rat and bat pestiviruses, we have expanded our knowledge of the ecology of pestiviruses. In this study we report the identification and characterization of the complete polyprotein sequence of the most divergent pestivirus described to date and importantly demonstrate its widespread circulation in the U.S. swine herd.

PPeV1 was identified in five swine serum samples that were part of a set of 182 (2.7%) that were analyzed by metagenomic sequencing aimed at elucidating complete PRRSV genomes directly from clinical samples. While clinical histories are unknown, all serum samples were submitted to Veterinary Diagnostic Laboratories for PRRSV qRT-PCR. Unexpectedly, PPeV1 qRT-PCR assays failed to detect PPeV1 in a collection of 292 PRRS negative sera. These results suggest that PPeV1 is more common in pigs infected with PRRSV. PRRSV establishes persistent infections in pigs and results in immunosuppression. Host immunosuppression and persistent infections are also hallmarks of pestivirus infections and similar viral strategies are employed by PRRSV and pestiviruses to evade host immunity including induction of tolerance by *in utero* infection, innate immunity antagonism and a high mutation rate. Similarly, recent work demonstrated an association between PRRSV infection and reverse zoonotic influenza B virus infection in pigs. Further research is needed to determine if there is synergy between PPeV1 and PRRSV infections.

We were unable to isolate PPeV1 on any cell line assayed despite attempting virus isolation on the qRT-PCR positive samples, preventing us from performing serum neutralization or indirect immunofluorescence assays to gauge seroprevalence. To expand upon the results from our molecular testing, we expressed two Erns peptides in *E. coli* and assayed PRRSV qRT-PCR positive sera collected from at least seven states as well as sera from one PRRSV antibody

positive and two PRRSV antibody negative farms. In contrast to our molecular assays, results suggest that PPeV1 infections can occur without PRRSV co-infections. Greater than 90% of serum samples collected from PRRSV qRT-PCR diagnostic sample submissions and from the PRRSV antibody positive and one PRRSV antibody negative farm were positive for antibodies that cross react with PPeV1 Erns. All pigs in the second PRRSV antibody negative farm were negative on the ELISA. This result suggests that the assay is specifically measuring antibodies cross reactivity with Erns and not measuring non-specific interactions. Not unexpectedly, a wide range of O.D. values were observed for the diverse collection of PRRSV qRT-PCR positive serum while much lower variability was observed in samples collected from a single site. These results suggest that PPeV1 infections are common in pigs and that the association with PRRSV co-infection suggested by molecular assay results is unclear. Similar high seroprevalence rates have been observed in cattle to influenza D virus with similar low qRT-PCR positivity rates. These serological results require substantiation by additional assays including control samples with known serological status.

The Npro protein is found only in the genus Pestivirus. Besides autocatalytic activity, Npro subverts cellular antiviral responses through degradation of IRF3 to prevent apoptosis and interferon production and also plays a role in viral RNA translation. While the protease catalytic residues and autoprotease cleavage sites were conserved, overall the Npro protein had no significant similarity to known proteins. Further experimentation is required to determine if the PPeV1 Npro can antagonize porcine cellular antiviral defenses. The Erns protein also acts to inhibit interferon production by degradation of viral double stranded RNA produced during viral replication. As the T2 RNase superfamily domain was identified in Erns, it appears likely that PPeV1 degrades viral dsRNA to prevent a cellular interferon response despite its relatively low ~40% a.a. identity to pestivirus Erns proteins.

Two PPeV1 proteins, E2 and NSP2, showed substantial deletions of over 100 a.a. as compared to other pestiviruses with the exception of RaPV1. The cell tropism of pestiviruses is determined by the E2 protein which binds to its receptor CD46. With the exception of NRPV1 and RaPV1, where virus has not been isolated, all pestiviruses are readily cultured in cells *in vitro*. Despite inclusion of multiple cells lines generally permissive to pestiviruses, we were unable to propagate PPeV1 *in vitro*. Further experimentation is needed to determine if the substantial reduction in E2 size and only ~30% identity to non-RaPV1 pestiviruses E2 proteins has resulted in a change in receptor utilization.

The finding of a divergent pestivirus that is widely distributed in pigs in the U.S. raises

numerous questions. Is PPeV1 a newly emerged swine virus or has it circulated unrecognized for some time? Does PPeV1 infection of swine result in pathology and what clinical symptoms, if any, result? The evolutionary relationship to RaPV1 also draws into question our understanding of the ecology, host range and natural reservoirs of pestiviruses, warranting further investigation.

EXAMPLE 2

Investigation of pestivirus as causative agent for pig intention tremors

Introduction

In this study, two field cases involving late-onset of intention tremors and mortality were investigated. The samples tested positive for APPV. The first case was from pigs exhibiting intention tremors, resulted in the death of nearly 700 pigs at age of 5-14 weeks from swine farms in North Carolina. The second field case with similar clinical symptoms in 10-16 weeks old pigs occurred in the same production system later that same year. Metagenomic sequencing of brain homogenate from the first field case identified the sample as APPV positive, and the result was further confirmed by quantitative real-time RT-PCR (qRT-PCR). Sera, lymph node, liver and spleen samples also identified as APPV positive by qRT-PCR. The existence of viral antigen (Erns protein) in tissue samples was further confirmed by immunohistochemistry (IHC) using an APPV Erns protein-specific monoclonal antibody. Lymph node samples from both field cases were identified to be positive for APPV by qRT-PCR, IHC and immunofluorescent assay. The data indicate that the novel pestivirus (APPV or APPV-like virus) could be a causal agent of neurological diseases in various aged pigs. The diagnostic reagents and assays developed in this study will be important tools for future control of APPV infection on swine farms.

Materials and Methods

RNA extraction and RT-PCR analysis. Viral RNA from clinical samples were extracted by using the MagMAX™-96 viral RNA isolation kit (Life Technologies) according to the manufacturer's instruction. Quantitative real-time (qRT-PCR) targeted the Erns gene of the virus was performed as previously described (Hause 2015).

Metagenomic sequencing. Pig brain tissues were homogenized in phosphate buffered saline (PBS) and clarified by centrifugation at 6,000 x g for 10 minutes. An aliquot of the supernatant was treated with nucleases at 37°C for 90 minutes to degrade unprotected nucleic acids as previously described (Hause 2015). Viral nucleic acids were extracted using the

MinElute Virus spin filter kit (Qiagen) according to the manufacturer's instruction. First-strand cDNA synthesis from viral RNA was performed using the Superscript III first-strand synthesis kit (Invitrogen) using previously described primers (Allander et al., 2005). Sequenase 2.0 DNA polymerase (Affymetrix) was used for second strand synthesis, followed by cDNA purification using the Agencourt AMPure XP beads (Agencourt Bioscience). The double-stranded cDNA was amplified with TaKaRa DNA polymerase (Clontech) using previously described primers (Allander et al 2005). Amplicons greater than 300 bp were purified using Agentcourt AMPure XP beads (Agencourt Bioscience). Amplicons were quantified using a Qubit fluorimeter (Thermofisher) and the Nextera XT library preparation kit (Illumina) was used to prepare sequencing libraries. Pooled libraries were sequenced on an Illumina Miseq instrument using paired 150 bp read chemistry.

Monoclonal antibody production for APPV Erns protein. The APPV Erns-specific monoclonal antibody (mAb) was produced by immunizing BALB/c mice with truncated Erns recombinant protein. The synthetic gene encoding amino acids 20-150 of Erns was cloned into pET-28a vector (Novagen) and recombinant proteins were expressed and purified using the method as we described previously (Brown et al., 2009). BALB/c mice were immunized with 50 ug Erns antigen mixed with Freund's incomplete adjuvant at 2-week intervals for 8 weeks. Mouse splenocytes were fused with NS-1 myeloma cells. Specific anti-Erns mAbs were screened by IFA using MARC-145 cells transfected with the plasmid p3xFLAG-Erns. Hybridomas secreting Erns-specific mAb were subcloned. The clone 96-11 generated the highest antibody titer on IFA was selected for subsequent experiments.

Indirect immunofluorescent assay (IFA). Since there was no APPV isolate available, IFA was developed using MARC-145 cells transfected with the plasmid p3xFLAG-Erns expressing FLAG-tagged APPV Erns protein. The plasmid p3xFLAG-Erns was constructed by cloning the synthesized APPV Erns gene into the pCMV24-3Flag vector (Sigma-Aldrich). Transfection on MARC-145 cells was performed using HD-FuGENE 6 transfection reagent followed the manufacturer's instruction (Roche Molecular Biochemicals). At 48 hours post transfection, cells were fixed in 4% paraformaldehyde and stained with primary mAb 96-11. After 1hour incubation at 37° C, cells were washed and then incubated with fluorescein isothiocyanate (FITC) labeled goat anti-mouse (KPL) for additional 1 hour. For IFA on lymph node, slide made from lymph node section of formalin-fixed and paraffin-embedded tissue (see below) was stained with primary mAb 96-11, and then incubated with FITC-labeled goat anti-mouse (KPL) as the secondary antibody. Immunostained cells or lymph node section were imaged with an

LSM880 Zeiss confocal microscope (Zeiss). Collected images were processed using Zen 2 and Adobe Photoshop CS3.

Western blot analysis. Cell lysates from transfected or nontransfected control HEK293T cells were lysed in Laemmli sample buffer and samples were heated at 95°C for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline (PBS) containing 5% nonfat dry milk. The membrane was then incubated overnight with primary anti-Erns mAb 96-11 at 4°C in PBS containing 0.1% Tween-20 (PBST). Following incubation with the primary mAb, the membrane was washed and IRDye 800CW-conjugated goat anti-mouse antibody (Li-Cor Biosciences, Lincoln, NE) was added. The blot was incubated for additional 2 hours at room temperature. Imaging of the blot was scanned under the appropriate fluorescent excitation wavelength using a digital imaging system (Odyssey infrared imaging system; Li-Cor Biosciences). Data were analyzed using Odyssey application software version 4.0 (LI-COR Bioscience).

Immunohistochemistry test. Immunohistochemical staining (IHC) was performed using formalin-fixed paraffin-embedded tissues that were sectioned at 4μm thickness onto positively charged slides. Slides were stained using the Leica Bond-Max autostainer with the Polymer Refine Red Detection kit (Leica Biosystems) followed the manufacturer's instruction. The anti-Erns primary antibody (mAb 96-11) was diluted to 1:400 with Bond Primary Antibody Diluent (Leica Biosystems). Heat meditated epitope retrieval was performed using citrate pH 6.0 for 20 minutes at 100°C. Tissue sections were incubated with the primary antibody for 15 minutes at ambient temperature. Polymerization was performed with Polymer-AP α-Rabbit (Leica Biosystems) for 25 minutes at ambient temperature. Specific staining was visualized using Fast Red chromogen and slides were counterstained with hematoxylin.

Results and Discussion

Case histories and clinical symptoms. The first field case was reported from nursery to finishing pigs that were originally from three separate sow farms in North Carolina. Initially, five-week-old pigs in two nursery facilities began to show intention tremor symptoms. It was described as "Parkinson's-like" disease. In addition to intention tremors, pigs displayed increased respiration rate. As tremors progressed more seriously, pigs lost the ability to control their movements, including difficulties to walk, standing up and inability to swallow or close their mouths. Mortality was 100% in those pigs demonstrating tremor symptom within four days

onset of disease. The first nursery lost 273 pigs and the second nursery lost 173 pigs. The remaining pigs were moved to four separate finishing facilities and new tremor cases continued to develop, resulting in additional death of 247 pigs by 14 weeks of age. A second field case of 10-16 weeks old pigs was reported experiencing similar clinical signs of intention tremors. This group of pigs was also originated from the same sow farms in North Carolina. Again, mortality rate was 100% in those pigs experiencing intention tremors.

Molecular detection of APPV RNA in serum and tissues from pigs experiencing intension tremor. Serum and tissue samples from both field cases were submitted to Kansas State Veterinary Diagnostic Laboratory (KSVDL). Initially, the brain homogenate from a 14 week-old pig showing intention tremor symptom (first field case) was analyzed by metagenomic sequencing. Following subtraction of reads mapping to the host *Sus scrofa*, sequences were assembled *de novo* into 159 contigs and analyzed by BLASTN. Two contigs were identified as APPV. Templated assembly using the APPV reference strain (Genbank accession KR011347) mapped 195 reads. Where read coverage was sufficient, consensus sequences were extracted and analyzed by BLASTN and showed approximately 87% identity to APPV.

Subsequently, serum and tissues from the first case of pigs were tested using qRT-PCR. Serum, brain, lymph nodes, liver, spleen tissue homogenates were determined to be positive for APPV [cycle threshold (Ct) 33.1-36.9]. The samples from the second case study were similarly analyzed. The results showed that lymph nodes from one pig was positive for APPV by qRT-PCR (Ct=31.5), while other tissues were negative.

Monoclonal antibody development and antigen detection in tissues from pigs experiencing intension tremor. Tissue samples from the first field case were initially submitted to the diagnostic lab of Iowa State University. No significant gross and histopathologic lesions were detected. When the samples were submitted to KSVDL, we also examined the tissue samples in hematoxylin and eosin slides; again, no significant pathological lesion was observed. To test the possibility of existing APPV antigen in those tissue samples, we created a mAb 96-11 that recognizes the APPV Erns protein. The reactivity of the mAb was first tested in IFA using transfected MARC-145 cells that expresses the full-length APPV Erns. MARC-145 cells were transfected with a plasmid DNA expressing pestivirus Erns protein or mock-transfected (control). At 48 hours post-transfection, cells were fixed and stained with anti-Erns mAb and FITC-conjugated goat anti-mouse was used as the secondary antibody. Cells were then stained with DAPI. As shown in Fig. 3A, specific fluorescent signal was detected in Erns-expressing MARC-145 cells using mAb 96-11, but not in MARC-145 control cells. Lymph node section

from formalin-fixed paraffin-embedded tissues was also analyzed. Tissue sections are shown from samples identified as APPV positive by qRT-PCR ("LN-APPV infection"), and from a healthy pig, identified as APPV negative by qRT-PCR ("LN-control"), as indicated. The tissue section was incubated with the primary anti-Erns mAb and FITC-conjugated goat anti-mouse was used as the secondary antibody. Nuclei were stained with DAPI. As shown in Fig. 3A, a large number of APPV positive cells were detected and APPV Erns protein was localized in the cytoplasm of virus-infected cell.

Specificity of the mAb was further determined in Western blot (Fig. 3B). HEK293T cells were mock-transfected (lane 2) or transfected with a plasmid DNA expressing pestivirus Erns protein (lane 3). At 48 hours post-transfection, cell lysate were analyzed by western blotting with anti-Erns mAb. IRDye 800CW-conjugated (green fluorescence) goat anti-mouse antibody was used as the secondary antibody.

Subsequently, this mAb was used to determine the virus distribution in tissues of APPV-infected pigs from first field case. Immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissues. Tissue sections were from samples identified as APPV positive by qRT-PCR, as compared to tissue sections from a healthy pig, identified as APPV negative by qRT-PCR. Slides were stained using the Leica Bond-Max autostainer with the Polymer Refine Red Detection kit (Leica Biosystems). Tissue sections were incubated with the primary anti-Erns mAb and polymerization was performed with Polymer-AP α-mouse. Colors were developed using Fast Red chromogen and slides were counterstained with hematoxylin. The IHC result showed that the viral antigen Erns was detected positive in the liver, spleen and lymph nodes, but was negative in the brain tissue (Fig. 4). We further used the mAb 96-11 to conduct IFA on tissue samples from the second field case. The result showed only the lymph node sample was positive for APPV Erns antigen among all the tissue samples, including brain, liver, kidney, spleen, intestine, and spinal cord (Fig. 3A, bottom panels).

The study present here is based on two separate outbreaks of disease typified by uncontrollable intention tremors, which led to 100% mortality in pigs shown the symptom. The finding of APPV RNA in the brain of a 14 week-old pig experienced intention tremor suggests that APPV is the causal agent for such disordered neurologic disease. The data suggests that APPV as a viral agent causative of neurological disease in pigs. Our data indicate that APPV distributed in many tissues/organs of pigs, including brain, lymph nodes, liver, and spleen. Although both metagenomic sequencing and real-time RT-PCR detected positive for APPV in brain tissue, IHC was negative for the viral antigen. The negative IHC result is most likely

caused by lower amount of antigens being present in brain tissue, since the lower amount of APPV RNA was detected by qRT-PCR (Ct value of 36.9). Compared to other tissue samples, the lymph nodes were consistently identified as APPV-positive in pigs from both case studies using qRT-PCR, IFA and IHC, which suggests the lymph node may be one of the sites for viral persistence. Furthermore, the results from both antigen detecting tests (IFA and IHC) are consistent with that of qRT-PCR results, indicating these tests and reagent (anti-Erns mAb) are reliable in clinical diagnosis of APPV infection; and they will be important tools for future control of APPV infection on swine farms.

CLAIMS:

1. An immunogenic composition to induce an immune response against porcine pestivirus infection in a pig, said composition comprising a therapeutically-effective amount of atypical porcine pestivirus (APPV) antigenic agents dispersed in a pharmaceutically-acceptable carrier, said APPV antigenic agents being selected from the group consisting of isolated whole virus having an mRNA complementary coding sequence according to Genbank accession no. KR011347.1 (SEQ ID NO:1), derivatives thereof, functional fragments thereof, and combinations of the foregoing.

2. The composition of claim 1, wherein said virus is an APPV isolate comprising a gene according to SEQ ID NO:3 encoding for an Erns protein.

3. The composition of claim 1, wherein said virus is an APPV isolate presenting an Erns epitope according to SEQ ID NO:4, or an epitope having at least 95% sequence identity to SEQ ID NO:4.

4. The composition of claim 1, said composition comprising from about 5% to about 95% by weight of APPV antigenic agents, based upon the total weight of the composition taken as 100% by weight.

5. The composition of claim 1, wherein said isolated whole virus is a live, attenuated virus.

6. The composition of claim 1, wherein said isolated whole virus is an inactivated virus.

7. The composition of claim 1, wherein said isolated whole virus is synthetically generated from said coding sequence.

8. The composition of claim 1, wherein said derivatives are selected from the group consisting of recombinant viral particles comprising said isolated virus, chimeras comprising said isolated virus, and functional fragments of the foregoing derivatives.

9. The composition of claim 1, wherein said functional fragments of said isolated whole virus are selected from the group consisting of virus subunits, purified antigens, surface proteins, recombinant viral proteins, and combinations thereof.

10. The composition of claim 9, wherein said purified antigen or surface protein are selected from the group consisting of Erns, E2, NS3, and combinations thereof..

11. The composition of claim 9, wherein said purified antigen or surface protein is an Erns epitope according to SEQ ID NO:4, or an epitope having at least 95% sequence identity to SEQ ID NO:4.

12. The composition of claim 9, wherein said functional fragments are coupled to a carrier protein.

13. The composition of claim 1, comprising a combination of at least two different APPV antigenic agents.

14. The composition of claim 1, wherein said carrier is selected from the group consisting of normal saline, phosphate buffered saline, sterile water, aqueous dextrose solutions, aqueous glycerol solutions, ethanol, normal allantoic fluid, oil-in-water or water-in-oil emulsions, dimethyl sulfoxide (DMSO), and mixtures thereof.

15. The composition of claim 1, further comprising an adjuvant.

16. The composition of claim 15, wherein said adjuvant is selected from the group consisting of aluminum salts, potassium aluminum sulfate, mixed aluminum salts, peptides, oil or hydrocarbon emulsions, and combinations thereof.

17. The composition of claim 1, further comprising secondary active agents, preservatives, buffering agents, salts, and mixtures thereof.

18. The composition of claim 17, wherein said secondary active agents are selected from the group consisting of antigenic components of a microorganism or infectious agent other than said APPV.

19. A method of vaccinating a pig to induce an immune response against porcine pestivirus infection, said method comprising administering an immunogenic composition according to any one of claims 1-18 to said pig.

20. The method of claim 19, wherein said pig after said administering has reduced incidence or severity of clinical symptoms of porcine pestivirus infection, said clinical symptoms being selected from the group consisting of tremors, increased respiration rate, lack of coordination, and/or inability to swallow or control mouth movements.

21. The method of claim 19, wherein said pig is a pregnant sow, wherein offspring subsequently born by said sow have reduced incidence or severity of clinical symptoms of porcine pestivirus infection in comparison to offspring born by a sow not receiving the immunogenic composition.

22. The method of claim 19, wherein said administering is selected from the group consisting of:

injecting said vaccine composition intramuscularly, subcutaneously, intradermally, or intravenously using a needle and syringe, or a needleless injection device; and

mucosal administration nasally or orally.

23. A kit for inducing an immune response against porcine pestivirus infection in a pig, said kit comprising:

an immunogenic composition according to any one of claims 1-18; and

instructions for administering said composition to said pig.

24. Use of an immunogenic composition according to any one of claims 1-18 for inducing an immune response against porcine pestivirus infection in a pig.

25. A method of detecting pestivirus antigen in a biological sample, said method comprising:

contacting a biological sample with a monoclonal antibody against an atypical porcine pestivirus (APPV), said pestivirus having an mRNA complementary coding sequence according to Genbank accession no. KR011347.1 (SEQ ID NO:1), said monoclonal antibody specifically binding to an antigen of said pestivirus if present in said sample; and

detecting antibody binding to said antigen in said sample.

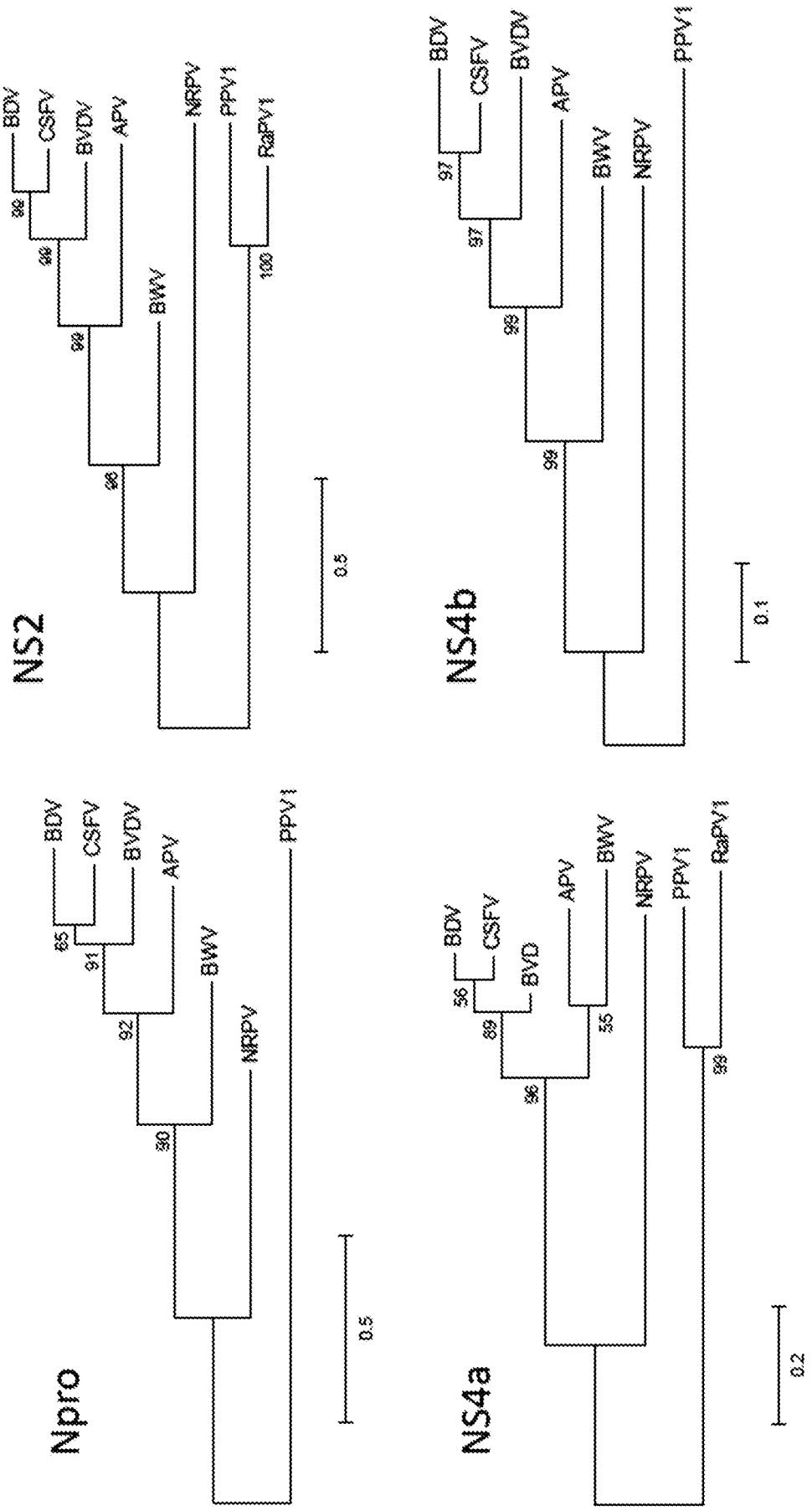
26. The method of claim 25, wherein said antigen is a surface protein of said APPV isolate, if present, in the sample.

27. The method of claim 25, wherein said antibody binds to an epitope having SEQ ID NO:4, or at least 95% sequence identity to SEQ ID NO:4.

28. The method of claim 25, wherein said antibody is conjugated with a detectable label selected from the group consisting of fluorophores, biotin, radioisotopes, and enzymes, wherein said detecting comprises detecting said detectable label in said sample.

29. The method of claim 25, wherein said antibody binding to said antigen is detected via enzyme-linked immunosorbent assay, immunofluorescence, immunohistochemistry, immunochromatography, flow cytometry, immunoprecipitation, and/or Western blot.

30. The method of claim 25, wherein said biological sample is selected from the group consisting of serum, brain, lymph nodes, liver, spleen, intestine, and spinal cord tissue.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CSFV
BVDV
APV
BWV
NRPV
PPV1
RaPV1
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99
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NS4b
BDV
CSFV
BVDV
APV
BWV
NRPV
PPV1
97
97
99
99
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BVDV
APV
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NS4a
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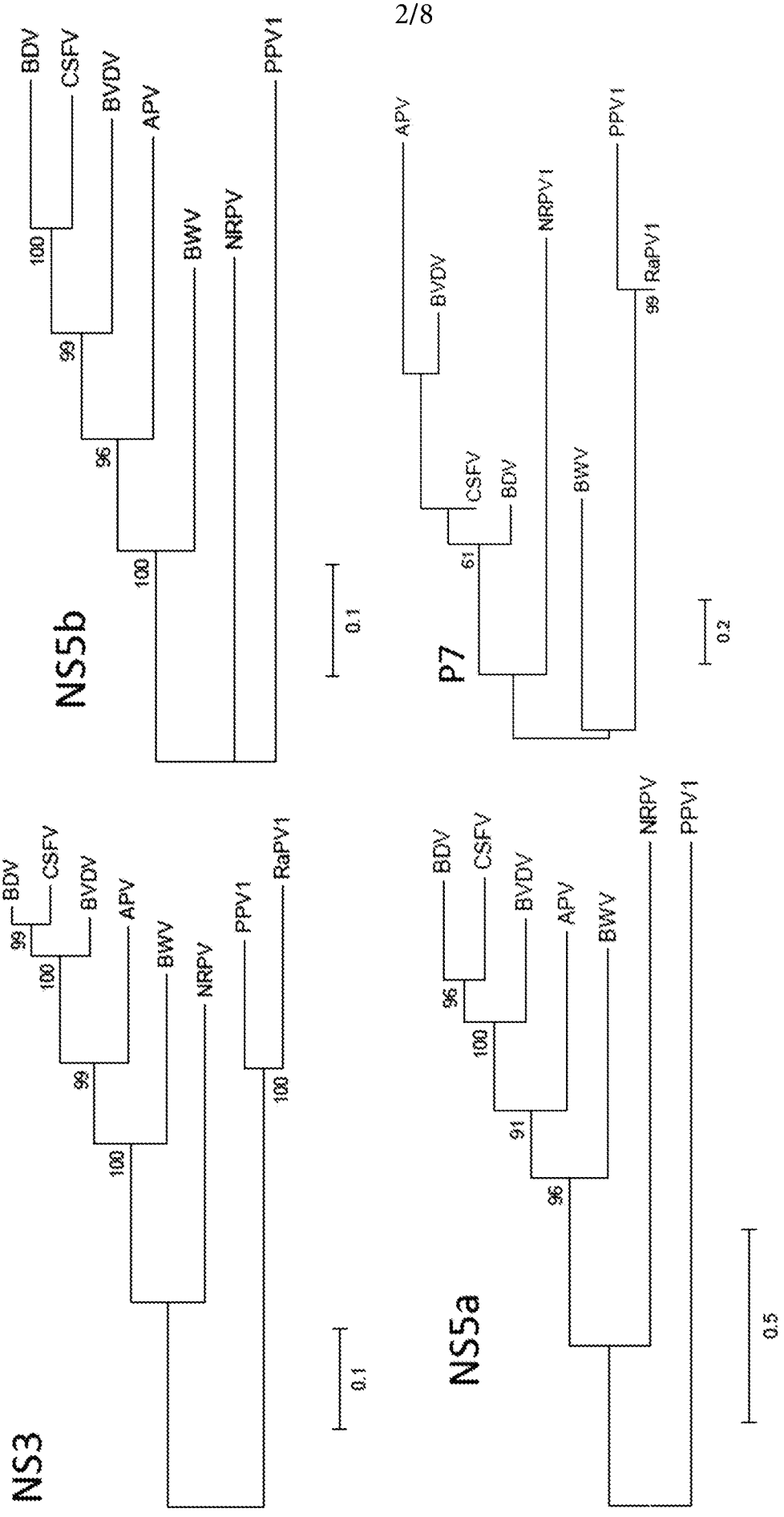
0.2

Fig. 1A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CSFV
BDV
BWV
NRPV
PPV1
RaPV1
61
99
0.2
NS3
BDV
CSFV
BVDV
APV
BWV
NRPV
PPV1
RaPV1
99
100
99
100
100
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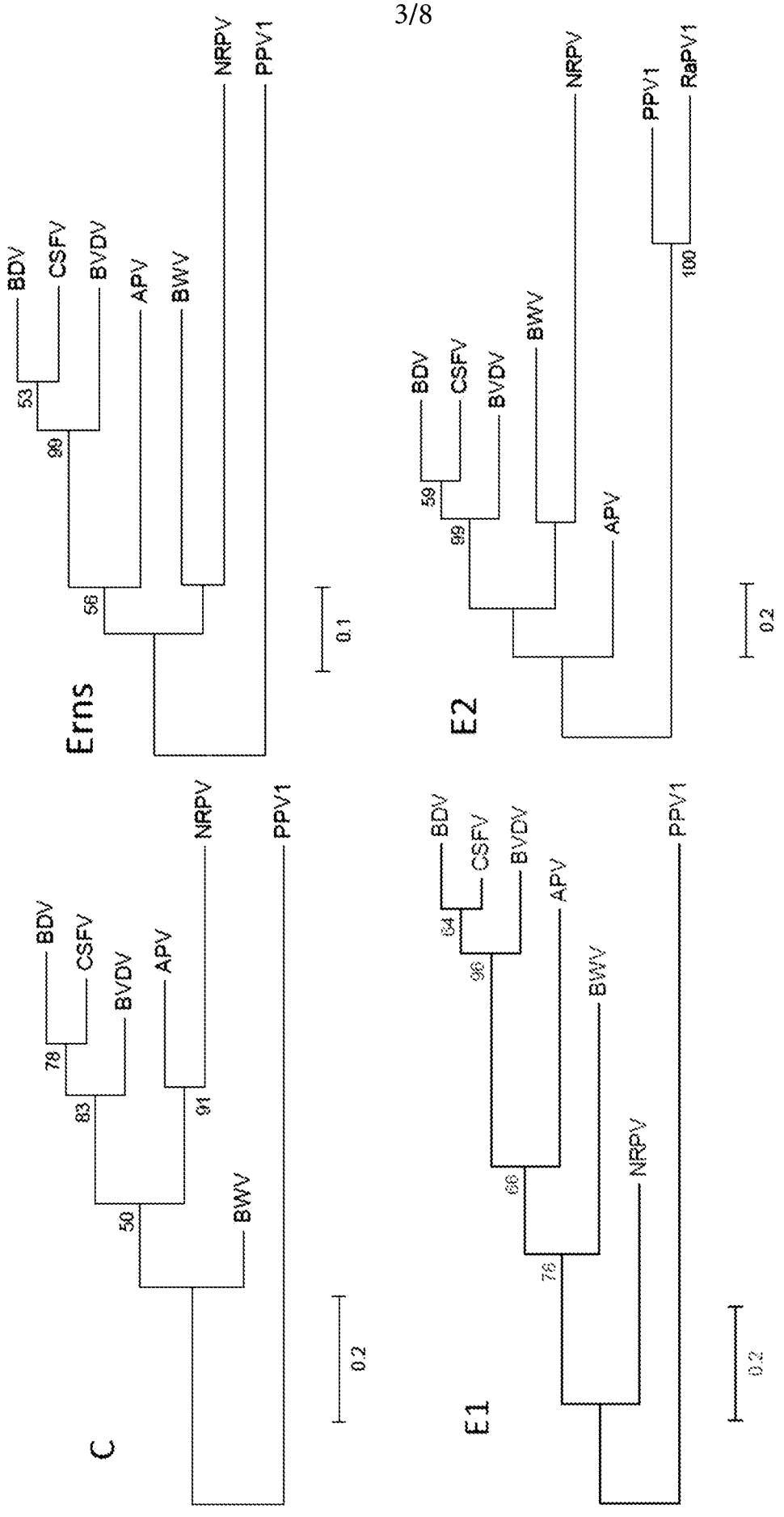
NRPV
PPV1
96
100
91
96
0.5

Fig. 1B


Erns
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CSFV
BVDV
APV
BWV
NRPV
PPV1
53
99
56
0.1
E2
BDV
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BVDV
BWV
NRPV
PPV1
RaPV1
59
99
100
0.2
C
BDV
CSFV
BVDV
APV
NRPV
PPV1
BWV
78
83
91
50
0.2
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CSFV
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APV
BWV
NRPV
PPV1
84
96
66
76
0.2

Fig. 1C

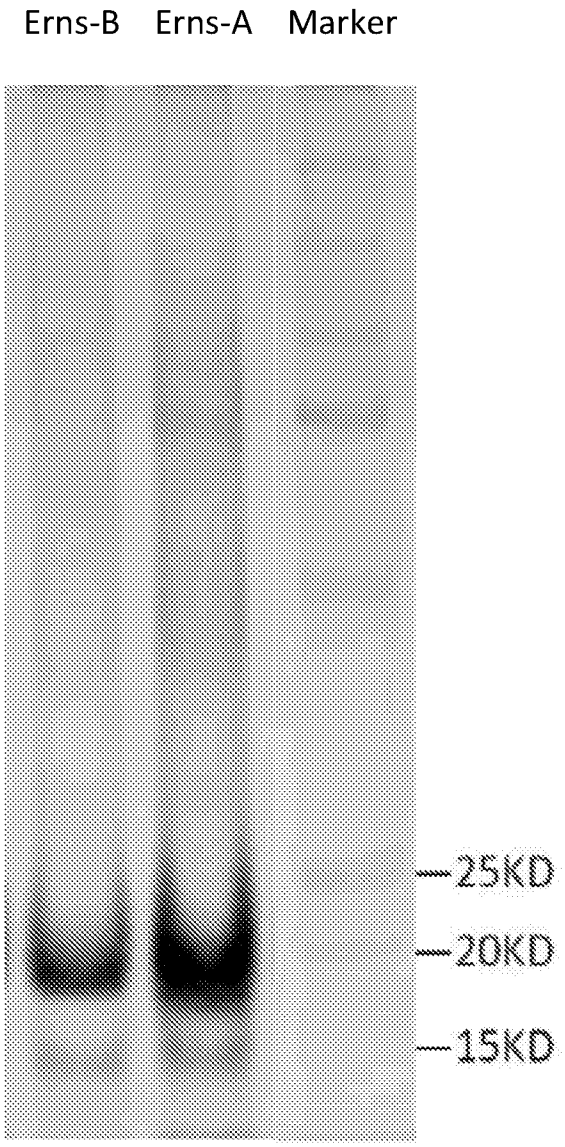
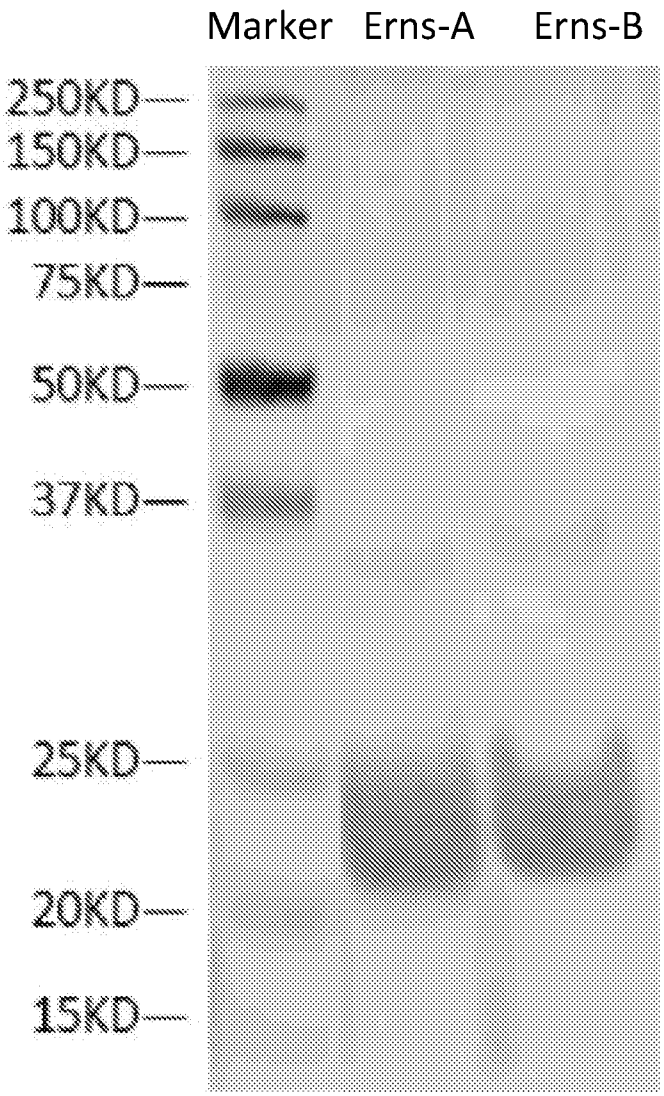


Fig. 2A

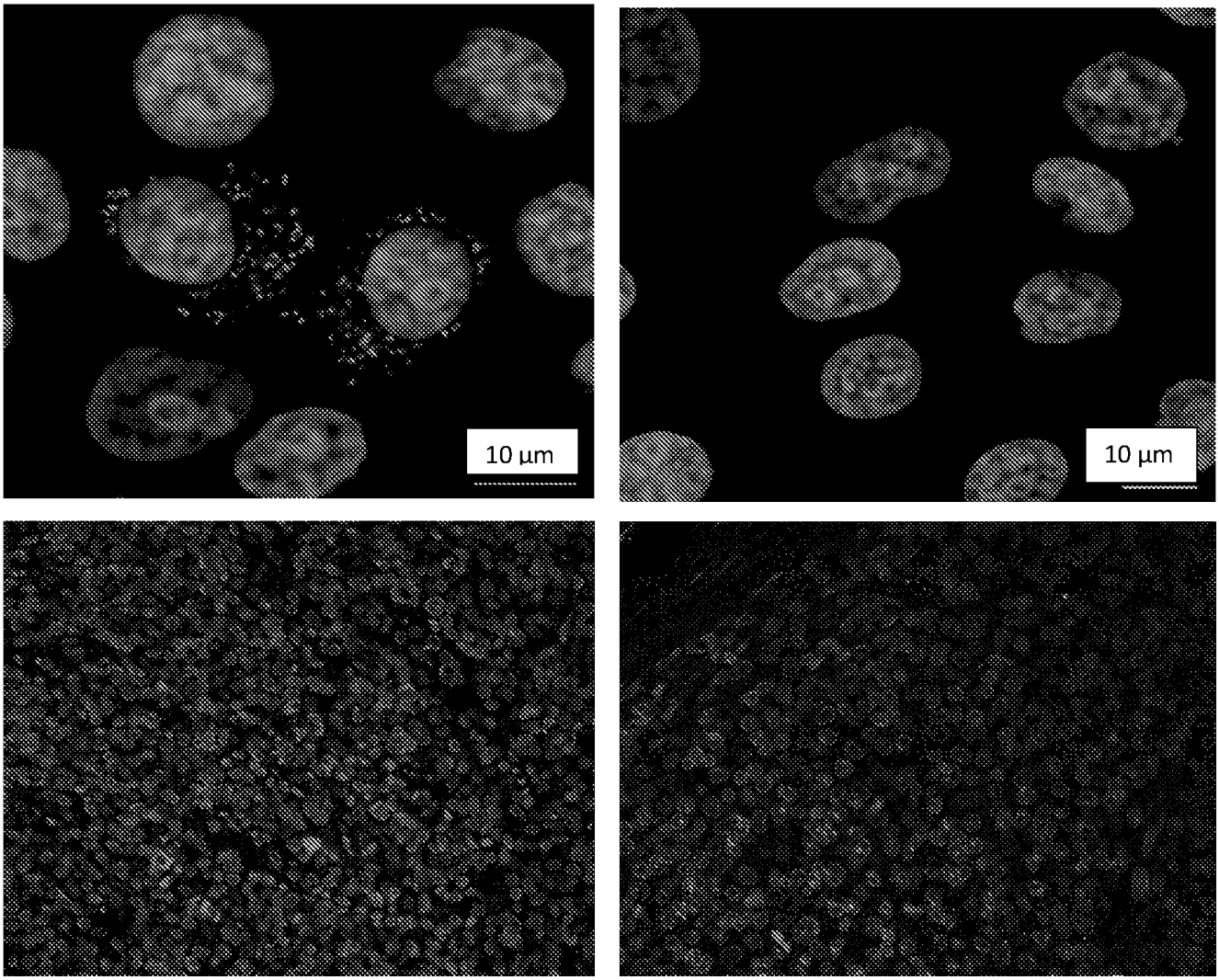


Western Blot

Fig. 2B

MARC-145 cells expressing APPV-Erns

Control MARC-145 cells



LN-APPV infection

LN-control

Fig. 3A

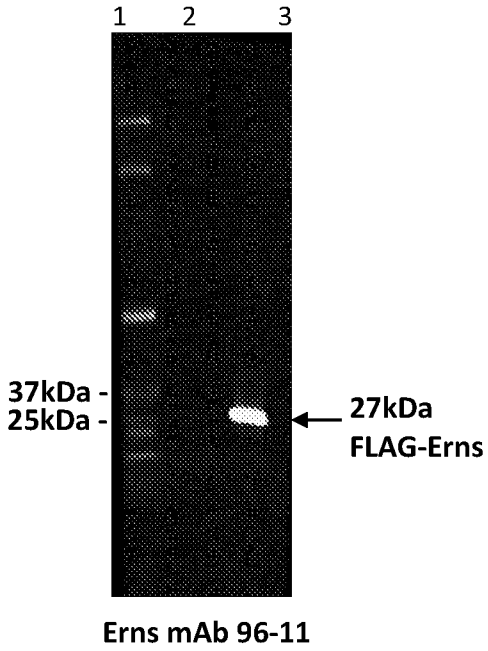


Fig. 3B

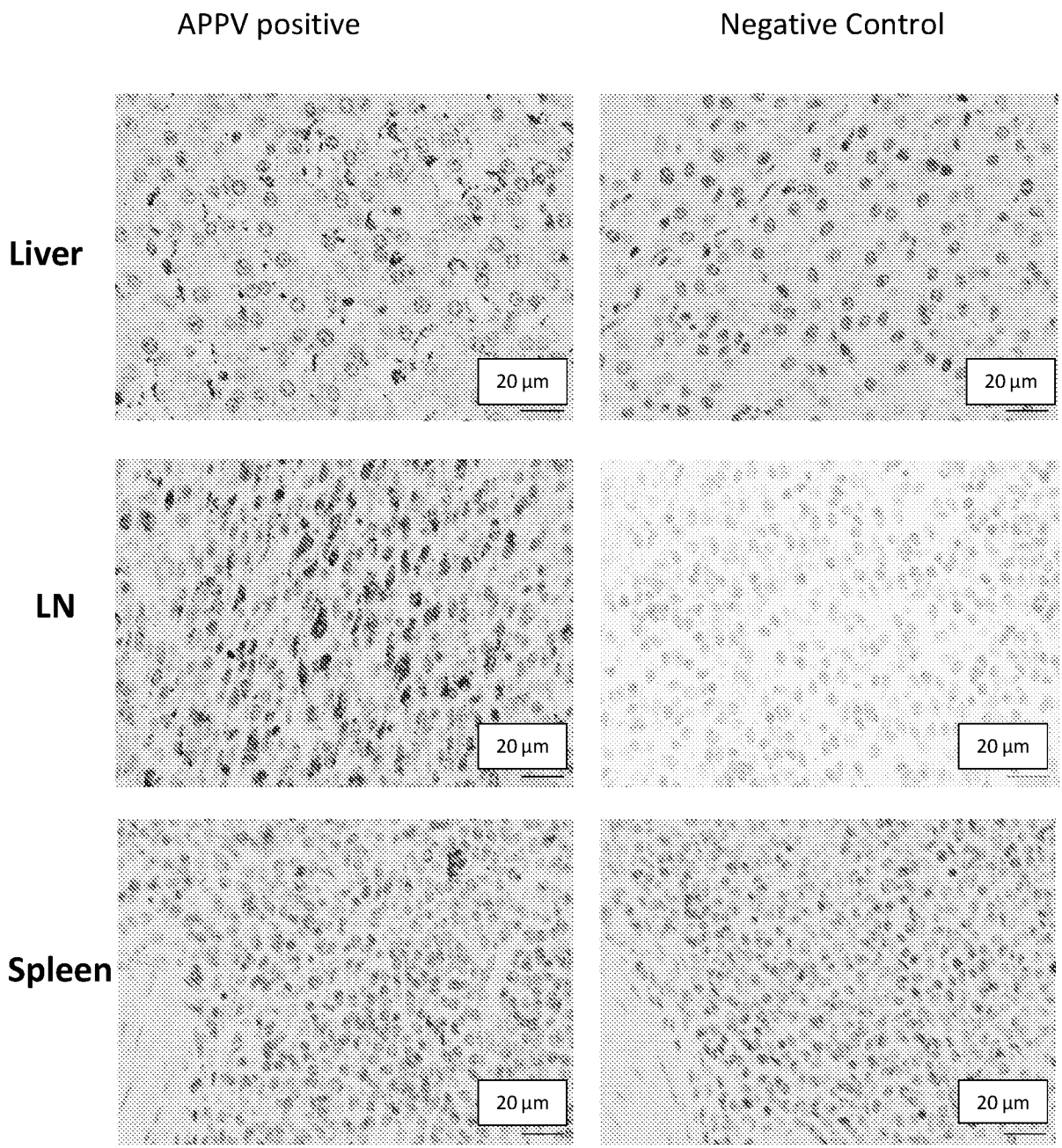

APPV positive
Negative Control
Liver
LN
Spleen
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm

Fig. 4