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

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Porcine deltacoronavirus (PDCoV) is a novel coronavirus that causes diarrhea in nursing piglets. Following its first detection in the United States in February 2014, additional PDCoV strains have been identified in the United States and Canada. Currently, no treatments or vaccines for PDCoV are available. In this study, U.S. PDCoV strain OH-FD22 from intestinal contents of a diarrheic pig from Ohio was isolated in swine testicular (ST) and LLC porcine kidney (LLC-PK) cell cultures by using various medium additives. We also isolated PDCoV [OH-FD22(DC44) strain] in LLC-PK cells from intestinal contents of PDCoV OH-FD22 strain-inoculated gnotobiotic (Gn) pigs. Cell culture isolation and propagation were optimized, and the isolates were 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 (passage 11 [P11] and P20). Genetically, the S and N genes of the PDCoV isolates were relatively stable during the first 20 passages in cell culture, with only 5 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 to other U.S. PDCoV strains, with the highest sequence similarity to South Korean strain KNU14-04. This is the first report describing cell culture isolation, serial propagation, and biological and genetic characterization of cell-adapted PDCoV strains. The information presented in this study is important for the development of diagnostic reagents, assays, and potential vaccines against emergent PDCoV strains.

Porcine deltacoronaviruses (PDCoVs) belong to the *Deltacoronavirus* genus of the *Coronaviridae* family (1–3). They appear to be newly emerging coronaviruses (CoVs) in pigs in the United States and were isolated 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 found only in nursing pigs. The disease was clinically similar to, but reportedly milder than, disease caused by porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV), with lower mortality rates in 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 new deltacoronaviruses in a variety of mammalian and avian species, with 10% of 169 swine fecal samples tested being positive for PDCoV. 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 United States in early 2014. Marthaler et al. (5) sequenced the genome of the SDCV/USA/Illinois121/2014 (Illinois121/2014) strain (GenBank accession no. KJ481931.1), which had ~99% nucleotide identity to the two Hong Kong PDCoV strains. Another U.S. PDCoV strain from Iowa (USA/IA/2014/8734 [GenBank accession no. KJ567050]) had 98.9% nucleotide identity to the HKU15-44 strain and 99.2% nucleotide identity to the HKU15-155 strain (6). Additionally, PDCoV strain HKU15-OH1987 (GenBank accession no. KJ462462) was identified in fecal and intestinal samples from pigs with diarrheal disease in Ohio and had 99% nucleotide identity to PDCoV strains HKU15-44 and HKU15-155 (4). Subsequently, PDCoVs were detected in 9 other U.S. states, and they share a high level of nucleotide similar-

ity (≥99.8%) with each other and 98.9% to 99.2% nucleotide similarity with the HKU15-44 and HKU15-155 strains (8). Apart from the United States, PDCoV was also detected in 6 farms in Ontario, Canada, in March 2014 (9). Recently, PDCoV strain KUN14-04 (GenBank accession no. KM820765) was also identified in feces from diarrheic piglets in South Korea. This South Korean strain has nucleotide identities of 98.8% to 99.0% to strains HKU15-44 and HKU15-155 and 99.6% to 99.8% to eight U.S. strains (10). Other research groups (8, 9) and our molecular surveillance studies indicated that PDCoV was a common viral pathogen of pigs in the Midwestern United States and that PDCoV coinfections were common, especially with rotavirus group C (Rota C) and PEDV. Our recent study confirmed that PDCoV is enteropathogenic in young pigs, as evident by severe watery diarrhea and/or vomiting and severe atrophic enteritis in all 11- to 14-day-old gnotobiotic (Gn) pigs inoculated with 2 PDCoV strains, OH-FD22 and OH-FD100 (11).

A real-time reverse transcription-PCR (RT-PCR) method was developed by Marthaler et al. (9) to detect PDCoV and has been used to diagnose PDCoV field infections. However, other virological and serological diagnostic assays are lacking. A critical step in

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TABLE 1 Detection of PDCoV and other porcine enteric viruses in field samples from swine herds with suspected PDCoV diarrhea outbreaks in 2014^a

Farm ^b	Original sample type ^c	Pig age	No. of samples	No. (%) of samples positive for PDCoV and porcine enteric virus				
				PDCoV	PEDV	Rota C	TGEV/PRCV	Calicivirus
SF-OH	IC	Nursing piglet	12	7	3	1		
	Serum	Sow	6					
	Colostrum	Sow	6					
MR-OH	IC	Nursing piglet	3	2		1		
	Feces	Nursing piglet	4	1		1		
	Feces	Sow	5	4				
PV-OH	IC	Nursing piglet	16					
CF-OH	IC	Nursing piglet	5	1				
WH-IN	Feces	Nursing piglet	2	2		1		
Total			59	17 (29) ^d	3	4	0	0

^a PDCoV and PEDV were detected by qRT-PCR; Rota A to Rota C, TGEV, PRCV, and calicivirus were detected by conventional RT-PCR.

^b OH, Ohio; IN, Indiana.

^c IC, intestinal contents.

^d Excluding 17 nonclinical sow samples, the percentage of PDCoV-positive piglet clinical samples (13/42) was 31%.

the development of PDCoV diagnostic assays and potential future vaccines is the isolation of PDCoV in cell culture.

Here, we report the first isolation, to our knowledge, of a PDCoV strain from intestinal contents collected from a diarrheic pig from Ohio, in swine testicular (ST) and LLC porcine kidney (LLC-PK) cell cultures. We also isolated PDCoV from the intestinal contents of Gn pigs inoculated orally with the original sample, OH-FD22, in cell culture. The cell culture isolation and propagation procedures were optimized, and the isolates were successfully serially propagated in cell culture for >20 passages. In addition to characterizing virus growth during serial passage in cell culture, the spike (S) and nucleocapsid (N) gene sequences from the original sample, the Gn pig-passaged virus, and selected cell culture passages were determined to compare their genetic sequences with other PDCoV sequences by phylogenetic analysis. The results of this study are critical for the development of 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 (aged 1 to 7 days) on different farms with diarrhea outbreaks in Ohio and Indiana (Table 1). On 2 farms, acute-phase 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 TaqMan real-time quantitative RT-PCR (qRT-PCR) targeting the membrane (M) gene (nucleotides [nt] 23395 to 23466), as reported previously (9). All PDCoV-positive samples were tested for other swine enteric viruses, including PEDV, rotavirus group A (Rota A) to Rota 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 (C_T) 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. PDCoV strain OH-FD22 from farm SF-OH, 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 $1,847 \times g$ at 4°C for 10 min. The supernatant was filtered through a 0.22- μ m syringe filter (Millipore, USA) and used as the inoculum for the Gn pigs or for virus isolation in cell culture.

Inoculation of Gn pigs with PDCoV strain OH-FD22. 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 log₁₀ genomic equivalents (GE) per pig. Clinical signs were monitored, and viral shedding in rectal swab samples was tested by using qRT-PCR. Pig 1 was euthanized after the 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 OH-FD22(DC44) and was also used as the 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 (p.i.) day 30, pig 2 was immunized intramuscularly with the semi-purified PDCoV from Gn pig-passaged OH-FD22 after mixing with an equal volume of Freund's complete adjuvant (Sigma-Aldrich) (17). On p.i. day 44, the pig was reinoculated 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 OH-DC97.

The Institutional Animal Care and Use Committee (IACUC) of the Ohio State University approved all protocols related to the animal experiments in this study.

The OH-FD22(DC44) sample was diluted 10-fold with minimum essential medium (MEM) (Gibco, USA), mixed, and centrifuged at $1,847 \times g$ at 4°C for 10 min. The supernatant was filtered by using a 0.22- μ m syringe filter and used as the inoculum for cell cultures to isolate PDCoV.

Virus isolation and propagation in cell lines of swine origin. The ST cell line (ATCC CRL1746) and the 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 se-

rum, 1% MEM nonessential 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) and then inoculated with the filtered samples. After adsorption for 60 min at 37°C in 5% CO₂, cells were washed 3 times, and maintenance medium was added. The cell cultures were observed for cytopathic effect (CPE).

For the first inoculation, cells were cultured in 6-well plates, and 300 µl of the inoculum was added to each well. When >80% CPE was evident in the inoculated cell monolayers (around p.i. day 5), the plates were frozen at -80°C and thawed twice. The cells and supernatants were harvested together, the 0-h postinoculation (hpi) and p.i. day 5 samples were tested by qRT-PCR, and the difference in cycle threshold (ΔC_T) values was calculated. These samples were used as seed stocks (passage zero [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 (TCID₅₀), 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-virus inoculation, and (iii) different concentrations (1%, 10%, and 20%) of SIC from healthy uninfected Gn pigs were added pre- or post-virus inoculation. The SIC were prepared in our laboratory as described previously by Flynn and Saif (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 by using the 5×MagMAX-96 virus isolation kit (Ambion by Life Technologies, USA) and the RNA extraction robot MagMax Express (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The viral RNA was eluted with 50 µl of elution buffer and used as the template for RT-PCR and qRT-PCR.

RT-PCR and qRT-PCR based on the PDCoV M gene. Initial screening for the M gene of PDCoV was performed by qRT-PCR as reported previously by Marthaler et al. (5, 9). qRT-PCR was conducted by 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 using the system software. The RT-PCR method was also applied by amplifying a 541-bp fragment of the M gene that covered the qRT-PCR-amplified fragment. Primers (5'-CGCGTAATCGTGTGATCTATGT-3' and 5'-CCGGCCTTTGAAGTGGTTAT-3') were designed according to the sequence of a U.S. strain, Illinois121/2014 (GenBank accession no. [KJ481931](#)). The PCR products were purified by using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA), sequenced, and then used as the template to construct a qRT-PCR standard curve. The detection limit of qRT-PCR was 10 GE per reaction, corresponding to 4.6 log₁₀ GE/ml.

Virus titration and purification by a plaque assay. A plaque assay for PDCoV was developed with modifications of an assay for PEDV described previously by Oka et al. (16). 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 6-well plates and grown to 100% confluence after 24 h. The growth medium was replaced with maintenance medium (without trypsin). Following 1 h of incubation at 37°C, the cells were washed once with maintenance medium. Duplicate wells were then inoculated with 10-fold serially diluted virus (0.3 ml/well) and incubated for 1 h at 37°C in an atmosphere of 5% CO₂. The virus inoculum was removed,

and cells were washed 2 times with Dulbecco's PBS (DPBS) without Mg²⁺ and Ca²⁺ (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 was added to each well. The plates were stained with 0.01% neutral red (Sigma) for 3 h at 37°C on p.i. days 2 to 3. The plaques were counted under oblique light and confirmed by using a light microscope (CK2; Olympus, Japan). After the viral plaques were enumerated by counting, the plaque titers were expressed as PFU per milliliter.

For virus plaque purification, uniform and clear plaques were picked by 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 to 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 a TCID₅₀ assay. LLC-PK cells were seeded into 96-well plates, and after confluence, the monolayers were washed once with maintenance medium with 10 µg/ml of trypsin (MMT). One hundred microliters of 10-fold dilutions of PDCoV was inoculated in eight replicates per dilution. After absorption for 1 h, another 100 µl of MMT was added to each well. Viral CPE was monitored for 5 to 7 days, and virus titers were calculated by using the Reed-Muench method (19) and expressed as TCID₅₀ per milliliter.

Immunofluorescence (IF) assay. PDCoV-infected ST cells and LLC-PK cells in 6-well plates were fixed with 100% ethanol at 4°C overnight and then washed 5 times with PBS and blocked with 5% bovine serum albumin (BSA) at 37°C for 1 h. The OH-DC97 hyperimmune antiserum (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). A 1:100 dilution of affinity-purified fluorescein-labeled goat anti-pig IgG(H+L) (Kpl, MD, USA) was then added, and the plates were incubated for 1 h at 37°C and then washed 6 times with PBST. Cell staining was examined by using a fluorescence microscope (IX-70; Olympus, Japan).

Immunoelectron microscopy. Immunoelectron microscopy (IEM) was conducted by incubating virus samples with the OH-DC97 Gn pig antiserum, as described previously (18). For visualization of the virion particles in infected-cell culture medium, 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 by using an ultracentrifuge (Beckman Coulter, Miami, FL, USA). Virus pellets were resuspended in MEM. The purified samples were incubated with the OH-DC97 antiserum (diluted 20-fold) overnight at 4°C.

For negative-staining IEM, the prepared cell culture samples and antiserum mixtures were stained with an equal volume of 3% phosphotungstic acid (PTA) (pH 7.0) in 0.4% sucrose for 1 min and then applied onto 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 passage 20 (OH-FD22-P20-ST and OH-FD22-P20-LLC-PK) samples were amplified, cloned, and sequenced. The S genes were amplified by using primers PDCoV-SF2 (5'-AGCGTTGACACCAACCTATT-3') and PDCoV-SR2 (5'-TCGTCGACTACCATTCTTCAAC-3'). The N genes were amplified with primers PDCoV-NF1 (5'-CCATC GCTCCAAGTCATTCT-3') and PDCoV-NR1 (5'-TGGGTGGGTTTAA

TABLE 2 Summary of titers of PDCoV isolates grown in cell culture and after serial passage

Passage	OH-FD22 on ST cells			OH-FD22 on LLC-PK cells			OH-FD22(DC44) on LLC-PK cells		
	Viral RNA titer (log ₁₀ GE/ml)	Infectious titer (log ₁₀ TCID ₅₀ /ml)	Virus titer (log ₁₀ PFU/ml)	Viral RNA titer (log ₁₀ GE/ml)	Infectious titer (log ₁₀ TCID ₅₀ /ml)	Virus titer (log ₁₀ PFU/ml)	Viral RNA titer (log ₁₀ GE/ml)	Infectious titer (log ₁₀ TCID ₅₀ /ml)	Virus titer (log ₁₀ PFU/ml)
P0	9.9	NT ^a	NT	10.5	NT	NT	11.1	NT	NT
P1	9.9	NT	NT	10.6	NT	NT	10	NT	NT
P5	10.7	NT	NT	10.2	8.1	7.3	10	NT	NT
P6	10.3	NT	7.2 ^b	10.5	NT	NT	10.5	NT	NT
P8	10.5	7	7	10.5	7.6	8.6	9.7	6.5	5.3
P9	NT	NT	NT	10	NT	NT	10.4	NT	7.4 ^b
P11	9.4	7.3	6.5	9.7	8.6	9.2 ^b	10.9	NT	NT
P12	NT	NT	NT	10.1	9.5	NT	NT	9.1	8.5
P13	9	8.3	8.5	10.1	9.1	8.6	8.8	NT	NT
P14	8.9	NT	NT	NT	NT	NT	10	10.5	8.8
P15	9.5	8	7.8	10.6	9.1	8.6	9.9	NT	NT
P16	9.9	9.9	7.9	10	10.5	7.2	9.9	10.5	8.5
P19	9.1	8.4	7.6	9.8	10.5	9.6			
P20	8.9	7.5	7.1	10.2	10.9	9.2			

^a NT, not tested.^b This passage of virus was used for plaque assays.

CAGACATAG-3'). All the primers were designed according to the sequence of the U.S. PDCoV strain Illinois121/2014.

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

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

Nucleotide sequence accession numbers. The complete S and N genes of the OH-FD22, OH-FD22(DC44), OH-FD22-P11-ST, OH-FD22-P11-LLC-PK, OH-FD22(DC44)-P11-LLC-PK, OH-FD22-P20-ST, and OH-FD22-P20-LLC-PK strains were deposited in GenBank under accession numbers [KP995356](#) to [KP995369](#).

RESULTS

Pathogen detection. A total of 59 samples were collected from 5 pig farms in Ohio and Indiana 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 nonclinical sow samples (colostrum, serum, and feces), the percentage of PDCoV-positive samples 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, Rota B, and caliciviruses. Seven (41.2%) of the PDCoV-positive samples were positive for PEDV (3 samples) or Rota C (4 samples) (Table 1).

Virus isolation and propagation in LLC-PK cell monolayers.

LLC-PK cell monolayers were inoculated with 10 of the samples that were positive for PDCoV only. Only the OH-FD22-inoculated cell monolayers showed visible CPE in the form of enlarged, rounded cells at p.i. day 2 that rapidly detached from the monolayers on p.i. day 3. To confirm PDCoV replication on LLC-PK cells, viral RNA was extracted from the inoculated cells at p.i. day 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, PDCoV (OH-FD22 strain) was successfully isolated in cell culture from the intestinal contents of a piglet from farm SF-OH (collected in February 2014). PDCoV from the first passage on LLC-PK cells was designated OH-FD22-P0.

The OH-FD22-P0 virus was further serially passaged in LLC-PK cells for a total of 20 passages (P1 to P20). During the passages, different culture conditions were compared: cultures with or without exogenous trypsin, with the addition of SIC from healthy Gn pigs pre- or postinoculation, with the addition of different concentrations of pancreatin pre- or postinoculation, and with or without washing of the cell monolayers after virus incubation. The results showed that the OH-FD22 strain replicated in LLC-PK cells under several of these conditions. The optimal cell culture conditions used to propagate PDCoV on LLC-PK cells were as follows: washing of cells with MMT 2 times, virus incubation for an hour, and then washing (with MMT) and the addition of MMT. CPE was usually observed by p.i. day 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 (~5 log₁₀ PFU/ml) was lower than that with trypsin (10 µg/ml) treatment (~9 log₁₀ PFU/ml). The pancreatin or SIC/trypsin-treated groups

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 μ g/ml trypsin, and 10 μ g/ml trypsin). At p.i. day 1 or 2, cytopathic effects were examined. (A) Mock-inoculated LLC-PK cell culture showing normal cells. (B) OH-FD22-inoculated LLC-PK cells with the addition of 1% pancreatin at p.i. day 1 showing rounded and clustered cells (arrows). (C) OH-FD22-inoculated LLC-PK cells with the addition of 10% SIC plus 10 μ g/ml trypsin at p.i. day 1 showing densely granular cells (arrows) and detached cells. (D) Mock-inoculated ST cell culture showing normal cells. (E) OH-FD22-inoculated ST cells with 1% pancreatin at p.i. day 2 showing cytopathic effects characterized by cell rounding, with cells clumping together in clusters (arrows). (F) OH-FD22-inoculated ST cells with 10% SIC plus 10 μ g/ml trypsin at p.i. day 2, where most of the cells were detached (arrows). (G) OH-FD22-inoculated ST cells with 10 μ g/ml trypsin at p.i. day 2 showing no CPE. (H) OH-FD22-inoculated LLC-PK cells with 10 μ g/ml trypsin at p.i. day 1 showing no CPE. (I) OH-FD22-inoculated LLC-PK cells with 10 μ g/ml trypsin at p.i. day 2 showing rounded and detached cells. Original magnification for all panels, $\times 200$.

showed earlier evident CPE than did the trypsin treatment group (Fig. 1B and C), but the virus titers ($\sim 7 \log_{10}$ PFU/ml) were lower than those for the trypsin (10 μ g/ml) treatment group ($\sim 9 \log_{10}$ PFU/ml). Virus growth was confirmed by IF staining using the OH-DC97 antiserum. The PDCoV antigens were located mostly

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 ~ 150 to 180 nm in diameter with typical spike surface

FIG 2 Detection of PDCoV isolate OH-FD22 in LLC-PK and ST cells by IF staining using hyperimmune pig antiserum against PDCoV. (A) IF staining of OH-FD22-inoculated LLC-PK cells showing large numbers of IF-stained cells. Original magnification, $\times 400$. (B) IF staining of OH-FD22-inoculated ST cells showing IF-positive staining mainly in the cytoplasm of infected cells. Original magnification, $\times 400$. (C) IF staining of mock-inoculated LLC-PK cells showing no IF-positive cells. Original magnification, $\times 400$. ST and LLC-PK cells inoculated with OH-FD22 were fixed at p.i. day 1.

FIG 3 Electron micrographs of 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. Bar, 100 nm.

projections were visible in the samples, as confirmed by negative-staining IEM.

OH-FD22(DC44) virus (LIC of Gn pigs inoculated with OH-FD22) was also inoculated onto cell monolayers under the same culture conditions as those for the OH-FD22 strain. The OH-FD22(DC44) virus was successfully isolated in LLC-PK cells and induced CPE similar to that induced by the OH-FD22 strain. The OH-FD22(DC44) virus was serially passaged in LLC-PK cells for a total of 16 passages. The viral RNA titers of OH-FD22(DC44) propagated in LLC-PK cells were similar (~ 8.8 to $11.1 \log_{10}$ GE/ml) to the OH-FD22 viral RNA titers (Table 2).

Virus isolation and propagation in ST cell monolayers. The original OH-FD22 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 p.i. day 7, and the virus RNA titer was increased compared to that at 0 hpi, with a ΔC_T of 7 and detachment of cells as the major visible CPE. The virus harvested from the ST cells was designated OH-FD22-P0. 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: culture with or without trypsin, with the addition of SIC from healthy Gn pigs pre- or postinoculation, and with the addition of different concentrations of pancreatin pre- or postinoculation. PDCoV replicated in ST cells only when 10% Gn pig 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 as follows: washing in maintenance medium 2 times, incubation of virus for 1 h, and then washing of monolayers and the addition of maintenance medium with 1% pancreatin. The OH-FD22 isolate was further serially passaged 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 on p.i. days 2 to 3 (Fig. 1E and F). The trypsin-alone group showed

no CPE (Fig. 1G), whereas the pancreatin- or SIC/trypsin-treated groups showed evident CPE (Fig. 1E and F). Virus titers in the pancreatin- or SIC/trypsin-treated groups tested at p.i. day 3 were similar. Virus growth was also confirmed by qRT-PCR (Table 2), an immunofluorescence assay (IFA) (Fig. 2B), and IEM (Fig. 3B), as noted for virus propagated in LLC-PK cells.

Virus titration and purification by a plaque assay. During 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 compared with those at 0 hpi (data not shown). However, the RNA virus titers in LLC-PK cells (9.7 to $10.6 \log_{10}$ GE/ml; average of $10.2 \log_{10}$ GE/ml) were usually higher than those in ST cells (8.9 to $10.7 \log_{10}$ GE/ml; average of $9.7 \log_{10}$ GE/ml). The infectious titers of some passages were determined by a TCID₅₀ assay on LLC-PK cells. The TCID₅₀ titers of OH-FD22 ranged from 7.6 to $10.9 \log_{10}$ TCID₅₀/ml in LLC-PK cells, which were higher than those in ST cells (range of 7.0 to $9.9 \log_{10}$ TCID₅₀/ml) (Table 2). The virus titers for some passages were also determined by a 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 those of the OH-FD22 strain in LLC-PK cells (Table 2).

A plaque assay was used to isolate and purify PDCoV on ST cells. OH-FD22-P6 virus in ST cells ($7.2 \log_{10}$ PFU/ml) was used for 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 the 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).

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

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 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 passage 11 [OH-FD22-P11-ST, OH-FD22-P11-LLC-PK, and OH-FD22(DC44)-P11-LLC-PK] and passage 20 (OH-FD22-P20-ST and OH-FD22-P20-LLC-PK) strains were amplified and sequenced. All the sequenced S genes were 3,483 nucleotides long, encoding a protein of 1,161 amino acids (aa). The S genes examined in this study shared 99.9% to 100% nucleotide identities with each other, and they shared 98.5% to 100% nucleotide identities with the other 20 PDCoV strains available in GenBank. The S gene of the field strain OH-FD22 had the highest nucleotide similarity (100%) with the Ohio HKU15-OH1987 strain. Compared with the OH-FD22 strain, OH-FD22-P11 and -P20, passed in both ST and LLC-PK cells, each had five nucleotide changes, at positions 430, 466, 1191, 2456, and 3331 (nucleotides are numbered according to the S genes of the PDCoV HKU15-OH1987 and OH-FD22 sequences). These nucleotide changes all induced the corresponding amino acid 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 1111) (Fig. 4). OH-FD22(DC44)-P11 acquired two nucleotide changes, at positions 487 and 1890, compared with OH-FD22(DC44), and these two sites also induced amino acid 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 sustained through P20, with 100% nucleotide identity in P11 and P20. The PDCoV S protein contained 27 potential glycosylation sites (Asn-Xaa-Ser/Thr sequences). Further analysis demonstrated that no amino acid changes were located in any of the predicted N-linked glycosylation sites.

A phylogenetic tree was constructed by using the entire S gene sequences of 27 PDCoVs (20 obtained from GenBank and 7 new sequences generated in this study), some newly detected avian deltacoronaviruses, and several TGEV/PRCV and PEDV isolates (alphacoronaviruses). Phylogenetic analysis of the S genes showed that all PDCoV strains clustered together in one clade of the *Deltacoronavirus* genus and were distinct from all avian deltacoronaviruses. Furthermore, analysis of the S genes of PDCoV strains indicated that all the U.S. strains clustered into a subclade with a PDCoV strain, KNU14-04, from South Korea isolated in 2014, while PDCoV strains HKU15-44 and HKU15-155, isolated in Hong Kong in 2012, were clustered separately. Additionally, the

OH-FD22-related strains in the current study were most closely related to two other PDCoV strains from Ohio (HKU15-OH1987 and OhioCVM1 strains), whereas strain Ohio137 clustered in another sublineage with most of the U.S. strains (Fig. 5).

Phylogenetic analysis of the N genes of PDCoV isolates before and after serial passages in cell cultures and in Gn pigs. The N genes of these sequenced samples were determined to be 1,029 nucleotides long, coding for a polypeptide of 342 amino acids. The N genes shared 100% nucleotide identities with each other, and they shared 98.9% to 99.9% nucleotide identities with the other 20 PDCoV strains available in GenBank. Phylogenetic analyses of the N genes revealed that they belonged to the same groups as in 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 subcluster in the S gene tree (Fig. 5).

DISCUSSION

Following the first detection of PDCoV in the United States in February 2014, additional PDCoV strains have been identified in swine farms in Canada and the United States. The prevalence of PDCoV in pigs in North America was reported to be >25% on the farms surveyed (4, 8, 9). Higher rates of PDCoV positivity were reported in Ohio. Wang et al. (4) noted a rate of PDCoV positivity of 92.9% for 5 farms in Ohio. Our results showed a rate of PDCoV positivity of 29% for 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 this 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 Gn pig-passaged PDCoV in cell culture. Propagation of PDCoV was confirmed by the detection of 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 vaccine development.

We attempted to isolate virus from 4 fecal and 6 intestinal content samples that were positive for PDCoV, but only the PDCoV OH-FD22 strain was successfully isolated in ST and LLC-PK cells. This low rate of success of PDCoV isolation might be associated with the poor quality of the original samples. Samples positive by qRT-PCR may contain noninfectious or low-titer virus. Also, storage conditions that were suboptimal for the early

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

FIG 5 continued

samples and freezing-thawing may disrupt the PDCoVs. None of the attempts at virus isolation from fecal samples were successful. Based on the limited numbers of clinical samples, we cannot explain the difference in the successful isolation of PDCoV from intestinal contents versus the failure to isolate PDCoV from fecal samples. During virus isolation, cell toxicity was observed for 2 of the intestinal content samples. These results indicate that sample types, virus viability, infectious-virus titers, and substances in intestinal contents may be important factors that influence virus isolation. Furthermore, isolation procedures need to be improved to increase the rate of success of PDCoV isolation, particularly with 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 p.i. days 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 low. During CoV infection, the cleavage of the 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 the PDCoV S protein or for virus entry, replication, or release. The conditions required for the propagation of PDCoV in ST cells differed from those of LLC-PK cells. OH-FD22-P0 grew well in ST cells with trypsin, but when serially passaged in ST cell cultures without trypsin in the medium, replication ceased. Based on the extensive experience in isolating swine enteric viruses in our laboratory (18), SIC from a noninoculated 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. Substances in SIC may be beneficial for PDCoV attachment to its receptor and/or virus entry or for 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. Thus, commercial pancreatin, reported to be an important factor in adapting a porcine group C rotavirus to primary porcine kidney (PPK) cell cultures (18, 22), was used for PDCoV growth by adding it to the cell maintenance medium (no trypsin) postinoculation, and successful serial propagation of PDCoV in ST cells was accomplished. Thus, pancreatin or SIC in the cell maintenance medium was critical for PDCoV propagation in ST cells. Even at later passages, when pancreatin or SIC was excluded from the medium, PDCoV infectivity was reduced (data not shown). Since the exact roles of these growth-promoting factors in 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 addition of SIC or pancreatin.

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

To characterize the virus isolates, the complete S and N genes were sequenced and analyzed, and the phylogenetic relationships among the PDCoV strains were determined. The seven PDCoVs examined in this study are genetically closely related, with 99.9% to 100% nucleotide identities at the S and N gene levels. They also shared 98.5% to 100% nucleotide identities with 20 other PDCoV strains available in GenBank. Phylogenetic trees constructed by using the entire S and N gene sequences showed that all PDCoV strains clustered into one clade in the genus *Deltacoronavirus*, and they were distinct from the avian deltacoronaviruses (Fig. 5). Our results are consistent with data from previous reports (4, 8). These findings suggest that PDCoV strains currently circulating in multiple U.S. states are closely related. Notably, PDCoV strain KNU14-04, isolated from South Korea in 2014, was most closely related to the emerging U.S. PDCoV strains, suggesting that they may be derived from a similar ancestral strain. Furthermore, the PDCoV HKU15-155 and HKU-44 strains isolated in Hong Kong in 2012 belong to a different subcluster, suggesting that the U.S. PDCoVs may have emerged independently from the initially reported PDCoV strains from 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 U.S. PDCoVs 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 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 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. OH-FD22(DC44)-P11 had acquired two nucleotide changes compared with OH-FD22(DC44). These nucleotide changes all induced the corresponding amino acid changes. Our study revealed that the five amino acid changes in the S gene of the OH-FD22 strain were retained after serial passages in both cell cultures, suggesting that common mechanisms may govern PDCoV cell culture adaptation in both swine cell lines. Additionally, the nucleotide and the corresponding amino acid 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

virus adaptation to replication *in vitro*. However, whether these amino acid changes could alter the efficiency of viral replication and the viral pathogenicity of the emergent U.S. PDCoV strains needs to be investigated further. Since there were no amino acid changes in the predicted N-glycosylation sites of the S genes, they may be important for virus replication *in vitro*.

In conclusion, PDCoV strains OH-FD22 and OH-FD22(DC44), 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 cell culture-adapted PDCoV strains. The information presented in this study is important for the development of diagnostic reagents, assays, and potential vaccines against emergent PDCoV strains.

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We declare that we have no conflicts of interest.

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