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Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets

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ABSTRACT

Porcine deltacoronavirus (PDCoV) was first identified in Hong Kong in 2009–2010 and reported in United States swine for the first time in February 2014. However, diagnostic tools other than polymerase chain reaction for PDCoV detection were lacking and Koch's postulates had not been fulfilled to confirm the pathogenic potential of PDCoV. In the present study, PDCoV peptide-specific rabbit antisera were developed and used in immunofluorescence and immunohistochemistry assays to assist PDCoV diagnostics. The pathogenicity and pathogenesis of PDCoV was investigated following orogastric inoculation of 5-day-old piglets with a plaque-purified PDCoV cell culture isolate (3×10^4 $TCID_{50}$ per pig). The PDCoV-inoculated piglets developed mild to moderate diarrhea, shed increasing amount of virus in rectal swabs from 2 to 7 days post inoculation, and developed macroscopic and microscopic lesions in small intestines with viral antigen confirmed by immunohistochemistry staining. This study experimentally confirmed PDCoV pathogenicity and characterized PDCoV pathogenesis in neonatal piglets.

Introduction

Coronaviruses (CoV) are enveloped, positive-sense, single-stranded RNA viruses in the family *Coronaviridae* of the order *Nidovirales* and have the largest RNA genomes among the recognized RNA viruses thus far (Woo et al., 2010). The traditional group 1, 2, and 3 coronaviruses have been replaced with three genera designated *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus*, respectively. In recent years, a group of novel coronaviruses was identified in Asian leopard cats and some avian species (Dong et al., 2007; Woo et al., 2009) and they were proposed to represent a new coronavirus genus, i.e. the fourth genus *Deltacoronavirus* (Woo et al., 2010). During a molecular surveillance study conducted by a Hong Kong group, additional deltacoronaviruses were identified in avian and mammalian species including two porcine CoVs (HKU-15-44 and HKU-15-155) detected in pig samples collected in 2009 (Woo et al., 2012). Hereby, five coronaviruses have been identified in pigs: porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine respiratory coronavirus (PRCV) in the genus *Alphacoronavirus*; porcine hemagglutinating encephalomyelitis virus (PHEV) in the genus *Betacoronavirus*; and porcine deltacoronavirus (PDCoV) in the genus *Deltacoronavirus*.

PDCoV was first detected in US swine in the state of Ohio in February 2014 from pigs with diarrhea (Wang et al., 2014a) and has been confirmed in 17 US states as of December 2014 (www.aasv.org). PDCoV has also recently been detected in South Korea (Lee and Lee, 2014). It remains unclear when PDCoV was introduced into the US, but a recent PCR-based retrospective evaluation of diagnostic samples revealed that PDCoV could be detected as early as August 2013 in pig samples from the US (Sinha et al., unpublished data). The US PDCoV sequences determined thus far share high nucleotide identity ($\geq$ 99.8%), with 98.9–99.2% nucleotide identity to the Hong Kong strains, and 99.6–99.8% nucleotide identity to the Korean strain, at the whole genome level (Lee and Lee, 2014; Li et al., 2014; Marthaler et al., 2014a, 2014b; Wanget al., 2014a, 2014b; Woo et al., 2012). The genome organization and arrangement of PDCoV are in the order of: 5′ untranslated region (UTR), open reading frame 1a/1b (ORF1a/1b), spike (S), envelope (E), membrane (M), nonstructural protein 6 (NS 6), nucleocapsid (N), nonstructural protein 7 (NS 7), and 3′ UTR (Li et al., 2014; Woo et al., 2012).

Thus far, all peer-reviewed literature related to PDCoV has focused on polymerase chain reaction (PCR) detection and genetic analyses. There are no other published assays for diagnosing

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PDCoV infection worldwide. Although PDCoV has been detected in swine samples, its clinical significance as an etiological pathogen has not been experimentally confirmed in pigs. In this paper, the development of PDCoV peptide-specific rabbit antisera and the use of these antisera in immunofluorescence and immunohistochemistry assays for PDCoV detection are described. Additionally, 5-day-old piglets were experimentally inoculated with a plaque-purified US PDCoV cell culture isolate to characterize the pathogenicity and pathogenesis of this virus.

Results

PDCoV propagation

Swine testicle (ST) cells infected with the plaque-purified PDCoV cell culture isolate USA/IL/2014 at a multiplication of infection (MOI) of 1 developed $>80\%$ cytopathic effects (CPE) at 24 h post infection (hpi), characterized by syncytia formation and cell detachment (Fig. 1A). Virus harvested at 24 hpi had a titer of 1.8×10^7 $TCID_{50}$/ml and was stored at $-80\,^{\circ}C$ until being diluted to 3×10^3 $TCID_{50}$/ml for pig inoculation.

PDCoV peptide-specific rabbit antisera

Rabbit antisera against the PDCoV M-peptide, N-peptide, and S-peptide collected at Day 0, 28, 56, 72 and 90 post immunization were diluted (1:100, 1:200, 1:500, and 1:1000) and evaluated by IFA on PDCoV-infected ST cells. Sera collected at Day 28, 56, 72 and 90 from one rabbit (#8005) immunized with the PDCoV M-peptide yielded positive IFA staining at 1:100, 1:200 or 1:500 dilutions. There were no marked staining differences for sera collected at Day 28, 56, 72 or 90. However, 1:200 dilutions gave best IFA staining signals with less background staining. One representative image is shown in Fig. 1B. Sera at Day 0 were IFA negative. Similarly, sera collected at Day 28, 56, 72 and 90 from one rabbit (#8049) immunized with the PDCoV S-peptide were IFA positive (Fig. 1D). However, even after optimization of serum dilutions, the PDCoV S-peptide rabbit antisera still produced stronger background staining in the negative control ST cells (Fig. 1H) compared to the negative control ST cells tested by the PDCoV M-peptide rabbit antisera (Fig. 1F). Sera collected from the second rabbit immunized with the PDCoV M-peptide, from the second rabbit immunized with the PDCoV S-peptide, and from two rabbits immunized with the PDCoV N peptide, were always IFA negative regardless of collection date or dilutions. Thus, the PDCoV M-peptide rabbit antisera (#8005) were selected for subsequent evaluation and assay development.

We further evaluated potential cross staining of PDCoV M-peptide rabbit antisera, PEDV monoclonal antibody conjugate SD6-29, and a TGEV polyclonal antibody conjugate on PDCoV-, PEDV-, TGEV- and PRCV-infected cells. As shown in Fig. 2A, B, C and D, PDCoV M-peptide rabbit antisera specifically stained PDCoV-infected ST cells but did not stain PEDV-infected Vero cells or TGEV- or PRCV-infected ST cells. PEDV N-based monoclonal antibody specifically stained PEDV-infected cells but not in PDCoV-, TGEV-, or PRCV-infected cells (Fig. 2E, F, G and H). The TGEV polyclonal antibody conjugate stained TGEV- and PRCV-infected cells but not in PDCoV- or PEDV-infected cells (Fig. 2I, J, K and L).

Clinical assessment

In PDCoV-inoculated pigs, 2/10 pigs had soft feces during 2-4 DPI, 5/5 pigs developed mild diarrhea at 5 DPI, 5/5 pigs had profuse watery diarrhea at 6 DPI, and 3 of 5 pigs recovered to mild diarrhea at 7 DPI (Fig. 3A and B). Inoculated pigs remained active but had fecal staining on the skin (Fig. 3C). No vomiting, dehydration, severe loss in body condition, lethargy, loss of appetite, or mortality was observed in PDCoV-inoculated pigs in spite of the presence of diarrhea. The negative control pigs were active and fleshy throughout the study period with no observed clinical signs.

There were no significant differences in average body weights between the PDCoV-inoculated and the negative control pigs at (−1) DPI (P-value=0.62), 4 DPI (P-value=0.74), or 7 DPI (P-value=0.65) (Fig. 3D). Average daily gain was lower in the PDCoV-inoculated compared to the negative control pigs between (−1) and 4 DPI but the differences were not statistically significant (P-value=0.06). Average daily gain between (−1) and 7 DPI of the two groups was also not significantly different (P-value=0.22).

Virus shedding and distribution

Fecal virus shedding of the PDCoV-inoculated pigs is summarized in Table 2 and Fig. 4A. PDCoV RNA was detected in rectal swab samples from 1/10 pigs at 2 DPI with a Ct value of 37.4 (equivalent to 3 $TCID_{50}$/ml), from 4/10 pigs at 3 DPI with Ct ranges of 28.99–32.23 (10^2–10^3 $TCID_{50}$/ml), and from 8/10 pigs at 4 DPI with wide Ct ranges of 19.05–37.53 ($10^{0.5}$–10^5 $TCID_{50}$/ml). All of the 5 remaining pigs after the first necropsy were PDCoV PCR positive

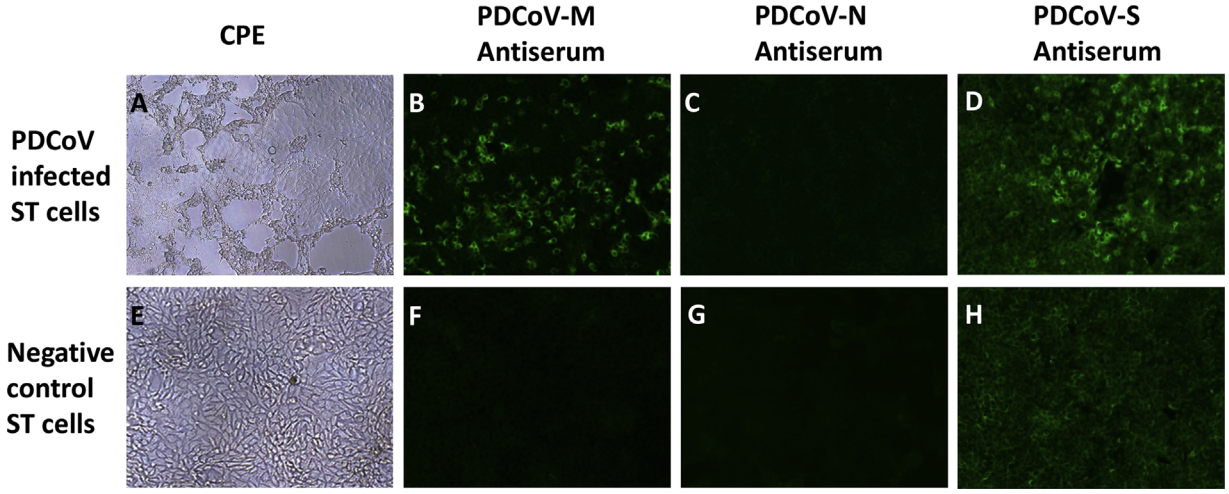

Fig. 1. Cytopathic effect (CPE) and IFA staining on PDCoV-infected ST cells or negative control ST cells (100× magnifications). (A) PDCoV CPE on ST cells at 24 h post infection; (E) negative control ST cells; (B and F) IFA staining with PDCoV M-peptide rabbit antiserum; (C and G) IFA staining with PDCoV N-peptide rabbit antiserum; (D and H) IFA staining with PDCoV S-peptide rabbit antiserum.