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Isolation and Characterization of *Porcine Deltacoronavirus* from Pigs with Diarrhea in the United States

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Abstract

Porcine deltacoronavirus (PDCoV) is a novel coronavirus that causes diarrhea in nursing piglets. Following its first detection in the United States (US) in February 2014, additional PDCoV strains have been identified in the US and Canada. Currently no treatments or vaccines for PDCoV are available. In this study, the US PDCoV OH-FD22 strain from intestinal contents of a diarrheic pig from Ohio was isolated in swine testicular (ST) and LLC-porcine kidney (LLC-PK) cell cultures using various media additives. We also isolated PDCoV [OH-FD22 (DC44) strain] in LLC-PK cells from intestinal contents of the PDCoV OH-FD22 strain inoculated into gnotobiotic (Gn) pigs. Cell culture isolation and propagation were optimized, and the isolates have been serially propagated in cell culture for >20 passages. The full-length S and N genes were sequenced to study PDCoV genetic changes after passage in Gn pigs and cell culture (P11 and P20). Genetically, the S and N genes of PDCoV isolates were relatively stable during the first 20 passages in cell culture with only five nucleotide changes, each corresponding to an amino acid change. The S and N genes of our sequenced strains were genetically closely related to each other and other US PDCoV strains, with the highest sequence similarity to South Korean KNU14-04 strain. This is the first report describing the cell culture isolation, serial propagation, biologic and genetic characterization of the cell adapted PDCoV strains. The information presented in this study is important for the development of diagnostic reagents, assays and potential vaccines against the emergent PDCoV strains.

***Keywords*:** Porcine deltacoronavirus (PDCoV); Isolation; Serial propagation; Pig; Cell culture

Introduction

Porcine deltacoronaviruses (PDCoV) belong to the deltacoronavirus genus of the *Coronaviridae* family (1-3). They appear to be newly emerging CoVs in pigs in the US and were reported from clinical cases of diarrhea in young pigs in 2014 by Wang *et al*. in Ohio (4), Marthaler *et al*. in Illinois (5), and Li *et al*. in Iowa (6). Infected herds had clinical signs of acute watery diarrhea in sows and nursing pigs, but mortality was shown only in the nursing pigs. The disease was clinically similar to, but reportedly milder than porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV), and with lower mortality rates in the affected nursing pigs. Like PEDV, there is no evidence that PDCoV is transmissible to humans. There are currently no treatments or vaccines available for PDCoV.

PDCoV was initially reported in pigs in Hong Kong in 2012. Woo *et al.* (7) detected the new deltacoronaviruses in a variety of mammalian and avian species, with a 10% positive rate for PDCoV in the 169 swine fecal samples tested. Complete genome sequences were reported for 2 PDCoV strains (HKU15-44 and HKU15-155, GenBank accession no. JQ065042 and JQ065043, respectively) (7). PDCoV was first detected in a swine herd in the US in early 2014. Mathaler *et al.* (5) sequenced the genome of SDCV/USA/Illinois121/2014 strain (GenBank accession no: KJ481931.1) which had about 99% nucleotide (nt) identity to the two Hong Kong PDCoV strains. Another US PDCoV strain from Iowa (USA/IA/2014/8734, GenBank accession no: KJ567050) had 98.9% nt identity to the HKU15-44 strain and 99.2% nt identity to the HKU15-155 strain (6). Additionally, the PDCoV HKU15-OH1987 strain (GenBank accession no. KJ462462) was identified in feces and intestinal samples from pigs with diarrheal disease in Ohio, which had a 99% nt identity to PDCoV HKU15-44 and HKU15-155 (4). Subsequently, PDCoVs were

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66 detected in other 9 US states, and they share a high nt similarity (≥99.8%) with each other, and
67 98.9%-99.2% nt similarity with the HKU15-44 and HKU15-155 strains (8). Apart from the US,
68 PDCoV was also detected in 6 Ontario farms in Canada in March 2014 (9). Recently, PDCoV
69 KUN14-04 strain (GenBank accession no. KM820765) was also identified in feces from
70 diarrheic piglets in South Korea. This Korean strain has nt identities of 98.8%-99.0% to
71 HKU15-44 and HKU15-155 strains and 99.6%-99.8% to eight US strains (10). Other research
72 groups (8, 9) and our molecular surveillance studies indicated that PDCoV was a common viral
73 pathogen of pigs in the Midwestern US, and that PDCoV co-infections were common, especially
74 with rotavirus C and PEDV. Our recent study confirmed that PDCoV is enteropathogenic in
75 young pigs, as evident by severe watery diarrhea and/or vomiting and severe atrophic enteritis in
76 all 11- to 14-day-old gnotobiotic (Gn) pigs inoculated with 2 PDCoV strains, OH-FD22 and
77 OH-FD100 (11).

78 A real-time RT-PCR has been developed by Marthaler *et al*. (9) to detect PDCoV and has
79 been used to diagnose PDCoV field infections. However, other virological and serological
80 diagnostic assays are lacking. A critical step to develop PDCoV diagnostic assays and potential
81 future vaccines is the isolation of PDCoV in cell culture.

82 Here, we report, to our knowledge, the first isolation of a PDCoV strain from intestinal
83 contents collected from a diarrheic pig from Ohio, in swine testicular (ST) and LLC-porcine
84 kidney (LLC-PK) cell cultures. We also isolated PDCoV in cell culture from the intestinal
85 contents of Gn pigs inoculated orally with the original sample, OH-FD22. The cell culture
86 isolation and propagation procedures were optimized, and the isolates have been successfully
87 serially propagated in cell culture over 20 passages. In addition to characterizing the virus growth

during serial passage in cell culture, the spike (S) and nucleocapsid (N) gene sequences were determined from the original sample, the Gn pig passaged virus, and selected cell culture passages to compare their genetic sequences with other PDCoV by phylogenetic analysis. The results of this study are critical to develop new serologic tests for PDCoV and to advance our knowledge of the biology and epidemiology of PDCoV in swine.

Materials and methods

Sample collection and testing

From February to July 2014, 42 clinical samples (including feces and intestinal contents) were collected from young nursing piglets (ages 1-7 days) on different farms with diarrhea outbreaks in Ohio and Indiana, USA (see Table 1). On 2 farms, acute serum (n=6) and colostrum (n=6) or feces (n=5) were also collected from subclinically affected sows. The collected samples were tested for PDCoV by using a TaqMan real-time RT-PCR (qRT-PCR) targeting the membrane (M) gene (23395-23466 nt) as reported previously (9). All PDCoV positive samples were tested for other swine enteric viruses including PEDV, rotavirus groups (Rota) A-C, TGEV/porcine respiratory coronavirus (PRCV), and caliciviruses (noroviruses, sapoviruses, and St-Valerien-like viruses) by RT-PCR as reported previously (12-16). Based on the qRT-PCR cycle threshold (Ct) values for PDCoV and the testing results for the other swine enteric viruses, 10 samples (PDCoV positive only) were selected for isolation of PDCoV in cell culture. The PDCoV strain OH-FD22 from the SF-OH farm, which had the highest viral RNA titer of these 10 samples, was selected for inoculation of Gn pigs.

The original samples were diluted 10-fold with phosphate-buffered saline (PBS), vortexed and centrifuged at 1,847×g at 4 °C for 10 min. The supernatant was filtered through a 0.22-μm syringe filter (Millipore, USA), and used as inoculum for the Gn pigs or for virus isolation in cell culture.

Inoculation of Gn pigs with PDCoV OH-FD22 strain

Gn pigs were delivered aseptically by hysterectomy from a specific pathogen-free sow. Two 14-day-old pigs were inoculated orally with the original OH-FD22 filtered intestinal contents using 8.8 $\log_{10}$ genomic equivalents (GE) per pig. Clinical signs were monitored and viral shedding in rectal swab samples was tested using qRT-PCR. Pig 1 was euthanized after onset of clinical signs. Large intestinal contents (LIC) and small intestinal contents (SIC) were collected and tested by qRT-PCR for PDCoV and by RT-PCR for other enteric viruses. The LIC of pig 1 was designated as OH-FD22 (DC44) and also was used as inoculum for virus isolation in cell culture. Pig 2 was monitored for longer-term clinical signs and virus shedding. To obtain hyperimmune antiserum against PDCoV, at postinoculation day (PID) 30 pig 2 was immunized intramuscularly with the semipurified PDCoV from the Gn pig-passaged OH-FD22 after mixing with an equal volume of Freund's complete adjuvant (Sigma, Aldrich) (17). On PID 44, the pig was re-inoculated intramuscularly with the virus mixed with Freund's incomplete adjuvant (Sigma, Aldrich). The pig was euthanized after 1 week, and PDCoV antiserum was collected and designated as OH-DC97.

The OH-FD22 (DC44) sample was diluted 10 fold with Minimum Essential Medium (MEM) (Gibco, USA), mixed and centrifuged at 1,847×g at 4 °C for 10 min. The supernatant was filtered by using a 0.22-μm syringe filter, and used as inoculum for cell cultures to isolate PDCoV.

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Virus isolation and propagation in the cell lines of swine origin

The ST cell line (ATCC: CRL1746) and LLC-PK cell line (ATCC: CL-101) were used to isolate PDCoV from the original field and pig-passaged OH-FD22 samples. The growth medium for ST cells was Advanced MEM (Gibco, USA), supplemented with 5% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 1% Antibiotic-antimycotic (Gibco, USA), 1% HEPES (Gibco, USA), and 1% L-Glutamine (Gibco, USA). LLC-PK cells were grown in MEM supplemented with 5% heat-inactivated fetal bovine serum, 1% MEM non-essential amino acids (NEAA, Gibco), 1% Antibiotic-antimycotic, and 1% HEPES.

One or two-day-old, 80% confluent cell monolayers were used for virus inoculation. Briefly, cells were washed twice with maintenance medium (advanced MEM supplemented with 1% Antibiotic-antimycotic and 1% HEPES for ST cells, and MEM supplemented with 1% Antibiotic-antimycotic, 1% NEAA and 1% HEPES for LLC-PK cells), then inoculated with the filtered samples. After adsorption for 60 min at 37 °C in 5% CO_2, cells were washed 3 times and maintenance medium was added. The cell cultures were observed for cytopathic effects (CPE).

For the first inoculation, cells were cultured in 6-well plates and 300 µl of inoculum were added to each well. When over 80% CPE was evident in the inoculated cell monolayers (around PID 5), the plates were frozen at - 80 °C and thawed twice. The cells and supernatants were harvested together, the 0 HPI and PID 5 samples were tested by qRT-PCR, and the difference of cycle threshold (ΔCt) values was calculated. These samples were used as seed stocks (P0) for the next passage.

For serial passage, T25 or T75 flasks were used for PDCoV propagation. Virus titration was performed by qRT-PCR, 50% tissue culture infectious dose ($TCID_{50}$) and plaque assays. During

the serial passages, various additives were incorporated into the maintenance medium to promote PDCoV propagation. The additives and conditions were as follows: (i) trypsin (Gibco, USA) was added at a final concentration of 10 μg/ml in maintenance medium; (ii) different concentrations (1% and 10%) of pancreatin (Sigma, USA) were added pre- or post-viral inoculation; and (iii) different concentrations (1%, 10% and 20%) of SIC from healthy uninfected Gn pigs were added pre- or post-viral inoculation. The SIC were prepared in our lab as described by Flynn et al. (18). Briefly, the small intestinal contents of a 9-day-old, uninfected Gn pig were collected aseptically, diluted 1:10 in PBS, clarified by low-speed centrifugation (650×g for 30 min at 4°C), and filtered through a 0.45 μm (pore size) filter.

Viral RNA extraction

Viral RNA was extracted from the intestinal content suspensions, rectal swab fluids, feces, and cell culture samples using the 5×MagMAX^{TM}-96 Viral Isolation kit (Ambion by Life Technologies, USA) and the RNA extraction robot MagMaxTM Express (Applied Biosystems, Foster, CA) according to the manufacturer's instructions. The viral RNA was eluted with 50 μl of elution buffer and was used as the template for RT-PCR and qRT-PCR.

RT-PCR and qRT-PCR based on PDCoV M gene

Initial screening for PDCoV was performed for the M gene by qRT-PCR as reported by Marthaler *et al*. (5, 9). The qRT-PCR was conducted using the QIAgen OneStep RT-PCR Kit (Qiagen Inc., Valencia, CA, USA) on a real-time thermocycler (RealPlex, Eppendorf, Germany) and the results were analyzed by the system software. The RT-PCR method was also applied by amplifying a 541-bp fragment of the M gene which covered the qRT-PCR amplified fragment. The primers (5'-CGCGTAATCGTGTGATCTATGT-3' and

5'-CCGGCCTTTGAAGTGGTTAT-3') were designed according to the sequence of a US strain Illinois121/2014 (GenBank accession no: KJ481931). The PCR products were purified using QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA, USA) and sequenced, then used as template to construct the qRT-PCR standard curve. The detection limit of qRT-PCR was 10 GE per reaction, corresponding to 4.6 $\log_{10}$ GE/ml.

Virus titration and purification by plaque assay

A plaque assay for PDCoV was developed with modifications of that reported for PEDV as described by Oka *et al*. (16). The ST cells in 6-well plates were used for all plaque assays for PDCoV propagated in both ST cells and LLC-PK cells. Briefly, cells were seeded into the 6-well plates and grown to 100% confluency after 24 h. The growth medium was replaced with maintenance medium (without trypsin). Following 1 h incubation at 37 °C, the cells were washed once with maintenance media. Then duplicate wells were inoculated with 10-fold serially diluted virus (0.3 ml/well) and incubated for 1 h at 37 °C in an atmosphere of 5% CO_2. The virus inoculum was removed and cells were washed 2 times with Dulbecco's PBS (DPBS) without Mg^{2+} and Ca^{2+} (Sigma, St. Louis, MO). An agarose overlay was prepared as follows: an equal volume of 3% SeaPlaque agarose (Lonza, Rockland, ME) was mixed with 2×MEM (Gibco, USA) containing 1% Antibiotic-antimycotic, HEPES, NEAA, and 2% pancreatin. Two milliliters of the agarose/MEM mixture were added to each well. The plates were stained with 0.01% neutral red (Sigma) for 3 h at 37°C at PID 2-3. The plaques were counted under oblique light, and confirmed by using a light microscope (Olympus CK2, Japan). After the viral plaques were enumerated by counting, the plaque titers were expressed as plaque forming units (PFU)/ml.

For virus plaque purification, uniform and clear plaques were picked using sterile pipette

tips, and the agarose plug was placed into a microcentrifuge tube containing 0.5 ml maintenance medium. The selected plaques in maintenance medium were stored at -80°C or used to inoculate 6-well plates directly. After inoculation with the selected plaque clones, the cells were observed for CPE for 4-5 days. After the positive clones were harvested and the viral titers were determined, the clones with the highest titers were used for further passage of the plaque-isolated PDCoV clones.

Infectious virus titrations by $TCID_{50}$

The LLC-PK cells were seeded into 96-well plates and after confluency the monolayers were washed once with maintenance medium with 10 μg/ml of trypsin (MMT), One hundred microliters of 10-fold dilutions of the PDCoV were inoculated in eight replicates per dilution. After absorption for 1h, another 100μl of MMT was added to each well. Viral CPE were monitored for 5 to 7 days, and virus titers were calculated using the Reed-Muench method (19), and expressed as $TCID_{50}$/ml.

Immunofluorescence assay

PDCoV-infected ST cells and LLC-PK cells in 6-well plates were fixed with 100% ethanol at 4 °C overnight, then washed 5 times with PBS, and blocked with 5% bovine serum albumin (BSA) at 37 °C for 1h. The hyperimmune antiserum OH-DC97 (diluted 1:100) was used as the primary antibody. After overnight incubation at 4 °C, plates were washed 6 times with PBS containing 0.05% Tween-20 (PBST). Then a 1:100 dilution of affinity purified Fluorescein Labeled Goat anti-pig IgG (H+L) (Kpl, MD, USA) was added and incubated for 1h at 37 °C, then plates were washed 6 times with PBST. Cell staining was examined using a fluorescence microscope (Olympus IX-70, Japan).

Immune electron microscopy (IEM)

IEM was conducted by incubating virus samples with the Gn pig antisera OH-DC97 as described previously (18). For visualizing the virion particles in infected cell culture media, PDCoV-infected ST and LLC-PK cell culture media were clarified by centrifugation at 1,847 × g for 30 min at 4 °C. After filtration through 0.45 μm filters, the virus medium was further ultracentrifuged at 106,750 × g for 2 h at 4 °C using an ultracentrifuge (Beckman Coulter, Miami, FL, USA). Virus pellets were resuspended in MEM. The purified samples were incubated with the antiserum OH-DC97 (diluted 20-fold) overnight at 4°C.

For negative staining for IEM, the prepared cell culture samples and antiserum mixtures were stained with an equal volume of 3% phosphotungstic acid (PTA, pH7.0) in 0.4% sucrose for 1 min, then applied to a 300-mesh formvar and carbon-coated copper grid for 5 min. After blotting and drying, the grids were examined with an H7500 electron microscope (Hitachi High Technologies, Tokyo, Japan)

PDCoV S and N gene sequencing and phylogenetic analysis

The complete S and N genes of PDCoV in the original OH-FD22, Gn pig-passaged OH-FD22 (DC44), and cell culture adapted passage 11 (OH-FD22-P11-ST, OH-FD22-P11-LLC-PK, and OH-FD22 (DC44)-P11-LLC-PK) and 20 (OH-FD22-P20-ST, OH-FD22-P20-LLC-PK) were amplified, cloned and sequenced. The S genes were amplified using primers PDCoV-SF2 (5'-AGCGTTGACACCAACCTATT-3) and PDCoV-SR2 (5'-TCGTCGACTACCATTCCTTAAAC-3'). The N genes were amplified with the primers PDCoV-NF1 (5'-CCATCGCTCCAAGTCATTCT-3') and PDCoV-NR1 (5'-TGGGTGGGTTTAACAGACATAG-3'). All the primers were designed according the

sequence of the US PDCoV strain Illinois121/2014.

The RNA was converted to cDNA by oligo (dT)-priming strategy (Invitrogen). The genes were amplified using PrimeSTAR GXL DNA Polymerase (Takara Bio Inc. Japan). The PCR mixture (50 µl) contained cDNA 2µl, 5 × PCR buffer 10µl, dNTP (2.5mM each) 4µl, PCR enzyme GXL 1µl, forward and reverse primers (50µM stock) 1µl each. The PCR program was: 95 °C for 5 min, 40 cycles of 98 °C for 10 S, 55 °C for 15 S, 68 °C for 5 min of S gene and 2 min of N gene, and 68 °C for 15 min. The PCR products were ligated with linearized pMiniT Vector by using the NEB PCR Cloning Kit (Ipswich, MA, USA). The cloning was conducted according to the manufacturer's instructions. The recombinant plasmids were extracted and verified by PCR, then sequenced.

Sequence data were assembled and analyzed using the DNAStar 7.0 green (DNAstar, Madison, WI). The PDCoV S and N gene nucleotide sequences of this study as well as other PDCoV strain sequences available in GenBank were subjected to phylogenetic analysis. Phylogenetic trees were constructed using the maximum likelihood method of MEGA6.06 software (http://www.megasoftware.net). Bootstrap analysis was carried out on 1,000 replicate data sets. N-linked glycosylation sites of PDCoV S protein was predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Results

Pathogen detection

A total of 59 samples were collected from 5 pig farms in Ohio and Indiana, USA, and were tested for PDCoV by qRT-PCR (Table 1). Of these tested samples, 17 (29%) were positive for

PDCoV. Four (80%) of the farms had at least 1 PDCoV-positive sample. Seven of 11 (63.6%) fecal samples and 10 of 36 (27.8%) intestinal content samples were PDCoV positive. Four of 5 (80%) sow fecal samples were positive for PDCoV. Excluding the 17 non-clinical sow samples (colostrum, serum, and feces), the percent of PDCoV positives was 31% of the 42 samples from young nursing pigs on the farms with diarrhea. Of the 17 PDCoV positive samples, all were negative for TGEV, PRCV, Rota A and B, and caliciviruses. Seven (41.2%) of the PDCoV-positive samples were positive for PEDV (3 samples) or Rota C (4 samples), respectively (Table 1).

Virus isolation and propagation in LLC-PK cell monolayers

The LLC-PK cell monolayers were inoculated with 10 of the PDCoV only positive samples. Only the OH-FD22 inoculated cell monolayers showed visible CPE, in the form of enlarged, rounded cells at PID 2 that rapidly detached from the monolayers on PID 3. To confirm PDCoV replication on LLC-PK cells, viral RNA was extracted from the inoculated cells at PID 7, and tested by qRT-PCR (Table 2). Upon testing by RT-PCR this cell culture passaged sample was negative for other swine enteric viruses. Thus the PDCoV (OH-FD22 strain) was successfully isolated in cell culture from the intestinal contents of a piglet from the SF-OH Farm (collected in Feb. 2014). The first PDCoV passage on LLC-PK cells was designated as OH-FD22-P0.

The OH-FD22-P0 virus was further serially passed in LLC-PK cells for a total of 20 passages (P1 to P20). During the passages, different culture conditions were compared: with or without exogenous trypsin, adding SIC from healthy Gn pigs pre or post-inoculation, adding different concentrations of pancreatin pre or post-inoculation, and washing or not washing the cell monolayers after virus incubation. The results showed that the OH-FD22 strain replicated in

LLC-PK cells under several of these different conditions. The optimal cell culture conditions used to propagate PDCoV on LLC-PK cells were as follows: MMT washed cells 2 times, virus incubated for an hour, then washed (with MMT) and MMT added. CPE was usually observed within PID 2. The morphological changes in the PDCoV-infected cells were characterized by enlarged, rounded, and densely granular cells that occurred singly or in clusters (Fig. 1I). The infected cells eventually detached from the monolayer. PDCoV replicated in LLC-PK cells without trypsin, but it did not induce visible CPE, and the virus titer (around 5 $\log_{10}$ PFU/ml) was lower than with trypsin (10 μg/ml) treatment (around 9 $\log_{10}$ PFU/ml). The pancreatin or SIC/trypsin treated groups showed earlier evident CPE compared to the trypsin treatment group (Fig. 1B and C), but the virus titers (around 7 $\log_{10}$ PFU/ml) were lower than for the trypsin (10 μg/ml) treatment group (around 9 $\log_{10}$ PFU/ml). Virus growth was confirmed by IF staining using the antiserum OH-DC97. The PDCoV antigens were mostly located in the cytoplasm (Fig. 2A). The levels of PDCoV RNA in the culture supernatants were also determined by qRT-PCR. The presence of PDCoV particles in the supernatant from the infected cells was also examined by IEM. As shown in Fig. 3A, multiple virus particles around 150 to 180 nm in diameter with typical spike surface projections were visible in the samples as confirmed by negative-staining IEM.

OH-FD22 (DC44) virus (LIC of OH-FD22 passaged on Gn pigs) was also inoculated onto cell monolayers by using the same culture conditions as for the OH-FD22 strain. The OH-FD22 (DC44) virus was successfully isolated in LLC-PK cells, and induced similar CPE compared with the OH-FD22 strain. The OH-FD22 (DC44) virus has been serially passaged in LLC-PK cells for a total of 16 passages. The viral RNA titers of the OH-FD22 (DC44) propagated in LLC-PK cells

were similar (around 8.8-11.1 $\log_{10}$ GE/mL) to the OH-FD22 viral RNA titers (Table 2).

Virus isolation and propagation in ST cell monolayers

The OH-FD22 original sample was also inoculated onto ST cell monolayers. Virus growth was detected by qRT-PCR (Table 2). The OH-FD22 viral RNA titer was 9.9 $\log_{10}$ GE/mL at PID 7, and the virus RNA titer was increased compared to 0 HPI, with a ΔCt of 7, and detachment of cells as the major visible CPE. The harvested virus from the ST cells was designated OH-FD22-P0 in ST cells. When the OH-FD22-P0 virus was further passaged in ST cells, no viral RNA titer increase was detected for the next 5 passages, and the cells did not show CPE compared with the negative control (data not shown). Different culture conditions were applied for the OH-FD22-P0 passages in ST cells: with or without trypsin, adding SIC from healthy Gn pigs pre or post-inoculation, and adding different concentrations of pancreatin pre or post-inoculation. The PDCoV replicated in ST cells only when 10% Gn pig's SIC or 1% pancreatin was added. CPE was induced only after incubation with pancreatin or SIC. The optimal propagation conditions for PDCoV in ST cells were: maintenance medium washed cells 2 times, virus incubated for 1h, then monolayers washed and maintenance medium with 1% pancreatin added. The OH-FD22 isolate was further serially passed on ST cells for a total of 20 passages. Distinct CPE, characterized by cell rounding, clumping together in clusters, and eventual detachment of cells, was usually observed at PID 2-3 (Fig. 1E and F). The trypsin alone group showed no CPE (Fig. 1G), whereas pancreatin or SIC/trypsin treated groups showed evident CPE (Fig. 1E and F). Virus titers in pancreatin or SIC/trypsin treated groups tested at PID 3 were similar. Virus growth was also confirmed by qRT-PCR (Table 2), IFA (Fig. 2B) and IEM (Fig. 3B) as noted for virus propagated in the LLC-PK cells.

Virus titration and purification by plaque assay

During the serial passages, the PDCoV RNA titers for both LLC-PK and ST cell cultures were assessed by qRT-PCR (Table 2). Significant increases in virus RNA titers were observed following each cell passage when compared with that at 0 HPI (data not shown). However, the RNA virus titers in LLC-PK cells (9.7-10.6 $\log_{10}$ GE/mL, average of 10.2 $\log_{10}$ GE/mL) were usually higher than that in ST cells (8.9-10.7 $\log_{10}$ GE/mL, average of 9.7 $\log_{10}$ GE/mL). The infectious titers of some passages were determined by $TCID_{50}$ on LLC-PK cells. The $TCID_{50}$ titers of OH-FD22 ranged from 7.6 to 10.9 $\log_{10}$ $TCID_{50}$/ml in LLC-PK cells, which were higher than that in ST cells (range of 7.0 to 9.9 $\log_{10}$ $TCID_{50}$/ml) (Table 2). The virus titers of some passages were also determined by plaque assay on ST cells. The virus titers ranged from 7.2 to 9.6 $\log_{10}$ PFU/ml in LLC-PK cells and from 6.5 to 8.5 $\log_{10}$ PFU/ml in ST cells. The OH-FD22 (DC44) virus infectious titers were similar to that of the OH-FD22 strain in LLC-PK cells (Table 2).

A plaque assay was used to plaque isolate and purify PDCoV on ST cells. OH-FD22-P6 virus in ST cells (7.2 $\log_{10}$ PFU/ml) was used for the plaque isolation. Large clear plaques were evident under an agar overlay medium on the cells. The ST plaque cloned OH-FD22-P7 was further serially passaged to P20 on ST cells (7.1 $\log_{10}$ PFU/ml). The OH-FD22-P11 virus in LLC-PK cells (9.2 $\log_{10}$ PFU/ml) was used for plaque purification on ST cells since these latter cells showed the best PDCoV plaque morphology. The cloned virus OH-FD22-P12 was further serially passaged to P20 on LLC-PK cells (9.2 $\log_{10}$ PFU/ml). The OH-FD22 (DC44)-P9 virus was also tested in a plaque assay on ST cells (7.4 $\log_{10}$ PFU/ml). The plaque purified virus

OH-FD22 (DC44)-P10 was serially passaged to P16 on LLC-PK cells (8.5 $\log_{10}$ PFU/ml) (Table 2).

Phylogenetic analysis of the S genes of PDCoV isolates before and after serial passages in cell cultures and in Gn pigs

To examine if genetic changes occurred in the S gene of PDCoV during the serial passages in cell cultures and Gn pigs, the complete S genes of PDCoV in the original OH-FD22, Gn pig-passaged OH-FD22, and cell adapted passages 11 (OH-FD22-P11-ST, OH-FD22-P11-LLC-PK, and OH-FD22 (DC44)-P11-LLC-PK) and passages 20 (OH-FD22-P20-ST, OH-FD22-P20-LLC-PK) were amplified and sequenced. All the sequenced S genes were 3,483 nucleotides in length, encoding a protein of 1,161 amino acids. The S genes examined in this study shared 99.9%-100% nt identities with each other, and they shared 98.5% -100% nt identities with the other 20 PDCoV strains available in GenBank. The S gene of the field OH-FD22 strain had the highest nt similarity (100%) with the Ohio HKU15-OH1987 strain. Compared with the OH-FD22 strain, OH-FD22 P11 and P20, passaged in both ST and LLC-PK cells, each had five nt changes at positions 430, 466, 1191, 2456, and 3331 (nts were numbered according to the S genes of PDCoV HKU15-OH1987 and OH-FD22 sequences). These nt changes all induced corresponding amino acid (aa) changes (Glu changed to Gln at position 144; Val changed to Phe at position 156; Asn changed to Lys at position 397; Thr changed to Ile at position 819; and Ala changed to Thr at position 1,111) (Fig. 4). The OH-FD22 (DC44)-P11 acquired two nt changes at positions 487 and 1,890 when compared with OH-FD22 (DC44), and these two sites also induced aa changes (Tyr changed to His at position 163 and Val changed to Ala at position 630) (Fig. 4). In both cell culture lines, the mutations observed at P11 were

372 sustained through P20, with a 100% nt identity in P11 and P20. The PDCoV S protein contained
373 27 potential glycosylation sites (Asn-Xaa-Ser/Thr sequences). Further analysis demonstrated that
374 no aa changes were located in any of the predicted N-linked glycosylation sites.

375 A phylogenetic tree was constructed using the entire S gene sequences of 27 PDCoV (20
376 obtained from GenBank and 7 new sequences generated in this study), some newly detected
377 avian delta CoVs, and several TGEV/PRCV and PEDV (Alpha CoVs). Phylogenetic analysis of
378 the S genes showed all PDCoV strains clustered together in one clade of the Delta CoV genus,
379 and were distinct from all avian delta CoVs. Further, analysis of the S gene of PDCoV strains
380 indicated that all the US strains clustered into a subclade with a PDCoV strain KNU14-04 from
381 South Korea isolated in 2014, while the PDCoV HKU15-44 and HKU15-155 strains isolated in
382 Hong Kong in 2012 were clustered separately. Additionally, the OH-FD22 related strains in the
383 current study were most closely related to two other PDCoV strains from Ohio (HKU15-OH1987
384 and OhioCVM1 strains), whereas the strain Ohio137 clustered in another sublineage with most of
385 the US strains (Fig. 5).

386 **Phylogenetic analysis of the N genes of PDCoV isolates before and after serial passages in**
387 **cell cultures and in Gn pigs**

388 The N genes of these sequenced samples were determined to be 1,029 nucleotides in length,
389 coding for a polypeptide of 342 amino acids. The N genes shared 100% nt identities with each
390 other, and they shared 98.9% -99.9% nt identities with the other 20 PDCoV strains available in
391 GenBank. Phylogenetic analyses of the N genes revealed that they belonged to the same groups
392 as the tree based on the S genes, except for the following: on the basis of the N gene tree, the

Ohio137 strain showed a close relationship with our seven newly determined sequences and the HKU15-OH1987 strains, which were in a different sub-cluster in the S gene tree (Fig. 5).

Discussion

Following detection of the first PDCoV in Feb. 2014 in the US, additional PDCoV have been identified in swine farms in Canada and the US. The prevalence of PDCoV in pigs in North America was reported to be over 25% on those farms surveyed (4, 8, 9). Higher PDCoV positive rates were reported in Ohio. Wang *et al*. (4) noted a PDCoV positive rate of 92.9% from 5 farms in Ohio. Our results showed a 29% PDCoV positive rate within 5 pig farms. Despite the rapid detection of PDCoV RNA sequences in the last 8 months, to our knowledge, there are no published papers reporting the successful isolation of PDCoV. In current study, the PDCoV OH-FD22 strain was isolated from intestinal contents collected from a diarrheic pig in ST and LLC-PK cell cultures. We also isolated the Gn pig-passaged PDCoV in cell culture. Propagation of PDCoV was confirmed by CPE development, IF staining, infectious virus titration, IEM, and sequencing of the PDCoV S and N genes. All of these results clearly demonstrated that the PDCoV isolates replicated during serial passage in cell culture. This cell adapted PDCoV strain can be used for PDCoV pathogenesis studies, virological and serological assay development, and warranted for vaccine development.

We attempted to isolate virus from 4 fecal and 6 intestinal content PDCoV positive samples, but only the PDCoV OH-FD22 strain was successfully isolated in the swine ST and LLC-PK cells. This low success rate of PDCoV isolation might be assoicated with the poor quality of the original samples. Samples positive by qRT-PCR may contain non-infectious or low titer virus.

Also suboptimal storage conditions for the early samples and freezing/thawing may disrupt the PDCoV. None of the virus isolation attempts from feces were successful. Based on limited numbers of clinical samples, we can not explain the difference in successful isolation of PDCoV from the intestinal contents versus failure to isolate PDCoV from feces samples. During virus isolation, cell toxicity was observed for 2 of the intestinal content samples. These results indicate that the sample types, virus viability and infectious virus titer, and substances in intestinal contents may be important factors that influence virus isolation. Further, the isolation procedures need to be improved to increase the success rate of PDCoV isolation, particularly from fecal samples.

PDCoV OH-FD22 replicated in both ST and LLC-PK cells, with cell enlargement, rounding and rapid detachment from the cell monolayers at PID 2 to 3. Trypsin was beneficial, but not essential for the propagation of PDCoV in LLC-PK cells. PDCoV replicated in LLC-PK cells without trypsin, however, it did not induce visible CPE, and virus titers were lower. During the CoV infection, the cleavage of S protein by endogenous or exogenous proteases into two subunits is the important for cell culture adaptation of CoVs. This cleavage is essential for the induction of cell-to-cell fusion and virus entry into cells (20, 21). Further studies are needed to investigate whether trypsin or pancreatin is essential for the cleavage of PDCoV S protein or for the virus entry, replication, or release. The conditions required for propagation of PDCoV in ST cells differed from that for LLC-PK cells. The OH-FD22-P0 grew well in ST cells with trypsin, but when serially passaged in ST cell cultures without trypsin in the medium, the replication ceased. Based on the extensive experience in isolating swine enteric viruses in our lab (18), SIC from a non-inoculated Gn pig and trypsin were incorporated into the cell culture medium (serum-free

advanced MEM) to support PDCoV propagation in ST cells. PDCoV could be serially propagated in ST cells with the addition of SIC from uninoculated Gn pigs. The factors in SIC may be beneficial to PDCoV attachment to its receptor and/or virus entry or to other stages of the viral replication cycle. The SIC of healthy Gn pigs is a complex mixture and we did not determine which components exerted growth effects. So commercial pancreatin, reported as an important factor in adapting a porcine group C rotavirus to PPK cell cultures (18, 22), was used for PDCoV growth by adding it to the cell maintenance medium (no trypsin) post-inoculation, successful serial propagation of PDCoV in ST cells was accomplished. Thus pancreatin or SIC in the maintenance medium were critical factors for PDCoV propagation in ST cells. Even at higher passage levels, when they were excluded from the medium, PDCoV infectivity was reduced (data not shown). Since, the exact roles of these growth-promoting factors on PDCoV replication remain unknown, and the mechanisms used by the virus to enter the host cells are unclear, we could not determine the stages of the virus replication cycle that were affected by the SIC or pancreatin additives.

PDCoV infection showed similar clinical signs to those associated with PEDV infection of pigs, and many groups have reported that PEDV was isolated successfully and propagated in Vero cell cultures in the presence of trypsin (2, 16, 23, 24). We initially attempted to isolate PDCoV on Vero cells; however, we were unsuccessful. PDCoV still failed to grow in Vero cells, even when high titer PDCoV obtained from infected LLC-PK cells was used to inoculate Vero cells, and the medium was supplemented with different additives (SIC, trypsin, and pancreatin),. Recently, Zhao *et al*. (25) have reported that porcine intestinal epithelial cells (IPEC-J2) were susceptible to TGEV and PEDV infection. Isolation of PDCoV in the IPEC-J2 cell line was also

459 unsuccessful. These results suggest PDCoV, TGEV and PEDV share different cellular tropisms
460 in vitro. Whereas porcine aminopeptidase N (pAPN) is the cellular receptor for TGEV and PEDV
461 (26, 27), but the receptor for PDCoV is unknown. It was also demonstrated for PEDV that
462 soluble pAPN could facilitate the replication of PEDV in Vero cells (28). However, PEDV could
463 not be propagated in ST or other cell lines which are commonly used for TGEV propagation (23)
464 and now shown to support PDCoV replication as well. These dissimilarities may be due to
465 variations in the viral spike proteins and their receptors, and/or target cells. Further research is
466 needed to evaluate if pAPN serves as a cellular receptor for PDCoV.

467 To characterize the virus isolates, the complete S and N genes were sequenced and analyzed,
468 and the phylogenetic relationships among the PDCoV strains were determined. The seven
469 PDCoVs examined in this study are genetically closely related, with 99.9% to 100% nt identities
470 at the S and N gene levels. They also shared 98.5% -100% nt identities with the other 20 PDCoV
471 strains available in GenBank. Phylogenetic trees constructed using the entire S and N gene
472 sequences showed that all PDCoV strains clustered in one clade in the genus *Deltacoronavirus*,
473 and they were distinct from the avian delta CoVs (Fig. 5). Our results are consistent with the
474 previous reports (4, 8). These findings suggest that PDCoV strains currently circulating in
475 multiple US states are closely related. Notably, the PDCoV strain KNU14-04 isolated from South
476 Korea in 2014 was most closely related to the emerging US PDCoV strains, suggesting that they
477 could be derived from a similar ancestral strain. Furthermore, the PDCoV HKU15-155 and
478 HKU-44 strains from Hong Kong from 2012 belong to a different subcluster, suggesting that the
479 US PDCoV may have emerged independently from the initially reported PDCoV strains from
480 Hong Kong. Alternatively, our analysis may be reflective of temporal clustering, which can be

confirmed by analyzing additional PDCoV strains from Hong Kong, South Korea and other geographical regions. The exact origin of the US PDCoV is difficult to identify at this time. Continuous surveillance studies will be important to monitor the genetic evolution of PDCoV in swine.

The S protein of CoVs is responsible for receptor binding and host adaptation and is therefore among the most variable regions within the CoV genomes (29). Many studies of CoVs have shown that changes in the S protein could influence CoV cross-species transmission and emergence in new host populations (30, 31). Comparison of the sequenced S genes in our study revealed five nucleotide changes in the OH-FD22-P11 in ST cells and LLC-PK cells compared with the original OH-FD22 strain. Moreover, the mutations acquired at P11 were sustained through P20. The OH-FD22 (DC44)-P11 had acquired two nucleotide changes when compared with OH-FD22 (DC44). These nucleotide changes all induced corresponding amino acid changes. Our study revealed that the 5 aa changes in the S gene of OH-FD22 strain were retained after serial passages on both cell cultures, suggesting that common mechanisms may govern PDCoV cell culture adaptation in both swine cell lines. Additionally, the nt and the corresponding aa changes occurred within the first 11 passages in both cell lines suggesting that these mutations may be of primary importance in the initial steps of the virus adaptation to replication in vitro. However, whether these aa changes could alter the efficiency of the viral replication and the viral pathogenicity of the emergent US PDCoV strains needs to be investigated further. Since there were no aa changes in the predicted N-glycosylation sites of the S genes, they may be important for virus replication in vitro.

In conclusion, PDCoV OH-FD22 and OH-FD22 (DC44) strains, associated with diarrheic swine in Ohio, were isolated and serially passaged in cell culture and characterized. The full-length S and N genes were sequenced to study PDCoV genetic changes during passage in cell culture and Gn pigs. To our knowledge, this is the first report describing the isolation, serial propagation, and genetic characterization of S and N genes of the cell culture adapted PDCoV strains. The information presented in this study is important for the development of diagnostic reagents, assays and potential vaccines against the emergent PDCoV strains.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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Table 1. Detection of PDCoV and other porcine enteric viruses from field samples from swine herds with suspect PDCoV diarrhea outbreaks in 2014[a]

Farm code	Original samples	Pig age	Sample No.	PDCoV and porcine enteric virus detection No. (% positive)				
				PDCoV	PEDV	Rota C	TGEV /PRCV	Calicivirus
SF-OH[b]	IC[c]	Nursing piglets	12	7	3	1	-	-
	Serum	Sow	6	-	-	-	-	-
	Colostrum	Sow	6	-	-	-	-	-
MR-OH	IC	Nursing piglets	3	2	-	1	-	-
	Fecal	Nursing piglets	4	1	-	1	-	-
	Fecal	Sow	5	4	-	-	-	-
PV-OH	IC	Nursing piglets	16	-	-	-	-	-
CF-OH	IC	Nursing piglets	5	1	-	-	-	-
WH-IN[b]	Fecal	Nursing piglets	2	2	-	1	-	-
Total			**59**	**17 (29%)**[d]	**3**	**4**	**0**	**0**

[a] PDCoV and PEDV were detected by qRT-PCR, Rota A-C, TGEV, PRCV, and Calicivirus were detected by conventional RT-PCR.

[b] OH, Ohio, US; IN, Indiana, US.

[c] IC, intestinal contents.

[d] Excluding 17 non-clinical sow samples, the percent of PDCoV positives (13/42) in the piglet clinical samples was 31%.

609 **Table 2. Summary of titers of PDCoV isolates grown in cell culture and after serial passage**

Isolates and parameter	Passage numbers													
	P0	P1	P5	P6	P8	P9	P11	P12	P13	P14	P15	P16	P19	P20
OH-FD22 on ST cells														
Viral RNA titers (log_{10} GE/mL)	9.9	9.9	10.7	10.3	10.5	NT[a]	9.4	NT	9.0	8.9	9.5	9.9	9.1	8.9
Infectious titer (log_{10} $TCID_{50}$/ml)	NT	NT	NT	NT	7.0	NT	7.3	NT	8.3	NT	8.0	9.9	8.4	7.5
Virus titer (log_{10} PFU/ml)	NT	NT	NT	7.2*	7.0	NT	6.5	NT	8.5	NT	7.8	7.9	7.6	7.1
OH-FD22 on LLC-PK cells														
Viral RNA titers (log_{10} GE/mL)	10.5	10.6	10.2	10.5	10.5	10.0	9.7	10.1	10.1	NT	10.6	10.0	9.8	10.2
Infectious titer (log_{10} $TCID_{50}$/ml)	NT	NT	8.1	NT	7.6	NT	8.6	9.5	9.1	NT	9.1	10.5	10.5	10.9
Virus titer (log_{10} PFU/ml)	NT	NT	7.3	NT	8.6	NT	9.2*	NT	8.6	NT	8.6	7.2	9.6	9.2
OH-FD22 (DC44) on LLC-PK cells														
Viral RNA titers (log_{10} GE/mL)	11.1	10.0	10.0	10.5	9.7	10.4	10.9	NT	8.8	10.0	9.9	9.9		
Infectious titer (log_{10} $TCID_{50}$/ml)	NT	NT	NT	NT	6.5	NT	NT	9.1	NT	10.5	NT	10.5		
Virus titer (log_{10} PFU/ml)	NT	NT	NT	NT	5.3	7.4*	NT	8.5	NT	8.8	NT	8.5		

610 Note: [a] NT, not tested.

611 *, that passage of virus was used for plaque assay.

Figure Legends

Fig. 1 Cytopathic effects of PDCoV isolates in inoculated LLC-PK and ST cells. LLC-PK or ST cells were inoculated with PDCoV OH-FD22-P19 with different additives (1% pancreatin, 10% SIC plus 10 μg/ml trypsin, and 10 μg/ml trypsin). At PID 1 or 2, cytopathic effects were examined. (A) Mock-inoculated LLC-PK cells, showing normal cells. (B) OH-FD22 inoculated LLC-PK cells with addition of 1% pancreatin at PID 1, showing rounded and clustered cells (arrows). (C) OH-FD22 inoculated LLC-PK cells with addition of 10% SIC plus 10 μg/ml trypsin at PID 1, showing densely granular cells (arrows) and detached cells. (D) Mock-inoculated ST cells, showing normal cells. (E) OH-FD22 inoculated ST cells with 1% pancreatin at PID 2, showing cytopathic effects characterized by cell rounding, clumping together in clusters (arrows). (F) OH-FD22 inoculated ST cells with 10% SIC plus 10 μg/ml trypsin at PID 2, most of cells were detached (arrows). (G) OH-FD22 inoculated ST cells with 10 μg/ml trypsin at PID 2, showing no CPE. (H) OH-FD22 inoculated LLC-PK cells with 10 μg/ml trypsin at PID 1, showing no CPE. (I) OH-FD22 inoculated LLC-PK cells with 10 μg/ml trypsin at PID 2, showing rounded and detached cells. Original magnification, all ×200.

Fig. 2 Detection of PDCoV OH-FD22 isolate in LLC-PK and ST cells by immunofluorescent (IF) staining using hyperimmune pig antiserum against PDCoV. (A) IF staining in OH-FD22-inoculated LLC-PK cells, showing large numbers of IF-stained cells. Original magnification, ×400. (B) IF staining in OH-FD22-inoculated ST cells, showing IF-positive staining mainly evident in the cytoplasm of infected cells. Original magnification, ×400. (C) IF staining in mock-inoculated LLC-PK cells, showing no

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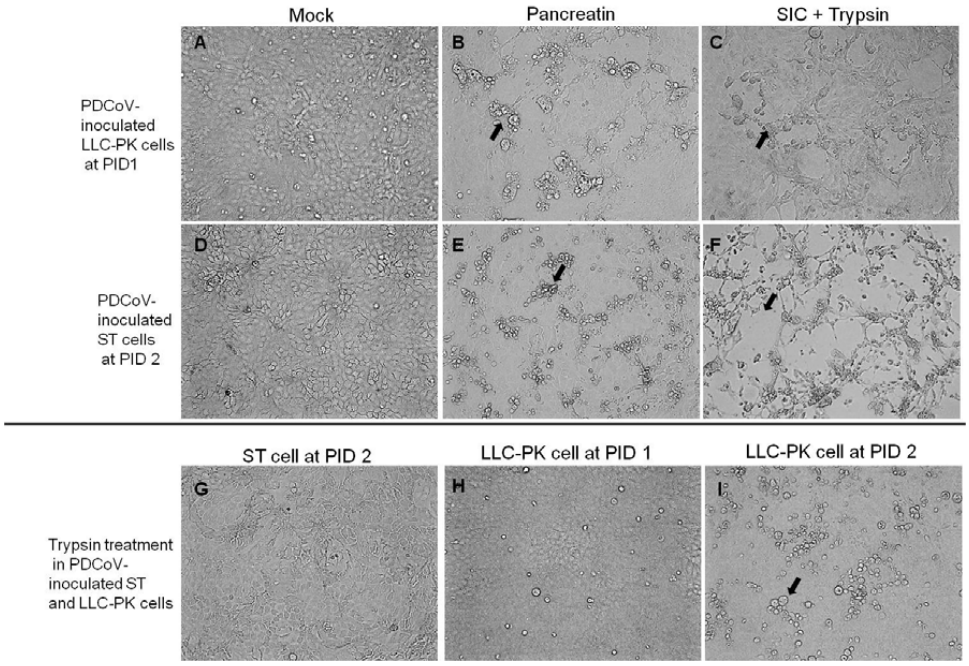
IF-positive cells. Original magnification, ×400. The ST and LLC-PK cells inoculated with OH-FD22 were fixed at PID 1.

Fig. 3 Electron micrographs on PDCoV OH-FD22-inoculated LLC-PK (A) and ST (B) cells. Crown-shaped spikes are visible. The samples were negatively stained with 3% phosphotungstic acid. The magnification bar in the picture represents 100 nm in length.

Fig. 4 The aa locations mapped onto the PDCoV S protein of the mutations that developed upon serial virus passage in LLC-PK and ST cells.

Fig. 5 Phylogenetic analyses of complete S and N gene nucleotide sequences of 7 PDCoV from this study (indicated with triangle) and other published PDCoV sequences, avian Delta CoV, and swine diarrhea related Alpha CoVs (TGEV and PEDV). Reference sequences obtained from GenBank are indicated by strain names and accession numbers. The trees were constructed using the maximum likelihood method of the software MEGA6.06 (http://www.megasoftware.net). Bootstrap analysis was carried out on 1,000 replicate data sets, and values are indicated adjacent to the branching points. Scale bars represent 0.1 (CoV-S) or 0.2 (CoV-N) nucleotide substitutions per site.



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652 Mock-inoculated LLC-PK cells, showing normal cells. (B) OH-FD22 inoculated LLC-PK cells with
653 addition of 1% pancreatin at PID 1, showing rounded and clustered cells (arrows). (C) OH-FD22
654 inoculated LLC-PK cells with addition of 10% SIC plus 10 μg/ml trypsin at PID 1, showing densely
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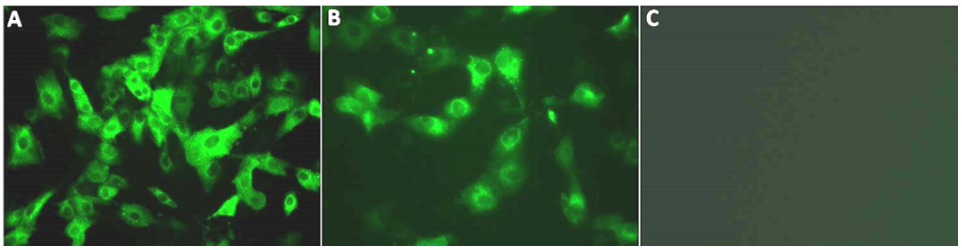

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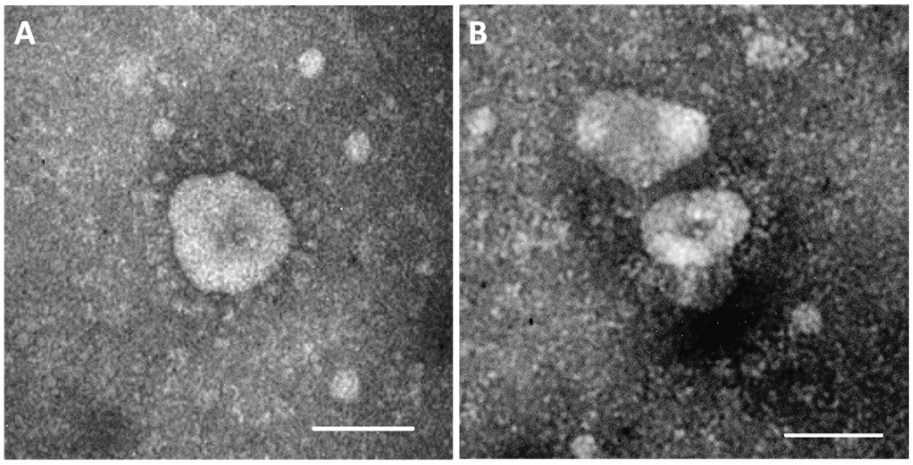

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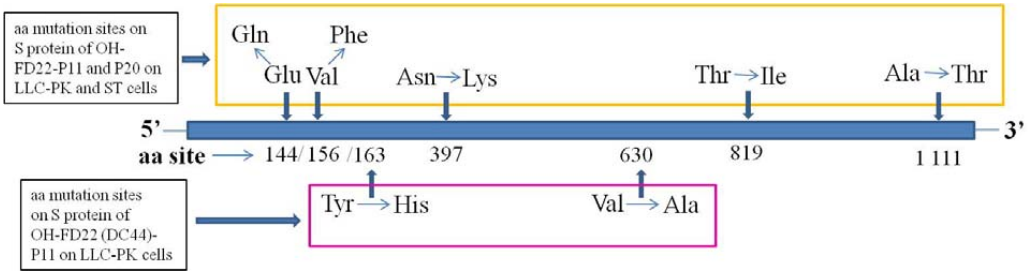

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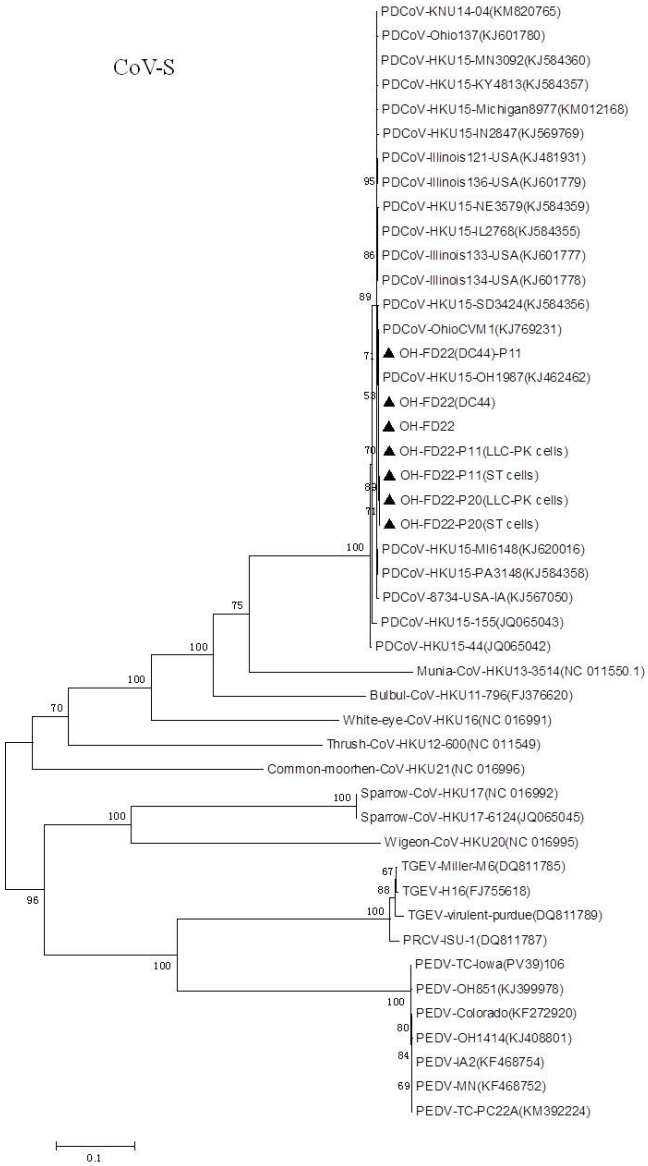

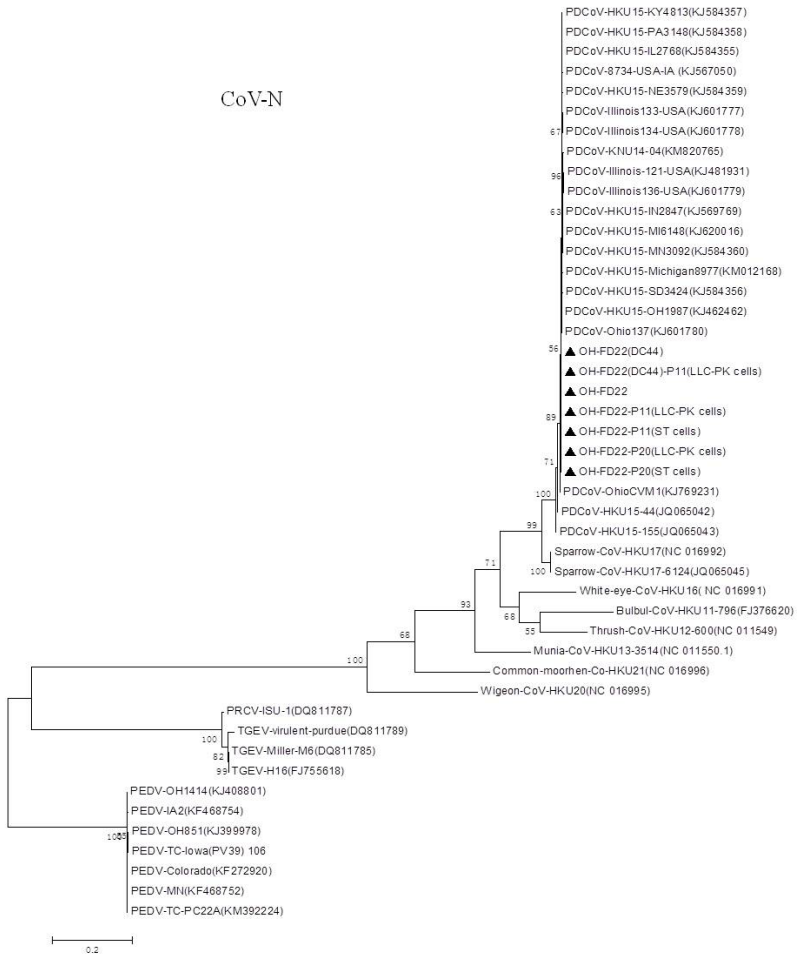

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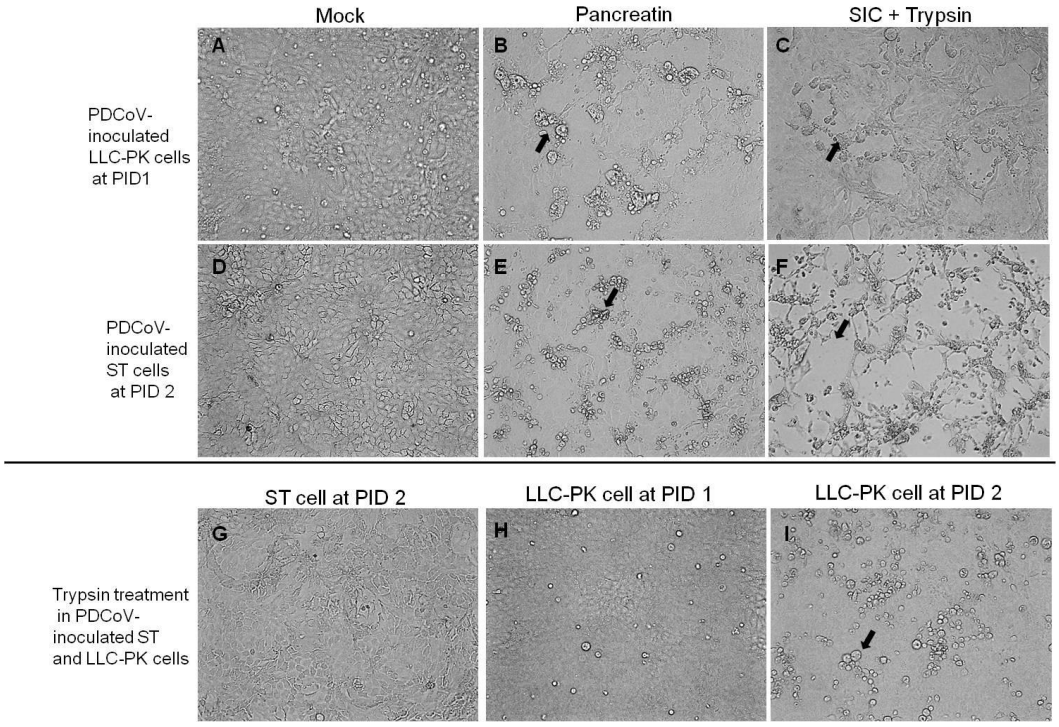
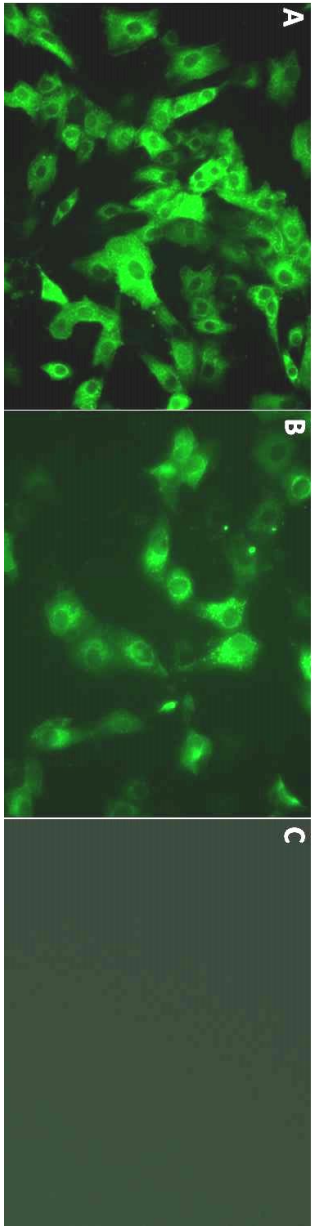
679 carried out on 1,000 replicate data sets, and values are indicated adjacent to the branching points.
680 Scale bars represent 0.1 (CoV-S) or 0.2 (CoV-N) nucleotide substitutions per site.




A
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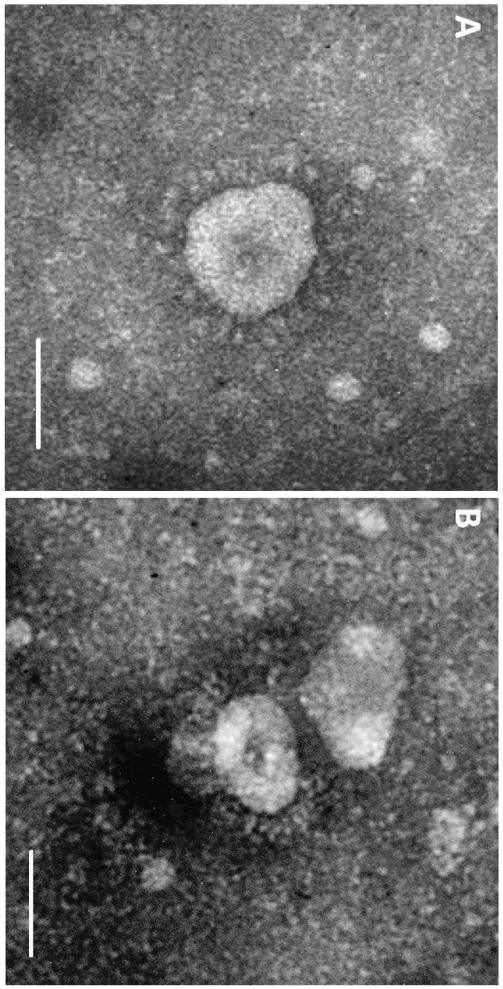
A
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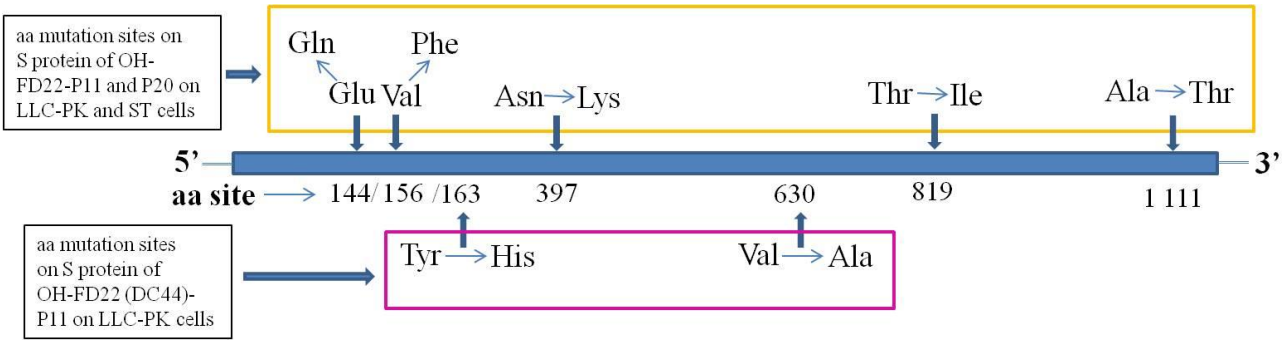

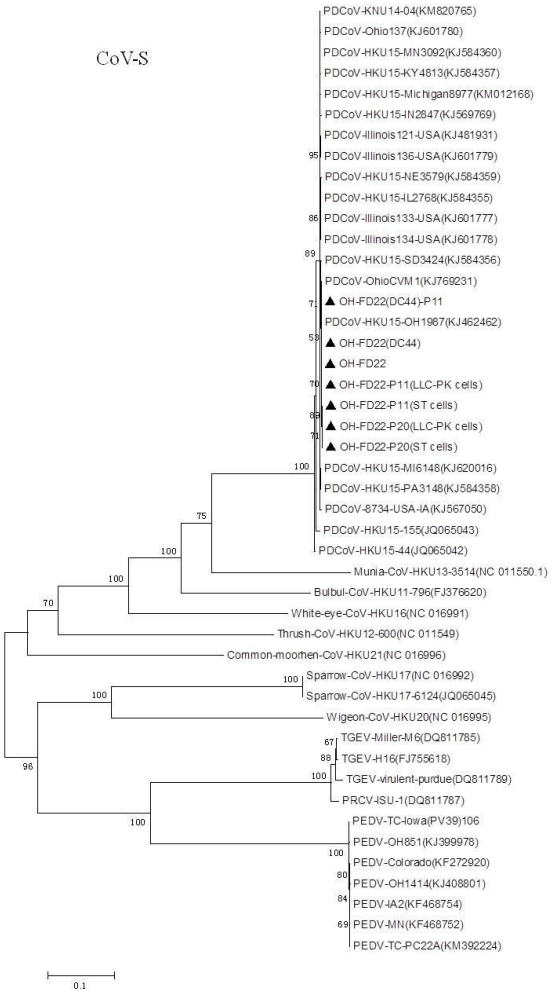

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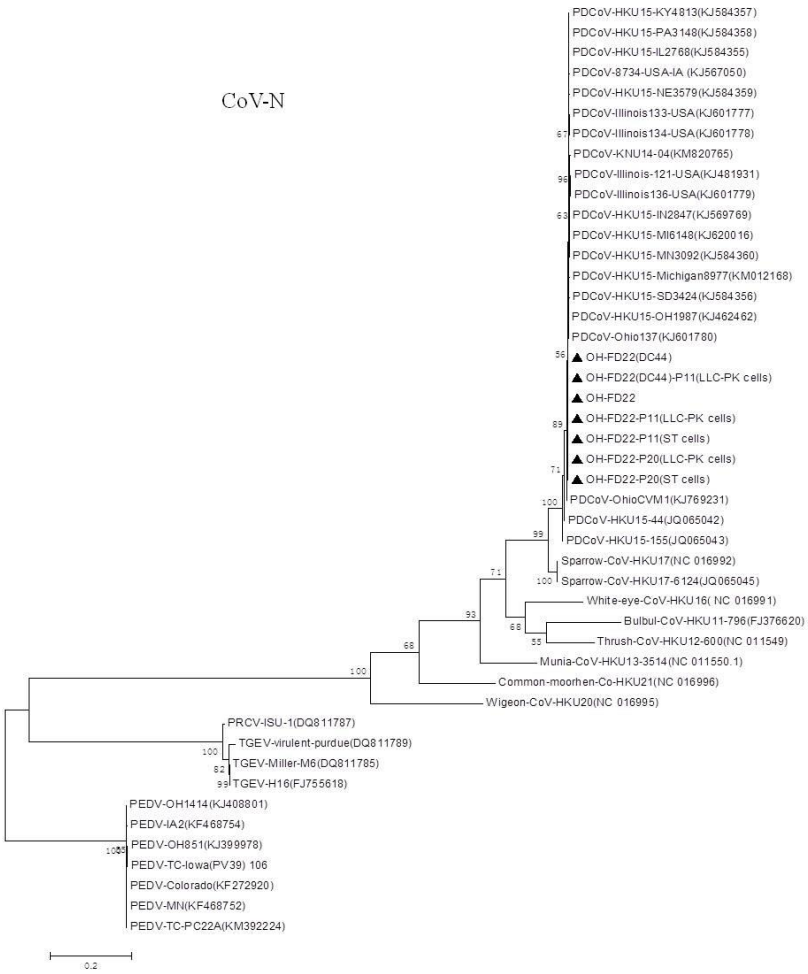

Table 1. Detection of PDCoV and other porcine enteric viruses from field samples from swine herds with suspect PDCoV diarrhea outbreaks in 2014[a]

Farm code	Original samples	Pig age	Sample No.	PDCoV and porcine enteric virus detection No. (% positive)				
				PDCoV	PEDV	Rota C	TGEV /PRCV	Calicivirus
SF-OH[b]	IC[c]	Nursing piglets	12	7	3	1	-	-
	Serum	Sow	6	-	-	-	-	-
	Colostrum	Sow	6	-	-	-	-	-
MR-OH	IC	Nursing piglets	3	2	-	1	-	-
	Fecal	Nursing piglets	4	1	-	1	-	-
	Fecal	Sow	5	4	-	-	-	-
PV-OH	IC	Nursing piglets	16	-	-	-	-	-
CF-OH	IC	Nursing piglets	5	1	-	-	-	-
WH-IN[b]	Fecal	Nursing piglets	2	2	-	1	-	-
Total			**59**	**17 (29%)[d]**	**3**	**4**	**0**	**0**

[a] PDCoV and PEDV were detected by qRT-PCR, Rota A-C, TGEV, PRCV, and Calicivirus were detected by conventional RT-PCR.

[b] OH, Ohio, US; IN, Indiana, US.

[c] IC, intestinal contents.

[d] Excluding 17 non-clinical sow samples, the percent of PDCoV positives (13/42) in the piglet clinical samples was 31%.