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Correlation of viral load with lesion severity in field pigs affected with porcine circovirus type 2

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Porcine circovirus type 2 (PCV2) is an important infection factor causing post weaning multisystemic wasting syndrome (PMWS). The amount of PCV2 viral load may mainly give rise to clinical symptom and pathological lesion of PMWS. In order to investigate the relationship between PCV2 viral load and the lesion severity of lymph tissues, and search further for some clues of pathologic diagnosis of PMWS, thirty pig cases affected with PCV2 and aged 30～90 days old were collected from swine farms and their lymph tissues were treated by quantitative Real Time PCR, immunohistochemistry (IHC) and histopathology examination. Four various groups were classified according to their evaluation scores for lesion severity, and it is shown that the higher the score for pathological lesion of lymph tissues, the more there is viral load in tonsil. Especially, the amount of PCV2 DNA in group one was 1/1000 lower than other three groups. It is supposed that group one is considered as subclinical case and the other three groups as clinical PMWS cases. Furthermore, it is likely presumed whether PCV2 infection is subclinical or clinical PMWS case can be helpfully diagnosed by these criteria.

Key words: Correlation, PCV2 load, Real Time polymerase chain reaction (PCR), immunohistochemistry (IHC).

INTRODUCTION

Post weaning multisystemic wasting syndrome (PMWS) is a kind of complex disease causing late nursery and fattening pigs affected by main pathogen PCV2. It is characterized by clinical fever, progressive weight loss and respiratory and digestive disorders (Clark, 1997; Harding, 1997). The disease has been reported worldwide, including Spain (Segales et al., 1997; Segales et al., 2005), France (Blanchard et al., 2003), United States (Allan et al., 1998; Yu et al., 2007), and other countries.

As a member of the *Circoviridae* family, porcine circovirus type 2 (PCV2), a widespread, circular and single-stranded DNA virus (Allan and Ellis, 2000), is a ubiquitous agent which can infect domestic swine as a crucial infectious cause of PMWS (Segales et al., 2005). PMWS is considered as a multifactorial disease in which the occurrence of PCV2 infection is necessary but not sufficient in a number of cases (Segales et al., 2005). PCV2 can infect most tissues in domestic pigs and give

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rise to an extensive lesion. The identification results displayed that PCV2-positive cells are presented in heart, liver, lung, spleen and lymph node (Sanchez et al., 2003). Nowadays, PCV2 detection is not sufficient to establish a diagnosis of PMWS which sometimes is complicated and difficult. An accurate diagnosis for individual pigs is currently based on the presence of all of the following three conditions: clinical signs including progressive wasting after weaning, characteristic microscopic lesions, and detection of PCV2 within the lesions. Quantitative Real Time PCRs have recently been described in experimental and field cases of PCV2 infections (Brunborg et al., 2004; Olvera et al., 2004; Reiner et al., 2010). The more severe lesions and the higher amounts of viral genome by *in situ* hybridization were correlated with the higher PCV2 load in serum and swab specimens in the quantification of Real Time PCR (Segales et al., 2005). Given the complexity of PMWS diagnosis, it is essential to find a more beneficial and subsidiary diagnostic or evaluation method. The objective of this study is to assess the correlation of viral load in the tonsil with the lesion severity of pig cases affected with PCV2 by combining the results of quantitative Real Time PCR with histopathology as well as immunohistochemistry (IHC).

MATERIALS AND METHODS

Tissue samples

Thirty pig cases affected with PCV2 by PCR diagnosis, aged 30～90 days old, were collected from swine farms and used in this study. Seven kinds of tissues including tonsil, mesenteric lymph node, mandibular lymph node, ileum, spleen, pulmonary lymph node and inguinal lymph node were taken and fixed by immersion in 10% neutral buffered formalin and embedded in paraffin; these tissues were made sections for examination of PCV2 antigen and histopathology by IHC and hematoxylin and eosin (H&E) staining respectively. In addition, tonsil in each case was taken out and stored in refrigerator at minus 20°C and was used for the quantitation of PCV2 DNA by Real Time PCR.

Histopathology examination

According to the conventional staining procedure, H&E staining was conducted to observe histopathology in these tissues. On the basis of lesion content which contains lymphocyte depletion, necrosis, inclusion bodies, and so on, a set of evaluation criteria was made in order to evaluate the lesion severity as below. Briefly, these tissue sections were evaluated by the presence of lymphocyte depletion ranging from 0 to 3 (0, Normal; 1, Mild lymphocyte depletion or dispersed single cell necrosis of histiocyte or macrophage linage cells in lymphoid follicle; 2, Moderate lymphocyte depletion or aggregated necrotic cells in follicles; 3, Severe lymphoid depletion with loss of lymphoid follicle structure) (Opriessnig et al., 2004). Moreover, cytoplasm inclusion bodies or histiocytic-to-granulomatous inflammation in these tissues were scored, and evaluation on presence of cytoplasm inclusion bodies ranged from 0 to 3 (0, No detected; 1, A few number of inclusion bodies or mild histiocytic-to-granulomatous inflammation; 2, Inclusion bodies in multiple follicles or moderate histiocytic-to-granulomatous inflammation; 3, Inclusion bodies in almost all of follicles or severe histiocytic-to-granulomatous inflammation with replacement of follicles).

IHC detection

In order to detect PCV2 antigen of these cases, IHC for PCV2-specific antigen was performed on tissue sections of formalin-fixed, paraffin-embedded blocks of selected organ samples. The fixed tissues were pretreated with protease and antiserum as previously (Onuki et al., 1999; Kawashima et al., 2003), then the staining assessment on PCV2 antigen was run in a blinded fashion and its scores ranged from 0 to 3 (0, Negative; 1, Less than 10% of the lymphoid follicles contain cells with PCV2 antigen staining; 2, 10～50% of the lymphoid follicles contain cells with PCV2 antigen staining; 3, More than 50% of the lymphoid follicles contain cells with PCV2 antigen staining) (Opriessnig et al., 2004).

The total score for evaluation of tissue lesion can be expressed by the following format: Score = (A + B) / 2 + C (A: The score from the presence of lymphocyte depletion; B: The score from presence of cytoplasm inclusion bodies; C: The score from the presence of PCV2 antigen).

Extraction of DNA

Among these lymph tissues, tonsils were especially selected to detect the amount of PCV2 DNA. Firstly, the stable suspension was made from these tonsils by weighing 100 mg exactly, shocking with shocker at 2000 rpm for 10 s, centrifuging at 2000 rpm for 30 s, homogenizing with shocker at 2000 rpm for 10 s, centrifuging at 2000 rpm for 30 s. Then DNA was extracted from the stable suspension using QIAampR DNA Mini Kit according to the manufacturer's instructions (QIAGEN GmbH, Germany). At last the lysate was taken and stored in refrigerator at minus 20 °C.

Quantitation by Real Time PCR

The lysate extracted from tonsils was run to quantify PCV2 DNA by an optimized TaqMan PCR. Reactions were carried out in 96-well plates, including sample and standard (from 10^{-5} to 10^{-1} PCV2 plasmid copies/ml), both by duplicates. A negative control was used by DNase and RNase-free distilled water. Amplification and detection of fluorescence was carried out in Sequence Detection System (ABI PRISM 7000, USA).

The TaqMan PCR reaction was performed in 25 μl reaction volume containing 12.5 μl universal master mixtures, 2.25 μL forward primer, 2.25 μl reverse primer, 0.5 μl TaqMan probe, 5 μl template of DNA and 2.5 μl distilled water. The concentrations of primers and TaqMan probe were 0.9 pM and 0.25 μM respectively. The reaction parameters were set at 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.

TaqMan MGB probe: 5'-FAM-CTGTAGTATTCAAAGGGT-MGB-3'
The forward primer: 5'-GAGCAGGGCCAGAATTCAAC-3'
The reverse primer: 5'-TCCCGCACCTTCGGATATACT-3'

Statistical analyses

In order to normalize the data for statistical comparisons, the PCV2 Cap protein mRNA copy numbers and viral DNA copy numbers obtained from Real Time PCR and its assays, respectively, were transformed by $\log_{10}^{n}$ (n: PCV2 DNA in tonsil). Data were assessed by analysis of variance (ANOVA) software.

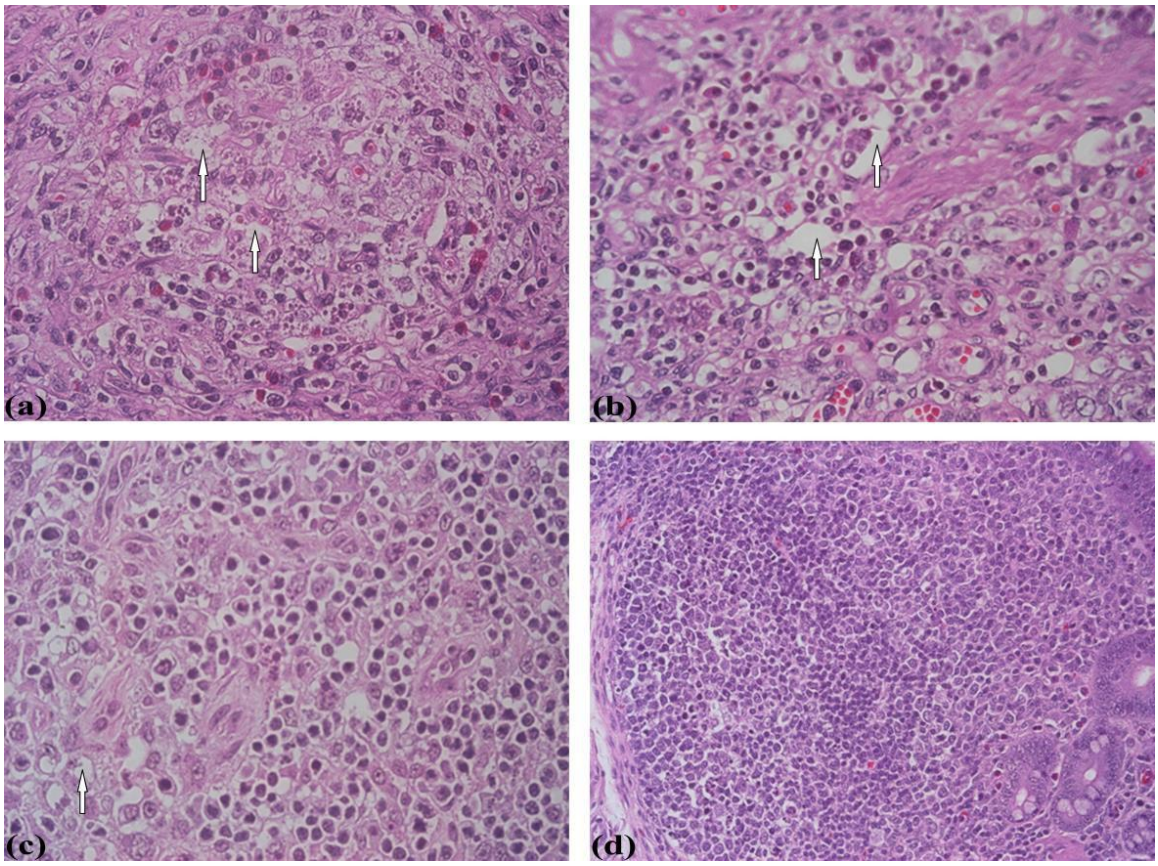

Figure 1. Various lesions of lymph tissues in PMWS cases with HE stain, showing lymphocyte depletion and necrotic cells as arrows. (a) Ileum (severe lesion: +3); (b) Mesenteric lymph node (moderate lesion: +2); (c) Inguinal lymph node (light lesion: +1); (d) Control: ileum (normal case: 0). Magnification: (a) 40×10; (b) 40×10; (c) 40×10; (d) 10×10.

RESULTS

Histopathologic lesion

Various lesions were present in these detected tissues according to microscope observation (Figure 1). Firstly, lymphocytes in these tissues disappeared at different extent, for some severe cases, most lymphocytes disappeared in groups, and it was difficult to find intact tissues because of severity. Furthermore, inclusion bodies emerged with different global sizes in the cytoplasma of these tissues. Epithelioid cell also presented in the cytoplasma with groups or single. The cytoplasma started to dissolve and disappear; it only remained the membrane. In addition, while karyopycnosis emerged, the nucleus was much smaller than normal cells, leading to lots of vacuoles in these tissues.

Antigen distribution

According to observation and analysis on IHC of six kinds of tissues, the extent of tissue staining color was coincident to the lesion extent of these tissues. The extent and depth varied accordingly with the lesion severity (Figure 2). Three kinds of groups can be classified according to the positive staining color and represent various extents infected by PCV2 viral antigen. The staining region appeared commonly in the lymph follicles or center of the lymph tissues from severe and moderate organs. Moreover, there is a trend for the positive staining being present in the center whether it is slight or severe samples.

The correlation between pathologic lesion and PCV2 load

With the purpose of investigating the exact correlation between pathologic lesion and PCV2 load for field pig cases, these tested samples were divided into four groups according to the integrative evaluation score (Table 1). The first group was lower than 10, the second was between 10 and 20, the third was between 20 and 30, the last group was between 30 and 40. The analysis result shows a significant association in four groups (Figure 3),

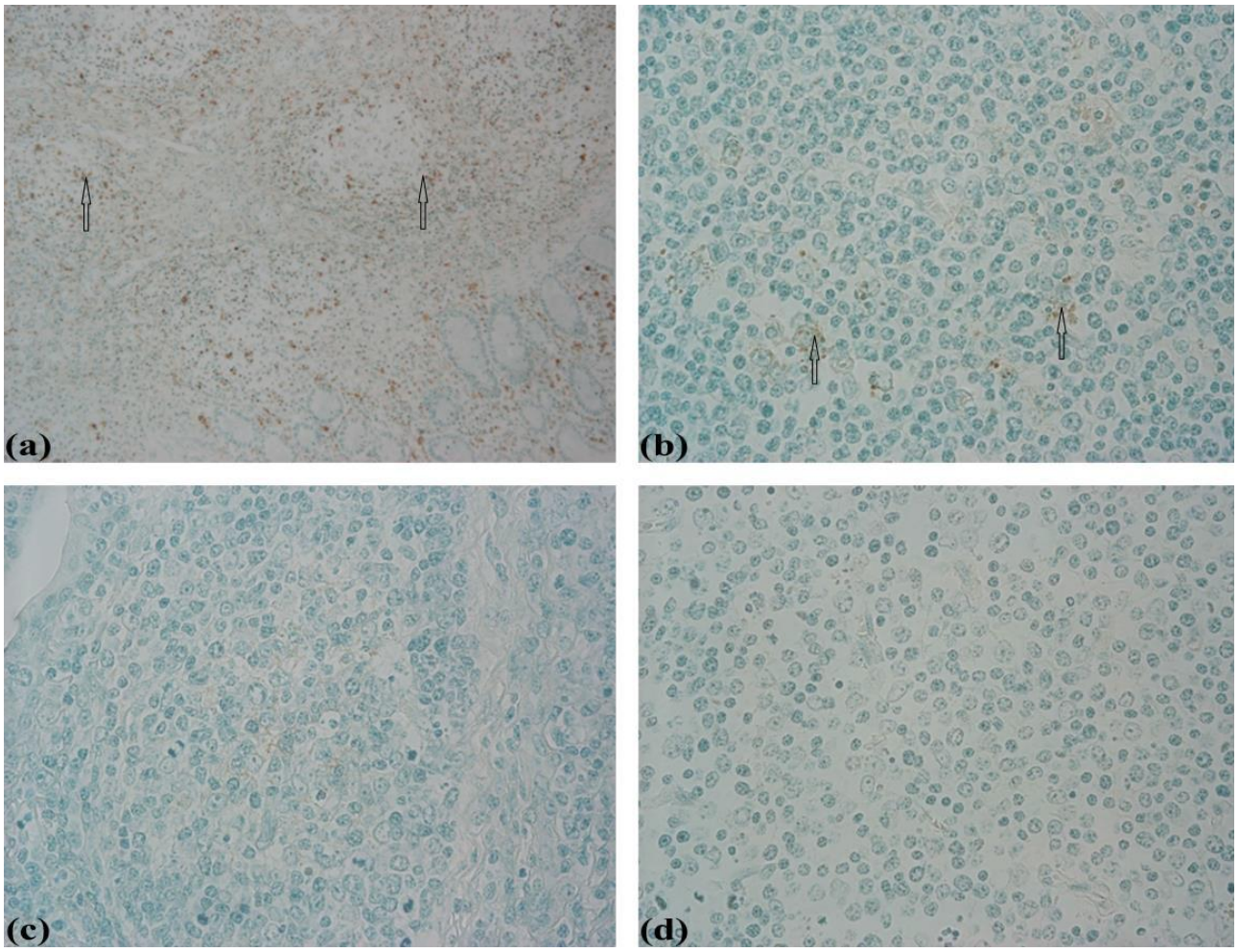

Figure 2. The various extent of color stain in PMWS cases with IHC, showing positive PCV2 antigen as arrows. (a) Ileum (severe lesion: +3); (b) Ileum (moderate lesion: +2); (c) Tonsil (light lesion: +1); (d) Control: ileum (normal case: 0). Magnification: (a) 10×10; (b) 40×10; (c) 40×10; (d) 40×10.

Table 1. Four various groups classified by the evaluating score for pathological lesion.

Groups (Total score)	Group 1 (0～10)	Group 2 (10～20)	Group 3 (20～30)	Group 4 (30～40)
No. of pigs	6	8	7	9
The average score	7 ± 2.22	15 ± 2.82	25.8 ± 2.42	38.2 ± 3.12
Log_{10}PCV2 DNA(*)	－0.18 ± －0.35	2.94 ± 1.33	4.17 ± 1.24	5.76 ± 1.19

An asterix (*) indicates the values with $p < 0.001$ among four groups.

which indicates that there is more viral load in tonsil with higher score for pathologic lesion ($P < 0.001$, ANOVA). It can provide a cue that PCV2 can directly cause the severe pathology with increasing viral load. Furthermore, comparing with other three groups, the integrative evaluation score for the first group is 1/1000 lower than other three groups. It is shown that there is a significant difference between group one and the other three groups.

DISCUSSION

PCV2 is associated with PMWS and other syndrome diseases collectively known as porcine circovirus-associated disease (PCVAD). PCV2 infection and PMWS have great impact on pig production (Ge et al., 2012; Chae, 2012). PCVAD continues to be an important differential diagnosis on pig farms (Opriessnig et al.,

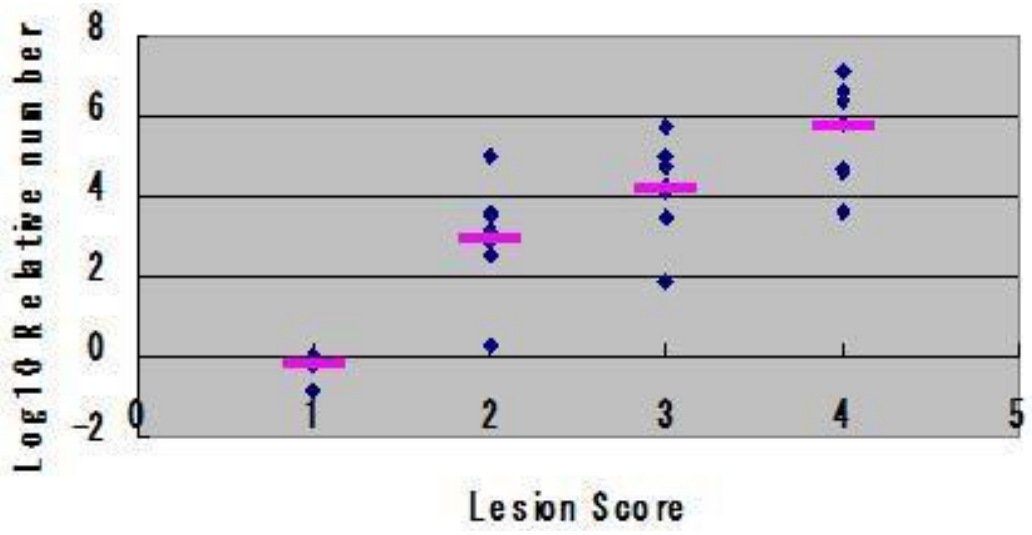

Figure 3. Correlation between the lesion severity and the amount of log_{10}PCV2 DNA in tonsil. r = 0.84 (P < 0.001, n = 30). Amount of log_{10}PCV2 DNA in tonsil from four groups divided by lesion severity (P < 0.05, ANOVA).

2007). The precise mechanisms by which a PCV2 infected pig develops a PCV2 subclinical infection or a clinical PCVAD are still to be fully elucidated, but inferences based upon clinical, gross and histologic findings from field cases of disease have been useful to suggest the pathogenesis of this viral infection (Segales, 2012). The ubiquity of PCV2 and the lack of specificity of the PCV2 tests indicate that PCV2 may be a necessary but not sufficient cause of PMWS disease (Turner et al., 2009).

In pathogenesis of PCV2 infection aspect, organelles may play an important role in the replication of PCV2, indicating that virus replicates within the histiocytes of lymph nodes (Rodriguez-Carino et al., 2010). A certain proportion of macrophages may support PCV2 replication, because Cap antigen was shown to be present in macrophages and less often in lymphocytes (Becskei et al., 2010); but main cells where PCV2 replicates are of epithelial/endothelial origin (Perez-Martin et al., 2007). Meanwhile, the presence of typical granulomatous lesions with multinuclear giant cells was also recorded in the lymphatic tissue (Becskei et al., 2010). PCV2 inclusion bodies were demonstrated to be located in the cytoplasm of epithelial cells by immunohistochemical staining for PCV2 and cytokeratin antigens and by ultrastructural demonstration of viral particles in the inclusion bodies within renal tubular epithelium (Huang et al., 2008).

Some recent advance on diagnosis of PCV2 infection is as follows: besides tissues were examined by pathohistology, IHC, nested PCR and quantitative PCR (Reiner et al., 2010), a method for detection of both strands of PCV2 in situ can be useful for studies of replication and in situ detection of PCV2 as well as of DNA viruses in general (Henriksson et al., 2011). Indirect *in situ* PCR is a more effective, cell-based diagnostic tool with good specificity to detect limited PCV2 infection in formalin-fixed and paraffin-embedded tissue specimens and it would be a useful tool to further explore the pathogenesis of PCV2 infection (Lin et al., 2009). *In situ* hybridization was also demonstrated to prove more sensitive than IHC for the detection of PCV2 in formalin-fixed, paraffin-embedded lymph node tissues (Kim et al., 2009). Due to better image quality after staining, IHC became a reliable and useful technique for PMWS diagnosis (Szczotka et al., 2011). Microarray results validated by quantitative Real Time PCR show the characterization of the early and late molecular events taking place in response to a subclinical PCV2 infection (Tomas et al., 2010).

In reality, PMWS was usually caused due to diverse clinical symptoms when pigs are affected by PCV2 and other factors; otherwise pigs only are in infected condition. Owing to complexity of PMWS diagnosis, some various criteria on PMWS diagnosis were established as following. PMWS case definition contains three different criteria including clinical signs, moderate to severe lymphoid lesions and moderate to high amount of PCV2 antigen or genome in these lymphoid lesions (Segales, 2002). The suggested 10^7 PCV2 genome copies/ml of serum was reinforced as a threshold for PMWS diagnosis (Brunborg et al., 2004; Olvera et al., 2004). Besides, mean viral concentration values of 7, 6 and 5 log_{10} DNA copies/ng (10^7, 10^6 and 10^5 PCV2 DNA copies/ng of total DNA) from tracheo-bronchial, tonsillar and faecal swabs, respectively, could also be considered potential thresholds in these locations to help establish PMWS diagnosis. This approach would not be useful for the results from the nasal cavity and urine, since subclinically infected pigs

had similar or higher viral loads than PMWS affected pigs (Segales et al., 2005).

Given PMWS caused by PCV2 belong to a kind of respiratory disease; tonsil is a critical immune organ at the entrance of piglets' respiratory tract. Therefore, its viral load indicates quite considerable for diagnosis on PMWS. According to experimental results, these samples show various pathologic character and presence of PCV2 antigen. When pig cases were divided into four groups according to the lesion severity, there is the significant correlation between the lesion severity and the amount of $\log_{10}$ PCV2 DNA in tonsil (R=0.84, $P < 0.001$). The amount of PCV2 DNA in group one (score < 10) was 1/1000 lower than the other three groups. Pig case in group one was thought as in subclinical infection on account of having slight lymphoid lesions. Therefore, it is supposed that group one with lower 10 is considered as subclinical cases and other three groups with higher 10 as clinical PMWS cases by this significant correlation. It is likely presumed that whether it is subclinical and clinical PMWS case can be helpfully diagnosed by these criteria.

Conflict of Interests

The authors have not declared any conflict of interests.

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