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Pancreatic lesions of pigs with post-weaning multisystemic wasting syndrome

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Abstract : Post-weaning multisystemic wasting syndrome (PMWS) is a new emerging disease affecting nursery and growing pigs in worldwide. Porcine circovirus type 2 (PCV-2) is a most important pathogen associated with PMWS. This study was carried out to investigate the pathological changes in the pancreas of pigs diagnosed as PMWS. To detect the PCV-2 antigen and nucleic acid in the tissue, immunohistochemistry and polymerase chain reaction (PCR) was conducted, respectively. 24 pigs of 4-10 weeks old showed clinical signs of PMWS such as chronic wasting, respiratory distress and diarrhea were examined. Histopathologically, interstitial and periductular mononuclear cells infiltration were observed in pancreas. Multifocal to diffuse necrosis of acinar tissues or necrotizing to granulomatous pancreastitis with numerous syncytial cells infiltration were examined in severe cases. PCV-2 nucleic acid was detected from all tested pancreas using PCR. The PCV-2 antigen in 12 pancreas sections was detected by immunohistochemical staining. PCV-2 has a tropism for vascular endothelial cells and infiltrated macrophages. Although gross lesions are uncommon in the pancreas of pigs with PMWS, histopathological changes and the presence of PCV-2 in this tissue may be related to clinical signs associated with digestive disorders.

Keywords : immunohistochemistry, pancreas, PCR, PCV-2, PMWS

Introduction

Porcine circovirus type 1 and type 2 (PCV-1 and PCV-2) are a member of the family *Circoviridae* and genus *Circovirus* [5]. The PCV virion is nonenveloped agents containing a unique single strand circular DNA genome of 1.76 kb to 2.31 kb [5, 12]. PCV-1 was originally detected as a contaminant of a continuous pig kidney cell line (PK-15). Although serological surveys have indicated that antibodies to PCV-1 are very common in the swine population, PCV-1 is regarded as a nonpathogenic virus [3]. In contrast, PCV-2 antigen has been demonstrated in association with lesions in pigs with proliferating and necrotizing pneumonia, sow abortion and mortality syndrome, porcine dermatitis and nephropathy syndrome, and primarily post-weaning multisystemic wasting syndrome (PMWS) [1, 20].

PMWS is a new emerging disease affecting nursery and growing pigs [3, 5, 17]. PMWS was first observed in Canada in 1991, has been a major pig health problem in worldwide. The diagnosis of PMWS is based on the characteristic clinical signs and histopathologic lesions, as well as demonstration of the virus within the tissues of affected pigs [1, 3, 4, 7, 17]. Clinical signs of PMWS include progressive weigh loss, respiratory distress, lymphadenopathy, skin pallor, diarrhea, and occasionally jaundice [9, 10, 11]. Affected pigs have characteristic gross lesions in many organs, mainly the presence of enlarged superficial inguinal lymph nodes, and non-collapsed lungs with surface mottling [17]. Histopathological lesions include macrophage infiltration,

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lymphocyte depletion, multinucleated giant cells formation and numerous cytoplasmic and nuclear basophilic inclusion bodies in lymphoid tissues [1, 9, 17]. Non-lymphoid lesions include pneumonia, hepatitis, nephritis, myocarditis, encephalitis, enteritis, and pancreatitis [1, 9, 14, 17, 20].

Until recently, no detailed pathogenic studies have been focused on the occurrence of pancreatic lesions in PMWS-affected pigs. Therefore this study was carried out to investigate the pathological changes and to detect the PCV-2 antigen and nucleic acid in the pancreas of pigs with the clinical signs of diarrhea from Jeju diagnosed as PMWS.

Materials and Methods

Tissue samples and histopathology

A total of 24 pigs with diarrhea from 16 farms were selected on the basis of PMWS infection. For all of them PMWS was diagnosed by histological findings and by polymerase chain reaction (PCR) identification of the PCV-2 nucleic acid in samples from the superficial inguinal lymph node and lung. The ages of pigs ranged from 4 to 10-weeks. Negative control pancreas tissues were prepared from 6-week-old PCV-2 negative pigs in Jeju.

All pancreas samples were collected from pigs at necropsy. One-third of each pancreas was frozen at –70°C for PCR, while the other part was fixed in 10% neutral buffered formalin for histopathology. Formalin-fixed pancreas were embedded in paraffin wax by standard methods, cut into 3 μm sections, and stained with hematoxylin and eosin (H&E).

PCR

PCR analysis was conducted to detect viral DNA in pancreas tissue essentially as described previously [16]. Frozen pancreases were washed thoroughly with physiological saline until complete removal from blood. Small tissue blocks from pancreas (0.5 g) were homogenized with 5 mL of DNase RNase free distilled water (Invitrogen, USA). After centrifugation, 200 μL of supernatant were processed using the G-spin Genomic DNA extraction kit (iNtRON Biotech, Korea) to extract the DNA. PCR primers were selected according to the published sequences of PCV-2. The primers CF8 (5′-TAGGTTAGGGCTGTGGCCTT-3′) and CR8 (5′-CCGCACCTTCGGATATACTG-3′) were used for the PCR amplification (263 bp products). The reaction conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C during 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min. A final 10 min extension step at 72°C was included. All PCR analyses were performed on the Thermal Cycler Dice TP600 (TakaRa, Japan). The amplified products were run in a 1.5% agarose gel, and visualized by staining with 0.5 μg/mL of ethidium bromide.

Immunohistochemistry (IHC)

Immunohistochemical identification of PCV-2 was performed with a streptavidin-biotin-complex immuno-peroxidase method. The sections were placed on silane-coated slides, dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase was removed by 10 min reaction of 3% hydrogen peroxide in phosphate-buffered saline (PBS), and digested with 0.05% protease (Sigma, USA) for 10 min at 37°C. After rinsing in PBS, they were incubated in 10% normal goat serum for 20 min at 37°C to prevent background labeling. Primary rabbit anti-PCV2 antibody (Iowa State University, USA) was diluted (1 : 1,000) in antibody diluent solution (Dako, USA), and incubated on a slide at 37°C for 1 h. After washing in PBS, a solution of biotinylated goat anti-mouse and anti-rabbit antibody (Dako, USA) was applied to the sections for 40 min at 37°C. They were then washed in PBS and a streptavidin-horseradish peroxidase (Dako, USA) was applied for 30 min at 37°C. Sections were finally incubated in 3,3′-diaminobenzidine tetrahydrochloride (Dako, USA) for 1-2 min, and counterstained with Mayer's hematoxylin.

Bacteriology

If necessary, both aerobic and anaerobic bacterial cultures were performed using aseptically taken intestinal contents. Isolated bacteria were identified using a Vitek system automatic identification apparatus (Vitek, USA).

Results

Clinical signs and gross findings

All pigs showed typical clinical signs of PMWS including wasting or unthriftiness, dyspnea, and enlarged lymph nodes. In addition, all pigs showed digestive symptoms such as watery diarrhea (13 pigs) or digestive

Table 1. Results of histopathological lesions, PCR and IHC in the pancreas of 24 pigs diagnosed as by PMWS

Lesion severity	Microscopic lesions	No. of pig	PCR +	IHC +
No	No typical lesion	9	9	1
Mild	Vacuolar degeneration	4	4	1
Moderate	Pancreatic acinar necrosis with mild (peri)vasculitis	7	7	6
Severe	Pancreatitis with (peri)vasculitis	4	4	4
Total		24	24	12

PCR: polymerase chain reaction, IHC: Immunohistochemistry, PMWS: post-weaning multisystemic wasting syndrome.

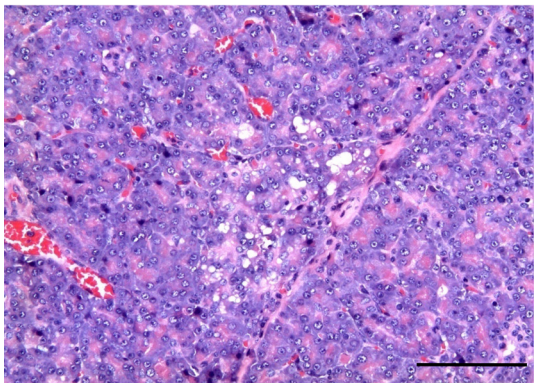

Fig. 1. Pancreas. Note vacuolar degeneration of pancreatic acinar. H&E stain, Bar = 100 μm.

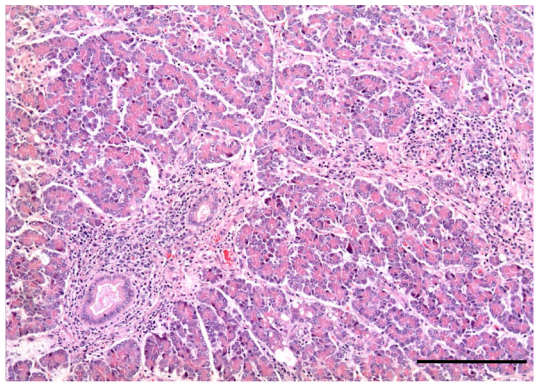

Fig. 2. Pancreas. Note interstitial and periductular mononuclear cells infiltration and degeneration of acinar cells. H&E stain, Bar = 200 μm.

disorder with soft stool (11 pigs). At post-mortem examination, gross findings were similar to those described in previous reports of PMWS, but typical gross lesions were not observed in the pancreas.

Histopathological lesions

Pancreatitis is characterized by varying degrees of edema, hemorrhage, necrosis, inflammation, and perivascular infiltration of inflammatory cells of the pancreas [13]. Because there is no adequate evaluating system for pancreatic lesions in pigs, three categories of pancreatic lesions were applied with the modification of histopathologic grading for pancreatitis [13]. The microscopic lesions observed in the pancreas from PMWS-affected pigs were summarized in Table 1.

Histopathologically, pancreatic lesions of PMWS-affected pigs were in variable severity. In mild cases, focal to multifocal epithelial atrophy or vacuolar degeneration of acinar cells without inflammatory reactions was observed in some sections (Fig. 1). In moderate cases, multifocal necrosis of acinar cells and mild inflammation characterized by periductular, perivascular and interstitial mononuclear cell infiltration were observed (Fig. 2). The distinct lesions of severe cases were multifocal cluster of mononuclear inflammatory cells within the pancreatic parenchyma and severe parenchymal atrophy or necrosis of acinar tissue and adjacent fibrosis. The zymogen granules were disappeared in necrotic acinar epithelial cells. Non-suppurative vasculitis or perivasculitis were observed in many cases. Mild to moderate fibroblast-like cells were proliferated in periductular or vascular areas. Severe necrotizing or granulomatous pancreatitis characterized by diffuse lymphohistiocytic infiltration and numerous syncytial cell formations were observed in 2 pigs (Fig. 3). Interstitial adipose tissue showed severe fat necrosis. However we could not find any typical lesions in 9 pigs. Unfortunately we could not detect any PCV-2 specific inclusions in all tested pancreas samples.

PCR and IHC

For the detection of PCV-2 nucleic acids, PCR was performed on DNA extracted from pancreas samples

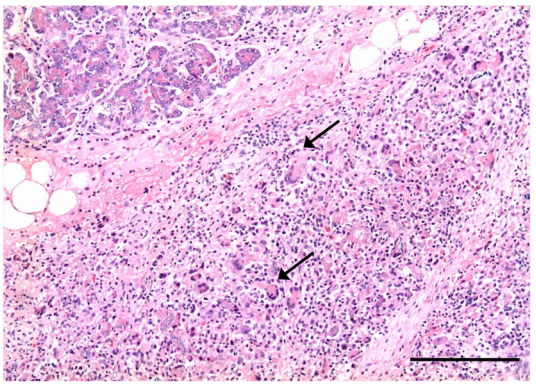

Fig. 3. Pancreas. Severe necrosis of acinar tissues with numerous mononuclear cells infiltration and syncytial formation (arrows). H&E stain, Bar = 200 μm.

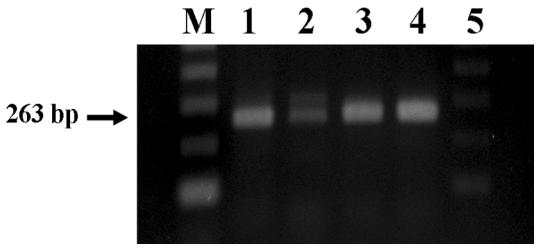

Fig. 4. PCR products of porcine circovirus type 2 (PCV-2) from pancreas homogenate. Lane M: 100 bp DNA ladder, Lanes 1-3: 263 bp field samples, Lane 4: PCV-2 positive control, Lane 5: PCV-2 negative control.

of 24 pigs. The second reaction was expected to produce 263 bp products (Fig. 4). PCV-2 nucleic acids were detected in all 24 pancreas samples examined, but not in negative control pigs.

Immunohistochemical detection of PCV-2 antigen in pancreases has been attempted in 24 pigs. PCV-2 antigens were identified in pancreas tissues of 12 pigs. Large amounts of PCV-2 antigen were labeled in the cytoplasm or nuclei of infiltrated or perivascular macrophages in 8 pancreases (Fig. 5). These macrophages had typical morphologic features such as central or eccentric nuclei and abundant foamy cytoplasm. Very strong immunohistochemical signals were widely distributed in infiltrated mononuclear cells. Occasionally, PCV-2 antigens were found in vascular endothelial cells of 6 cases and fibroblast-like cells in interstitium. The amount of PCV-2 antigens in pancreatic tissues increased with the severity of lesions.

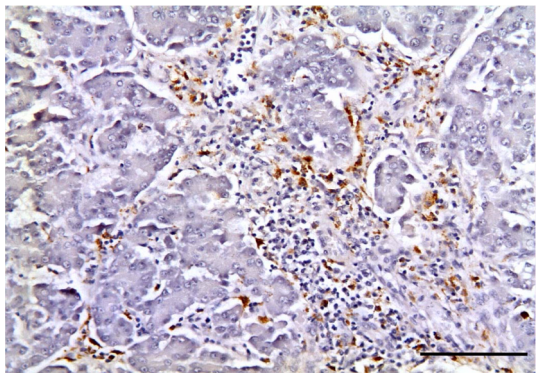

Fig. 5. Pancreas. Abundant PCV-2 antigens in infiltrated macrophages. IHC, Bar = 100 μm.

Bacteriology and other enteric lesions

In the 24 pigs, *Salmonella* spp. was isolated in 8 cases. According to gross and histopathologic findings, gastric ulcer, enteric coccidiosis, and coltitis cystica profunda were detected in 4 cases, 2 cases, and 2 cases, respectively. However, 8 pigs were free from enteric pathogens or gastrointestinal lesions.

Discussion

Pancreatitis as a specific disease is not frequent in animals with the exception of the dog, however in all species inflammation of the pancreas can occur in association with other diseases [8]. Acute pancreatitis is a condition characterized primarily by necrosis and varying degree of inflammation of the pancreas.

Although lesions and PCV-2 antigen in pancreas associated with PMWS had been demonstrated in natural cases [1, 12, 17, 20], little is known about the prevalence of pancreatic lesions, frequency of PCV-2 in pancreas and correlation with clinical signs. Because of low importance for the diagnosis of pig diseases, tissue sampling of pancreas was not usually included in necropsy procedure. According to previous report for the viral distribution in pancreas, PCV-2 nucleic acids were detected in 5 pancreases (45.5%) out of 11 PMWS-affected pigs [20]. Although typical gross findings were not examined in all pancreases, 15 pancreas samples (52.5%) of 24 pigs had variable microscopic lesions in the present study. PCV-2 induced pancreatic lesions were classified into 3 categories such as mild vacuolar degeneration in acinar cells (16.7%), moderate acinar necrosis (29.1%), and severe necrotizing to

granulomatous pancreatitis (16.7%), respectively. Most of the histopathologic lesions in this study were similar with previous reports [1, 12, 17, 20], but interestingly severe necrotizing or granulomatous pancreatitis was observed in two 70-day-old pigs. The microscopic lesions observed in necrotizing or granulomatous pancreatitis were different from those seen with PMWS. These lesions were characterized by diffuse lymphohistiocytic and numerous multinucleated syncytial cell infiltrations previously described in lungs and lymph nodes of PMWS cases [1, 4, 20].

According to the PCR method, PCV-2 nucleic acids were detected in all pancreas samples of this study. However IHC methods have been routinely used to detect PCV2 in affected tissues and to correlate its detection with the presence of lesions [4, 16, 20]. The use of IHC helped to establish the cell tropism and the pathogenesis of PCV-2 in cases of PMWS [17]. Although the overall detection rate of PCV-2 using IHC technique was lower than PCR, 12 pancreases (50%) of 24 pigs showed strong positive signals for PCV-2 antigen. The IHC positive signals were detected in 1 sample (25%) of 4 mild cases, 6 samples (85.7%) of 7 moderate cases, and 4 samples (100%) of 4 severe cases, respectively. There was close relationship between the severity of histologic lesions and the amount of PCV-2 antigens in the pancreas. This is a very similar feature, the hallmark in lymphoid tissues of PMWS-affected pigs [4]: the more severe the lesions, the higher the amount of PCV-2.

In the present study, both infiltrated inflammatory cells and vascular endothelial cells contained PCV-2. Therefore PCV-2 should be considered as new pancreatitis or pancreatic necrosis-inducing agent in pigs. Potential mechanism is viral direct damage to endothelial cells and secondary inflammation on the pancreas. The endotheliotropism of PCV-2, detected by IHC in 6 cases, and the lack of detectable immune complexes in affected vessels pointed towards a direct PCV-2-mediated vascular injury. In cases of PCV-2-associated cardiovascular lesions in piglets, a similar pathogenic endotheliotropism of the virus has been described [14]. In addition, previous research suggested that the direct virus-induced apoptosis of endothelial cells plays a role in the pathogenesis of PCV-2 associated vasculitis [19]. The vascular endothelial cells were one of the target cells of porcine reproductive and respiratory syndrome virus (PRRSV) [18]. We also performed the IHC for PRRSV previously described [6], but we could not find any evidences of PRRSV infection in all tested pancreases of this study (data not shown). Based on the IHC results, PCV-2 might be reached pancreas via blood stream, destroyed endothelial cells, and then virus-laden inflammatory cells extended pancreas. Therefore endothelial cells may play an important role in viral load of pancreas and the pathogenesis of PCV-2 related pancreatic lesions.

Pancreatic edema, mesenteric fat edema, perirenal edema, peritoneal exudate, and bowel distension were observed in piglets with necrotizing pancreatitis [15]. Although the clinical signs associated with pancreatitis are well described in dogs, these are not clearly visible in pig cases. Although enteric pathogens such as *Salmonella* spp. (8 cases) and coccidian (2 cases) or other gastrointestinal lesions (6 cases) were detected in the tested pigs, the other 8 cases failed to detect the enteric pathogens. In this study, PCV-2 nucleic acid was detected in all tested 24 pancreases and the pancreatic damage was a frequent microscopic lesion in case of PMWS with clinical signs of diarrhea. These results suggested that PCV-2 induced pancreatic lesions would be directly or indirectly related with diarrhea of pigs in this study. Although many viral, bacterial, and fungal pathogens were closely related with gastrointestinal problems in pigs, pancreatic lesions associated with PCV-2 might be as possible cause of diarrhea in PMWS cases.

Porcine cells, tissues, and organs are the primary animal tissues being considered for human transplantation for more than two decades, because of similar anatomical and physiological features in humans and pigs [2, 15]. Therefore many porcine cells such as neuron, hepatocytes, and pancreatic cells are in various stages of trials for transplantation into humans, and the results are encouraging [2]. Transmission of pig pathogens is the main risk associated with xenotransplantation using pig as donor. Recently, the Korean Food and Drug Administration listed 32 pig-related viral pathogens including PCV-2 that need to be excluded from the organ-source herd for their use in xenotransplantation. And pancreatic enzymes supplements widely used in human medicine for the treatment of exocrine pancreatic insufficiency are porcine derived. Hence the possibility of contamination of the starting material with viruses potentially present in swine and capable of infecting humans has to be considered. Application of monitoring system and

preventing xonozoonotic disease is warranted for the successful development of both xenotransplantation and replacement therapy using porcine tissues.

Conclusion

In summary, both infiltrated inflammatory cells and vascular endothelial cells in porcine pancreas contained PCV-2 antigens. Therefore PCV-2 should be considered as new pancreatitis or pancreatic necrosis-inducing agent in pigs. Although many viral, bacterial, and fungal pathogens were closely related with gastrointestinal problems in pigs, pancreatic lesions associated with PCV-2 might be as possible cause of diarrhea in PMWS cases.

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