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Detection, isolation, and in vitro characterization of porcine parainfluenza virus type 1 isolated from respiratory diagnostic specimens in swine

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ABSTRACT

Porcine parainfluenza virus type 1 (PPIV-1) is a member of the genus *Respirovirus* in the family *Paramyxoviridae*. The PPIV-1 was initially detected in 2013 from slaughter pigs in Hong Kong, China although its role in respiratory disease has remained unknown without virus isolates for experimental inoculation in swine. The objective of this study was to determine the relative frequency of PPIV-1 detection in diagnostic samples collected from swine in the United States, describe the cell culture isolation of PPIV-1, and characterize PPIV-1 cell culture isolates in vitro. Among 842 porcine specimens submitted to the Iowa State University Veterinary Diagnostic Laboratory during 2016–2017, 43.3% were PPIV-1 positive by a real-time, reverse transcriptase PCR suggesting PPIV-1 may be common in swine. Two strains of PPIV-1 were successfully isolated in an LLC-MK2 cell line from a PPIV-1 RT-qPCR positive nasal swab (USA/MN25890NS/2016) and lung (USA/IA84915LG/2017). The PPIV-1 cytopathic effect was demonstrated in tissue culture and enveloped viral particles were observed by electron microscopy. The whole genome, F, and HN gene sequences of both isolates share 98.2%, 98.5%, and 98.2% nucleotide homology, respectively, and phylogenetic analysis indicated they are closely related to other PPIV-1 strains detected in swine from the United States. Whole virus PPIV-1-specific monoclonal antibodies were generated for PPIV-1 detection in infected LLC-MK2 cells by indirect immunofluorescence and immunocytochemistry assays. The virus isolates and monoclonal antibodies obtained in the present study can be used to investigate the pathogenesis of PPIV-1 and develop new diagnostic tests.

1. Introduction

Paramyxoviruses have been detected in humans and various animal species, including swine, and may cross host species barriers (Philbey et al., 1998; Thibault et al., 2017; Zeltina et al., 2016). Swine are the primary reservoir of porcine rubulavirus associated with encephalomyelitis, reproductive disorders, and corneal opacity (blue-eye disease) in pigs (Stephan et al., 1988). Nipah virus and Menangle virus have also been detected in swine and caused disease outbreaks in humans as well as various species of domestic animals (Chua et al., 1999; Murray et al., 1995). Recently, a new paramyxovirus, porcine parainfluenza virus type 1 (PPIV-1), has been detected in swine diagnostic specimens and may have the potential to be associated with the porcine respiratory disease complex (Palinski et al., 2016; Sun et al., 2013).

Porcine parainfluenza virus type 1 (species *Porcine respirovirus 1*) is a member of the genus *Respirovirus* in the family *Paramyxoviridae* and is an enveloped, single-stranded, negative sense RNA virus. PPIV-1 was first detected in 2013 by reverse transcription PCR (RT-PCR) from nasopharyngeal and rectal swabs collected from slaughterhouse pigs in Hong Kong, China (Lau et al., 2013). The virus was proposed a novel porcine paramyxovirus. The whole genome of PPIV-1 is approximately 15 kb in length and encodes six major proteins (3'-N-P/C/V-M-F-HN-L-5') and two accessory proteins associated with the phosphoprotein (Henrickson, 2003; Lau et al., 2013; Palinski et al., 2016; Park et al., 2017). Two PPIV-1 proteins important for virus replication in host cells include the hemagglutinin-neuraminidase (HN) and fusion (F) protein, which facilitate attachment and entry into the host cell and fusion between viral envelope and plasma cell membrane, respectively (Breker-Klassen et al., 1996; Henrickson, 2003; Klenk et al., 1977; Morrison, 2003).

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Porcine parainfluenza type 1 is most closely related to human parainfluenza virus 1 (HPIV-1) and Sendai virus (SeV) based on full genome sequence analysis (Lau et al., 2013; Palinski et al., 2016). However, little is known about the zoonotic potential or detection of cross-reactive antibodies between PPIV-1 and other parainfluenza viruses. Currently, PPIV-1 is circulating in swine in the United States (US) and was initially detected by a pan-Paramyxovirus PCR in swine of all ages, but may be more common in nursery and grow-finish pigs (Sun et al., 2013). PPIV-1 has been implicated as a potential cause of porcine respiratory disease with clinical signs that may include coughing, minor sneezing, and serous nasal discharge in naturally infected pigs (Palinski et al., 2016). However, controlled experimental infections using a pure cell culture isolate or a virus rescued from infectious cDNA clones have not been reported or applied to characterize the pathogenesis of PPIV-1 infection and clinical implications in porcine respiratory disease.

In this study, we report the detection and genetic diversity of PPIV-1 circulating in swine in the US and the isolation of the virus in a cell culture system. In addition, we generated monoclonal antibodies for the rapid and accurate detection of PPIV-1 in infected cells.

2. Materials and methods

2.1. Diagnostic samples and PPIV-1 real-time reverse transcription-polymerase chain reaction

Among routine diagnostic cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) from 2016 through 2017, 842 porcine diagnostic specimens including lungs, nasal swabs, bronchoalveolar lavage, nasal turbinate, respiratory tract swabs (laryngeal, tracheal, bronchial) and assorted tissue representing 583 individual swine and 259 oral fluids from various populations of pigs were randomly selected and tested by a PPIV-1 real-time reverse transcriptase PCR (RT-qPCR) (Table 1). The samples were processed per routine procedures at the ISU VDL and the RNA was extracted using the MagMAX viral RNA isolation kit (ThermoFisher Scientific, Waltham, MA, USA) and a Kingfisher 96 instrument (ThermoFisher Scientific). The PPIV-1 RT-qPCR was performed with primers and probe targeting the nucleocapsid gene (Table 2). The assay was performed using the Path-ID Multiplex One-Step RT-PCR kit (ThermoFisher Scientific) under the following conditions: 10 min at 48 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 45 s at 60 °C. Internal control XIPC was included in the extraction (50,000 copies per sample) with appropriate primers and probe included in the master mix to monitor PCR amplification and inhibition (Schroeder et al., 2012). One positive extraction control, one positive amplification control, one negative extraction control, and one negative amplification control were also included with each extraction and PCR run. Assays conducted on the ABI 7500 Fast Real Time PCR System (ThermoFisher Scientific) used the "auto baseline" to determine fluorescence baselines with thresholds set at 0.1. The internal control XIPC threshold was set at 10% of the maximum amplification curve.

Table 1
Summary of 842 porcine diagnostic specimens tested by porcine parainfluenza virus type 1 RT-qPCR at the Iowa State University Veterinary Diagnostic Laboratory from 2016 and 2017.

	Total specimens tested by RT-qPCR		PPIV-1 RT-qPCR positive	
Specimen Type	# of specimens	% of specimens	# positive	% positive
Lung	355	42.2	49	13.8
Oral fluid	259	30.8	175	67.6
Nasal swab	156	18.5	100	64.1
Respiratory swab (other)	59	7.0	35	59.3
Assorted tissue	7	0.8	3	42.9
Nasal Turbinate	4	0.5	2	50.0
Bronchoalveolar lavage	2	0.2	1	50.0
Total	842		365	43.3

Table 2
Real-time, reverse transcriptase PCR primers and probe targeting the PPIV-1 nucleoprotein and primers for amplification and sequencing of the Fusion (F) and Hemagglutinin-neuraminidase (HN) gene.

Oligo name	Sequence (5'-3')	Application
PPIV-1RTF	CGGATACTTCATCGTCAGTGTT	N-RT-qPCR
PPIV-1RTR	TGGAGACAACAAAGGGAGAATAG	N-RT-qPCR
PPIV-1RTP	FAM-AGCAGAGGAGATGGGAAACAACCA-Iowa Black	N-RT-qPCR
F-Rev	TCGTGCACCCTAAGTTTTCTTTA	F PCR
F-For	ACTTAGGGTACAAGTTATCCAAAAAA	F PCR
F-For int 1	GAGAGAAGAGCTTAACATTACAGGC	F sequencing
F-Rev int 1	TCATAAATATCTGTYTTCCCGAGATT	F sequencing
F-For int 2	GGTGGAGTAGTTGCRAACTGTATAGC	F sequencing
HN for 1	TTAGGGTGCACGACAGTAAC	HN PCR
HN rev1	GTCCACAGGTCACTTATC	HN PCR
HN for 2	TTAGGGTGCACGACAGTAACTC	HN sequencing
HN rev 2	CCACAGATCACCTGTCTCTAAG	HN sequencing
HN int 1	CGGTGAGAAAGGATGA	HN sequencing
HN int 2	CAAAGGGTCCTCTAGAAG	HN sequencing

2.2. Cell culture, virus isolation, and growth kinetics

LLC-MK2 cells (ATCC® CCL-7™) obtained from ATCC (Manassas, VA, USA) were maintained in M199 medium (ThermoFisher Scientific) supplemented with 1% horse serum (ThermoFisher Scientific) and 1% penicillin and streptomycin (ThermoFisher Scientific). Lung and tissue homogenates, swabs, and oral fluids were resuspended to 10% (w/v) in Earle's balanced salt solution (Sigma-Aldrich, St. Louis, MO). The suspensions were clarified at 11,000 × *g* for 5 min, filtered through 0.2 µm membrane filter (Corning, Germany), and then inoculated onto LLC-MK2 monolayers. After 2 h at 37 °C in a humidified 5% CO_2 incubator, the inoculum was removed and minimum essential medium (MEM) supplemented with 1% Penicillin-Streptomycin and 2 µg/ml of TPCK-trypsin was added. Samples without a cytopathic effect (CPE) and RT-qPCR negative after four blind serial passages were considered negative for virus isolation. Virus growth curves were conducted using LLC-MK2 monolayers infected with USA/MN25890NS/2016 PPIV-1 at a multiplicity of infection (MOI) of 0.05. The virus growth curves were conducted in duplicate in 24-well plates and harvested at 0, 1, 2, 3, and 4 days post inoculation (DPI). The virus titration was performed in 96-well plates with 10-fold serial dilutions. Virus titers were calculated as the 50% tissue culture infectious dose ($TCID_{50}$) per ml according to the Reed-Muench method (Reed and Muench, 1938).

2.3. Electron microscopy

USA/MN25890NS/2016 PPIV-1 was cultured in LLC-MK2 cells and ultra-centrifuged at 100,000 × *g* for 90 min on a 20% sucrose cushion. The pellet was resuspended in MEM media and stained with 2% phosphotungstic acid (PTA; pH 7.0) (Electron Microscopy Sciences, Hatfield, PA). Negative electron microscopy was performed with a FEI Tecnai G^2 BioTWIN electron microscope (FEI Co., Hillsboro, OR).

2.4. Whole genome sequencing

Whole genomes of USA/MN25890NS/2016 and USA/IA84915LG/2017 isolates were determined by next-generation sequencing (NGS) technology using an Illumina MiSeq (Illumina, San Diego, CA, USA) following previously established procedures (Chen et al., 2014; Zhang et al., 2017). Briefly, total DNA/RNA was extracted from virus cell

culture and purified with MagMAX viral RNA isolation kit and a Kingfisher 96 instrument. DNA was then removed with RNase-Free DNase Set (Qiagen, Valencia, CA, USA) from the total DNA/RNA, and the remaining RNA was purified with an Agencourt RNAClean XP kit (Beckman Coulter, Indianapolis, IN, USA). Purified RNAs were reverse transcribed to single stranded cDNA and then double stranded DNAs were synthesized using NEXTflex Rapid RNA-Seq Kit. A DNA sequencing library was constructed with Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). Sequencing was performed on MiSeq with 300-cycle MiSeq Reagent Micro Kit v2 (Illumina, San Diego, CA, USA) to generate paired-end 2 × 150 bp reads for each sample. The PPIV-1 sequencing reads were extracted from raw sequencing output with BWA-MEM (v 0.7.15). Specifically, the reference genome library in the analysis pipeline was built by searching against NCBI nucleotide database with query '(complete genome) AND "porcine parainfluenza virus" (porgn: txid1357321)'. Extracted PPIV reads were assembled with ABySS (v 1.5.2) and SeqMan Pro version 11.2.1 (DNASTAR, Inc., Madison, WI, USA).

2.5. Sanger sequencing and phylogenetic analyses

The full-length Fusion (F) and Hemagglutinin-Neuraminidase (HN) genes were amplified from 18 randomly selected RT-qPCR PPIV-1 positive samples using a Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA). Amplification was performed using the ABI 2720 Thermal Cycler (ThermoFisher Scientific) using the following conditions: 20 min at 48 °C, 3 min at 94 °C, 40 cycles of 30 s at 94 °C, 50 s at 55 °C, and 2 min at 68 °C, 7 min at 68 °C. The PCR products were sequenced by the Sanger method at the Iowa State University DNA facility. Sequencing primers are described in Table 2.

A phylogenetic analysis was conducted using the whole genome sequences, F, and HN genes aligned using MAFFT in Geneious 11.1.5 (https://www.geneious.com) and maximum-likelihood trees were constructed using RAxML (Geneious 11.1.5). The bootstrap values were determined by 100 replicates.

2.6. Generation of monoclonal antibodies

Hybridomas were established using three, six-week-old Balb/c mice that were immunized three times, two weeks apart, with 200 µl of purified PPIV-1 at 10^6 $TCID_{50}$. Five days after the third immunization, the spleens were removed from the mice and the splenocytes were fused with a Myeloma cell line SP2/0 with selection per manufacturer's instructions (ClonaCell-HY, STEMCELL, Cambridge, MA, USA). After 2 weeks, hybridoma supernatants were screened in an indirect enzyme-linked immunosorbent assay (ELISA) coated with whole virus and in a Cell-ELISA using PPIV-1 infected LLC-MK2 cells as the antigen. Monoclonal antibody (Mab) isotyping was performed using a mouse Mab isotyping kit (ThermoFisher Scientific). The mouse experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Iowa State University.

2.7. Indirect immunofluorescence assay and immunocytochemistry

An indirect immunofluorescence assay (IFA) and immunocytochemistry (ICC) were conducted using PPIV-1 infected LLC-MK2 cells fixed in 80% acetone for 15 min at −20 °C and dried at room temperature. The cells were rinsed with phosphate-buffered saline (PBS), blocked with 2% bovine serum albumin (BSA) in PBS-0.05% tween 20 (PBS-T) for 30 min at 37 °C, and washed with PBS-T. Both assays used 50 µl of 100-fold diluted PPIV-1 Mab (34 A) as the primary antibody added to the infected cell monolayer and incubated for 1 h at room temperature followed by washing 6 times with PBS-T. For IFA, the cells were incubated with 1:100 diluted goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (ThermoFisher Scientific) for 1 h at room temperature. After a final wash, infected cells were visualized under a fluorescence microscope (Olympus, Tokyo, Japan). For ICC, 50 µl of 1:250 diluted goat anti-mouse IgG Fc-Biotin conjugate (ThermoFisher Scientific) was added to the cells for 50 min at 37 °C. Plates were washed 6 times with PBS-T and incubated with 50 µl of 1:100 dilution of Ultra Streptavidin-Horseradish peroxidase conjugate (ThermoFisher Scientific) for 50 min at 37 °C. Plates were washed 6 times with PBS-T and cell staining carried out by the addition of 50 µl of a chromogenic substrate aminoethyl carbazole (AEC) (Sigma-Aldrich) for approximately 8–10 min at room temperature and rinsed with deionized water to allow visualization under a microscope.

2.8. Accession numbers

The complete genome sequence of USA/MN25890NS/2016 has been deposited in GenBank under the accession number MF681710 and USA/IA84915LG/2017 under the accession number MG753974.

3. Results

3.1. Detection of PPIV-1 in diagnostic specimens

Eight hundred forty-two swine samples were tested for PPIV-1 by RT-qPCR regardless of clinical history and with a broad range of pig ages (suckling to adult). Samples were randomly collected from diagnostic cases submitted to the ISU VDL in 2016–2017 and originated from twenty US states including IA (272 cases), IL (158), MN (60), KS (55), OH (52), MO (45), IN (44), NC (42), NE (31), PA (18), SD (17), OK (14), GA (10), AZ (6), WI (4), TX (4), MI (3), MD (3), CO (3), and AR (1). Among all specimen types, 43.3% (365/842) were positive for PPIV-1 by RT-qPCR (Table 1). Lungs, oral fluids and nasal swabs represented the largest proportion of specimens evaluated. The highest detection rate was observed in oral fluids and nasal swabs at 67.6% and 64.1%, respectively (Table 1). Lung demonstrated the lowest positive rate (13.8%). These data suggest that PPIV-1 may be common in US swine and may replicate in the upper and lower respiratory tract.

3.2. PPIV-1 isolation in cell culture

Virus isolation was attempted on RT-qPCR positive porcine samples with C_q values of approximately 20–25 that included four lung homogenates, five nasal swabs, eight oral fluids, and three bronchial swabs. Two PPIV-1 strains, USA/MN25890NS/2016 and USA/IA84915LG/2017, were successfully isolated in LLC-MK2 cells (ATCC® CCL-7™) from a nasal swab and a lung sample, respectively. Inoculated cells demonstrated a distinct cytopathic effect including rounding and cellular detachment in addition to formation of syncytia and presence of multinucleate cells after two or three cell culture passages compared to mock-infected LLC-MK2 cells (Fig. 1A, B). When USA/MN25890NS/2016 PPIV-1 viral particles cultured in LLC-MK2 cells were examined by negative-stain electron microscopy, enveloped virus particles of approximately 200 nm in diameter were observed (Fig. 1C). Growth kinetics of PPIV-1 isolate USA/MN25890NS/2016 were evaluated in LLC-MK2. The highest virus titer of $10^{7.3}$ $TCID_{50}$/ml was demonstrated at 2 DPI (Fig. 1D).

3.3. Genome analysis of PPIV-1 isolates

Whole genome sequences were generated using NGS technology on the two PPIV-1 isolates. The whole genome of USA/MN25890NS/2016 was approximately 15,334 bp and USA/IA84915LG/2017 was approximately 15,332 bp. Both PPIV-1 sequences encoded six open reading frames (3'-N-P/C/V-M-F-HN-L-5'). Currently, seven PPIV-1 whole genome sequences are available in GenBank. These include three Chinese strains (JX857409.1, JX857410.1, and JX857411.1), and four U.S. strains (KT749882.1 and KT749883.1) reported previously as well as the two current ISU VDL isolates MF681710 and MG753974. The

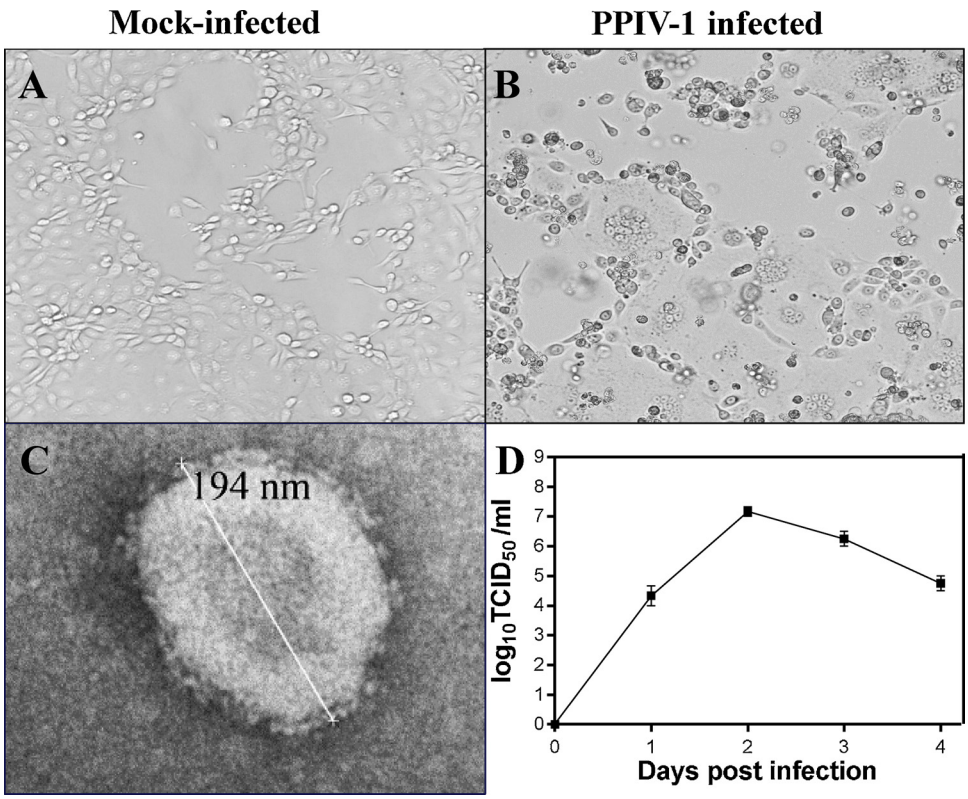

Fig. 1. PPIV-1 infection of LLC-MK2 cells. (A) Mock-infected LLC-MK2 cells, (B) USA/MN25890NS/2016 PPIV-1-infected LLC-MK2 cells, (C) Negative stain electron micrograph of USA/MN25890NS/2016 PPIV-1 virus cultured in LLC-MK2 cells, and (D) Growth curve of USA/MN25890NS/2016 PPIV-1 in LLC-MK2 cells in serum-free media supplemented with TPCK-trypsin.

whole genome sequences of the two PPIV-1 isolates described in the present study were more closely related to PPIV-1 strains detected in the US in 2016 (KT749882.1 and KT749883.1), forming a clade with 98.1%–98.3% nucleotide homology (Fig. 2A). In contrast, the US strains demonstrated 91.5%–96.7% identity with the PPIV-1 detected in Hong Kong in 2013 (JX857409.1, JX857410.1, and JX857411.1) (Fig. 2A). One Chinese isolate, S033N (JX857410.1), did not cluster with any PPIV-1 (Fig. 2A).

To provide further analysis of the PPIV-1 genomes circulating on US swine farms, a phylogenetic analysis of the F and HN genes of additional PPIV-1 strains was performed. The F and HN proteins are the major envelope glycoproteins on the viral surface suspected to be important for receptor binding, entry, and fusion (Henrickson, 2003; Vainionpaa and Hyypia, 1994). Eighteen porcine respiratory cases that were RT-qPCR positive for PPIV-1 at the ISU VDL were selected for F and HN gene sequencing by the Sanger method. A total of 16 F and 15 HN sequences were successfully obtained and compared with additional F and HN sequences available in GenBank. A phylogenetic analysis of 23 F gene sequences (cds region; 1674 nt) included 7 F sequences from whole genomes reported in GenBank and 16 sequences obtained from diagnostic samples submitted to the ISU VDL. The phylogenetic tree based on the F gene demonstrated 14 US strains, including isolates USA/MN25890NS/2016 and USA/IA84915LG/2017, clustered together, whereas 6 US strains were positioned in a distant cluster with two Chinese strains (JX857409, JX857411) (Fig. 2B). The two US isolates formed a cluster with 98.5% identity to each other in contrast to 94.1–98.7% nt identity to the other US strains. However, the two US strains described in this study had only 89.8–95.9% nt identity to three Chinese stains (Fig. 2B).

A similar phylogenetic tree was also constructed using the HN gene (cds region; 1731 nt) of 32 sequences including 17 sequences deposited in GenBank and 15 sequences originating from diagnostic samples submitted to the ISU VDL. The two US isolates were 98.2% identical to each other and only 95.8–99.0% identical to 27 US strains and only 90.6–96.7% to the three Chinese strains, which did not cluster with any US strains (Fig. 2C).

3.4. Generation and application of monoclonal antibodies

Twenty-nine Mabs were selected by screening approximately 500 hybridomas of fused mouse myeloma cells and splenocytes from mice immunized with USA/MN25890NS/2016. Among the 29 Mabs, IgG1 isotype 34A was used to conduct the IFA and ICC assays on PPIV-1 infected LLC-MK2 cells. Positive intracytoplasmic immunostaining in PPIV-1-infected cells but not in mock-inoculated cells were observed using the IFA assay (Fig. 3A, B) and ICC assay (Fig. 3C, D).

4. Discussion

Porcine parainfluenza virus type 1 has been detected in diagnostic specimens from swine with respiratory disease although PPIV-1 has not been experimentally confirmed as a component of the porcine respiratory disease complex due to the lack of virus isolates for experimental inoculation. The virus was initially detected by RT-PCR in nasopharyngeal and rectal swab samples collected from pigs at a slaughterhouse in China in 2013 (Lau et al., 2013). Recent studies conducted by Kansas State University reported that natural PPIV-1 infection may be associated with clinical signs of respiratory disease such as mild coughing, minor sneezing, and serous nasal discharge. However, clinical signs were not observed in some PPIV-1 RT-qPCR positive pigs in the same study (Palinski et al., 2016). These data may suggest that PPIV-1 alone is not sufficient to cause respiratory disease in swine. Bovine parainfluenza virus-3 (BPIV-3) belongs to the genus *Respirovirus* along with PPIV-1 and is associated with mild clinical signs (Ellis, 2010). However, a combination of BPIV-3, secondary bacterial infections and environmental stress factors may result in severe respiratory disease in cattle (Ghram et al., 1989; Lazic et al., 2009). Currently, the pathogenesis and epidemiology of PPIV-1 infection is largely unknown in pigs. Additionally, since PPIV-1 shares genetic and antigenic similarity with HPIV-1 and SeV, the zoonotic potential of PPIV-1 should be considered (Lau et al., 2013). Studies evaluating HPIV-1 infectivity in swine are currently in progress.

In this study, we surveyed the frequency of PPIV-1 detection in

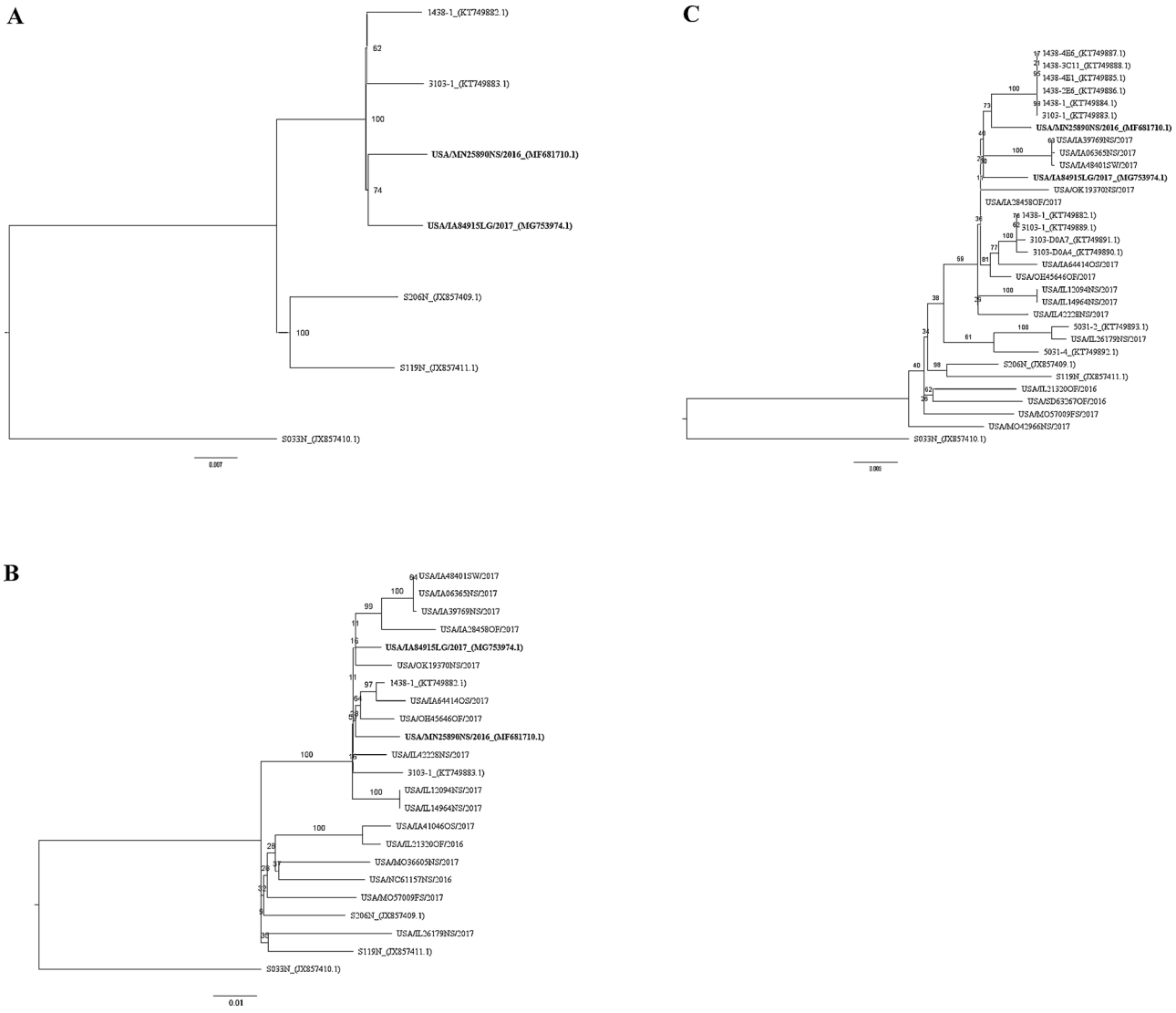

Fig. 2. Phylogenetic analysis of PPIV-1. Phylogenetic tree generated from (A) whole genome nucleotide sequences of two ISU VDL US isolates (MF681710.1 and MF681710.1), two additional US strains of PPIV-1 (KT749882.1 and KT749883.1), and three Chinese PPIV-1 (JX857410.1, JX857411.1, JX857409.1); (B) F gene nucleotide sequences including 16 strains obtained from the ISU VDL and 7 strains available in GenBank; C) HN gene nucleotide sequences including 15 strains obtained from the ISU VDL and 17 strains available in GenBank. The sequences were aligned in Geneious version 11.1.5 via MAFFT and maximum-likelihood trees were inferred using RAxML with 100 rapid bootstrap replicates and 20 maximum likelihood searches. Trees were created using FigTree version 1.3.1. Bootstrap values are indicated at each node. The bold indicates each of two ISU VDL US isolates. GenBank accession numbers for each sequence are given next to strain name.

porcine samples collected from 2016 through 2017 submitted to the ISU VDL. Porcine respiratory samples including lung, oral fluids, nasal swabs, nasal turbinate, bronchoalveolar lavage fluid and respiratory tract swabs such as tracheal, laryngeal and bronchial originating from 20 states in the US were evaluated. Interestingly, from 842 diagnostic specimens, 43.3% were RT-qPCR positive for PPIV-1 nucleic acid. Although the samples were randomly selected regardless of a history of respiratory disease, the results demonstrate PPIV-1 is commonly detected in swine in the US. The frequency of detection was higher in porcine oral fluids and nasal swabs, which were among the three most predominant sample types evaluated including lung. In contrast, lung samples had the lowest PPIV-1 positive rate at 13.8%. This suggests porcine lung, nasal swabs and oral fluids are appropriate sample types for detecting PPIV-1 in swine. In a previous study, influenza A virus (IAV) was experimentally infected in pigs and the virus was detected in oral fluids and nasal swabs with 94% and 67% RT-qPCR positive, respectively. In contrast, IAV isolation was only successful in nasal swabs as viable virus was not isolated from the oral fluid samples (Detmer et al., 2011). In this study, we attempted virus isolation from 20 diagnostic samples submitted to the ISU VDL that tested PPIV-1 RT-qPCR positive (including 4 lungs, 5 nasal swabs, 8 oral fluids, and 3 bronchial swabs) that had C_q ranges of 20–25. Two PPIV-1 were successfully isolated from a nasal swab and a lung with no successful isolation from oral fluids. Oral fluids may be one of the most appropriate samples to detect PPIV-1 by PCR but prove to be more difficult for successful virus isolation. Oral fluids contain many contaminants from the environment, such as feces, feed, and non-specific debris, that may be cytotoxic to LLC-MK2 cells. Nonetheless, oral fluids have been used to detect nucleic acid by PCR or antibodies by ELISA for other swine pathogens, such as porcine circovirus 2, porcine reproductive and respiratory syndrome virus, influenza A virus in swine, and porcine epidemic diarrhea virus among others (Bjustrom-Kraft et al., 2016; Detmer et al., 2011; Olsen et al., 2013b; Prickett et al., 2011; Senthilkumaran et al., 2017; Vosloo et al., 2015). Oral fluids have significant diagnostic advantages in terms of less stress to pigs during collection and are appropriate to survey large numbers of swine increasing the probability of detection suggesting its applicability to survey pig pathogens in a population (Olsen et al., 2013a; Rotolo et al.,

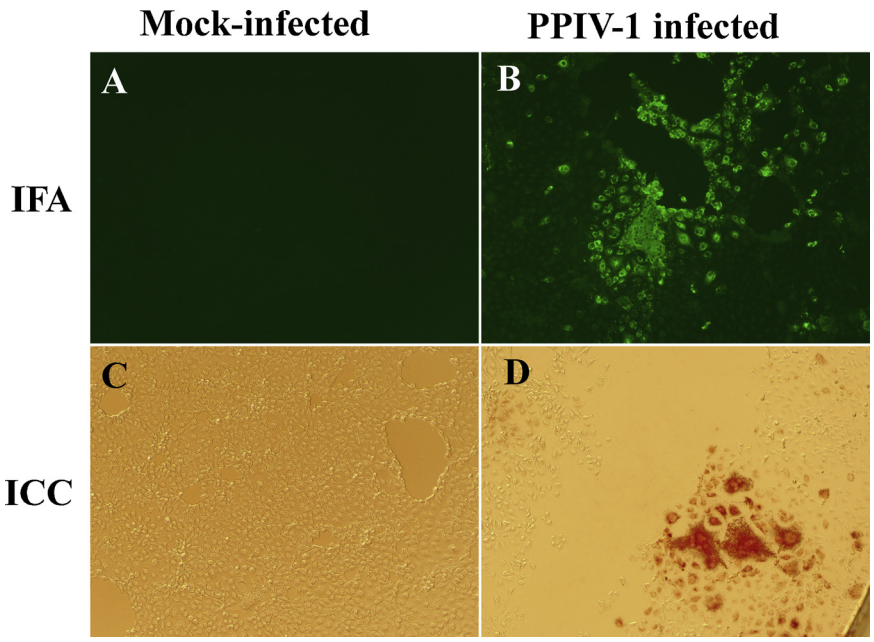

Fig. 3. Generation of monoclonal antibody. (A, C) Mock-infected LLC-MK2 cells; (B, D) USA/MN25890NS/2016 PPIV-1-infected LLC-MK2 cells. A monoclonal antibody, Mab 34A, demonstrated positive immunostaining in the infected cell monolayers using an indirect immunofluorescence assay (B) or by immunocytochemistry (D) compared to mock-infected cells (A, C).

2017).

In spite of the high PPIV-1 detection rate in US swine, a low number of whole genomes, HN, or F gene sequences are available for phylogenetic analysis. The two US isolates were more closely related with PPIV-1 previously detected in the US compared to the Chinese strains, which may suggest the presence of different genetic strains of PPIV-1 based on geographic locations. Phylogenetic analysis of the F and HN genes may be useful to evaluate the epidemiology of the virus in addition to antigenic or virulence features. Genetic and antigenic diversity of parainfluenza viruses have been previously evaluated using these genes (Almajhdi, 2015; Ambrose et al., 1995; Chow et al., 2016; Henrickson and Savatski, 1992; Rima et al., 2014; Tsutsui et al., 2017). Phylogenetic analysis of contemporary F and HN genes available in GenBank and the ISU VDL suggests the F gene is more genetically diverse compared to the HN gene and may be more appropriate for detecting differences in PPIV-1 strains. In contrast, the F gene showed less overall diversity than either HN or P gene in HPIV-1 studies (Ambrose et al., 1995; Beck et al., 2012). Continuous epidemiologic surveillance and genomic analysis of the currently circulating PPIV-1 based on spatial and temporal differences is necessary to understand the genetic and antigenic diversity of the virus.

In addition to clinical signs, a diagnosis of respiratory disease should include the presence of lung lesions and positive ancillary tests such as RT-qPCR, virus isolation or antibody-based assays. These laboratory techniques have been used successfully to detect and identify viruses or provide evidence of prior infection (Burgesser et al., 1999; Loeffelholz and Chonmaitree, 2010; Reis et al., 2008). Detection by PCR is a sensitive and specific method used routinely in veterinary diagnostic laboratories. However, the detection of virus antigens in diagnostic specimens could be a rapid and less expensive method compared to PCR and help confirm the presence of virus in cell culture (Grandien, 1996; Halonen et al., 1996; Madeley and Peiris, 2002; Payne et al., 1988). The PPIV-1 specific monoclonal antibodies described in this report are well adapted for IFA and ICC to detect PPIV-1 antigens in LLC-MK2 cells providing sensitive identification of PPIV-1. In future studies, the PPIV-1 monoclonal antibodies can be applied to detect viral antigens in clinical tissues. Studies evaluating the pathogenesis of USA/MN25890NS/2016 and USA/IA84915LG/2017 are currently in progress in swine with development of an IHC assay using the 34A monoclonal antibody to detect PPIV-1 in formalin-fixed tissues. In addition, the monoclonal antibody will be used to evaluate cross-reactivity with other strains of PPIV-1 as well as parainfluenza viruses from other species including HPIV-1, SeV, and BPIV-3.

5. Conclusion

In summary, the present study describes for the first time a cell culture method for isolation and characterization of two strains of PPIV-1 in swine. The PPIV-1 isolates can be used for pathogenesis studies to determine the role of PPIV-1 in respiratory disease in swine and to evaluate vaccine efficacy using appropriate challenge models. PPIV-1 monoclonal antibodies can be applied to confirm virus isolation results in cell culture, detect viral antigens in clinical specimens, and to develop serological assays.

Conflicts of interest statement

We declare that we have no conflicts of interest with respect to the research authorship and/or publication of this article.

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