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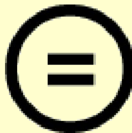
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수의학석사학위논문

Comparative study of the virulence of three major Korean porcine circovirus type 2 genotypes (a, b, and d)

한국에서 분리한 돼지 써코바이러스 2형의 3가지 유전자형 (a, b, d) 병원성 비교분석

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수의학과 수의병인생물학 및 예방수의학 전공

조 혜 진

Abstract

Comparative study of the virulence of three major Korean porcine circovirus type 2 genotypes (a, b, and d)

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Porcine circovirus type2 (PCV2) is an economically important swine pathogen beacuse it is the most common causative agent of several disease and syndromes including porcine circovirus-associated disease (PCVAD). Based on its genotypes, PCV2 have been distinguished and identified as PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e. In this study, we compared virulence between the three major Korean PCV2 genotypes, PCV2a, PCV2b, and PCV2d based on clinical signs, PCV2 viremia and antibody titers, lymphoid lesion scores and numbers of PCV2 antigens. A population of forty piglets was selected as healthy and colostrum fed, 14 days old from sows in a commercial farm which was determined to be free of porcine reproductive and respiratory syndrome virus (PRRSV) and also *Mycoplasama hyopneumoniae*-free based on monitoring results, serological testing, and long term clinical history. We designed to 4 groups of pigs and 10 piglets per group. Each group was intranasally inoculated with 2mL of tissue culture supernatant containing 10^5 $TCID_{50}$/mL of PCV2a, PCV2b, and PCV2d. The negative group was intranasally inoculated with 2mL of uninfected cell culture supernatant. Compare to the negative group, all 3 of experimental groups had more severe clinical signs, but no significant difference among PCV2a-, PCV2b-, and PCV2d-infected groups. Throughout the time of challenge, average daily weight which was calculated over the time period between 49 and 77 days of age showed no significant difference in infected groups. There were no detection of genomic copies of PCV2 from all 4 groups, and all infected groups were

appeared to have similar PCV2 antibody levels, regardless of genotype. Regardless of infected PCV2- genotypes, PCV2 antibodies were come out into similar results for all four groups. For pathological part the negative control group, all three PCV2a-, PCV2b-, and PCV2d-infected groups showed significantly higher number of lymphoid PCV2-positive cells and lymphoid lesion scores than the negative control group. Therefore, this study exhibits that there is no significant difference in virulence between the three major Korean PCV2 genotypes, PCV2a, PCV2b, and PCV2d.

Keywords:

Porcine circovirus type 2 genotype, Porcine circovirus-associated disease, Porcine circovirus type 2, Virulence

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LIST OF ABBREVIATIONS

PCV	Porcine circovirus
PCVAD	Porcine circovirus associated diseases
ORFs	Open reading frames
ELISA	Enzyme-linked immune-sorbent assay
dpi	Days post-inoculation
IHC	Immunohistochemistry
PBS	Phosphate-buffered saline
$TCID_{50}$	Median tissue culture infective dose

Abstract

The objective of this study was to compare the virulence of three major PCV2 genotypes in terms of clinical signs, PCV2 viremia and antibody titers, lymphoid lesions, and PCV2-antigen within lymphoid lesions in experimentally infected pigs. Pigs were infected at 7 weeks with PCV2a, PCV2b, and PCV2d strains and necropsied at 28 days post infection. No statistical differences were observed in clinical signs, PCV2 viremia and antibody titers, lymphoid lesions scores, and numbers of PCV2 antigens among the three major Korean PCV2 genotypes. The results in this study indicate that the three major Korean PCV2 genotypes, PCV2a, PCV2b, and PCV2d have similar virulence.

Keywords:

Porcine circovirus type 2 genotype

Porcine circovirus-associated disease

Porcine circovirus type 2

Virulence

1. Introduction

Porcine circovirus type 2(PCV2) is the smallest non-enveloped, single-stranded, circular deoxyribonucleic acid (DNA) virus belonging to the genus *Circovirus* and the family *Circoviridae* (1). It is the most common etiological agent associated with several syndromes and diseases that are now collectively referred to as porcine circovirus-associated disease (PCVAD) (2).

Today, PCV2 is prevalent worldwide with five different genotypes recognized so far based on the sequence identity in open reading frame 2 (ORF2). The genotypes have been designated with lower case letters (a, b, c, d, and e) based on the order of the first identification, with PCV2a, PCV2b, and PCV2d being the major genotypes. Porcine circovirus type 2c (PCV2c) has only been isolated from Danish tissues archived in the late 1990s (3) and in feral pigs in Brazil (4). The 5th genotype, PCV2e, has been reported in the USA and China, but so far has a very low prevalence (5).

Currently, PCV2d has become predominant genotype in North America, Asia, and Europe. While all 3 major genotypes (a, b, and d) are considered virulent and etiological agents of PCVAD, the degree of virulence is somewhat controversial. A previous study concluded that Chinese PCV2d strains caused a more serious disease than PCV2a or PCV2b (6). In contrast, a North American PCV2d strain exhibited similar virulence to PCV2a and PCV2b strains in caesarean-derived, colostrum-deprived pigs (7). So far, no comparison studies have been done on the virulence of the Korean

genotypes. The objective of this study was to compare the virulence of 3 major Korean PCV2 genotypes in terms of clinical signs, PCV2 viremia and antibody titers, lymphoid lesion scores, and PCV2-antigen present within lymphoid lesions in experimentally infected pigs.

2. Materials and methods

2.1. PCV2 inocula

The strains used in this study were; PCV2a strain SNUVR000032 (GenBank no. KF871067) which shares 98.8% to 99.1% and 99.0% nucleotide sequence identity of the full genome with 3 US PCV2a strains (GeneBank no. AY099499, AF264041, and AJ223185) and 1 European PCV2a strain (GeneBank no. AJ293868), respectively; PCV2b strain SNUVR000463 (GenBank no. KF871068) which shares 99.3% to 99.5% and 99.6% to 99.8% nucleotide sequence identity of the full genome with 2 US PCV2b strains (GenBank no. HQ713495 and GU799576) and 3 European PCV2b strains (GenBank no. AY484416, DQ233257, and AF055393), respectively; and PCV2d strain SNUVR140004 (GenBank no. KJ437506) which shares 99.7% and 96.5% to 98.0% nucleotide sequence identity of the full genome with 2 US PCV2d strains (GenBank no JX535297 and JX535296) and 2 European PCV2d strains (GenBank no. AY484410 and AY713470), respectively.

2.2. Animals

Forty clinically healthy, colostrum-fed conventional pigs from sows that had not been previously vaccinated against PCV2 were purchased at 14 days old from a commercial farm that was free of porcine reproductive and respiratory syndrome virus (PRRSV). It was also determined that the farm

was free of *Mycoplasma hyopneumoniae* based on serological testing and long-term clinical and slaughter history. Pigs were also tested with commercially available enzyme-linked immunosorbent assay (ELISA) kits (PRRSV: HerdChek PRRS X3 Ab Test; IDEXX Laboratories Inc., Westbrook, Maine, USA; PCV2: Synbiotics, Lyon, France; *M. hyopneumoniae*: *M. hyo*. Ab test, IDEXX Laboratories) and were seronegative for PRRSV, PCV2, and *M. hyopneumoniae*.

2.3. Experimental design

For the study, pigs were allocated into 4 groups (10 pigs per group) using the random number generation function from Excel (Microsoft Corporation, Redmond, Washington, USA) (Table I). Pigs in each group were randomly assigned into 4 separate rooms. On the day of infection (7 weeks old), pigs in the PCV2a-infected group were inoculated intranasally with 2 mL (1 mL/nostril) of tissue culture supernatant containing 10^5 50% tissue culture infectious dose ($TCID_{50}$)/mL of PCV2a (SNUVR000032, GenBank no. KF871067, 5th passage in PCV-free PK-15 cell lines) (8). Pigs in the PCV2b-infected group were inoculated intranasally with 2 mL (1 mL/nostril) of tissue culture supernatant containing 10^5 $TCID_{50}$/mL of PCV2b (SNUVR000463, GenBank no. KF871068, 5th passage in PCV-free PK-15 cell lines) (8). Pigs in the PCV2d-infected group were inoculated intranasally with 2 mL (1 mL/nostril) of tissue culture supernatant

containing 10^5 $TCID_{50}$/mL of PCV2d (SNUVR140004, GenBank no. KJ437506, 5th passage in PCV-free PK-15 cell lines) (9). Pigs in the negative control group were inoculated intranasally with 2 mL (1 mL/nostril) of uninfected cell culture supernatant. Blood samples were collected from each pig by jugular venipuncture at −28, 0, 7, 14, 21, and 28 days post infection (dpi). All pigs were euthanized for necropsy at 28 dpi after dedation by intravenous (IV) azaperon (Stersnil; Janssen Pharmaceutica, Beerse, Belgium). All experimental protocols were approved before the study by the Seoul National University Institutional Animal Care and Use Committee.

2.4. Clinical observations

Pigs were monitored daily for clinical signs and scored weekly. Briefly, scoring was defined as follows: 0 = normal; 1 = rough hair coat; 2 = rough hair coat and dyspnea; 4 = severe dyspnea and abdominal breathing; 5 = rough hair coat with severe dyspnea and abdominal breathing; and 6 = death. The live weight of each pig was measured at 49 and 77 days old. The average daily weight gain (ADWG; gram/pig/day) was analyzed over the time period from 49 to 77 days old and was calculated during the different production stages as the difference between the starting and final weight divided by the duration of the stage. Data for dead pigs were included as well in the calculation.

2.5. Quantification of PCV2 DNA in blood

Serum samples were collected at −28, 0, 7, 14, 21, and 28 dpi and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genomic DNA copy numbers for PCV2a, PCV2b, and PCV2d were quantified by quantitative real-time polymerase chain reaction (RT-PCR) (10-12). The detection limit of the assay was 1.2×10^2 genomic copies for each of the 3 PCV2 genotypes.

2.6. Enzyme-linked immunosorbent assay

Serum samples were also tested for antibodies against PCV2 (PCV2 Ab Mono Blocking; Synbiotics). Samples were considered positive for PCV2 antibodies if the reciprocal ELISA titer was greater than 350 according to the manufacturer’s instructions.

2.7. Histopathology

For the morphometric analysis of histopathological changes in superficial inguinal lymph nodes, 3 sections of that lymph node were examined "blindly" (13). 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).

2.8. Immunohistochemistry

Immunohistochemistry (IHC) and morphometric analysis of IHC was carried out as previously described (14). To obtain quantitative data, analyses of the slides were performed with the NIH Image J 1.43m Program. 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.

2.9. Statistical analysis

Prior to statistical analysis, RT-PCR data were log-transformed to reduce variance and positive skewness. Statistics were calculated using IBM SPSS® Statistics 25 (IBM, Armonk, New York, USA). 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 4 groups, for each time point. When a test result from 1-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 conducted. When the result form Kruskal Wallis test showed statistical significance, the

Mann-Whitney test with Tukey's adjustment was done to compare the differences among the groups. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Clinical observations

Pigs in the PCV2a-, PCV2b-, and PCV2d-infected groups had significantly more severe ($P<0.05$) clinical signs than the negative control group at 21 and 28 dpi. There was no significant difference in clinical signs between PCV2a-, PCV2b-, and PCV2d-infected groups at 7, 14, 21, and 28 dpi. There was no significant difference in ADWG among PCV2a-, PCV2b-, and PCV2d-infected groups over the time period from 49 to 77 days old. There was a numerical, but not statistically significant difference in ADWG between PCV2 (PCV2a, PCV2b, and PCV2d)-infected groups and the negative control group (Table I).

Table I. Average daily weight gain and pathology data (mean ± standard deviation) of 10 pigs in each of 4 groups at 28 days post challenge.

Groups	PCV2a-infected	PCV2b-infected	PCV2d-infected	Negative control
Average daily weight gain (49-77 days old)	533.21±37.80	534.29±37.47	531.43±32.16	569.64±29.17
Lymphoid lesion scores	1.40±0.23[a]	1.52±0.19[a]	1.42±0.27[a]	0.00±0.00[b]

Different superscripts (a and b) indicate significant ($P < 0.05$) difference among 4 groups.

3.2. Quantification of PCV2 DNA in blood

At the time of challenge, no genomic copies of PCV2 could be detected in any of the serum samples collected from all 4 groups. At 7, 14, 21, and 28 dpi, the number of genomic copies of the respective PCV2a, PCV2b, and PCV2d DNA was similar among PCV2a-, PCV2b-, and PCV2d-infected groups (Figure 1). Pigs in the PCV2a-infected group were negative for PCV2b and PCV2d DNA, pigs in the PCV2b-infected group were negative for PCV2a and PCV2d DNA and pigs in the PCV2d-infected group were negative for PCV2a and PCV2b DNA throughout the experiment. No PCV2a, PCV2b, or PCV2d genomes were detected in the sera of pigs from the negative control group throughout the study.

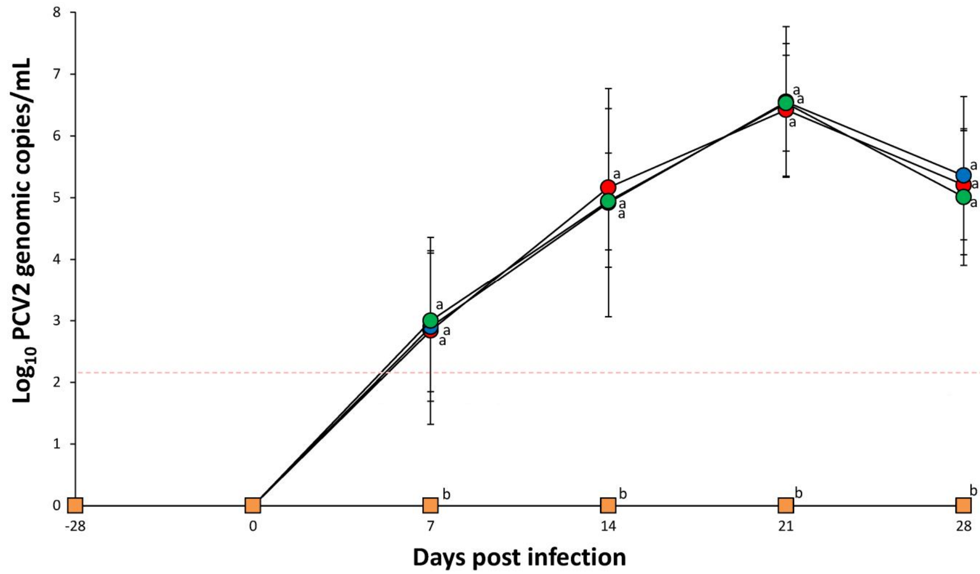

Figure 1. Mean values of the genomic copy number of PCV2 DNA in serum from PCV2a-infected (●, n = 10 pigs), PCV2b-infected (●, n = 10 pigs), PCV2d-infected (●, n = 10 pigs), and negative control (■, n = 10 pigs) groups. The detection limit of the assay was 1.2×10^2 genomic copies for each of the three PCV2 genotypes (red dotted line). Analysis of variance (ANOVA) was carried out to compare the 4 treatment groups. Tukey's adjustment was used for a post-hoc analysis. For not normally distributed data, Kruskal-Wallis test and consequently Mann-Whitney test were used to evaluate differences among the treatment groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant (P < 0.05) difference among 4 groups.

3.3 Enzyme-linked immunosorbent assay

At the time of challenge, serum samples collected from all 4 groups were negative for PCV2 antibodies. At 7, 14, 21, and 28 dpi pigs from all infected groups exhibited similar PCV2 antibody levels regardless of genotype. Pigs in the negative control group were negative for PCV2 antibodies throughout the study (Figure 2).

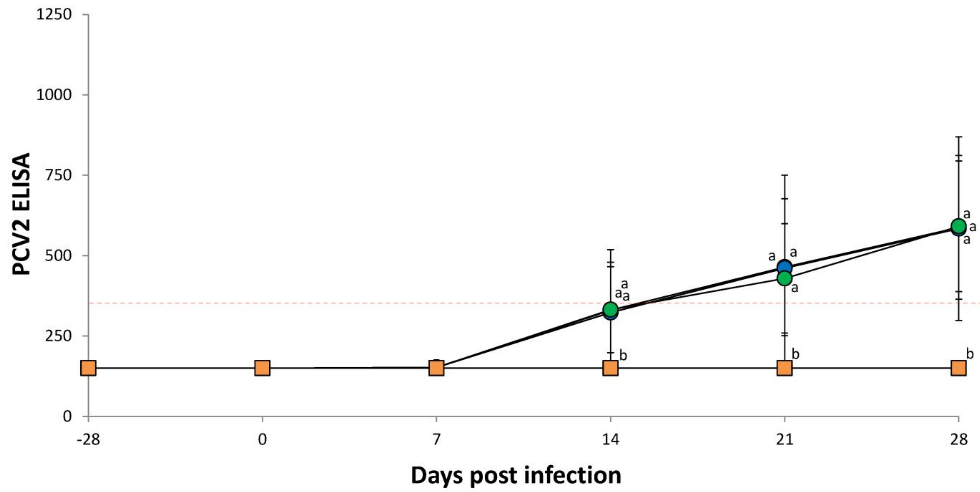

Figure 2. PCV2-specific enzyme-linked immunosorbent assay (ELISA) antibody levels in serum from PCV2a-infected (●, n = 10 pigs), PCV2b-infected (●, n = 10 pigs), PCV2d-infected (●, n = 10 pigs), and negative control (■, n = 10 pigs) groups. Serum samples are considered to be positive for anti-PCV2 antibody if the reciprocal ELISA titer is >350 (red dotted line). Analysis of variance (ANOVA) was carried out to compare the 4 treatment groups. Tukey's adjustment was used for post-hoc analysis. For not normally distributed data, the Kruskal-Wallis test and consequently Mann-Whitney tests were used to evaluate differences among the treatment groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant (P < 0.05) difference among 4 groups.

3.4. Pathology

Pigs in the PCV2a-, PCV2b-, and PCV2d-infected groups had significantly higher ($P<0.05$) lymphoid lesion scores (Table I). PCV2 antigen positive cells were detected in the PCV2a-infected (Figure 3A), PCV2b-infected (Figure 3B), and PCV2d-infected (Figure 3C) groups. Pigs in the PCV2a-, PCV2b-, and PCV2d-infected groups had significantly higher ($P<0.05$) number of lymphoid PCV2-positive cells than the negative control group (Figure 3D).

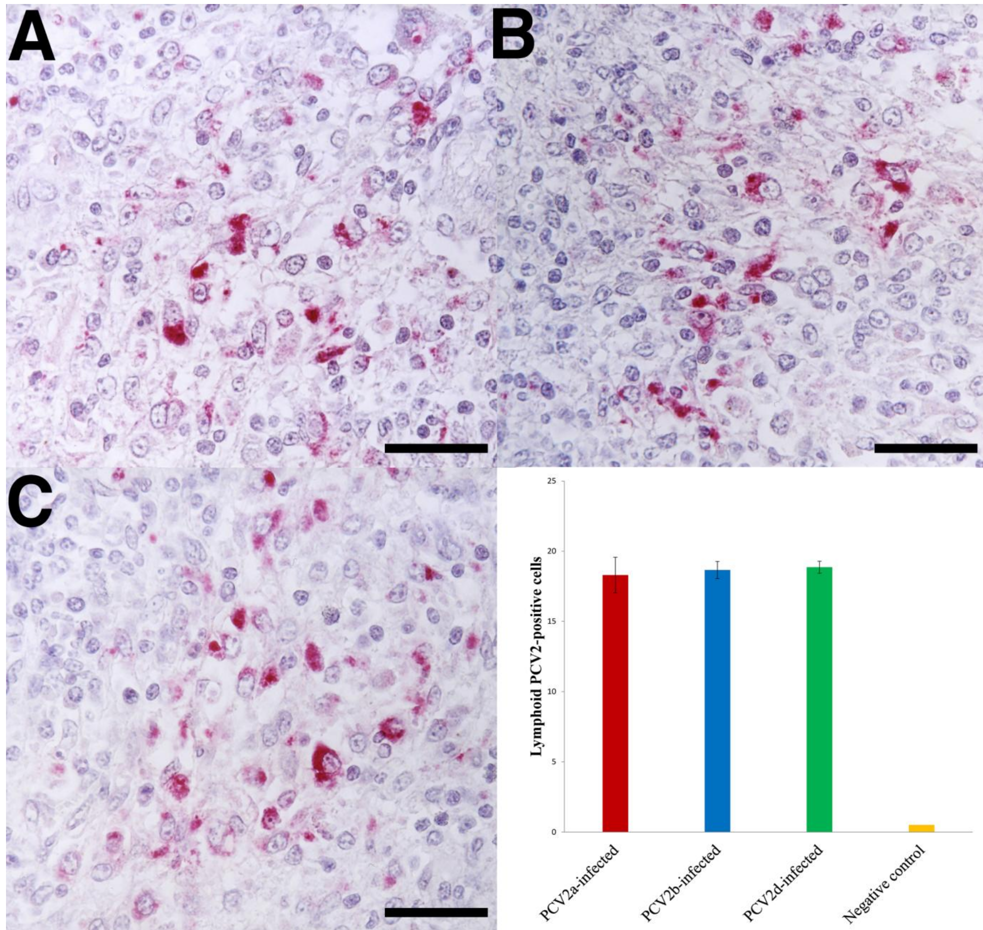

Figure 3. Immunohistochemistry (IHC) for the detection of PCV2 antigens in lymph node. **A** – Representative IHC for the detecting PCV2 antigens in lymph node from the PCV2a-infected group. Bar = 55 μm. **B** – Representative IHC for the detecting of PCV2 antigens in lymph node from the PCV2b-infected group. Bar = 55 μm. **C** – Representative IHC for the detecting of PCV2 antigens in lymph node from the PCV2d-infected group. Bar = 55 μm. **D** – Numbers of lymphoid PCV2 antigen-positive cells in lymph node from PCV2a-, PCV2b-, and PCV2d-infected groups. Numbers of PCV2 antigen-positive cells per unit are (0.25 mm^2) of lymph node from 10 pigs per group were counted using an NIH Image J 1.45s program (https://imagej.nih.gov/ij/download.html). Analysis of variance (ANOVA) was carried out to compare the 4 treatment groups. Tukey's adjustment was used for a post-hoc analysis. For not normally distributed data, the Kruskal-Wallis test and consequently Mann-Whitney tests were used to evaluate differences among the treatment groups. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among 4 groups.

4. Discussion

The evidence presented in this study demonstrates that there is no significant difference in virulence among the 3 major Korean PCV2 genotypes, PCV2a, PCV2b, and PCV2d. We should be cautious when interpreting these results, however, as even though PCV2 is the primary agent of PCVAD, a single infection with PCV2 does not always reproduce a full manifestation of clinical PCVAD observed in pig farms (15). Thus, a single infection model may not be the most representative when comparing the virulence of PCV2 genotypes. There is evidence that there are both mild and virulent PCV2 strains for each genotype (16, 17). In addition, co-infection of PCV2 with other viruses, such as porcine reproductive respiratory syndrome (PRRSV), could potentiate the virulence of mild virulent PCV2 strains. Therefore, further studies are needed to accurately compare the virulence of each PCV2 genotype by analyzing both single-infection and co-infection models.

Potential differences in virulence among the various genotypes have been a subject of recent debate. A previous study showed that infection with a Chinese PCV2d strain can result in more severe disease than PCV2a or PCV2b infections (7). In contrast, a study that used North American isolates for all 3 genotypes (a, b, d) reported no significant difference in virulence (8). In our study, we compared Korean isolates for each genotype and observed no significant differences in virulence similar to the North

American isolates. It is interesting to note, however, that even though no significant differences in virulence were observed among the 3 genotypes for Korean and North American isolates, the pattern of PCV2 viremia among genotypes is somewhat different when comparing isolates from Korea and North America. When comparing levels of PCV2 viremia with the North American isolates, the amount of PCV2 DNA in serum samples at 7dpi was significantly higher in pigs infected with PCV2d compared to PCV2b (8). In contrast, no statistical differences were observed in levels of viremia among all 3 major Korean PCV2 genotypes throughout our study. It should be noted, however, that the route of infection in this study was through intranasal inoculation only, whereas the previous 2 studies (7, 8) used both intranasal and intramuscular inoculations. It is possible that the route of infection could have an effect on the virulence. Further studies are needed to directly compare PCV2 genotypes using the same route of infection.

Even though PCV2a or PCV2b are still highly prevalent, the PCV2d genotype has now become the predominant genotype in Korea (18). Severe economic losses to the Korean pig industry due to PCVAD underscore the need for a comparative study of the virulence of the 3 major PCV2 genotypes. Additionally, most of the PCV2 vaccines are now available based on the PCV2a genotype. Cross-protection of the PCV2a vaccines against PCV2d infection is critical because, according to the Korean Animal Health Products Association, about 96.05% of total piglets farrowed in 2017

in Korea were vaccinated with PCV2a-based vaccines. While 2 commercial PCV2a-based vaccines have been shown to protect pigs against experimental challenge with a North American PCV2d strain (19, 20), further studies are needed to determine whether the current PCV2a vaccines can provide efficient cross-protection against Korean PCV2d strains.

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국 문 초 록

한국에서 분리한 돼지 써코바이러스 2형의 3가지 유전자형 (a, b, d)

병원성 비교분석

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돼지 써코바이러스는 양돈 산업에서 경제적 손실을 가져오는 가장 흔한 원인체이고, 주요 유전자형에 기반해 써코 바이러스는 PCV2a, PCV2b, PCV2c, PCV2d, PCV2e로 분류된다. 본 실험은 한국의 돼지 써코 바이러스 주요 유전자형인 PCV2a, PCV2b, PCV2d를 임상학적, 바이러스학적, 면역학적, 병리학적 기법을 통해 비교 분석한 것이다. 실험에 앞서 돼지 생식기 호흡기 증후군과 마이코 플라즈마성 폐렴 검사를 통해 감염 이력이 없는 돼지 농장에서 초유 수유가 이루어진 건강한 14일령 돼지 40마리를 선별하였다. 실험에는 14일령 돼지를 10마리씩 4개 그룹으로 나누어 진행하였다. 3개 그룹은 각각 PCV2a, PCV2b, PCV2d로 감염된 세포의 상층액을 2mL씩 비강 내 접종하였고, 대조군에는 감염되지 않은 세포의 상층액을 2mL 비강 접종하였다. 대조군은 접종 전후에 유의적 변화는 관찰되지 않은 것에 비해 감염된 상층액이 접종된 3그룹은

모두 접종 이후로 심한 임상학적 변화가 관찰되었다. 감염이 이루어진 3 그룹은 임상학적, 바이러스학적, 면역학적, 병리학적 측면에서 모두 유의한 차별점 없이 비슷한 변화 양상을 보였으며, 이를 통해 한국의 돼지 써코 바이러스 유전형, PCV2a, PCV2b, PCV2d의 병원성 정도에는 유의한 차이가 없음을 확인하였다.

주요어:

돼지 써코 바이러스 2형 유전자형, 돼지 써코 바이러스, 돼지 써코 바이러스 2형, 병원성

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