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The effects of transplacental porcine circovirus type 2 infection on porcine epidemic diarrhoea virus-induced enteritis in preweaning piglets

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Abstract

The effects of transplacental porcine circovirus type 2 (PCV2) infection on porcine epidemic diarrhoea virus (PEDV)-induced enteritis were examined in neonatal piglets. Six pregnant sows were randomly allocated to an infected ($n = 3$) or control group ($n = 3$). Three pregnant sows were inoculated intranasally with 6 mL of tissue culture fluid containing 1.2×10^5 tissue culture infective doses 50% ($TCID_{50}$)/mL of PCV2 strain SNUVR000470 three weeks before the expected farrowing date. Three control pregnant sows were similarly exposed to uninfected cell culture supernatants. Thirty piglets from PCV2-infected sows were randomly assigned to two groups (A and B) of 15 piglets each. Another 30 piglets from noninfected sows were randomly assigned to two groups (C and D) of 15 piglets each. The piglets in groups A and C were dosed orally at three days of age with 2 mL of virus stock ($1 \times 10^{6.5}$ $TCID_{50}$/mL) of the PEDV strain, SNUVR971496, at the third passage.

The mean villous height and crypt depth (VH:CD) ratio in PEDV-infected piglets from PCV2-infected sows (group A) were significantly different from those of the PEDV-infected piglets from PCV2 negative sows (group C) at 36, 48, and 72 h post-inoculation (hpi) ($P < 0.05$). In PEDV-infected piglets from PCV2-infected sows (group A), significantly more PEDV nucleic acid was detected in the jejunal tissues ($P < 0.05$) at 24 hpi than in the same tissues of the PEDV-infected piglets from PCV2 negative sows (group C). Thereafter, at 36, 48, 60, and 70 hpi significantly more PEDV nucleic acid ($P < 0.05$) was detected in the jejunal tissues of the PEDV-infected piglets from PCV2 negative sows (group C) than those of the PEDV-infected piglets from the PCV2-infected sows (group A). It is concluded that the clinical course of PEDV disease was markedly affected by transplacental infection of PCV2.

Keywords: Porcine epidemic diarrhoea virus; Pig; Porcine circovirus; Potentiation

1. Introduction

Porcine epidemic diarrhoea virus (PEDV), a member of the genus *Coronavirus*, family Coronaviridae, Order Nidovirales, is antigenically distinguishable from the other porcine coronaviruses such as transmissible gastroenteritis virus, porcine respiratory coronavirus and haemagglutinating encephalomyelitis virus (Pensaert et al., 1981; Zhou et al., 1988; Cavanagh, 1997). PEDV infection causes acute enteritis in swine of all ages and is often fatal in neonatal piglets. It causes destruction of villous enterocytes and villous atrophy within the jejunum and ileum (Pensaert and de Bouck, 1978; Kim and Chae, 2000), and there has been a high incidence of diarrhoea and death in neonatal piglets associated with PEDV infection which has become a major economic concern in Korea (Chae et al., 2000).

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Porcine circovirus type 2 (PCV2), a member of genus *Circovirus*, family Circoviridae, is the smallest virus that replicates autonomously in mammalian cells (Lukert et al., 1995; Mankertz et al., 1997) and shares the distinctive genomic structure of a covalently closed, circular, negative sense, single-stranded DNA molecule (Todd et al., 1991). PCV2 has been associated with a number of different syndromes and diseases such as postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure and, possibly, exudative epidermitis (Allan and Ellis, 2000; Chae, 2004, 2005).

Dual infection with PEDV and PCV2 has also been reported in neonatal piglets (Hirai et al., 2001). In addition, it has been suggested that PCV2 may be involved in the pathogenesis of PEDV infection in neonatal piglets (Jung et al., 2005). Field observations suggest that severe diarrhoea occurs with PEDV in PCV2-infected piglets (C. Chae, personal observation). Recently, transplacental transmission of PCV2 was demonstrated in pregnant sows (Park et al., 2005) but interaction between PEDV and PCV2 has not been demonstrated. The object of the present study was to determine if congenital infection with PCV2 produced a longer clinical course and more severe histopathological lesions in pigs infected post-natally with PEDV.

2. Materials and methods

2.1. Virus strains

PCV2 strain SNUVR000470 was isolated from lymph nodes in sows with respiratory disease which had been submitted to the diagnostic laboratory at the College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University. PEDV strain SNUVR971496 was isolated from the small intestine of a 3-day-old pig with severe diarrhoea.

2.2. Experimental design

Six pregnant sows with known breeding dates were purchased from a commercial herd believed to be free of PMWS based upon herd history, clinical signs, and diagnostic tests. All were second parity sows. The herd had sporadic colibacillosis in preweaning piglets. Pregnant sows were vaccinated with *Escherichia coli* $F4^+$, $F5^+$, $F6^+$ and $F41^+$ strains, *Clostridium perfringens*, transmissible gastroenteritis virus (TGEV), and rotavirus (groups A and B).

Six pregnant sows were identified that were free of PCV, porcine parvovirus (PPV), and porcine reproductive and respiratory syndrome virus (PRRSV)-specific antibody. PCV1 and PCV2 infections were tested by indirect fluorescent antibody test, and PPV and PRRSV infections were tested by enzyme-linked immunosorbent assay. The serological tests were performed twice at 3-week intervals prior to the start of the experiment. PCV1 and PCV2 were not detected in serum samples from six sows by polymerase chain reaction (PCR) (Kim et al., 2001; Kim and Chae, 2001). The sows were randomly allocated to an infected ($n = 3$) or control group ($n = 3$).

The viral inoculum contained isolate PCV2 strain SNUVR000470 at its third passage in PCV-free PK-15 cells. Three pregnant sows were inoculated intranasally with 6 mL of tissue culture fluid containing 1.2×10^5 tissue culture infective doses 50% ($TCID_{50}$)/mL of PCV2 strain SNUVR000470 at 93 days of gestation. The three control pregnant sows were similarly exposed to uninfected cell culture supernatants. Each inoculum was instilled over a period of 4–5 min into both nostrils.

The sows were housed in isolation facilities and allowed to farrow naturally, but the farrowings were attended. Thirty piglets from the PCV2-infected sows were randomly assigned to two groups (A and B) of 15 piglets each. Another 30 piglets from the noninfected sows were randomly assigned to two groups (C and D) each of 15 piglets. An equal number of animals from each litter were allotted to each group. The piglets in groups A and C were dosed orally at 3 days old with 2 mL of virus stock ($1 \times 10^{6.5}$ $TCID_{50}$/mL) of the PEDV strain SNUVR971496 at the third passage. The piglets in groups B and D were similarly treated with uninfected cell culture supernatants.

All piglets were maintained in sterile stainless steel isolators (three per isolator) and fed a commercial sterile milk substitute. The piglets were examined three times daily for clinical signs. Subgroups of three piglets from each group were euthanased and subjected to necropsy at 24, 36, 48, 60 and 72 h post-inoculation (hpi). Tissue specimens were collected as previously described (Kim and Chae, 2002a).

The experiment was approved by the Institutional Animal Care and Use Committee of Seoul National University.

2.3. In situ hybridization

A 412 base pair (bp) cDNA probe for the viral RNA encoding the membrane protein of PEDV was used as a probe. The forward and reverse primers were 5′-GGGCGCCTGTATAGAGTTTA-3′ (nucleotides 927–946) and 5′-AGACCACCAAGAATGTGTCC-3′ (nucleotides 1319–1338), respectively (Kim and Chae, 2002b). A 547 bp DNA fragment from open reading frame (ORF) 2 was used as a

PCV2 probe. The forward and reverse primers were 5′-CAGTTCGTCACCC-TTTCCC-3′ (nucleotides 939–957) and 5′-GGGGGACCAACAAAATCTCT-3′ (nucleotides 1466–1485), respectively (Kim et al., 2001). The PCR for both viruses was carried out as previously described (Kim et al., 2001; Kim and Chae, 2002b).

The PCR products from PEDV and PCV2 were purified with Wizard PCR Preps (Promega Biotech) and labeled by random priming with digoxigenin-dUTP with a commercial kit (Boehringer Mannheim), according to the manufacturer's instructions. In situ hybridization was carried out as previously described (Kim and Chae, 2000; Kim et al., 2003). The digoxigenin-labelled cDNA probe of TGEV and PCV1 was used as negative probe (Kim and Chae, 2001, 2002a). Negative jejunal tissue controls were collected from three 1-day-old colostrum-deprived piglets that had been experimentally infected with TGEV (Kim and Chae, 2002a).

2.4. Morphometric analysis

Three pieces of formalin-fixed jejunum were taken from each virus-infected and control piglet for morphometric analysis. Only well-orientated sections were measured and care was taken to ensure that only transverse sections cut perpendicularly from villous enterocytes to the muscularis mucosa were included. Villous height, crypt depth, and number of positive cells for ISH were estimated by measuring 10 villi and crypts throughout the section. Villous height and crypt depth (VH:CD) ratios were calculated as previously described (Kim and Chae, 2002a). The mean number of positive cells for in situ hybridization per villous was determined by dividing the number of positive cells by the number of villi examined.

Three pieces of formalin-fixed inguinal lymph node were taken from the piglets of PCV-infected sows for morphometric analysis as previously described (Kim et al., 2003). To obtain quantitative data, the morphometric analysis of in situ hybridization slides was performed with the NIH Image J Program (National Institutes of Health). In each case, three fields were randomly selected, the number of positive cells per unit area (0.25 mm^2) were counted, and the mean values were calculated.

2.5. Statistical analysis

The Wilcoxon matched pairs test was used to determine the significance of differences between infected and control piglets in terms of (1) VH:CD ratio, (2) mean number of PEDV-positive cells per villous, (3) mean number of PCV2-positive cells per lymph node.

3. Results

3.1. Clinical signs

The signs observed in PEDV-infected piglets from PCV2-infected sows (group A) and PEDV-infected piglets from PCV2 negative sows (group C) ranged from diarrhoea to vomiting and dehydration. PEDV-infected piglets from PCV2-infected sows (group A) developed signs of anorexia, vomiting and diarrhoea within 12 hpi. In pigs in group C, diarrhoea was seen in all piglets at 12 hpi. Anorexia was seen in some piglets at the onset of diarrhoea, but others continued to eat until they became too weak to do so. Vomiting was seen in some piglets at 24 hpi but by 36 hpi all infected animals were vomiting. In addition, they appeared to be severely dehydrated at 36 hpi. No clinical signs were seen in the piglets from PCV2-infected sows (group B) and negative control piglets (group D).

3.2. VH:CD ratio

The results are summarised in Fig. 1. At 24 hpi, the mean VH:CD ratio in groups A, B, and C was not significantly different from that of group D. At 36, 48 60, and 72 hpi, however, the mean value in PEDV-infected piglets (groups A and C) was significantly different from that of the controls (group D). The mean VH:CD ratio in PEDV-infected piglets from PCV2-infected sows (group A) was significantly different from that of the PEDV-infected piglets from PCV2 negative sows (group

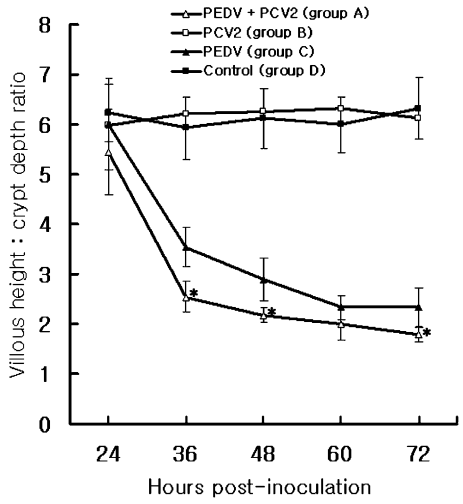

Fig. 1. Mean villous height and crypt depth (VH:CD) ratios from porcine epidemic diarrhoea virus (PEDV)-infected piglets from porcine circovirus type 2 (PCV2)-infected sows (group A), piglets from PCV2-infected sows (group B), PEDV-infected piglets from PCV2 negative sows (group C), and piglets from PCV2 negative sows (group D). Bars represent standard deviation. *Significant ($P < 0.05$) decrease in VH:CD ratio in PEDV-infected piglets from PCV2-infected sows compared to piglets from PCV2 negative sows.

C) at 36, 48, and 72 hpi ($P < 0.05$). The mean VH:CD ratio in piglets from PCV2-infected sows (group B) was not significantly different from that of the negative control pigs (group D) throughout the experiment (Fig. 1).

3.3. PEDV distribution in infected piglets

PEDV nucleic acid was detected at 24, 36, 48, 60 and 72 hpi, being seen almost exclusively in the cytoplasm of jejunal villous enterocytes from PEDV-infected pigs from PCV2-infected sows (group A) and PCV2 negative sows (group C). Intense and specific hybridization and immunohistochemical signals were most often seen within jejunal villous enterocytes. Strong hybridization signals were seen in areas of moderate to severe villous atrophy or vacuolation with positive cells arranged continuously over the villi. In the lumen, exfoliated enterocytes were strongly positive. No hybridization signal was consistently seen in tissue sections pretreated with RNase A. The cDNA probes for TGEV were consistently negative in tissues tested from both infected and uninfected pigs. PEDV nucleic acid was not detected in the intestinal tissues from uninfected control piglets.

Fig. 1 summarises the number of positive results (PEDV nucleic acid) in four specific tissues from the two groups (A and C). Positive cells typically exhibited a dark black reaction product in the cytoplasm, without background staining. In PEDV-infected piglets from PCV2-infected sows (group A), significantly more PEDV nucleic acid was detected in the jejunal tissues at 24 hpi ($P < 0.05$) than in the same tissues of the PEDV-infected piglets from PCV2 negative sows (group C). Thereafter, at 36, 48, 60, and 70 hpi significantly more PEDV nucleic acid ($P < 0.05$) was detected in the jejunal tissues of the PEDV-infected piglets from PCV2 negative sows (group C) than of PEDV-infected piglets from PCV2-infected sows (group A) (Fig. 2).

3.4. PCV2 distribution in infected piglets

PCV2 DNA was detected in lymph nodes, spleen, tonsil and small intestines harvested from all piglets from PCV2-infected sows (groups A and B). Positive cells typically exhibited a dark black reaction product in the nucleus and cytoplasm, without background staining. Hybridization signals of PCV2 were seen occasionally in lymphoid tissues in pig with congenital PCV2 infection. The granulomatous inflammatory reaction and lymphoid depletion that are typical lesions associated with PCV2 infection in pigs with PMWS were not observed in the liver and lymph nodes taken from piglets from PCV2-infected sows. The DNA probes for PCV1 were consistently negative in tissues tested from pigs from PCV2-infected sows. The mean number of PCV2 positive cells in PEDV-infected piglets from PCV2-infected sows (group A) was not significantly different from that of the piglets from PCV2-infected sows (group B) throughout the experiment.

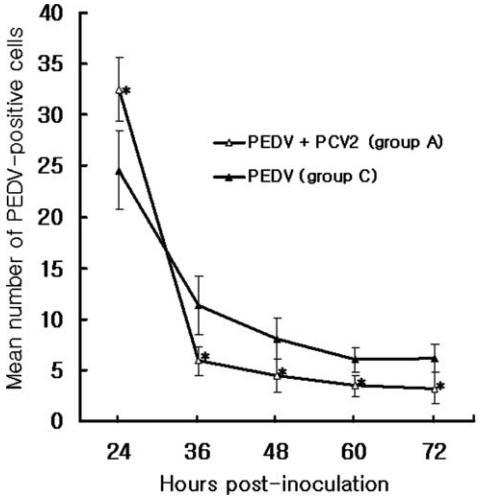

Fig. 2. Mean porcine epidemic diarrhoea virus (PEDV)-positive cells in the jejunal tissues from PEDV-infected piglets from porcine circovirus type 2 (PCV2)-infected sows (group A) and PEDV-infected piglets from PCV2 negative sows (group C). Bars represent standard deviation. * Significant ($P < 0.05$) difference between groups A and B in numbers of PEDV-positive cells.

4. Discussion

This study was designed to determine any interaction between PEDV and PCV2. Congenital infection with PCV2 produced a longer clinical course and more severe histopathological lesions in pigs infected post-natally with PEDV. Coinfection with PEDV and PCV2 resulted in more severe lesions compared to animals infected with either PEDV or PCV2 alone. Thus a possible explanation for the difference between the dually and singularly infected pigs in terms of lesions could be the effect of PCV2. Our results indicate that PCV2 infection potentiates PEDV-induced disease and lesions and clearly this may be important with respect to the control of enteric disease.

Clinical syndromes and diseases associated with PCV2 infections are divided into pre- and post-natal manifestations (Chae, 2005). In the former, PCV2 infection is linked to reproductive failure and perhaps even a subclinical infection (West et al., 1999; O'Connor et al., 2001). Pre-natal subclinical infection is most frequently asymptomatic as shown in this study. However, prior transplacental subclinical infection of piglets with PCV2 affects the severity of enteric disease when the pigs were challenged three days later with PEDV. In field observations, severe, prolonged and recurrent diarrhoea was reported for preweaning pigs infected with PEDV. The severe diarrhoea in piglets naturally infected with PEDV in the field was similar to the finding in our study.

Numbers of PEDV-positive cells in intestinal tissues were significantly increased in PEDV-infected piglets from PCV2-infected sows in early infection compared to piglets from PCV2 negative sows. In contrast, the number of PEDV-positive cells was significantly decreased in PEDV-infected piglets from PCV2-infected sows in late infection compared to piglets from PCV2 negative sows. There was villous atrophy and fusion of the enterocytes in which PEDV was shown to be replicating, indicating a direct pathogenic effect of the virus. As a result, PEDV replicated rapidly, destroying the villous enterocytes within 24 h of the initial infection. These changes decrease the intestinal surface and thereby limit number of available cells for replication of PEDV. Thus, after destroying the villous enterocytes, levels of PEDV in intestinal tissues were significantly decreased in later stages of the infection.

Once PEDV infection occurs on Korean farms, most pig producers purposely feed the intestines of sick piglets to pregnant sows to control PEDV infection. PCV2 typically causes subclinical infections, since many (if not all) farms worldwide are seropositive, whereas just a small proportion of farms show cases of PMWS (Larochelle et al., 2003; Rose et al., 2003). In addition, subclinically infected sows could be more prevalent than clinical reproductive failures suggests, although PCV2 infection is linked to abortion, stillbirths, and mummified fetuses (West et al., 1999; O'Connor et al., 2001). Therefore, exposure of virulent PEDV from the intestines of sick piglets is not recommended for the control of PEDV infection not least because of the concurrent infection of PCV2.

The number of PEDV-positive cells in PEDV-infected pigs from PCV2-infected sows was significantly higher than that in PEDV-infected piglets from PCV2 negative sows. In contrast, there was no difference in the number of PCV2-positive cells between PEDV-infected piglets and PEDV negative piglets from PCV2-infected sows. These results suggest a direct effect of PCV2 on the replication of PEDV.

In the present study, no lymphoid lesions (lymphocyte depletion together with granulomatous inflammation) compatible with PMWS were observed in the PEDV-infected piglets from PCV2-infected sows probably because it was too early for this to have occurred.

Although no other indicators of immunosuppression were measured, our data may suggest that transplacental infection with PCV2 could affect in some unknown way the outcome of PEDV infection in post-natal pigs. The synergism between PCV2 and PEDV could result because each of the viruses infects different types of enteric cells and thus they may combine to produce a more severe disease. It is concluded that in this study the clinical course of PEDV disease was markedly affected by transplacental infection of PCV2.

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