RESEARCH ARTICLE

Origin, Evolution, and Virulence of Porcine Deltacoronaviruses in the United States

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ABSTRACT **A novel porcine deltacoronavirus (PdCV) was first discovered in Ohio and Indiana in February 2014, rapidly spread to other states in the United States and Canada, and caused significant economic loss in the swine industry. The origin and virulence of this novel porcine coronavirus are not known. Here, we characterized U.S. PdCV isolates and determined their virulence in gnotobiotic and conventional piglets. Genome analyses revealed that U.S. PdCV isolates possess unique genetic characteristics and share a close relationship with Hong Kong and South Korean PdCV strains and coronaviruses (CoVs) of Asian leopard cats and Chinese ferret-badgers. The PdCV-positive intestinal content (Ohio CVM1) and the cell culture-adapted PdCV Michigan (MI) strain were orally inoculated into gnotobiotic and/or conventional piglets. Within 1 to 3 days postinfection, profuse watery diarrhea, vomiting, and dehydration were observed. Clinical signs were associated with epithelial necrosis in the gastric pits and small intestine, the latter resulting in severe villous atrophy. Mild interstitial pneumonia was identified in the lungs of PdCV-infected piglets. High levels of viral RNA (8 to 11 log RNA copies/g) were detected in intestinal tissues/luminal contents and feces of infected piglets, whereas moderate RNA levels (2 to 5 log RNA copies/g) were detected in blood, lung, liver, and kidney, indicating multisystemic dissemination of the virus. Polyclonal immune serum against PdCV but not immune serum against porcine epidemic diarrhea virus (PEDV) reacted with PdCV-infected small-intestinal epithelial cells, indicating that PdCV is antigenically distinct from PEDV. Collectively, we demonstrate for the first time that PdCV caused severe gastrointestinal diseases in swine.**

IMPORTANCE **Porcine coronaviruses (CoVs) are major viral infectious diseases of swine. Examples of porcine CoVs include porcine transmissible gastroenteritis coronavirus (TGEV), porcine epidemic diarrhea virus (PEDV), and porcine respiratory coronavirus (PRCV). In February 2014, another porcine CoV, porcine deltacoronavirus (PdCV), emerged in Ohio and Indiana and subsequently spread rapidly across the United States and Canada, causing significant economic losses. Here, we report the detailed genetic characterization, phylogeny, and virulence of emergent PdCV strains in the United States. We found that PdCV caused severe diarrhea, vomiting, and dehydration in gnotobiotic and conventional piglets, signs that were clinically indistinguishable from those caused by PEDV and TGEV. In addition to extensive intestinal lesions, PdCV caused significant lesions in the stomach and mild pulmonary lesions that have not been reported for TGEV and PEDV. The finding that PdCV is a significant enteric disease of swine highlights the need to develop effective measures to control this disease.**

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Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses in the family *Coronaviridae* that is within the order *Nidovirales*. The *Coronaviridae* contain at least four major genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. Porcine CoVs are significant enteric and respiratory pathogens of swine. In 1946, porcine transmissible gastroenteritis virus (TGEV), an alphacoronavirus (α-CoV), was identified as the cause of a devastating enteric disease of pigs in the United States (1). A second U.S. porcine α-CoV, porcine respiratory coronavirus (PRCV), was officially identified in 1984 (2). PRCV is a deletion mutant of TGEV that alters viral tropism from intestinal to respiratory epithelia (3, 4). In swine, PRCV replicates almost exclusively in the respiratory tract and closely resembles other porcine viral pneumonias (2–4). In 1971, a new α-CoV, porcine epidemic diarrhea virus (PEDV), was identified in the United Kingdom (5). Both TGEV and PEDV replicate in small-intestinal enterocytes, causing life-threatening acute enteric disease in suckling piglets that is characterized by profuse watery diarrhea, emesis, and resultant dehydration (1, 6, 7). Morbidity rates are high (80 to 100%), as are mortality rates (50 to 90%) (1, 8). Since 2010, variant strains of PEDV differing in sequences from the classic European strain (CV777) have appeared in China, South Korea, Japan, and many Asian countries, causing up to 100% mortality in suckling piglets (8, 9). In May 2013, PEDV was identified as a new cause of neonatal diarrhea in Iowa (10, 11). Infection rapidly spread to more than 30 states, Canada, and Mexico and caused significant economic losses in the swine industry (12–14). Sequence analyses suggest that U.S. PEDV strains originated from China (12).

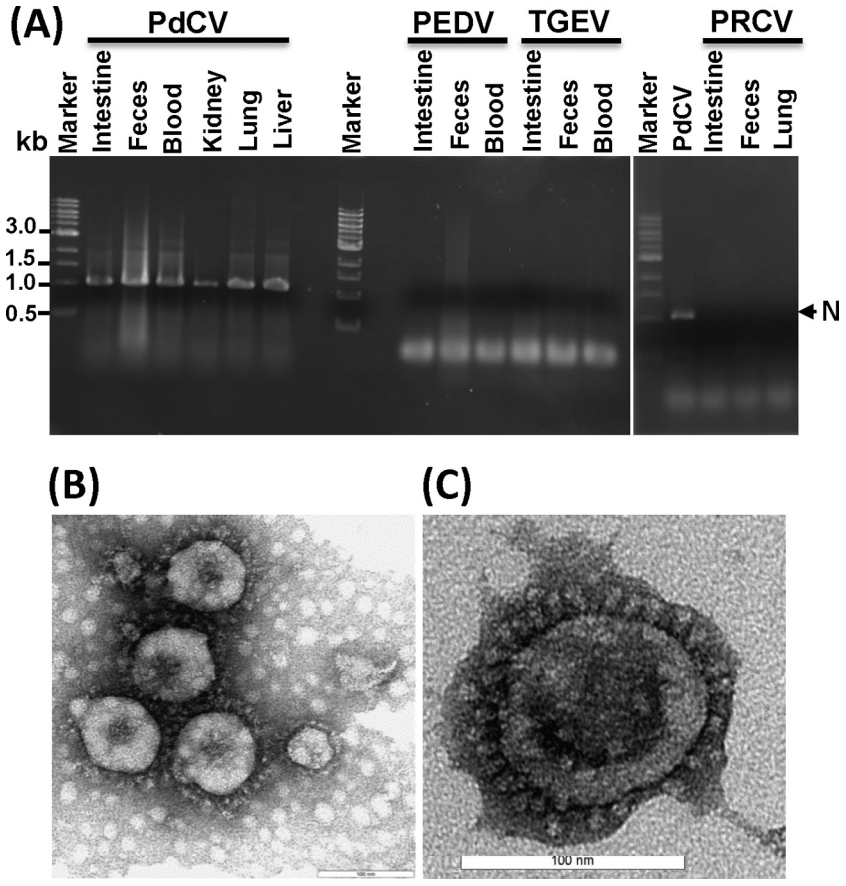

FIG 1 Detection of PdCV in a 7-day-old piglet with severe diarrhea and vomiting from an Ohio farm. (A) RT-PCR analysis of PdCV samples. The N gene was amplified by RT-PCR using primers specific for PdCV, PEDV, TGEV, or PRCV. The PCR products were detected on a 1% agarose gel. (B and C) Electron microscopy analysis of PdCV. Filtered intestinal contents were analyzed by electron microscopy.

The *Deltacoronavirus* genus has been only recently defined by genomic sequence analysis of both pig and avian isolates (15). Since 2009, avian deltacoronaviruses have been detected in a wide range of domestic and wild birds (15–17). The porcine deltacoronaviruses (PdCV) Hong Kong (HK) strains HKU15-155 and HKU15-44 were recently detected in rectal swabs from pigs (15). The potential of the HKU15-155 and HKU15-44 PdCVs to cause significant clinical disease in swine is not known. In mid-January 2014, PdCV was identified in fatal cases of PEDV-negative diarrhea in Ohio piglets (18). Field reports document a history and clinical presentation (vomiting, diarrhea, and dehydration) typical of TGEV and PEDV; like for the other swine enteric CoVs, morbidity and mortality rates at PdCV-infected farms are high (18–21). In some instances, PdCV is the only virus recovered from these diarrhea outbreaks; in others, it may be present as a coinfection with PEDV. To date, outbreaks have been documented in more than 20 states in the United States. In April 2014, a PdCV strain (KOR/KNU14-04/2014) was also identified in feces from diarrheic piglets in South Korea (22).

Aside from genomic sequence data, nothing is known about the U.S. PdCV. Although the virus is identified in diarrhea outbreaks, experimental studies that directly demonstrate its disease-causing potential remain to be shown. The objective of this study is to characterize the U.S. PdCV isolate in terms of genetic characteristics, phylogeny, and virulence in gnotobiotic (Gn) and conventional piglets. Genomic sequencing found that U.S. PdCV strains share 99.6 to 99.7% homology with each other and 99.0 to 99.1% homology with Hong Kong isolates (HKU15-155 and HKU15-44). We found that the U.S. PdCV strains caused severe diarrhea, vomiting, and dehydration clinically indistinguishable from those caused by PEDV and TGEV. Histologically, the PdCV strains caused severe lesions in the stomach and small intestine and mild interstitial pneumonia in lungs. Collectively, our study provides the first proof that PdCV causes significant enteric disease in swine.

RESULTS

Characterization of PdCV Ohio CVM1. To determine the causative agent of the diarrhea outbreaks at Ohio pig farms, one live pig with severe diarrhea was euthanized, and fecal, intestinal content, liver, kidney, lung, and blood samples were collected. Total RNA was extracted from these samples and subjected to reverse transcription (RT)-PCR. Results showed that these samples were positive for PdCV but negative for PEDV, TGEV, and PRCV (Fig. 1A). Feces and intestinal contents were filtered and subsequently analyzed by electron microscopy. Typical coronavirus-like particles with diameters ranging from 60 to 180 nm were observed (Fig. 1B and C). No other viral particles were observed. Subsequently, the filtered duodenum contents were subjected to deep sequencing. No other known viral sequences were identified,

except for PdCV. The virus identified was named PdCV Ohio CVM1.

Complete genome sequence of PdCV Ohio CVM1. The genomic sequence of PdCV Ohio CVM1 is 25,422 nucleotides (nt) in length, excluding the 3′ poly(A) tail, which is 1 nt and 6 nt longer than those of the PdCV Hong Kong strains HKU15-44 and HKU15-155, respectively. The genome organization of PdCV Ohio CVM1 is typical of CoVs. Starting from the 5′ end, approximately three-fourths of the viral genome encodes two overlapping open reading frames (ORF1a and ORF1b), producing up to 16 nonstructural proteins (NSP). Downstream of ORF1, PdCV contains six additional ORFs (ORF2 to ORF7) that code for the structural proteins spike (S), envelope (E), membrane (M), nonstructural protein 6 (NS6), nucleocapsid (N), and NS7. Similar to avian deltacoronaviruses, NS6 is located between M and N. However, ORF7 (NS7) of PdCV differs from that of avian deltacoronaviruses. NS7 of PdCV overlaps with the N protein, whereas avian deltacoronaviruses contain 2 to 4 additional ORFs that encode NS7a, NS7b, NS7c, and NS7d, which overlap or are downstream of the N protein (15, 17). Both 5′ and 3′ ends of PdCV contain short untranslated regions (UTR) with lengths of 539 and 395 nt, respectively. The genome of Ohio CVM1 shares 99% and 99.1% identity with Hong Kong PdCV strains HKU15-44 and HKU15-155, representing 249- and 206-nt differences, respectively. Compared to the HKU15-44 strain, PdCV Ohio CVM1 has a 1-nt (T) insertion at position 25267 located at the 3′ UTR. PdCV Ohio CVM1 also differs by having a 3-nt (AAT) insertion at positions 19477 to 19479, which encodes the N terminus of the S protein, and a 3-nt (GTT) insertion at positions 25047 to 25049, located at the 3′ UTR.

Unique genetic features of the emergent PdCV strains in the United States. To date, there are eleven complete genomic sequences of U.S. PdCVs available in GenBank. The genome sizes (25,422 nt) of all U.S. PdCV strains are identical. The genomic sequence of PdCV Ohio CVM1 shares 99.6 to 99.7% identity with other U.S. PdCV isolates and 99.7% homology with a South Korean isolate (see Table S1 in the supplemental material). The South Korean strain shares more homology with U.S. strains than Hong Kong strains. Within U.S. PdCV isolates, PdCV Ohio CVM1 shares the highest identity (99.8%) with the OH1987, PA3148, and IL2768 strains, with only 41-, 52-, and 58-nt differences, respectively, across the entire genome. A total of 94 nt differences, accounting for 14 unique amino acid changes, were found between the U.S. PdCV strains and the two Hong Kong strains. Specifically, 10 amino acids (F1019, I1320, I1414, V1483, I1647, I3120, F5442, V5816, H5871, and E5875) in ORF1ab were found to be unique to the U.S. isolates. In addition, 4 amino acids (V551, I670, S698, and I1016) were found to be unique to the S proteins of all known U.S. isolates. These unique amino acids can potentially serve as genetic markers to discriminate between U.S. and Hong Kong strains. Notably, HKU15-155 has a 1-amino-acid deletion at position 52 in the S protein, whereas all other strains have "N" at this position. Interestingly, all the U.S., Hong Kong, and South Korean strains share 100% identity in the E protein (see Table S1). Amino acid sequences of M proteins are identical in all strains, with the exception of an A83P substitution in HKU15-155. Only 3 amino acid differences were found in the NS6 proteins among all PdCV strains.

A unique feature of PdCV Ohio CVM1. Sequence analysis showed that the nucleotide sequences of the N and NS7 genes of PdCV Ohio CVM1 share higher homology with those of Hong Kong strain HKU15-44 than all other U.S. strains. Four continuous nucleotides (405C, 534C, 558A, and 564A) in the N gene are unique to Hong Kong strain HKU15-44 and the Ohio CVM1 strain (see Fig. S1 in the supplemental material). The corresponding nucleotides in all other U.S. strains are 405T, 534A, 558G, and 564G, all of which are silent mutations for N protein. However, the first three nucleotide changes result in 3 amino acid substitutions in the protein encoded by the NS7 gene, which overlaps with the N gene (see Fig. S1). Specifically, the shared amino acids in the predicted NS7 protein sequence of the Ohio CVM1 and Hong Kong strains are P104, P147, and N155, whereas all other U.S. strains at the same positions have L, Q, and S, respectively. Importantly, all other viral protein genes of Ohio CVM1 share higher homology with genes of other U.S. strains than with genes of the Hong Kong strain. This finding suggests that Ohio CVM1 may represent a recombinant strain in which the NS7 gene is derived from Hong Kong strains and the rest of the genome is derived from the U.S. strains. In addition, PdCV Ohio CVM1 has six other unique amino acid changes across the genome. M5309 and R5354 were unique for ORF1ab, I553 and K642 were unique for S protein, and S24 was unique for N protein.

Phylogenetic analysis. Phylogenetic trees were constructed by the neighbor-joining method in the MEGA 6 program using 11 U.S. PdCV strains, two Hong Kong PdCV strains, one South Korean PdCV strain, and nine avian deltacoronavirus strains. Sequence BLAST found that CoVs from Asian leopard cats and Chinese ferret-badgers also belongs to the genus *Deltacoronavirus*, for which partial genomic sequences encoded by the 3CLpro, RdRp, Hel, S, and N genes are available (23). Thus, known viral protein genes of Asian leopard cat and Chinese ferret-badger CoVs were included in the phylogenetic analysis. All U.S. PdCV strains, together with two Hong Kong strains, HKU15-44 and HKU15-155, and one Korean strain, fell into one cluster, forming the PdCV group (Fig. 2A). PdCV shares a closer relationship with other mammal deltacoronaviruses (i.e., Asian leopard cat and Chinese ferret-badger CoVs) than with avian deltacoronaviruses. This suggests that porcine, feline, and avian deltacoronaviruses may have a common ancestor (Fig. 2A). Similarly, PdCV shares higher homology with Asian leopard cat CoVs than with avian deltacoronaviruses in the phylogenetic tree analysis when the N, M, E, and NS6 protein gene sequences are used (data not shown). In addition, the phylogenetic tree of the NS7 protein gene showed that PdCV Ohio CVM1 is more closely related to HKU15-44 and HKU15-155 than to other U.S. PdCV strains (Fig. 2B).

Infection of gnotobiotic pigs with PdCV—clinical signs associated with infection by PdCV CVM1 and MI strains. We have successfully reproduced PdCV-associated diseases in Gn piglets. Twenty hours after oral inoculation with filtered intestinal content containing 10^6 genomic RNA copies of PdCV Ohio CVM1 (animal experiment 1), all three piglets developed sudden-onset, severe, persistent, and watery diarrhea (see Fig. S2 in the supplemental material), with diarrheal scores of 3 (Table 1). Vomiting was observed in the 48- and 72-h-PdCV-infected piglets (Table 1; see also Fig. S2). Core body temperatures remained within normal limits. Respiratory signs (coughing and nasal discharge) were not observed. In spite of the diarrhea and progressive dehydration, appetite was maintained. The most lethargic and severely affected piglet was terminated 24 h postinfection (p.i.), one piglet was terminated at 48 h p.i., and the third was terminated one day later

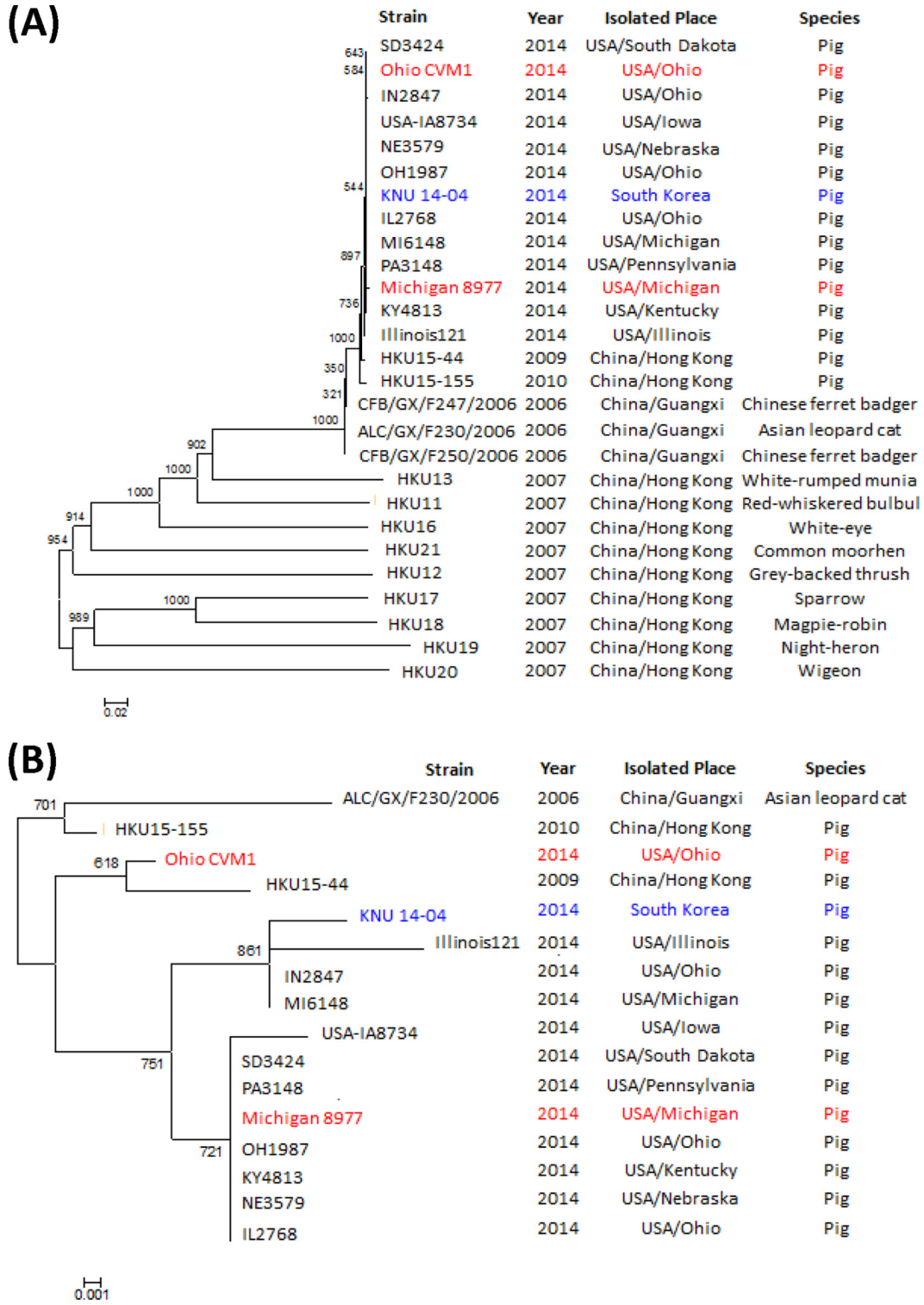

FIG 2 Phylogenetic analysis of mammalian and avian deltacoronaviruses. (A) Phylogenetic tree of the S protein; (B) phylogenetic tree of the NS7 protein. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA 6 program. The scale bar indicates the estimated number of substitutions per 20 amino acids. Virus strains used for phylogenetic analysis and accession numbers are shown in Table S1 in the supplemental material. ALC denotes Asian leopard cat, and CFB denotes Chinese ferret-badger. The two strains (Ohio CVM1 and Michigan 8977) used in this study are highlighted by red color. The South Korean strain is highlighted by blue color.

TABLE 1 Virulence and pathogenesis of PdCV in the piglets

Experiment	Pig type[a]	No. of piglets	Virus/dose[b]	Clinical sign score[c]		IT[d] (days)	Gross pathology score[e]	Histology score[f]				
				Vomiting	Diarrhea			Lung	Stomach	Duodenum	Jejunum	Ileum
1	Gn	3	CVM1/10^6 GRC	3	3	1	3	1	2	3	3	3
	Gn	2	Control	0	0	NA	0	0	0	0	0	0
2	Gn	3	MI/10^6 PFU	1	2	3	2	0.5	1	2	3	3
	Gn	2	MI/indirect	0	2	4	2	0.5	1	2	3	3
	Gn	3	Control	0	0	NA	0	0	0	0	0	0
3	Cv	4	MI/10^6 PFU	1	3	1	0	0	0	0	0	0
	Cv	4	Control	0	0	NA	0	0	0	0	0	0

[a] Gn, gnotobiotic piglets; Cv, conventional piglets.
[b] Piglets were orally inoculated with the indicated virus. GRC, genomic RNA copies; 10^6 PFU was equivalent to 10^9 GRC.
[c] Vomiting and diarrhea were scored for each piglet. 0 = no vomiting or diarrhea; 1 = mild; 2 = moderate; 3 = severe.
[d] IT, incubation time in days; NA, not applicable.
[e] Goss pathology was scored for each piglet. 0 = no change; 1 = mild; 2 = moderate; 3 = severe. Conventional piglets in the experiments were terminated 3 weeks postinoculation, and piglets have recovered from disease; thus, no gross and histologic lesions were found.
[f] Histology was scored for each tissue. 0 = no change; 1 = mild; 2 = moderate; 3 = severe.

(72 h p.i.). The two uninfected Gn piglets had normal feces (score of 0) and no clinical signs of illness.

To further confirm that PdCV is an enteric pathogen of swine, three out of five Gn piglets in one isolator (animal experiment 2) were orally inoculated with 10^6 PFU of the PdCV MI strain, a swine testicular (ST) cell-adapted, plaque-purified virus. The other two Gn piglets in the same isolator received Dulbecco's modified Eagle's medium (DMEM). No obvious clinical signs were observed on days 1 and 2 p.i. in all three PdCV-inoculated Gn piglets. All three piglets developed diarrhea (score of 2) on day 3 p.i. and were subsequently terminated. Interestingly, the two other piglets in the same isolator, which did not receive virus inoculation, developed diarrhea (score of 2) on day 6 and were terminated on day 7. No or only mild vomiting was observed in piglets infected with the PdCV MI strain. Results establish the potential of PdCV to serve as a transmissible agent of clinically significant diarrheal disease.

Gross pathological changes in gnotobiotic piglets infected with the PdCV CVM1 or MI strain. Gross findings were similar in all three Gn piglets inoculated with PdCV Ohio CVM1. The perineum, ventral abdomen, and hind legs were coated with yellow, adherent diarrheic feces (see Fig. S2). Dilated gas- and fluid-filled small intestines with thin, translucent walls were observed, and both the stomachs and small intestines contained coagulated liquid milk replacement diet (see Fig. S2). The cecum, spiral colon, and terminal colon were dilated and filled with yellow intestinal fluids. In addition to the intestinal changes, ascites, hydrothorax, and thymic atrophy were detected in the piglet terminated at 72 h p.i. The cell culture-adapted PdCV MI strain caused gross pathology similar to that of PdCV Ohio CVM1 in Gn piglets (see Fig. S3 in the supplemental material).

Histological changes in gnotobiotic piglets infected with the PdCV CVM1 or MI strain. Twenty-four hours after challenge with PdCV Ohio CVM1, severe villous atrophy of the duodenum, jejunum, and ileum was apparent (Fig. 3A). The villous changes were associated with extensive intestinal epithelial degeneration and necrosis. No intestinal lesions were found in uninfected piglets (Fig. 3B). In addition, focal areas of gastric epithelial cell degeneration and necrosis were observed in the gastric pits within the cardia, greater curvature of the fundus, and antrum of the stomach (Fig. 3D). No lesions were found in the stomachs of uninfected piglets (Fig. 3C). Epithelial cell necrosis was occasionally accompanied by syncytial giant cell formation in the gastric glandular mucosa, duodenum, jejunum, and ileum. Aside from edema of the lamina propria, the mucosal epithelial lining cells of the cecum and spiral colon were unchanged. No significant lesions were observed in extraintestinal tissues at this time.

By 48 h p.i., villous atrophy of the small intestine was further advanced; all divisions of small intestine contained only short blunted villi variably lined by flattened squamoid-to-cuboidal epithelial cells; goblet cells appeared unaffected by the direct viral cytopathic effect and rather were aggregated onto villus tips or sloughed into the intestinal lumen. Many mucosal epithelial cells were necrotic and lysed; nuclear changes consistent with cell death included pyknosis, karyorrhexis, and karyolysis (Fig. 3G). Epithelium-origin syncytial giant cells were still evident but were fewer in number. In the stomach (Fig. 3E), occasional dilated gastric pits were observed, presumably created as individual PdCV-infected cells lysed and were replaced by attenuated, immature squamoid epithelial cells. The underlying lamina propria was edematous and contained activated macrophages, occasional aggregates of lymphocytes, neutrophils, and a scattering of eosinophils. These modest inflammatory cell infiltrates were attributable to PdCV, as inflammatory cell infiltrates are notably absent in uninfected Gn swine. Peyer's patches of the ileum and mesenteric lymph nodes were moderately depleted of lymphocytes, as were mesenteric lymph nodes. As is typical of Gn swine, lymphoid follicles (i.e., sites of B-cell differentiation) were either not present or present only as inactive aggregates of lymphocytes.

By 72 h p.i., even though the villous atrophy was still present, limited reepithelization of villus tips was histologically evident throughout the small intestine; numerous mitotic figures in villus crypt epithelial lining cells accompanied this regenerative response (Fig. 3H). Regeneration was most prominent in the duodenum and least evident in the ileum. Duodenal villus tips were lined by columnar epithelial cells expressing microvilli. Multinucleated giant cells could still be seen, but these were largely necrotic and in the process of being sloughed into the gut lumen. Small foci of mononuclear cell infiltrates and occasional dilated gastric pits were seen in gastric mucosa (Fig. 3F). Syncytial giant cells were absent.

The histological lesions in the stomach and small intestine of

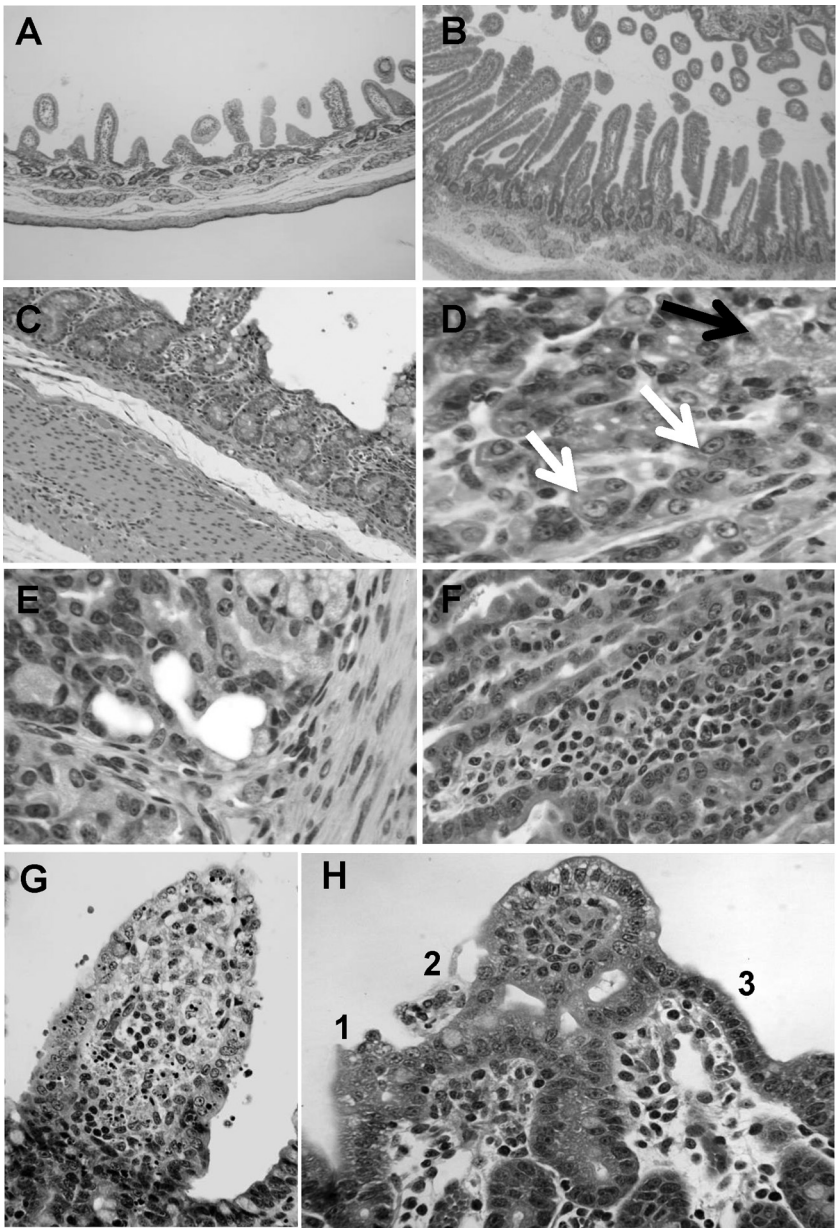

FIG 3 Histologic lesions of intestine and stomach caused by PdCV Ohio CVM1. (A) Severe villous atrophy in the duodenum of a PdCV-infected Gn piglet at 24 h p.i. (hematoxylin and eosin, ×300 magnification); (B) duodenum of uninfected Gn piglet; (C) normal gastric fundus (×400), uninfected Gn piglet; (D) syncytial giant cells are present (white arrows) as well as foci of cell necrosis (black arrow) in the fundic region of the stomach, PdCV Ohio CVM1-infected Gn piglet, 24 h p.i.; (E) dilated gastric pits are present by 48 h p.i.; (F) a focus of lymphocytic and monocytic inflammation in the gastric mucosa, 72 h p.i.; (G) illustrates necrosis of mucosal epithelial cells characterized by nuclear changes (pyknosis, karyorrhexis, and karyolysis) indicative of cell necrosis from a Gn piglet infected with PdCV Ohio CVM1, 48 h p.i. (×400); (H) multinucleated giant cell formation in jejunal epithelium from the Gn piglet infected with PdCV Ohio CVM1, 72 h p.i. (×400). A viable PdCV-induced syncytial giant cell in jejunal epithelia (1) lies adjacent to a necrotic syncytial giant cell that is in the process of being sloughed into the intestinal lumen (2). Unaffected jejunal mucosal epithelial cells (3) are also present.

Gn piglets infected by the PdCV MI strain were similar to those described for PdCV Ohio CVM1 (Table 1). Extensive villous atrophy, necrosis, and blunting were observed in the small intestine of all Gn piglets infected by the PdCV MI strain, including the two Gn piglets infected via indirect contact. Villous atrophy was more severe in the jejunum and ileum than in the duodenum for all PdCV MI-infected Gn piglets (Table 1; see also Fig. S4 in the supplemental material).

Histology of extraintestinal tissues. Histological changes associated with PdCV Ohio CVM1 infection were not seen in any other organs except the lung. In the lung, mild multifocal bronchial-to-bronchiolar-centric areas of nonsuppurative interstitial pneumonia were identified by 48 h p.i. (Fig. 4A and B). Convincing histologic evidence of epithelial cell necrosis or syncytia was not seen (data not shown). Similarly, the PdCV MI strain caused mild interstitial pneumonia in Gn piglets. No histologic

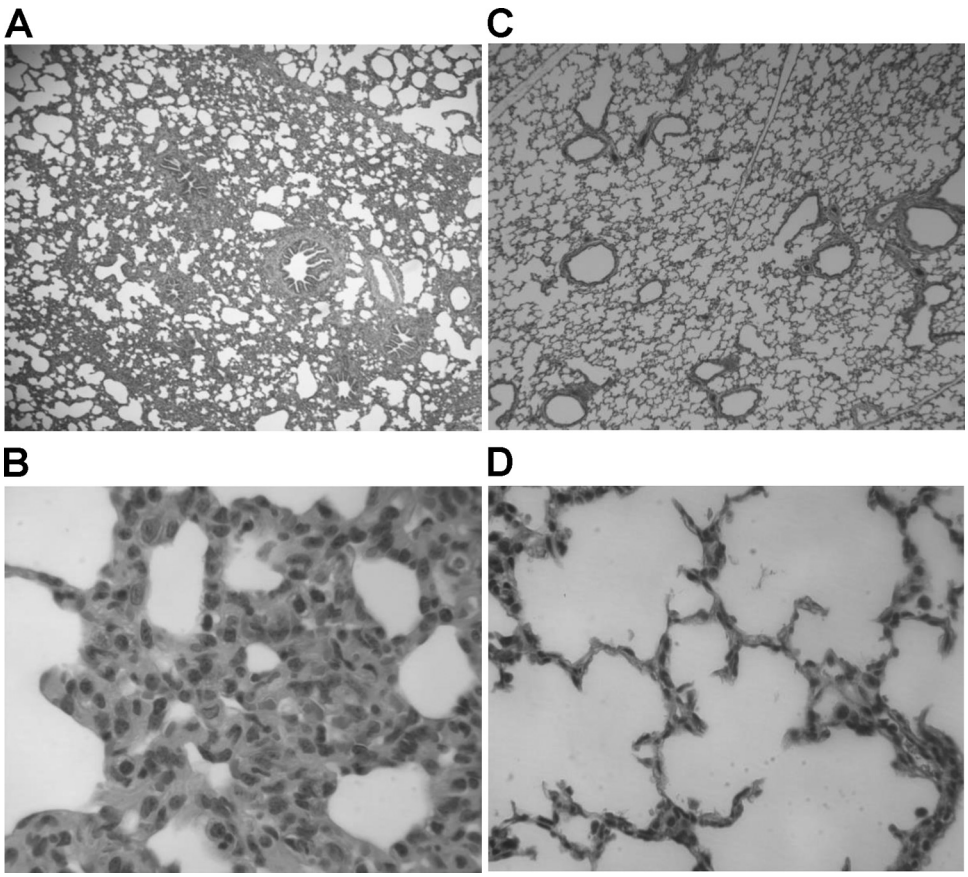

FIG 4 Mild (multifocal) interstitial pneumonia in a PdCV Ohio CVM1-infected Gn piglet, 48 h p.i. (A) Pulmonary tissue from PdCV-infected piglets (×50). The figure illustrates the bronchocentric distribution of the nonexudative histologic change in the lung. (B) Pulmonary tissue from PdCV-infected piglets (×400). The figure illustrates interstitial thickening of the alveolar cell walls. (C) Pulmonary tissue from an uninfected control (×50); (D) pulmonary tissue from an uninfected control (×400).

lesions were found in other organs of piglets infected by the PdCV MI strain.

Viral antigen distribution in gnotobiotic piglets infected with the PdCV CVM1 or MI strain. Deparaffinized PdCV CVM1-infected tissue sections or PEDV-infected tissue sections were stained with convalescent-phase serum from a PdCV-infected sow or monospecific polyclonal antiserum raised against PEDV in Gn piglets (Fig. 5). PdCV convalescent-phase serum stained infected mucosal epithelia from only PdCV-infected piglets (Fig. 5E); anti-PEDV stained infected epithelial cells from only PEDV-infected Gn piglets (Fig. 5A). Bronchial epithelial cells from PdCV-infected piglets contained a horseradish peroxidase (HRP) reaction product in the 72-h-p.i. lung sections (data not shown). A large number of antigens were detected in the jejunum and ileum in PdCV MI-infected Gn piglets on day 3 p.i. (see Fig. S5 in the supplemental material). These data demonstrate that (i) PdCV replicates in the epithelium of the small intestine and (ii) PdCV does not cross-react serologically with PEDV.

Viral RNA burdens and distribution in gnotobiotic piglets infected with the PdCV CVM1 or MI strain. PdCV viral RNAs were detected in all samples in Ohio CVM1-infected piglets (Fig. 6). At 24 h p.i., 9 to 11 $\log_{10}$ PdCV RNA copies/g or ml was detected in all samples of the small and large intestines. Viral RNA concentrations reached the highest levels at 48 h p.i. and decreased by 72 h p.i. In the duodenal tissues, viral RNAs declined from 24 to 72 h; only 4.7 $\log_{10}$ PdCV RNA copies/g was detected in the 72-h duodenal sample, correlating to histological evidence for mucosal epithelial cell regeneration. High levels (roughly 10 $\log_{10}$ viral RNA/g or ml) of viral RNAs were shed in fecal samples 24 to 72 h p.i. PdCV RNA levels in extraintestinal tissues (lung, liver, kidney, spleen, and blood) were relatively low, ranging from 3.1 to 6.27 $\log_{10}$ PdCV RNA copies/g of sample. Peripheral blood contained 3.4 to 4.1 $\log_{10}$ copies of PdCV RNA/ml, indicating viremia. These data demonstrated that viral RNA shedding in intestine and feces significantly exceeded that of the input virus (6 $\log_{10}$ RNA copies/piglet) and support the conclusion that PdCV caused enteric infection and replicated rapidly in the gastrointestinal tract.

PdCV MI-infected piglets resulted in a multisystemic distribution of virus, with the highest virus load in the intestinal tract and evidence of fecal viral shedding based upon real-time RT-PCR analysis of viral RNA levels (Fig. 7B and C). High levels of viral RNA (6 to 9 $\log_{10}$ viral RNA copies/g or ml) were detected in feces (Fig. 7B) and all sections of the small and large intestines, whereas a relatively low level of viral RNA (3 to 4 $\log_{10}$ viral RNA copies/g or ml) was detected in other tissues (Fig. 7C). The two uninoculated Gn piglets infected by indirect contact had levels of viral RNA (Fig. 7D) similar to those of the three virus-inoculated piglets.

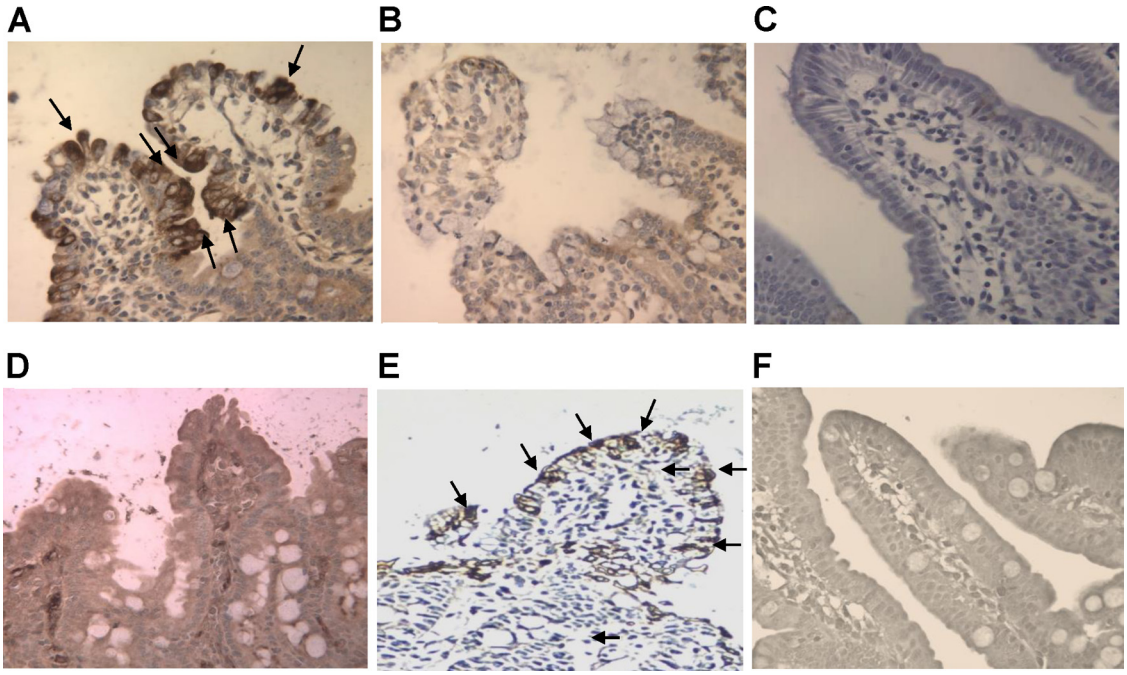

FIG 5 Immunohistochemistry (IHC) analysis of small-intestine sections from Gn piglets. PEDV VBS2-infected duodenal tissue at 48 h p.i. (A and B), PdCV Ohio CVM1-infected duodenal tissue at 24 h p.i. (D and E), and uninfected controls (C and F). Panels A to C were stained with hyperimmune serum against PEDV. Panels D to F were stained with hyperimmune serum against PdCV. Black arrows indicate positive antigens. Hematoxylin, ×300.

Infection of conventional piglets with the PdCV MI strain. We reproduced the PdCV-associated disease in conventional piglets. All four piglets inoculated with 10^6 PFU of PdCV MI developed sudden-onset, severe, and watery diarrhea (scores of 3) at day 1 p.i. The PdCV MI strain caused more severe clinical signs in conventional piglets than in Gn piglets at the same inoculation dose. Piglets were monitored through day 21 p.i. in order to define the duration of diarrhea and viral shedding in infected piglets. The diarrhea persisted for 7 to 10 days, and all four piglets were recovered from disease on day 10 p.i. These piglets lost approximately 10 to 15% of body weight on day 10 p.i. and started to gain weight after recovery from the disease. No body temperature changes were observed. The severity of diarrhea and kinetics of fecal RNA shedding are shown in Fig. 8. All piglets had severe watery diarrhea on day 1 p.i., but no or low viral RNA (1.0 $\log_{10}$ viral RNA copies/g) was detected in feces. Viral RNA peaked on day 7 p.i., gradually decreased after day 10 p.i., and was still detectable at day 21 p.i. No obvious gross and histologic lesions were observed in conventional piglets on the termination day (day 21 p.i.), consistent with recovery from disease. At this time point, the ileums, colons, blood, livers, and lungs of some infected piglets were still positive for PdCV RNA (Fig. 8B). These data suggest that PdCV was persistent in conventional piglets for at least 21 days.

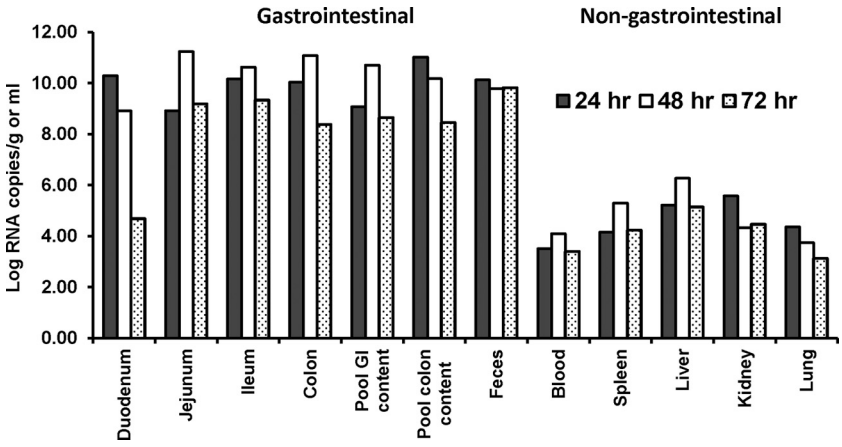

FIG 6 Viral RNA distribution of Gn piglets infected by PdCV Ohio CVM1. Three 19-day-old Gn piglets were orally inoculated with 10^6 genomic RNA copies of PdCV Ohio CVM1. Gn piglets were terminated at 24, 48, and 72 h p.i. Viral RNA genome copies in pig tissues were quantified by real-time RT-PCR. No PdCV-specific RNA was detected in mock-infected Gn piglets.

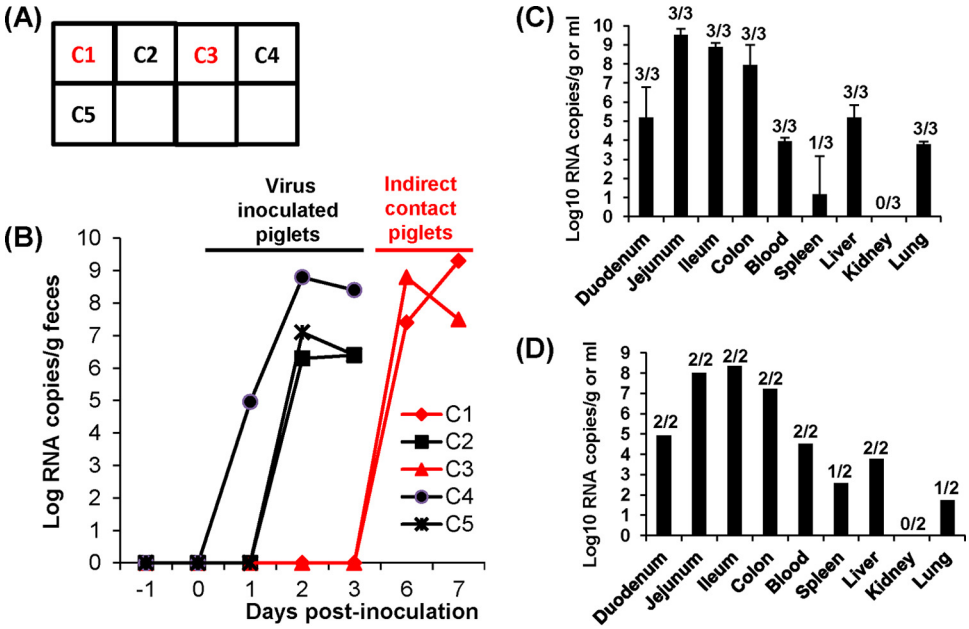

FIG 7 Viral RNA distribution of Gn piglets infected by the PdCV MI strain. (A) Location of each piglet in the germfree isolator, showing the enclosures that physically separated each piglet (C1 to C5). Three 10-day-old Gn piglets (C2, C4, and C5) were orally inoculated with 10^6 PFU of the PdCV MI strain and were terminated on day 3 p.i. Piglets C1 and C3 did not receive virus inoculation and were terminated on day 7 p.i. (B) Viral RNA shedding in feces after virus inoculation. (C) Viral RNA distribution in tissues from three PdCV-inoculated Gn piglets. The number of positive samples was indicated at the top of each column. (D) Viral RNA distribution in tissues from two Gn piglets infected via indirect contact.

DISCUSSION

In this study, we revealed unique genetic characteristics of newly emerged PdCV strains in the United States and determined their virulence in both Gn and conventional piglets. The clinical signs and lesions described in the gastrointestinal tracts of PdCV-infected piglets are very similar to those described in field cases of TGEV (24), for experimental TGEV in Gn swine (6, 25), for PEDV-associated disease in European strain-infected pigs, and for recent PEDV variant strains in U.S. swine operations (7, 12, 26, 27). For the porcine enteric CoVs, high mortality rates can be attributed to malabsorptive diarrhea and dehydration that is secondary to virus-mediated destruction of intestinal mucosal absorptive cells.

Emergence and epidemiology of PdCV. In February 2014, PdCV appeared in Ohio and Indiana, with epidemiological data supporting an association with diarrheal outbreaks in young swine (18). The rapid spread of PEDV across the United States in 2013 suggests that PdCV will follow a similar pattern. In fact, in 10 months, PdCV has spread to 20 contiguous states in the United States and Canada; both PdCV and PEDV have been declared as "reportable diseases" to the USDA and Canada. Available sequence data and our own demonstration of the lack of immunologic cross-reactivity between PdCV and PEDV suggest that there will be minimal to no immunologic cross-protection between PdCV and PEDV.

Interestingly, disease associated with PdCV has not been reported in China and other Asian countries in spite of being geographically closer to Hong Kong than is the United States. In 2012, Woo et al. analyzed a total of 169 pig rectal swabs, 17 of which were positive for PdCV HKU15 (15). However, clinical signs were not

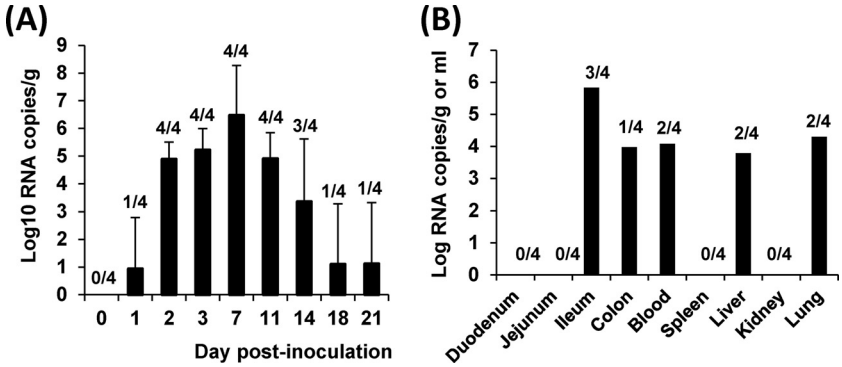

FIG 8 Viral RNA distribution in conventional piglets infected by the PdCV Michigan strain. Four 10-day-old conventional piglets were orally inoculated with 10^6 PFU of the PdCV MI strain and were terminated on day 21 p.i. (A) Viral RNA shedding in feces after virus inoculation; (B) viral RNA distribution in pig tissues.

described in their study. Aside from Woo's study, further outbreaks of HKU15-associated diseases in pigs have not been documented in Hong Kong and Asia, raising the possibility that HKU15 may not be a significant pathogen within its region of origin. Since PEDV is the dominant enteric disease in Asia and PdCV causes clinical signs indistinguishable from those of PEDV, it is also possible that clinically significant PdCV-induced disease has been overlooked. In contrast to the Asian experience, U.S. PdCV strains are associated with significant mortality (40 to 60%) in the field (18–20) and severe clinical disease and pathological changes in experimentally infected Gn and conventional piglets of the present study. In April 2014, PdCV was first identified in feces from diarrheic piglets in South Korea (22). The PdCV South Korean strain is more (99.7% homology) closely related to U.S. isolates than Hong Kong strains, suggesting that the South Korean strain may be virulent to piglets. Sequence analysis in the current work suggests that U.S. PdCV originated from Hong Kong strains. Unique amino acid substitutions exist in U.S. PdCV isolates that may account for the increased virulence of this virus in U.S. swine, a possibility that should be addressed in future studies.

Origin and evolution of deltacoronaviruses. In general, CoVs have high mutation rates and can easily undergo recombination and deletion events that lead to altered tissue tropism, transmission routes, and host specificity. Birds are reservoirs for avian deltacoronaviruses (15–17). These avian deltacoronaviruses may adapt and jump to some mammalian species. Interestingly, the first mammalian source of deltacoronavirus was detected in rectal swabs of Asian leopard cats at live-animal markets in Guangdong and Guangxi Provinces in China (23). It was found that 2.4% of Asian leopard cats (35 out of 1,453 samples) were positive for deltacoronavirus. It was also found that 1.1% of Chinese ferret-badgers (11 out of 934 samples) were also positive for deltacoronavirus, which shares 100% homology with the Asian leopard cat CoV based on the RdRp domain and the S gene sequence (23). Interestingly, the PdCVs of both Hong Kong strains and U.S. isolates share more homology with deltacoronaviruses of Asian leopard cats and Chinese ferret-badgers than avian deltacoronaviruses, suggesting that Asian leopard cats and Chinese ferret-badgers are intermediates for interspecies transmission of PdCV. At this time, we do not know if deltacoronaviruses are present in U.S. bird populations or other U.S. mammals (such as domestic cats). Clearly, a better understanding of the origin and evolution of the U.S. PdCV will await the detailed epidemiological study of deltacoronaviruses in the birds and mammals in the United States.

PdCV in Gn piglets: similarities with PEDV and TGEV. We have shown that both the PdCV Ohio CVM1 and MI strains induce significant disease in Gn pigs. The pathogenesis of TGEV and PEDV is well studied. Both TGEV and PEDV are members of the *Alphacoronavirus* genus and cause almost identical clinical signs and gross and histological lesions in young, susceptible pigs (6, 12, 24, 26), even though there is no serological cross-reactivity between TGEV and PEDV. In contrast, PdCV is a member of the genus *Deltacoronavirus* most closely related to avian CoVs (15). The overall clinical disease associated with PdCV infection is very similar to that induced by TGEV and PEDV, except that vomiting is more commonly seen in PdCV-infected piglets and the mortality rates are reported to be lower (on the order of 40 to 50%) than those for PEDV and TGEV (90 to 100%). Similar to TGEV and PEDV, PdCV replicates extensively in the small intestine. High levels of genomic RNA were detected in feces, intestinal contents, and tissues. A large number of antigen-positive cells were detected in all sections of the small intestine. Intestinal gross and histologic findings in PdCV-infected piglets are similar to those described for PEDV and TGEV. Finally, moderate levels of PdCV RNA were detected in blood and extraintestinal tissues, consistent with viremic dissemination similar to what has been observed in TGEV- and PEDV-infected piglets.

Unique features of PdCV infection in Gn piglets. Our study found that PdCV has two unique features of infection that are distinct from TGEV and PEDV infection. First, PdCV infection causes epithelial lesions in the glandular pits of the stomach. To our knowledge, involvement of gastric mucosal epithelial cells is not reported for either TGEV or PEDV. There is one report documenting gastric lesions associated with CoV RNAs in severe acute respiratory syndrome (SARS)-convalescent human patients, establishing a precedent for findings (28). Second, PdCV infection is associated with mild interstitial pneumonia, evident in formalin-inflated lungs. In this regard, mild and ill-defined interstitial lesions in pulmonary parenchyma suggest that PdCV may have an unrecognized PRCV-like respiratory component. Modest amounts PdCV viral RNA were found in lung tissue homogenates, and PdCV antigen was detected in bronchial mucosal epithelial cells. Pathological changes in the lung have not been reported for PEDV and TGEV.

The demonstrated pathogenicity in PdCV may reflect an emergent property inherent in sequence changes from otherwise closely related CoVs. For example, PRCV, a deletion mutant of TGEV, shifted the tissue tropism from intestine to lung (4, 29). Tropism for both respiratory and gastrointestinal tracts is also a feature of SARS-CoV. Although the respiratory tract is the main target for SARS-CoV infection, outbreaks in human patients were associated with gastrointestinal symptoms, and SARS-CoV was detected in the stool samples (30–32). Subsequent histologic characterization found that intestinal epithelia and mucosal lymphoid tissue are infected by SARS virus (30, 31). In contrast to SARS-CoV and PRCV, the gastrointestinal tract is the primary target for PdCV, whereas the respiratory tract is the minor target. PdCV shares the highest genomic homology with avian deltacoronaviruses, all of which were originally detected from the gastrointestinal tract via rectal swabs (15–17). Presumably, all these avian deltacoronaviruses were capable of replicating in the gastrointestinal tract, although it is not known if infection also involves a respiratory component. Further studies on this important point are urgently needed, particularly in light of the SARS and Middle East respiratory syndrome (MERS) experiences in humans.

Factors that may influence the severity of PdCV-associated disease. Clinically, Ohio CVM1 caused more severe disease in Gn piglets than the cell-adapted MI strain, despite the fact that the inoculation dose of Ohio CVM1 (10^6 genomic RNA copies) was less than that of the MI strain (10^6 PFU, equivalent to 10^9 genomic RNA copies). The reduced virulence of the MI strain may reflect a degree of attenuation secondary to cell culture adaptation. PdCV MI caused more severe disease in conventional piglets than in Gn piglet at the same inoculation dose. Conventional piglets developed severe sudden-onset diarrhea (score of 3) on day 1 p.i., whereas Gn piglets required 2 days of incubation time to develop diarrhea (score of 2). This finding raises the possibility that the gut microbial flora in conventional piglets facilitates development of diarrhea upon PdCV infection. The contributions of gut microflora or other physiological factors to virus-induced diarrhea are

also supported by the finding that the severity of diarrhea in conventional piglets was not correlated with the kinetics of viral RNA shedding in feces (Fig. 8). Findings in the current study also indicate that PdCV is highly transmissible among Gn piglets, most likely via aerosolized particles. Two uninoculated Gn piglets developed typical clinical signs and shed high levels of virus in feces despite the fact that they were physically separated from three virus-inoculated piglets. Viral RNA was detected in feces and in the ileum at day 21 p.i., suggesting that PdCV can be persistent in conventional piglets for a long time period, a fact that would enhance the potential for viral transmission. Collectively, these data demonstrated that PdCV is highly virulent and transmissible in piglets.

MATERIALS AND METHODS

PdCV-positive specimens. In early January 2014, an outbreak of TGEV- and PEDV-like disease was reported in several pig farms in Ohio; mortality rates ranged from 40 to 80%. Intestinal contents and feces were collected from a 7-day-old piglet with severe diarrhea and vomiting. Five grams of feces and intestine contents was homogenized in 50 ml of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA). The suspension was centrifuged at 5,000 × *g* for 10 min at 4°C. The supernatant was collected and filtered through 0.45-μm and 0.22-μm-pore-size filters. The filtered intestinal contents were negative for any bacteria or fungal growth when they were cultured on LB plates and medium in both anaerobic and aerophilic conditions.

Cell culture-adapted PdCV strain. The cell culture-adapted PdCV Michigan/8977/2014 strain (PdCV MI) was purchased from National Veterinary Services Laboratories at the USDA (Ames, IA). The PdCV MI strain was grown in swine testicular (ST) cells in DMEM containing 0.2 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Invitrogen). At 72 h postinfection, supernatant was harvested, and virus titer was determined by plaque assay in ST cells. This strain was passed 15 times in ST cells and used for the pig study.

RNA extraction, RT-PCR, and deep sequencing. Total RNA was extracted from feces and intestinal contents using an RNeasy minikit (Qiagen, Valencia, CA). Primers were designed to target the N gene based on the sequence of the U.S. PEDV Colorado strain (accession number KF272920), the conserved regions of the Miller M6 and M60, Purdue P115, and virulent Purdue TGEVs (accession numbers DQ811785, DQ811786, DQ811788, and DQ811789, respectively), PRCV (accession number DQ811787), and PdCV Hong Kong strain HKU15-44 (accession number JQ065042). Primers were also designed to target the VP1 gene of porcine rotavirus types A and C, porcine norovirus, and porcine sapovirus. RT-PCR was performed using a one-step RT-PCR kit (Qiagen), and the PCR products were visualized in 1% agarose gels.

Based upon the genomic sequence of strain HKU15-44 (15), eight sets of primers were designed to amplify the full-length genome of the PdCV Ohio CVM1 strain. RT-PCR was performed using a one-step RT-PCR kit (Qiagen). The PCR products were purified and sequenced at The Ohio State University Plant Microbe Genetics Facility. The total RNA of filtered intestinal contents was also submitted for Illumina TruSeq (Illumina, San Diego, CA) library preparation and next-generation sequencing (NGS) on Illumina MiSeq (Illumina) at the University of Minnesota Genomics Center, Minneapolis, MN. Using a template assembly (SeqMan NGen, DNASTAR version 11; Madison, WI), the PdCV sequences were assembled using the full-length genome of the U.S. PdCV MI strain.

Piglets. The animal protocol used in this study was approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University (IACUC-OSU). Gnotobiotic (Gn) piglets were derived in a sterile environment via caesarean section from a specific-pathogen-free gravid sow as previously described (33) and housed in germfree isolation units. Piglets were fed a milk-replacement diet (Permalat) and maintained as described elsewhere (33). Ten-day-old healthy conventional piglets were purchased from Hartley Farm, Circleville, OH. These piglets were seronegative for major porcine diseases, including PdCV, PEDV, transmissible gastroenteritis virus (TEGV), porcine circovirus (PCV), and porcine reproductive and respiratory syndrome virus (PRRSV).

Animal experiment 1: Gn piglets challenged with Ohio CVM intestinal contents. Five 19-day-old Gn piglets were randomly divided into two groups. Two piglets in group 1 served as uninfected controls. The three piglets in group 2 were infected with PdCV. Prior to oral challenge, preinoculation fecal and oronasal swabs and blood for serum were collected for virus detection from the three piglets of group 2. Piglets were pretreated with parenteral ranitidine (25 mg/kg of body weight). The three piglets in group 2 were orally challenged with 5.0 ml of filter-sterilized intestinal contents from the PdCV-positive piglet described above, representing 10^6 genomic RNA copies of PdCV Ohio CVM1. The two piglets in group 1 were challenged with 5 ml of filter-sterilized intestinal contents from normal piglets, negative for known porcine viruses.

Animal experiment 2: Gn piglets challenged with the PdCV MI strain. Eight 10-day-old Gn piglets were randomly divided into two isolators. There were a total of 5 Gn piglets (C1 to C5) in isolator 1, and each piglet was housed in an enclosure that prevented direct animal-to-animal contact (Fig. 7A). Three piglets (C1, C3, and C5) in isolator 1 were orally challenged with 5 ml of DMEM containing 10^6 PFU of the PdCV MI strain. The remaining two piglets (C2 and C4) in isolator 1 did not receive any treatment. This experiment was designed to determine whether PdCV can be transmitted from the virus-inoculated piglets to the uninoculated piglets. In isolator 2, three piglets were orally inoculated with 5 ml of DMEM and served as uninfected controls.

Animal experiment 3: conventional piglets challenged with the PdCV MI strain. Eight 10-day-old conventional piglets were randomly divided into two groups (4 piglets/group) and were housed in two separate rooms. Piglets in group 1 were orally inoculated with 5 ml of DMEM and served as uninfected controls. Piglets in group 2 were orally challenged with 5 ml of DMEM containing 10^6 PFU of the PdCV MI strain.

Pig challenge, sampling, and tissue collections. After virus challenge, the piglets were observed and evaluated daily for body weight and temperature changes and clinical signs of PdCV infection. Daily rectal mucosal/fecal swabs were collected from each piglet for virus detection. A diarrhea/fecal consistency score was assigned to each using a subjective scale wherein 0 is normal, 1 is pasty, 2 is creamy, and 3 is watery. Pigs with fecal consistency scores of 2 or 3 were scored as diarrhea-positive. Prior to termination, a blood sample for serum was collected from heavily sedated piglets; each piglet received Euthol solution intravenously to effect. Intestinal contents from the duodenum, proximal jejunum, ileum, transverse colon, spiral colon, and descending colon were collected from each pig. Whole blood and serum were collected first postmortem, followed by collection of duplicate tissues for RNA analysis and histological examination. The tissues sampled were glandular stomach, duodenum, proximal jejunum, distal jejunum, ileum, cecum, spiral colon, terminal colon, spleen, liver, kidney, and lung.

Quantification of viral RNAs by RT-qPCR. Feces and intestinal contents were diluted 1:5 in DMEM and centrifuged at 5,000 × *g* for 10 min at 4°C, and the supernatant was collected for viral RNA extraction. The total RNA was extracted by using an RNeasy minikit (Qiagen, Valencia, CA). Reverse transcription (RT) was conducted using a primer (5′ TTTT GCTCCATCCCCCCTATAAGC 3′) targeting the 3′-end UTR of PdCV and the Superscript III transcriptase kit (Invitrogen, Carlsbad, CA). The RT products were then used to perform real-time PCR using primers and probes specifically targeting the N gene of PdCV (forward, 5′ CGCTTAA CTCCGCCATCAA 3′; reverse, 5′ TCTGGTGTAACGCAGCCAGTA 3′; probe, 5′ 6FAM-CCCGTTGAAAACC-MGB 3′ [6FAM is 6-carboxyfluorescein] [Applied Biosystems, Foster City, CA]) in a StepOne real-time PCR system (Applied Biosystems). A standard plasmid for PdCV was constructed by inserting the sequence of the entire PdCV N gene into the

pGEM-T Easy vector (Promega, Madison, WI). Amplification cycles used were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The threshold for detection of fluorescence above the background was set within the exponential phase of the amplification curves. For each assay, 10-fold dilutions of standard plasmid were generated, and negative-control samples and double-distilled water (ddH_2O) were included in each assay.

Histology. Tissue samples of intestine, stomach, lung, kidney, spleen, and liver were fixed in 10% (vol/vol) phosphate-buffered formalin for 24 to 36 h, dehydrated in graded ethanol, embedded in paraffin, cut in 5-μm sections, and mounted onto glass slides. The sections were deparaffinized, rehydrated, and then stained with hematoxylin and eosin. Slides were examined by conventional light microscopy.

Immunohistochemical staining. Five-micron sections of paraffin-embedded tissues were placed onto positively charged slides. After deparaffinization, sections were incubated with target retrieval solution (Dako, Carpinteria, CA) for antigen retrieval. After blocking, a primary anti-PdCV serum antibody from PdCV-infected convalescent sows was incubated for 30 min at 22°C, followed by incubation with a biotinylated horse anti-pig IgG secondary antibody (Vector Laboratories, Burlingame, CA). Slides were further incubated with the ABC Elite complex to probe biotin (Vector Laboratories) and then developed using a 3,3′-diaminobenzidine (DAB) chromogen kit (Dako); hematoxylin was used as a counterstain. Tissue sections from PdCV-infected and uninfected samples were used as positive and negative controls, respectively. Tissue sections from PdCV-infected and uninfected samples were incubated with hyperimmune serum against PEDV in the immunohistochemical staining (IHC) assay. Reciprocally, IHC assays were also performed using intestinal tissue sections from PEDV-infected samples using anti-PdCV serum antibody.

Genome analysis. The nucleotide sequences of the genomes and the amino acid sequences of the open reading frames (ORFs) were compared to those of other CoVs using DNASTAR software (DNASTAR, Inc., Madison, WI). Phylogenetic tree construction was performed using the neighbor-joining method with the MEGA 6 program. Protein family analysis was performed using Pfam and InterProScan. Virus strains used for sequence and phylogenetic analysis and accession numbers are listed in Table S1 in the supplemental material.

Nucleotide sequence accession numbers. The complete genome sequence of the PdCV Ohio CVM1 has been deposited in GenBank under accession no. KJ769231.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00064-15/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.
Figure S2, TIF file, 2.6 MB.
Figure S3, TIF file, 2.3 MB.
Figure S4, TIF file, 1.7 MB.
Figure S5, TIF file, 2.8 MB.
Table S1, DOCX file, 0.03 MB.

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