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NOTE

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Comparison of pathogenicity of 4 porcine circovirus type 2 (PCV2) genotypes (2a, 2b, 2d, and 2e) in experimentally infected pigs

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ABSTRACT. The objective of the current study was to compare the virulence of four porcine circovirus type 2 (PCV2) genotypes (PCV2a, 2b, 2d, and 2e) in pigs. Pigs were inoculated at 42 days of age with one of four PCV2 genotypes, then necropsied at 63 days of age. PCV2 genotype groups were evaluated through a comparison of clinical outcomes, antibody titers, level of PCV2 loads in blood and lymph nodes, and lymphoid lesion severity. Statistical differences did not occur between the evaluated genotype groups. Pigs inoculated with PCV2a, PCV2b, or PCV2d had a significantly ($P<0.05$) higher levels of PCV2 loads in blood and lymph node compared to pigs inoculated with PCV2e. The results of this study indicated that the PCV2a, PCV2b, and PCV2d are more virulent than PCV2e based on blood and lymphoid viral load of PCV2.

KEYWORDS: porcine circovirus type 2a, porcine circovirus type 2b, porcine circovirus type 2d, porcine circovirus type 2e, virulence

Porcine circovirus type 2 (PCV2) is the smallest non-enveloped, circular, single-stranded DNA virus in existence and is categorized as a member of the genus *Circovirus* and of the family *Circoviridae* [19]. PCV2 was initially reported in Canada in the 1990s and is one of the most economically important viral pathogens within the global pork industry [3]. PCV2 is linked to a variety of clinical manifestations collectively named porcine circovirus associated disease (PCVAD) that includes postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failures, and enteritis [4].

To date, at least eight distinct genotypes (PCV2a to PCV2h) have been designated with lower case letters (a, b, c, d, e, etc) based on the order of the first identification [9]. Among these, PCV2a, PCV2b, and PCV2d are the three main genotypes currently found in global pig populations. Similarly, the same PCV2 genotypes (2a, 2b, and 2d) are in active circulation throughout Korean pig populations [22]. PCV2e has been first reported in the US and Mexico in 2015–2016 [6, 13] but it was first isolated from pigs in 2020 [23]. Previous comparison studies that evaluated the same clinical parameters have been conducted with the exclusion of PCV2e and concluded that PCV2a, 2b, and 2d all produce a similar virulence [5]. The objective of this study was to compare the virulence of four PCV2 genotypes (2a, 2b, 2d, and 2e) for the first time by evaluating experimentally infected pigs for each of the following clinical outcome, antibody titers, level of PCV2 loads in blood and lymph nodes, and lymphoid lesion severity.

Thirty clinically healthy, colostrum-fed conventional pigs from sows that had no history of vaccination against PCV2 were purchased at 40 days of age from a commercial porcine reproductive and respiratory syndrome virus (PRRSV)-free farm. The farm also tested *Mycoplasma hyopneumoniae*-free based on serology, and long term clinical and slaughter history. Pigs entered into the study were serologically evaluated with commercially available ELISA kits (PRRSV: HerdChek PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA; PCV2: INgezim CIRCO IgG, Ingenasa, Madrid, Spain; *M. hyopneumoniae: M. hyo*. Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA) and tested seronegative for PRRSV, PCV2, and *M. hyopneumoniae*. They were confirmed negative for PCV2 (2a, 2b, 2d, and 2e) and PRRSV viremia, and *M. hyopneumoniae* laryngeal shedding as evaluated by real-time polymerase chain reaction (PCR) testing upon arrival.

For the study, pigs were allocated into 5 groups (6 pigs per group) using the random number generator function from Excel (Microsoft Corp., Redmond, WA, USA) (Table 1). Pigs in each group were randomly assigned into five separate rooms. At 0 days post inoculation (dpi, 42 days of age), pigs in the PCV2a, PCV2b, PCV2d, and PCV2e groups were inoculated intranasally with 3 mL of their respective challenge strain; the PCV2a group received the PCV2a SNUVR100032 strain (GenBank no. KF871067), the PCV2b

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Table 1. Average daily weight gain (ADWG), lymphoid lesion and porcine circovirus type 2 (PCV2) antigen scores (mean ± SD) among different groups (n=6 per group)

Groups	ADWG (42–63 days of age)	Microscopic lymphoid lesion scores	No. of PCV2-antigen positive cells
PCV2a	368.25 ± 32.95	1.43 ± 0.29[a)]	17.78 ± 1.96[a)]
PCV2b	361.11 ± 24.91	1.57 ± 0.41[a)]	20.28 ± 1.02[a)]
PCV2d	369.05 ± 39.81	1.63 ± 0.39[a)]	18.56 ± 4.02[a)]
PCV2e	366.67 ± 47.90	1.40 ± 0.18[a)]	13.28 ± 3.45[b)]
Negative control	369.84 ± 38.65	0.00 ± 0.00[b)]	0.00 ± 0.00[c)]

Different letters (a, b, and c) indicate significant difference ($P<0.05$) among groups.

group received the PCV2b SNUVR202155 strain (GenBank no. MZ440696), the PCV2d group received the PCV2d SNUVR202003 strain (GenBank no. MZ440695), and the PCV2e group received the PCV2e SNUVR199707 strain (GenBank no. MN967003). PCV2e was isolated from superficial inguinal lymph node from an 82-day-old pig that had exhibited growth retardation [23]. Each strain of inoculum contained 1.2×10^5 50% tissue culture infective dose ($TCID_{50}$/mL) in a 5th passage of PCV-free PK15 cell line. Pigs in the negative control group were inoculated intranasally with 6 mL (3 mL/nostril) of uninfected cell culture supernatant.

Blood samples were collected from each pig by jugular venipuncture at 0, 7, 14, and 21 dpi. Pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 21 dpi as described previously [1]. Tissues were collected from each pig at necropsy. All experimental protocols were approved prior to the study by the Seoul National University Institutional Animal Care and Use Committee (SNU-210226-2).

Pigs were monitored daily for clinical signs and scored weekly using a score ranking system which ranged from 0 (normal) to 6 (severe dyspnea and abdominal breathing) [12]. All observers involved in these processes were blinded to type of challenge virus.

The pig was weighed at 42 (0 dpi) and 63 (21 dpi) days of age. The average daily weight gain (ADWG; gram/pig/day) was analyzed over the time period between 42 and 63 days of age. ADWG was calculated as the difference between the starting and final weight divided by the duration of the stage. Data for dead or removed pigs were included as well in the calculation.

A commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA) was use to extract DNA from serum samples for PCV2. Genomic DNA copy numbers for PCV2a, PCV2b, PCV2d, and PCV2e were quantified by real-time PCR [10, 14, 21, 24].

Serum samples were also tested for antibodies against PCV2 (INgezim CIRCO IgG, Ingenasa, Madrid, Spain). Samples were considered positive for PCV2 antibodies if the optical density (OD) was >0.3 according to the manufacturer's instructions.

For the morphometric analysis of histopathological changes in superficial inguinal lymph nodes, three sections of that lymph node were examined [16]. Lymph nodes were evaluated for presence of lymphoid depletion and inflammation, and given a score ranging from 0 to 5 (0 =normal; 1 =mild lymphoid depletion; 2 =mild to moderate lymphoid depletion and histiocytic replacement; 3 =moderate diffuse lymphoid depletion and histiocytic replacement; 4 =moderate to severe lymphoid depletion and histiocytic replacement; 5 =severe lymphoid depletion and histiocytic replacement).

Immunohistochemistry (IHC) and morphometric analysis of IHC was carried out as previously described [15]. Positive signal was quantified using the NIH Image J 1.45s Program (http://imagej.nih.gov/ij/download.html). For each slide of lymph node tissue, 10 fields were randomly selected, and the number of positive cells per unit area (0.25 mm^2) was counted. The mean values were also calculated [15].

Prior to statistical analysis, real-time PCR data were log-transformed to reduce variance and positive skewness. Data was tested for normal distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to examine whether there were statistically significant differences among the five groups, for each time point. When a test result from one-way ANOVA showed a statistical significance, a *post-hoc* test was conducted for a pairwise comparison with Tukey's adjustment. If the normality assumption was not met, the Kruskal-Wallis test was performed. When the result form Kruskal Wallis test showed statistical significance, Mann-Whitney test was performed to compare the differences among the groups. A value of $P<0.05$ was considered to be significant.

All pigs inoculated with any one of the four PCV2 genotypes remained clinically normal, meaning they were void of PCVAD-associated clinical signs such as anorexia, icterus, dyspnea, lethargy, depression and fever. Statistical differences in clinical signs did not occur between PCV2-infected and negative control pigs. There was no statistical difference in average body weight among all five groups (4 infected and 1 control) at the start of the experiment (42-day-old pigs). A statistical difference in ADWG from 42 to 63 days of age was not present among the five experiment groups (Table 1).

Prior to inoculation, all serum samples collected from the five groups tested negative for antibodies against PCV2. PCV2 ELISA antibody titers between pigs inoculated with PCV2a, PCV2b, PCV2d and PCV2e were not statistically different at any measured timepoint. Negative control pigs remained free of PCV2 antibodies at every timepoint (Fig. 1).

Prior to inoculation, all serum samples collected from the five groups tested negative for PCV2a, PCV2b, PCV2d, and PCV2e. Pigs inoculated with PCV2a, PCV2b, and PCV2d had a significantly ($P<0.05$) higher number of PCV2 genomic copies at 21 dpi compared with PCV2e-inoculated pigs. PCV2 genomic copies were not detected in the negative control pigs for the duration of the study (Fig. 2).

Mild lymphoid depletion was observed in pigs infected with PCV2a, PCV2b, PCV2d (Fig. 3A), PCV2e (Fig. 3B). Statistical differences in lymphoid lesion scores at 21 dpi between pigs inoculated with PCV2a, PCV2b, PCV2d, and PCV2e were not found (Table 1). Histopathological lesions were not present in the negative control pigs.

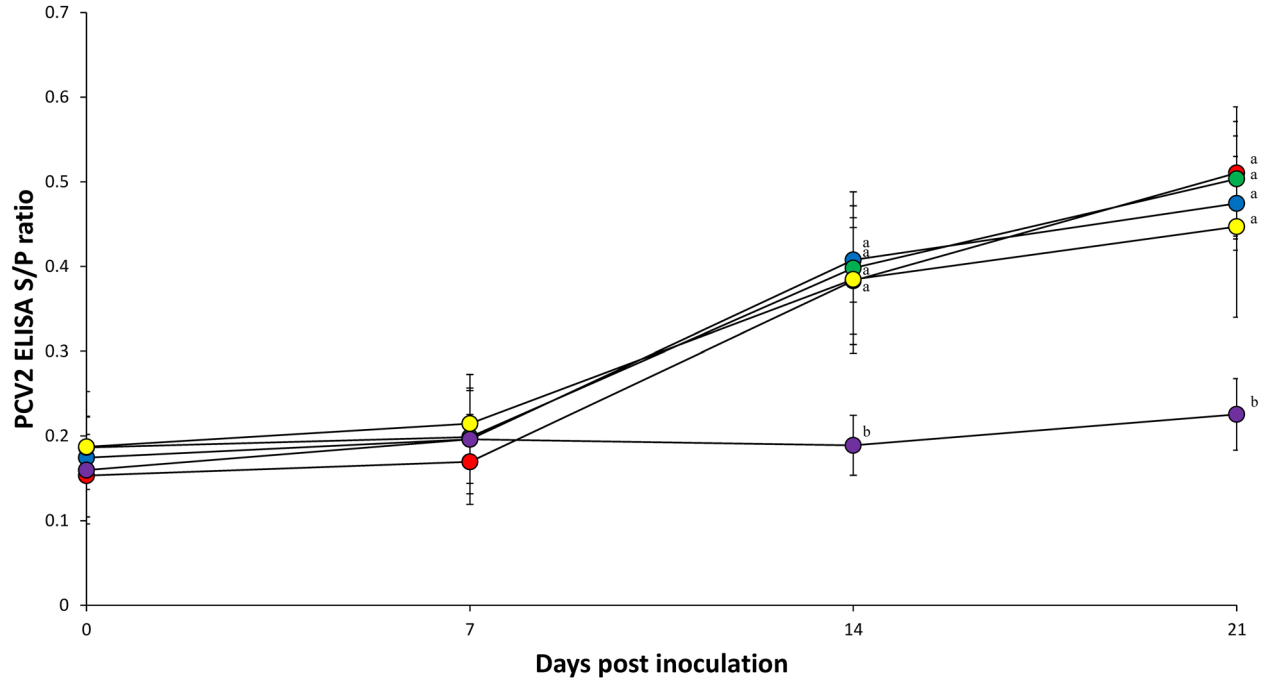

Fig. 1. Porcine circovirus type 2 (PCV2)-specific enzyme-linked immunosorbent assay (ELISA) antibody levels in serum of pigs inoculated with PCV2a (red circle), PCV2b (blue circle), PCV2d (green circle), and PCV2e (yellow circle) and negative control (purple circle) groups. Variation expressed as the standard deviation. Different superscripts (a and b) indicate significant (P<0.05) difference among the five groups.

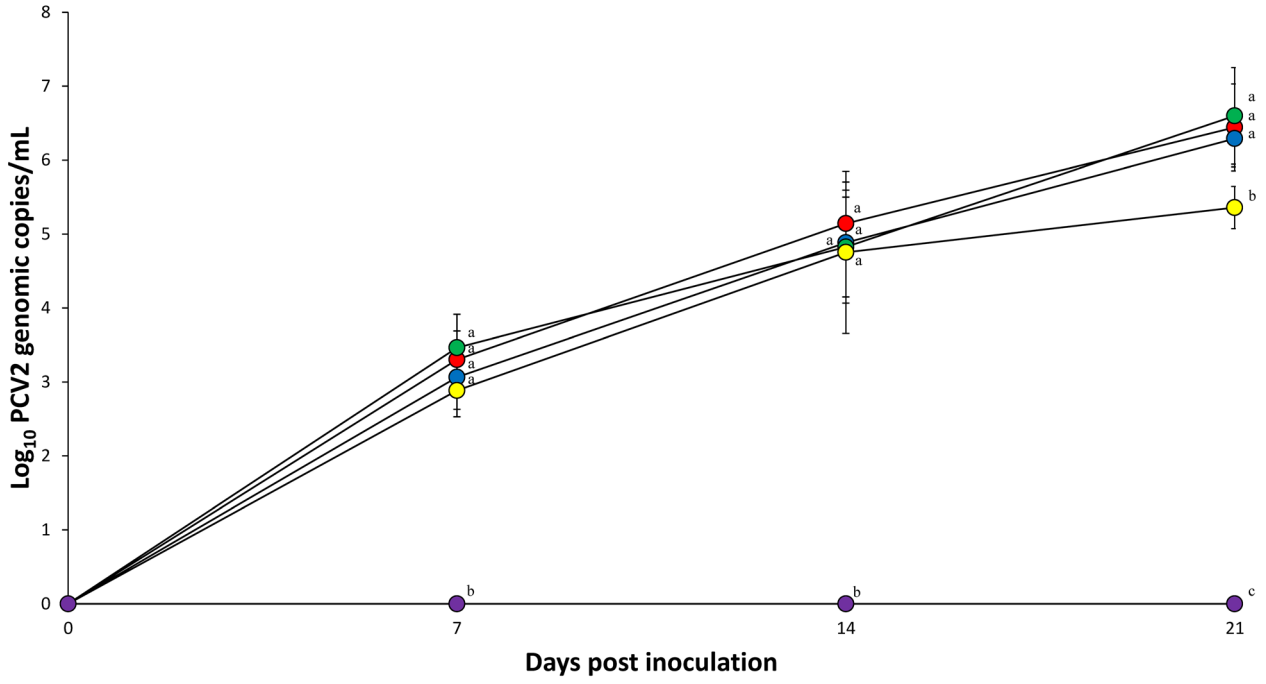

Fig. 2. Mean values of the genomic copy number of porcine circovirus type 2 (PCV2) DNA in serum of pigs inoculated with PCV2a (red circle), PCV2b (blue circle), PCV2d (green circle), and PCV2e (yellow circle) and negative control (purple circle) groups. Variation expressed as the standard deviation. Different superscripts (a, b and c) indicate significant (P<0.05) difference among the five groups.

All pigs infected with one of the four PCV2 genotypes were immunolabeled for PCV2 antigen. PCV2 antigens were observed, mainly in follicular macrophages. Pigs inoculated with PCV2a, PCV2b, and PCV2d (Fig. 3C) had a significantly higher (P<0.05) number of PCV2 antigen-positive cells per unit area (0.25 mm^2) in their lymph nodes than those of pigs inoculated with PCV2e (Fig. 3D). PCV2 antigen was not detected in any lymph node samples from the negative control pigs (Table 1).

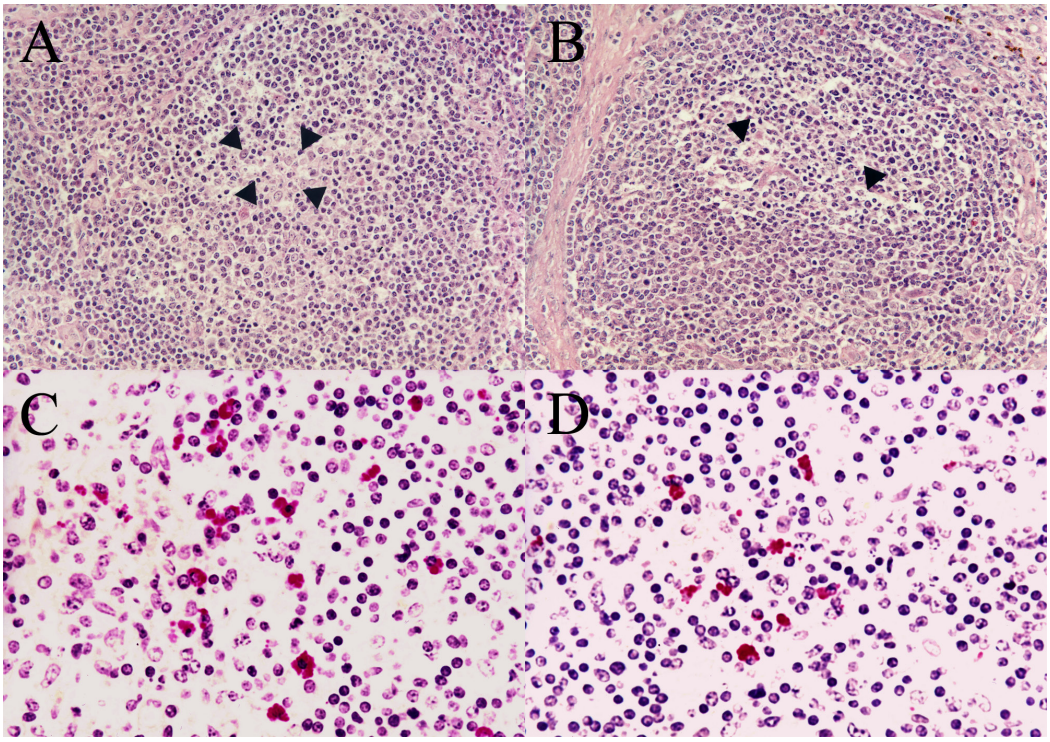

Fig. 3. Histopathology and immunohistochemistry, lymph node, pigs. (**A**) Mild lymphoid depletion (arrows) in tissues of pigs inoculated with porcine circovirus type 2d (PCV2d). HE. ×200. (**B**) Mild lymphoid depletion (arrows) in tissues of pigs inoculated with PCV2e. HE. ×200. (**C**) Porcine circovirus type 2 (PCV2) antigens in lymph node of pigs inoculated with PCV2d. Immunohistochemistry. ×200. (**D**) PCV2 antigens in lymph node of pigs inoculated with PCV2e. Immunohistochemistry. ×200.

Statistical differences were found in the virulence between the three major PCV2 genotypes (2a, 2b, and 2d) and PCV2e in experimentally infected pigs. PCV2a, PCV2b, and PCV2d were more virulent than PCV2e based on measured levels of PCV2 blood and lymphoid viral load. There were no significant differences in virulence among the three major PCV2 genotypes, concluding that the present results are consistent with previous Korean and US studies [5, 21]. One Chinese study concluded that PCV2d is more virulent than PCV2a and PCV2b which contradicts these study findings [11]. Therefore, there is a discrepancy in the virulence of the three major PCV2 genotypes isolated by different countries.

Pigs were experimentally infected with PCV2 at 42 days of age for this study as 42–49 days of age is the most common naturally occurring infection window for this virus as observed in Korean swine farms (C. Chae, personal observation). Previous studies observed inoculated pigs for 28 days [5], whereas this study reduced the observation period to 21 days. Pigs experimentally infected solely with PCV2 only do not develop the full manifestation of PCVAD, unlike the previously conducted studies that evaluated PCV2 under a co-infection with additional pathogens. Without additional pathogens, the shortened observation period was justifiable as it did not have a significantly effect on clinical sign and symptom outcomes.

The decreased ability of PCV2e replication after infection was evident. Reports have been filed that evaluated how subtle changes in the PCV2 capsid protein can increase the fitness level of the virus at the cellular level which leads to a virulence increase in infected pigs [7, 17]. Lower viral loads in serum and fewer PCV2 antigen-positive cells in the lymph nodes were observed in PCV2e-infected pigs when compared with those infected with the other three PCV2 genotypes. The viral structure of PCV2e contains 12 or 15 extra nucleotides of ORF2 sequences compared to those of PCV2a, PCV2b and PCV2d [6, 13, 18]. Due to the presence of these extra nucleotides at the 3′ end of ORF2, PCV2e was thought to be a progenitor of PCV2a, 2b, and 2d [6]. This distinct genetic characterization may affect the efficiency of replication *in vivo* and warrants further investigations.

The present study determined the virulence intensity of PCV2 by comparing the number of the PCV2 genomic copies or the number of the PCV2 antigen-positive cells between different infected and uninfected groups. This was an important evaluation, as statistical differences in clinical symptoms, antibody titers, and histological lesions in lymph nodes were not present among the genotypes. Real-time PCR values are also dependable during testing and are one of the only approximate epidemiologic measures of disease [2]. PCV2 infection is quite common in clinically healthy pigs, and the interpretation of a positive real-time PCR result is not always straightforward.

The appearance of new genotypes in the future is likely, since PCV2 is a single stranded DNA virus with a high nucleotide substitution rate (comparable to those of RNA viruses) which gives the genome a high mutation possibility [8]. PCV2e is the most recently emerged genotype and has been reported in several countries [6, 13, 18, 23]. Pigs inoculated with PCV2a, PCV2b, or PCV2d had a significantly higher levels of PCV2 loads in blood and lymph node compared to pigs inoculated with PCV2e. In experimental

study, all pigs co-infected with either *M. hyopneumoniae*/PCV2e or PCV2e/PRRSV developed mild PCVAD, whereas none of the pigs infected with PCV2e alone [20]. In the epidemiological survey, the results of prevalence identified PCV2d as the current dominant genotype, while the newly emerging PCV2e maintained the lowest prevalence among the evaluated swine farms [22]. Further studies are necessary to determine the clinical importance of this new PCV2 genotype.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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