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Classifying Host Susceptibility Using Porcine Circovirus Type 2 Viral Load and Antibody Titer

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Porcine circovirus type 2 (PCV2) is a notorious and ubiquitous virus in the swine industry. The susceptibility of the host to PCV2 infection is considered to be one factor associated with the dynamics of PCV2. The objective of this study was to verify the criteria for host susceptibility to PCV2, using blood parameters of post-weaned pigs naturally infected with the virus. The PCV2 DNA viral load, antibody titer, and leukopenia characteristics were measured in the serum extracted from the pigs at the 10th week. We classified the pigs into high (>5.0), intermediate (3.0 to 5.0), and low (<3.0) groups on the basis of the PCV2 viral load (log copies/ml), or as positive ($\leq$0.50) and negative (>0.50) groups on the basis of antibody titer (sample-to-negative corrected ratio). Moreover, using these two categorized parameters, we suggested the criteria for classification into the susceptible and resistant groups. Statistical analyses revealed that pigs in the susceptible group had a significantly higher viral load (p<0.001) and negative antibody titer (p<0.001), as well as significantly lower leukocyte counts (p=0.018) and lower amounts of several leukocyte components (p<0.05), than pigs in the resistant group. We concluded that the susceptible group could be considered to have PCV2-induced leukopenia. Therefore, we suggest that the combined classifications of viral loads and anti-PCV2 antibodies can be used to determine PCV2-induced leukopenia in the subclinical PCV2 infection of post-weaned pig populations.

Key words : Antibody titer, host susceptibility, leukopenia, pig, porcine circovirus type 2, viral load

Introduction

Porcine circovirus type 2 (PCV2) has been widely documented to wreak havoc in the swine industry [1, 8]. In particular, PCV2 is a major etiological agent of postweaning multisystemic wasting syndrome (PMWS), which occurs primarily in pigs between 8 and 12 wk of age, with clinical symptoms such as weight loss, respiratory signs, and jaundice [15]. In recent years, several combined PCV2-related diseases have been named porcine circovirus-associated disease (PCVAD) in North America and porcine circovirus disease (PCVD) in Europe, because PCV2 infection appears to cause a wide variety of clinical syndromes [12]. To overcome the serious problems related with these diseases, management strategy for controlling PCV2 have been recommended [8] and various vaccines against PCV2 have been used worldwide [2, 4]. However, PCV2 is still ubiquitous in swine farms and their infection cannot be completely prevented.

Previous studies focused on the host genetic differences in relation with PCVAD incidence and severity [11, 13]. In general, postmortem evaluation methods are used for the identification of individuals susceptible to PCV2. However, sacrifice of the tested pigs is inevitable in postmortem evaluation, and the tested pigs can no longer be used as seed stocks. For this reason, PCV2 infections cannot be measured in many individuals and controlled easily in piglet farms. Therefore, new evaluation methods are required that can accurately and immediately measure the virus in numerous live pigs.

Several measurable methodologies on living bodies that are related to the PCV2 infection dynamics have been suggested, for example, blood parameters like the serum viral load and PCV2 specific antibody titer [12]. Moreover, viremia, antibody response, and leukopenia are common features of the pathogenesis of PCV2 infection [5]. Alternatively, PCV2-induced leukopenia, which is related to lymphocyte depletion in lymphoid tissue, is widely observed in PMWS-

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affected piglets and is the primary characteristic for diagnosing PMWS [10]. However, those of previous studies had not been applied to estimate host susceptibilities using the combinational parameters. The objective of current study was to verify the potential use of blood parameters such as viral load, antibody level, and leukocyte count for the identification of individual susceptibility to PCV2 infection without necropsy in postweaning pigs. We used the PCV2 viral loads and antibody titers in the sera to develop criteria for the host susceptibility to PCV2 infection. The susceptibility criteria were confirmed by their associations with PCV2-induced leukopenia values.

Materials and Methods

Animals

A total of 155 crossbreds (82 females and 73 castrated males) generated from Yorkshire dams inseminated with semen from Landrace were randomly selected from 46 litters (average, 3.37±1.50 piglet per dam) at a farm with animals that had naturally occurring PCV2 infection. Although the vaccine program of the source farm had included PCV2 and porcine respiratory syndrome virus, all the animals used in this study were not vaccinated against PCV2, so as to determine the host response against natural PCV2 infection. The pigs were raised in four batches under the same condition. The experimental protocols and standard operating procedures were approved by the Institutional Animal Care and Use Committee at the National Institute of Animal Science (Wanju, Republic of Korea) in 2016(Ethic approval number: 2016-191). At the age of around 4 weeks (28.34±3.49 d), blood samples were collected from each pig and analyzed for the presence of PCV2 DNA in the serum, and 142 pigs were identified as seronegative for PCV2. We therefore used these 142 pigs to further determine how many had been exposed to natural PCV2 infection up to 10 weeks of age, which is predominant onset age of PMWS. The PCV2 viral load, anti-PCV2 antibody titer, and leukocyte counts were quantified in blood samples collected from the pigs at 10 wk of age (69.25±2.17d).

PCV2 viral load and antibody

The viral DNA from the serum samples was isolated using a commercial DNA extraction kit (Qiagen, Valencia, CA, USA). The PCV2 viral load was measured with TaqMan-based real-time polymerase chain reaction (PCR), with the following primers and probe designed from the complete PCV2 genome (GenBank Accession No. FR823451.1): forward primer, 5′-TCGATCTCAAGGACAACGGAGT-3′; reverse primer, 5′-TTGGTCTTCCAATCACGCTTCTGC-3′; and probe, 5′-CAGAGCAGCACCCTGTAACGTTTGTCA-3. The size of the amplified product was 173 bp. The reaction contained 500 nM of each primer, 250 nM of the probe, 25 μl of 2× TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), and 2.5 μl of the template. Nuclease-free water was added to bring the final volume to 50 μl. Amplification was performed under Universal cycling conditions (2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 58°C). The plasmid (pPCV2) used as the standard DNA was constructed by ligating a PCR fragment into the T Easy vector, according to the manufacturer's instructions (Promega, Madison, WI, USA). The cloned fragment included a 314 bp region of the PCV2 open reading frame 3. The plasmid was propagated in PK-15 porcine kidney cells (ATCC), purified using a Miniprep kit (Qiagen, Valencia, CA, USA), and quantified using a spectrophotometer (Thermo Scientific, Waltham, MA, USA). Ten-fold dilutions were made to obtain 10^{11} to 10^{1} plasmids per 2.5 μl sample for the real-time PCR. We transformed the unit of PCV2 DNA viral load (copies/ml) into log base 10.

Anti-PCV2 antibodies in the serum were measured with a commercial PCV2 Ab Mono Blocking kit (Synbiotics, Lyon, France), according to manufacturer's instructions. The anti-PCV2 antibody titers were expressed as a sample-to-negative corrected (SNc) ratio. Samples were considered negative if the SNc ratio was higher than 0.50 [17]. The indices for leukocytes including total count, neutrophils, lymphocytes, monocytes, eosinophils, and basophils were counted using a fully automatic hematology analyzer for animals (Drew Scientific Inc., Dallas, TX, USA) in accordance with the manufacturer's recommendations.

Statistical analysis

A total of 142 pigs at 10 wk of age were classified into three groups on the basis of the degree of viral load (high, intermediate, low), using the FASTCLUS procedure (SAS software). The pigs were also separated into two groups on the basis of the SNc ratio of the antibody titer. The SNc ratio of the positive group was $\leq$0.50, whereas that of the negative group was >0.50 (Fig. 1). Finally, we classified the susceptible and resistant groups among the studied population

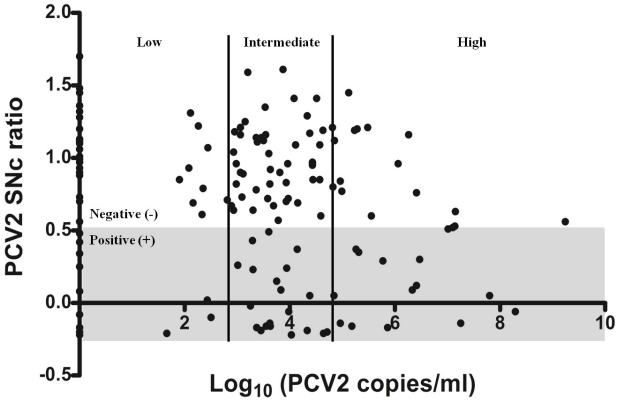

Fig. 1. Scatterplot representing the distribution of porcine circovirus type 2 (PCV2) viral loads and anti-PCV2 antibodies in 10-wk-old naturally infected pigs (n=142). The PCV2 viral loads in the sera were expressed as the logarithm of the PCV2 DNA copies/ml. The two vertical solid lines divide the PCV2 viral loads (log copies/ml) into the high (>5.0), intermediate (3.0 to 5.0), and low (<3.0) groups. PCV2 antibody titer levels were classified according to the sample-to-negative corrected (SNc) ratio (positive, ≤ 0.50; negative, >0.50). The positive (+) range of PCV2 antibody titer is represented by the gray background.

on the basis of the combined viral loads (log PCV2 copies/ml) and antibody titers (SNc ratio). Pigs were classified as susceptible if they showed a high PCV2 viral load (>5.0 log PCV2 copies/ml) with negative PCV2 antibody titer, whereas they were grouped as resistant if they showed a low PCV2 viral load (<3.0 log PCV2 copies/ml) with PCV2 antibody level.

The mixed model (SAS procedure MIXED; SAS software) was used to determine the relationships between the groups and measured traits. The model used was as follows: $y_{ijklm} = \mu + G_i + S_j + B_k + M_l + e_{ijklm}$, where y_{ijklm} is the observation of the traits, μ is the general mean, G_i is the fixed effect of group i, S_j is the fixed effect of sex j, B_k is the fixed effect of batch j, M_l is the random effect of mother l, and e_{jjklm} is the random error. When significant differences were determined, the mean values were separated by the probability difference (PDIFF) option.

Results and Discussion

In general, piglets are exposed through horizontal PCV2 infection, by direct or nose-to-nose contact with pigs that are already infected, after weaning and mingling in the post-weaning house [14]. Following PCV2 infection, viremia and seroconversion are noted in the serum. Along with the PCV2 viral load, the PCV2 specific antibody titer is generally considered to be informative of the immune status toward PCV2 infection. Therefore, the presence of PCV2 genomic DNA and PCV2 specific antibodies in the serum indicates that the tested pigs have an infectious level of PCV2 [7].

The distributions of the PCV2 viral loads (log PCV2 copies/ml) and anti-PCV2 antibody titers (SNc ratio) in the 142 pigs at 10 wk of age are shown in Fig. 1. The frequency of PCV2 DNA detected in the sera was 77% (n=110), and the average viral load was 4.22±1.43 log copies/ml. The viral loads ranged from 1.66 to 9.24 log copies/ml. Although the PCV2 viral loads were widely distributed in the study population, a relatively lower number of pigs (32%, n=46) showed a positive antibody titer against PCV2 (SNc ratio ≤0.50).

In a previous study, PCV2 genomic DNA and antibodies were detected in sera from both healthy pigs and PCVAD-diagnosed pigs [15]. However, other previous studies reported that the PCV2 viral loads in the case of PMWS in PCVAD-diagnosed pigs were higher (>7 log PCV2 copies/ml in the case of PMWS) than those in healthy pigs (<4 log PCV2 copies/ml) [3, 16]. In our population, eight pigs showed upper levels of PCV2 viral load (i.e., >7.0 log copies/ml). However, only three of these eight pigs showed positive PCV2 antibodies (SNc ratio ≤0.50). Moreover, we observed a weak negative correlation between the PCV2 viral load and antibody titer in our experimental population (r=-0.17, p=0.035; data was not shown). Based on the two-dimensional spread of PCV2 viral load and antibody titer, we postulate that the serum PCV2 viral load and antibody titer during the post-weaning period could be challenged as being useful parameters for the evaluation of host susceptibility to PCV2 infection, and there were high variations of host susceptibility in the population.

Classifications based on the PCV2 viral load and antibody titer were conducted to investigate the host susceptibility against PCV2 infection under the living conditions used (Table 1). The 10-wk-old pigs were classified by cluster analysis into three groups on the basis of PCV2 viral load (high, $>10^5$ copies/ml; intermediate, 10^5 to 10^3 copies/ml; and low, $<10^3$ copies/ml). The PCV2 viral loads were significantly different among the groups (p<0.001). However, no significant difference was observed in the SNc ratio of anti-PCV2 antibodies (p=0.908). Moreover, the leukocyte counts and all leukocyte components were not significantly different among the three groups.

Table 1. Classification of pigs according to the blood parameters of porcine circovirus type 2 (PCV2), viral load, antibody, and susceptibility and their associations

Traits	PCV2 viral load			*p-Value*	PCV2 antibody		*p-Value*	PCV2 susceptibility [1]		*p-Value*
	High (*n*=24)	Intermediate (*n*=68)	Low (*n*=50)		Positive (*n*=46)	Negative (*n*=96)		Susceptible (*n*=13)	Resistant (*n*=13)	
PCV2 viral load (log_{10} copies/ml)	6.19^{A}±0.21	3.90^{B}±0.12	1.04^{C}±0.14	<0.001	3.09±0.30	3.35±0.22	0.465	6.15^{A}±0.40	0.75^{B}±0.34	0.001
Anti-PCV2 antibody (SNc)	0.62±0.10	0.67±0.06	0.67±0.07	0.908	0.08^{B}±0.04	0.95^{A}±0.03	<0.001	1.04^{A}±0.08	-0.03^{B}±0.08	0.001
Leukocyte count (10^3/μl)	11.61±1.49	12.85±0.90	14.91±1.08	0.169	14.38±1.08	12.87±0.79	0.233	9.15^{B}±1.80	17.13^{A}±1.52	0.018
Neutrophils (10^3/μl)	5.03±0.77	5.26±0.46	6.58±0.56	0.127	6.05±0.55	5.50±0.41	0.395	3.27^{B}±0.88	6.99^{A}±0.75	0.021
Lymphocytes (10^3/μl)	5.23±0.70	6.23±0.42	6.72±0.51	0.247	6.78±0.51	5.97±0.38	0.173	5.04^{B}±0.90	8.55^{A}±0.76	0.029
Monocytes (10^3/μl)	0.46±0.08	0.55±0.04	0.48±0.05	0.391	0.52±0.05	0.51±0.04	0.784	0.36^{B}±0.06	0.61^{A}±0.05	0.021
Eosinophils (10^3/μl)	0.66±0.14	0.63±0.09	0.86±0.11	0.192	0.75±0.11	0.70±0.08	0.714	0.39±0.19	0.77±0.16	0.205
Basophils (10^3/μl)	0.19±0.04	0.19±0.02	0.22±0.03	0.559	0.23±0.03	0.18±0.02	0.178	0.10±0.05	0.22±0.04	0.090

[A,B,C]Different superscript letters within the row indicate significant differences (p<0.05).
[1]The susceptible group has a high PCV2 viral load with negative PCV2 antibody level, whereas the resistant group has a low PCV2 viral load with positive PCV2 antibody level.
SNc, sample-to-negative corrected ratio.

The alternative grouping was based on the antibody status as expressed by the SNc ratio (positive, ≤0.50; negative, >0.50). The associations of the groups were not significant with all of the measured traits, except for the anti-PCV2 antibody levels (p<0.001). Therefore, each classification had no significant effect on each of the other quantified parameters, as well as on any of the leukocyte characteristics. Based on each classification result, we concluded that either the PCV2 viral load or the antibody titer alone cannot be used to evaluate the host's susceptibility to PCV2 infection.

Next, the pigs were classified into PCV2-susceptible and PCV2-resistant groups by combining the two kinds of classification used above. The susceptible group had a high PCV2 viral load with negative PCV2 antibody level, whereas the resistant group had a low PCV2 viral load with positive PCV2 antibody level (Fig 1). As shown in Table 1, the association results were highly significant between the two groups with respect to the quantifiable parameters and leukocyte characteristics. The susceptible group showed a significantly higher viral load (p<0.001) and PCV2 SNc ratio (p<0.001) than did the resistant group. In addition, the total leukocyte counts were considerably lower in the susceptible group (p=0.018). Specifically, the mean value of leukocyte counts for the susceptible group was lower than the reference range (11 to 22 10^3/μl) for healthy pigs (Duncan et al., 1994). Among the leukocyte components, the neutrophils, lymphocytes, and monocytes were also significantly lower in the susceptible group (p=0.021, p=0.029, and p=0.021, respectively). Based on the significantly low levels of these leukocyte components, the susceptible group could be considered as having PCV2-induced leukopenia. Considering the association of leukopenia with PCV2, our criteria using the PCV2 viral load and antibody titer may be applicable for evaluating susceptibility against PCV2 infection.

Previous studies have also attempted challenge experiments using the PCV2 viral load and antibody titer as the blood parameters related with PCV2 infection, respectively [6, 9]. However, those studies used experimental pigs that were inoculated at the same time point, and they could measure the values under the control of the exact points of post day of infection. In the current study, a naturally PCV2-infected pig population was used. Consequently, it was difficult to control not only the point of PCV2 infection in each individual but also to control various concurrent co-infections by other pathogens. Therefore, the current study's suggested criteria of serum PCV2 viral load and antibody titer had limitations with respect to identifying the precise susceptibility at the individual level and having a direct relationship with the clinical PCVAD diagnosis (e.g., PMWS). However, we measured the leukopenia characteristics in the serum instead of making a simple PCVAD diagnosis and observations of clinical symptoms. Our results suggest that the optimum levels of the two parameters can be applicable to verifying PCV2-induced leukopenia in the

field, with a naturally PCV2-infected population. Moreover, it could be easily evaluated in the serum without the need to sacrifice the animals, so these parameters can be used as phenotypes for host susceptibility against PCV2 in further studies. In conclusion, we suggest that the combined criteria of a PCV2 viral load of >5.0 copies/ml and negative results for antibodies against PCV2 can be useful for determining PCV2-induced leukopenia in subclinical PCV2 infection of pig populations.

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초록 : 돼지 써코바이러스 2형 감염량과 항체가를 이용한 자돈의 저항성군 선발법

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양돈산업에 있어 돼지 써코바이러스 2형(PCV2)의 복합감염으로 인한 이유자돈의 질병 피해가 막대하다. PCV2 감염에 대한 숙주의 민감도는 상이한 것으로 알려져 있으며, 따라서 숙주의 민감도를 구분하는 것은, 이를 이용한 숙주의 저항성 향상 연구에 필수이다. 본 연구의 목적은 이유자돈군의 혈액 내에서 PCV2 바이러스에 대한 숙주의 민감도를 구분 짓고 구명하는데 있었다. 본 연구에서는 자연적으로 바이러스에 감염된 10주령의 이유자돈군으로부터 혈청을 채취하여 PCV2 바이러스량과 항체가를 측정하고 혈구분석을 실시했다. 또한, 측정된 PCV2 바이러스량과 항체가를 기준으로 자돈군 내에서 저항성군과 민감성군을 선정하였고, 통계분석결과 저항성군에 비해 민감성군에서 백혈구 수가 현저히 줄어든 것을 확인하였다. 본 연구를 통해서 PCV2 감염에 대한 돼지의 민감도를 구분짓기 위한 PCV2 바이러스량과 항체가를 이용한 복합기준을 제시할 수 있었으며, 이유자돈군의 PCV2 관련 질병저항성 및 백혈구감소증을 확인할 수 있는 방법을 마련하였다.