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Evaluation of a porcine circovirus type 2a (PCV2a) vaccine efficacy against experimental PCV2a, PCV2b, and PCV2d challenge

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ABSTRACT

The objective of this study was to compare the efficacy of a commercial porcine circovirus type 2a (PCV2a) subunit vaccine against experimental PCV2a, PCV2b, and PCV2d challenge. A total of 105 pigs were randomly divided into 7 groups (15 pigs per group). At 21 days old the pigs were intramuscularly administered the PCV2a vaccine as a 1.0 mL dose. Four weeks following vaccination, pigs were challenged with either Korean PCV2a, PCV2b, or PCV2d. All vaccinated pigs showed a significant ($P < 0.05$) reduction of clinical signs, PCV2 viremia, lymphoid lesions, and lymphoid PCV2 antigen levels compared to unvaccinated control pigs. Vaccination resulted also in significantly higher ($P < 0.05$) titers of neutralizing antibody against PCV2, and an increase in the frequency of PCV2-specific interferon-γ secreting cells (IFN-γ-SC). The vaccine showed similar protection among the vaccinated groups regardless of the genotype of the challenge. Interestingly, vaccinated pigs had higher levels of neutralizing antibody titers against PCV2a compared to PCV2b or PCV2d while the number of PCV2a-, PCV2b-, and PCV2d-specific IFN-γ-SC were similar. Taken together, the results presented here demonstrate that a PCV2a vaccine can be effective against experimental PCV2a, PCV2b, and PCV2d challenge.

1. Introduction

Porcine circovirus type 2 (PCV2) is a small single-stranded circular DNA virus that has been linked to a numbers of diseases (Mankertz et al., 1997; Chae, 2004, 2005). PCV2 is currently classified in five different genotypes designated PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e (Dupont et al., 2008; Segalés et al., 2008; Wang et al., 2009; Guo et al., 2012). Due to the variety of clinical conditions and symptoms associated with PCV2, disease is commonly referred to as porcine circovirus associated diseases (PCVAD) in Asia and North America (Chae, 2005; Opriessnig et al., 2007). In Europe, the term porcine circovirus disease (PCVD) is used instead (Segalés et al., 2005). PCVAD is responsible for production loss, mostly due to growth retardation and reduction in average daily weight gain. The most commonly used method in controlling PCVAD has been vaccination which has been successful in increasing weight gain, reduction of clinical signs, as well as reduction in the number of culls and runts and overall losses (Chae, 2012).

The initial vaccine development between 2002–2004 was based on the PCV2a genotype since it was the predominant genotype at the time (Fenaux et al., 2000; Larochelle et al., 2002). However, by the time the vaccine was introduced in 2007, PCV2b had overtaken PCV2a as the predominant genotype worldwide (Cheung et al., 2007; Gagnon et al., 2007; Dupont et al., 2008; Wiederkehr et al., 2009; Kim et al., 2011a). Nevertheless, the commercial PCV2a-based PCV2 vaccine is also effective against PCV2b (Fort et al., 2008; Seo et al., 2014a). Another genotypic shift has occurred more recently with PCV2d becoming the more prevalent genotype worldwide (Xiao et al., 2015). PCV2d is also currently the most prevalent genotype in the US and Korea (Xiao et al., 2016; Kwon et al., 2017). This second shift along with an outbreak of PCVAD caused by PCV2d in PCV2a-vaccinated herds (Opriessnig et al., 2013; Seo et al., 2014c; Ramos et al., 2015), has again raised questions about the efficacy of the PCV2a vaccine against the PCV2d genotype. Three commercial PCV2a-based vaccines were shown to protect finisher pigs naturally infected with PCV2b against experimental challenge with a PCV2d US isolate (Opriessnig et al., 2014a). Another study also showed that two commercial PCV2a-based vaccines were able to protect pigs against experimental challenge with PCV2d (Opriessnig et al., 2014b, 2017). However, no studies have been done so far to assess the efficacy of commercial PCV2a-based vaccines against Korean PCV2d strains. Cross-protection against PCV2d infection by currently commercially available PCV2a-based vaccines is very important because PCV2d is the most prevalent genotype causing PCVAD resulting in enormous economic losses in Asian pork industry. In addition, PCV2a-

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based vaccines are widely used covering to 96.05% of total piglets farrowed in 2017 in Korea according to Korea Animal Health Products Association (http://www.kahpha.or.kr). The objective of this study was to evaluate and compare the protection efficacy of a PCV2a-based vaccine against experimental challenge of PCV2a, PCV2b, and PCV2d strains isolated in Korea based on clinical, virological, immunological, and pathological analyses.

2. Materials and methods

2.1. Animals

One hundred five clinically healthy, colostrum-fed conventional pigs from sows that had not been previously vaccinated against PCV2 were purchased at 14 days of age from a commercial farm that was free of porcine reproductive and respiratory syndrome virus (PRRSV). The farm was determined to be *Mycoplasma hyopneumoniae*-free based on serological testing, and long term clinical and slaughter history. Pigs were also tested by ELISA (PRRSV: HerdCheck PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA; PCV2: Synbiotics, Lyon, France; *M. hyopneumoniae*: *M. hyo*. Ab test, IDEXX Laboratories Inc.) and were seronegative for PRRSV, PCV2, and *M. hyopneumoniae*. In addition, pigs were negative for PRRSV and PCV2 viremia, and nasal swabs were negative for *M. hyopneumoniae* as tested by real-time PCR (Dubosson et al., 2004; Wasilk et al., 2004; Gagnon et al., 2008).

2.2. Experimental design

Pigs were allocated into 7 groups (15 pigs per group) using the random number generation function from Excel (Microsoft Corporation, Redmond, WA, USA) (Table 1). Vaccinated and unvaccinated groups were assigned according to the challenge genotype (Vac/Ch2a with UnVac/Ch2a, Vac/Ch2b and UnVac/Ch2b, Vac/Ch2d and UnVac/Ch2d) and the pigs in each pair groups were randomly assigned into two separate rooms respectively. The pigs in UnVac/UnCh group were placed into the final room. Each room had 15 pens and each pig was housed individually in a pen for a welfare standpoint.

At −28 days post challenge (dpc, 3 weeks of age), pigs in the Vac/Ch2a, Vac/Ch2b, and Vac/Ch2d groups were injected intramuscularly with a 1.0 mL dose of CircoFLEX (Serial No. 3091124A, Manufacture date 20170320, Expiration date 20190319, Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA). Pigs in the UnVac/Ch2a, UnVac/Ch2b, UnVac/Ch2d, and UnVac/UnCh groups were similarly administered 1.0 mL of PBS.

At 0 dpc (7 weeks of age), pigs in the Vac/Ch2a and UnVac/Ch2a groups were inoculated intranasally with 2 mL (1 mL/nostril) of tissue culture supernatant containing 10^5 $TCID_{50}$/mL of PCV2a (SNUVR000032, GenBank no. KF871067, 5th passage in PCV-free PK-15 cell lines) (Seo et al., 2014b). Pigs in the Vac/Ch2b and UnVac/Ch2b groups 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) (Seo et al., 2014b). Pigs in the Vac/Ch2d and UnVac/Ch2d groups were inoculated intranasally with 2 mL (1 mL/nostril) of tissue culture supernatant containing 10^5 $TCID_{50}$/mL of PCV2d (SNUVR140004, GenBank no. KJ437 506, 5th passage in PCV-free PK-15 cell lines) (Seo et al., 2014c). Pigs in the UnVac/UnCh 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 dpc. All pigs were euthanized for necropsy at 28 dpc following tranquilization by IV azaperon (Stersnil, Janssen Pharmaceutica, Beerse, Belgium). All experimental protocols were approved by the Seoul National University Institutional Animal Care and Use Committee.

2.3. Clinical observation

Pigs were monitored and scored daily for clinical signs as previously described (Seo et al., 2012). Briefly, scoring was defined as follows 0 (normal), 1 (rough haircoat), 2 (rough haircoat and dyspnea), 4 (severe dyspnea and abdominal breathing), and 6 (death). Observers were blinded to vaccination status.

2.4. Quantification of PCV2 DNA in blood

Serum samples were collected at −28, 0, 7, 14, 21, and 28 dpc and DNA was extracted using the QIAamp DNA mini kit. Genomic DNA copy numbers for PCV2a, PCV2b, and PCV2d were quantified by quantitative real-time PCR (Gagnon et al., 2008; Opriessnig et al., 2013; Jeong et al., 2015). The detection limit of the assay was 1.2×10^2 genomic copy numbers of three PCV2 genotypes.

2.5. Serology

The serum samples were tested for antibodies to PCV2 using the commercial PCV2 ELISA (Synbiotics, Lyon, France) at −28, 0, 7, 14, 21, and 28 dpc. Serum samples were considered to be positive for anti-PCV2 antibody if the reciprocal ELISA titer was > 350, according to the manufacturer's instructions.

The serum samples were tested for serum virus neutralization against the same PCV2 genotype only as pig infected with PCV2 genotype. Each serum samples were serially diluted two-fold up to 1:512. An equal volume of each sample dilution was mixed with equal volume of PCV2 stock at the titer of 200 $TCID_{50}$/0.1 mL. Thus, the lowest dilution contained 25% (1:1 dilution + equal volume of PCV2 stock), thereby the detection limit of the assay was 2.0 $\log_2$ (Pogranichnyy et al., 2000; Fort et al., 2009; Shen et al., 2010).

2.6. PCV2-specific interferon-γ secreting cells

The numbers of PCV2a-, PCV2b-, and PCV2d-specific interferon-γ secreting cells (IFN-γ-SC) were determined in peripheral blood mononuclear cells (PBMC) at −28, 0, 7, 14, 21, and 28 dpc as previously described (Seo et al., 2012; Jeong et al., 2015). The frequency of PCV2-specific IFN-γ-SC was considered to be positive if the number of PCV2-specific IFN-γ-SC was greater than 20 cells/10^6 PBMC.

2.7. Pathology

For the morphometric analysis of histopathological changes in

Table 1
Pathology and immunohistochemistry (IHC) data (mean ± standard deviation) of pigs among 7 groups at 28 days post challenge.

Groups	Vac/Ch2a	Vac/Ch2b	Vac/Ch2d	UnVac/Ch2a	UnVac/Ch2b	UnVac/Ch2d	UnVac/UnCh
Vaccination	PCV2a	PCV2a	PCV2a	None	None	None	None
Challenge	PCV2a	PCV2b	PCV2d	PCV2a	PCV2b	PCV2d	None
Lymphoid lesion scores	0.48 ± 0.18[a]	0.51 ± 0.23 [a]	0.52 ± 0.10 [a]	1.37 ± 0.33[b]	1.44 ± 0.32 [b]	1.36 ± 0.50 [b]	0.00 ± 0.00[c]
Lymphoid PCV2-positive cells	2.96 ± 0.33 [a]	2.87 ± 0.45 [a]	2.75 ± 1.07 [a]	17.91 ± 1.29 [b]	18.29 ± 1.03 [b]	17.97 ± 4.23 [b]	0.00 ± 0.00 [c]
No of lymphoid PCV2 IHC positive pigs (all pigs)	15 (15)	15 (15)	15 (15)	0 (15)	0 (15)	0 (15)	0 (15)

Different superscripts (a–c) indicate significant ($P < 0.05$) difference among 7 groups.

superficial inguinal lymph nodes, three sections of that lymph node were examined "blindly" as previously described (Kim and Chae, 2004). Immunohistochemistry (IHC) and morphometric analysis of IHC was carried out as previously described (Kim et al., 2011b).

2.8. Statistical analysis

Prior to statistical analysis, real-time PCR data and neutralizing antibody data were transformed to $\log_{10}$ and $\log_2$ values, respectively. Continuous data (PCV2 DNA, ELISA, IFN-γ-SC, and PCV2 positive cells in lymph node) were analyzed by a one-way analysis of variance (ANOVA) for each time point. When a one-way ANOVA showed statistical significance, a pairwise test using Tukey's adjustment was performed to determine the significance of group differences at each time point. Discrete data (clinical signs, neutralizing antibody, microscopic lymphoid lesion scores) were analyzed by the non-parametric Kruskal-Wallis test. When the non-parametric Kruskal-Wallis test showed significance, the Mann-Whitney test was performed to determine the significant differences between the groups. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Clinical observation

No notable clinical signs and no local and systemic adverse reactions were observed in any of the groups throughout the study.

3.2. Quantification of PCV2 DNA in blood

Real-time PCR was used to quantify genomic copies in blood samples collected from each group. At 14, 21, and 28 dpc, pigs from the Vac/Ch2a, Vac/Ch2b, and Vac/Ch2d groups had significantly ($P < 0.05$) less genomic copies of PCV2a, PCV2b and PCV2d DNA respectively compared to the corresponding control groups. In addition, Vac/Ch2a, Vac/Ch2b, and Vac/Ch2d groups had similar number of genomic copies of PCV2a, PCV2b, and PCV2d DNA respectively. There was also no significant difference on genomic copies of PCV2a, PCV2b, and PCV2d DNA respectively between UnVac/Ch2a, UnVac/Ch2b, and UnVac/Ch2d groups (Fig. 1).

Pigs in the Vac/Ch2a group were negative for PCV2b and PCV2d DNA, pigs in the Vac/Ch2b group were negative for PCV2a and PCV2d DNA and pigs in the Vac/Ch2d 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 UnVac/UnCh group throughout the experiment.

3.3. Anti-PCV2 antibody

Pigs from the Vac/Ch2a, Vac/Ch2b, and Vac/Ch2d groups had significantly higher ($P < 0.05$) PCV2 antibody titers compared to the UnVac/Ch2a, UnVac/Ch2b, and UnVac/Ch2d groups at 21 and 28 dpc. No anti-PCV2 antibodies were detected in pigs from the UnVac/UnCh group (Fig. 2).

3.4. Neutralizing antibody titers against PCV2

At the time of vaccination, serum samples from pigs in all seven groups were negative for PCV2 NA. Pigs from the Vac/Ch2a group had significantly higher ($P < 0.05$) NA titers against PCV2a compared to the UnVac/Ch2a group between 0 and 28 dpc. Similarly, pigs from the Vac/Ch2b and Vac/Ch2d groups had significantly higher ($P < 0.05$) NA titers against PCV2b and PCV2d respectively, compared to their respective control group between 0 and 28 dpc. No neutralizing antibody titers against either PCV2a, PCV2b, or PCV2d were detected in serum samples collected from pigs in the UnVac/UnCh control group throughout the study (Fig. 3).

3.5. PCV2-specific interferon-γ secreting cells

Pigs from the Vac/Ch2a, Vac/Ch2b, and Vac/Ch2d groups had significantly higher ($P < 0.05$) numbers of PCV2a-, PCV2b-, and PCV2d-specific IFN-γ-SC respectively, compared to their respective unvaccinated control groups at 0, 7, 14, and 21 dpc. The frequency of PCV2-specific IFN-γ-SC remained at basal levels (< 20 cells/10^6 PBMC) in all the pigs from the UnVac/UnCh group throughout the experiment (Fig. 4).

3.6. Pathology

Pigs in the Vac/Ch2a, Vac/Ch2b, and Vac/Ch2d groups had significantly lower ($P < 0.05$) lymphoid lesion scores and smaller number of lymphoid PCV2-positive cells compared to the UnVac/Ch2a, UnVac/Ch2b, and UnVac/Ch2d groups (Table 1).

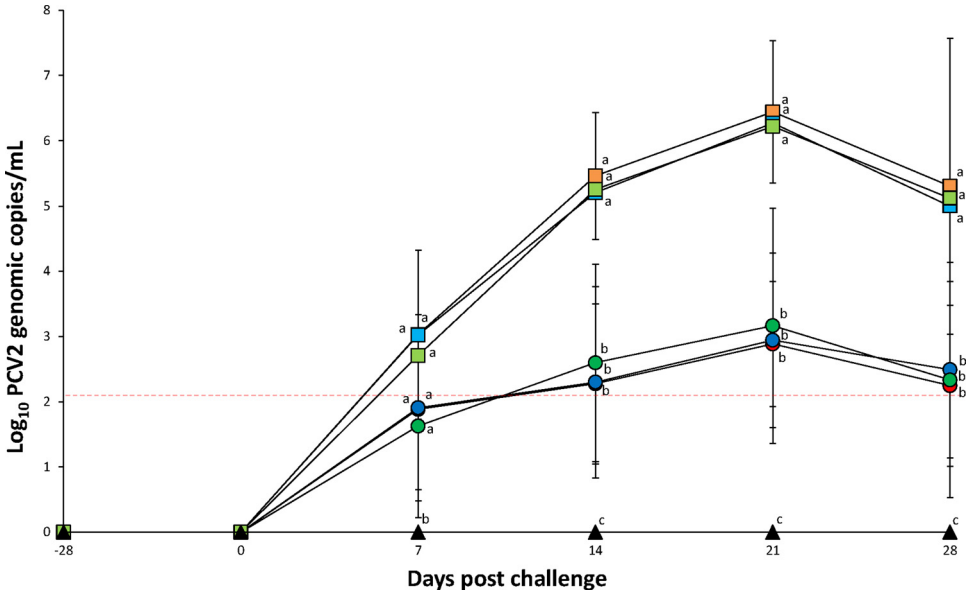

Fig. 1. Mean values of the genomic copy number of PCV2 DNA in serum from Vac/Ch2a (●), Vac/Ch2b (●), Vac/Ch2d (●), UnVac/Ch2a (■), UnVac/Ch2b (■), UnVac/Ch2d (■), and UnVac/UnCh (▲) groups. The detection limit of the assay is 1.2×10^2 genomic copy numbers of three PCV2 genotypes (red dotted line). Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among 7 groups (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

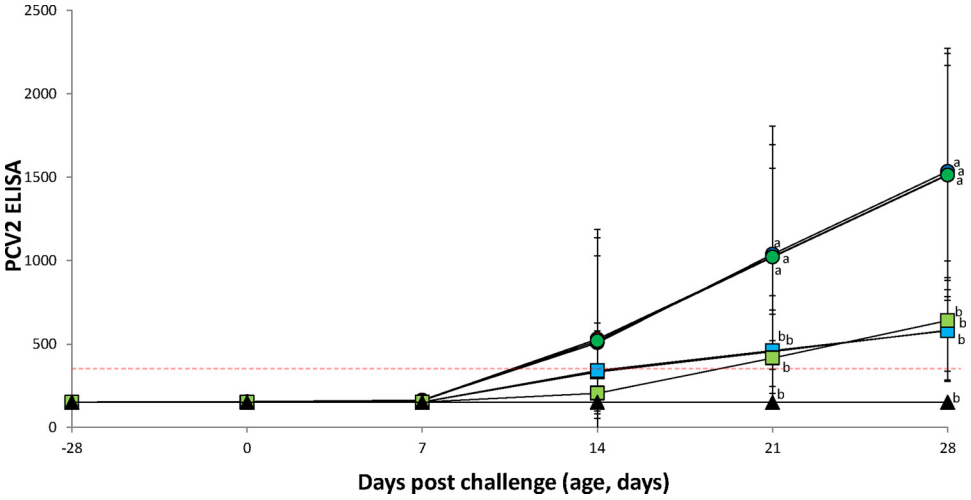

Fig. 2. PCV2-specific enzyme-linked immunosorbent assay (ELISA) antibody levels in serum from Vac/Ch2a (●), Vac/Ch2b (●), Vac/Ch2d (●), UnVac/Ch2a (■), UnVac/Ch2b (■), UnVac/Ch2d (■), and UnVac/UnCh (▲) groups. Serum samples are considered to be positive for anti-PCV2 antibody if the reciprocal ELISA titer is > 350 (red dotted line). Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among 7 groups (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

The results presented here demonstrate that PCV2a-based PCV2 vaccine is efficacious against experimental challenge with Korean PCV2a, PCV2b, and PCV2d strains. The same PCV2a-based PCV2 vaccine has been previously shown to provide good protection against experimental challenge with a PCV2b and a PCV2d strain isolated in the US (Shen et al., 2010; Seo et al., 2014b; Opriessnig et al., 2014a). These results are in agreement with other studies, where two other commercial PCV2a-based PCV2 vaccines were protective against PCV2d challenge (Opriessnig et al., 2014b, 2017).

Korean PCV2d strain used in this study has high levels of identity (99.5–99.7% for ORF1 and 99.8% for ORF2) in nucleotides sequences and (99.0–99.3% for ORF1 and 100% for ORF2) in deduced amino acids sequences with the two US PCV2d strains (Xiao et al., 2012). The genetic similarity between US and Korean PCV2d strains may not guarantee the same efficacy of PCV2 vaccine. However, previous (Opriessnig et al., 2014a) and present studies suggest that the same PCV2a-based subunit vaccine had a similar protective effect against both US and Korean strains. These results further indicated that, most probably, critical epitopes are not different between US and Korean strains.

Neutralizing antibodies have been shown to play a major role in inhibiting PCV2 replication, and preventing lymphoid lesions and clinical disease (Meerts et al., 2005, 2006; Chae, 2012). Interestingly, statistically significant difference on NA titers against PCV2a, PCV2b, and PCV2d is observed between vaccinated pigs at 28 days post vaccination and unvaccinated/changed pigs at 28 day post challenge (data not shown). These data would show that vaccination induce higher NA titers against PCV2a, PCV2b, and PCV2d than those in challenge (or infection) only. PCV2 vaccines elicit neutralizing antibodies which results in protection from PCVAD (Fort et al., 2008, 2009; Seo et al., 2014b). The PCV2a-vaccine in our study was able to elicit cross-reactive neutralizing antibody against PCV2a, PCV2b, PCV2d. Induction of neutralizing antibodies against PCV2a, PCV2b, and PCV2d challenge resulted in a reduction of PCV2a, PCV2b, and PCV2d viremia. However, neutralizing antibody titers against PCV2a seemed to be higher than those against PCV2b or PCV2d. Different neutralizing antibody responses may due to different neutralizing epitopes among PCV2 capsid protein (CP). Recent studies of the PCV2a capsid protein have identified two critical amino acid residues 59 (A) and 60 (T) that determine a conformational neutralizing epitope (Huang et al., 2011; Liu et al., 2013). Sequence analysis of the PCV2b and PCV2d challenge strains revealed point mutations of alanine to arginine (A-R) and alanine to lysine (A-K) at residue 59, respectively. These single mutations at residue 59 may result in a slightly different conformation affecting the

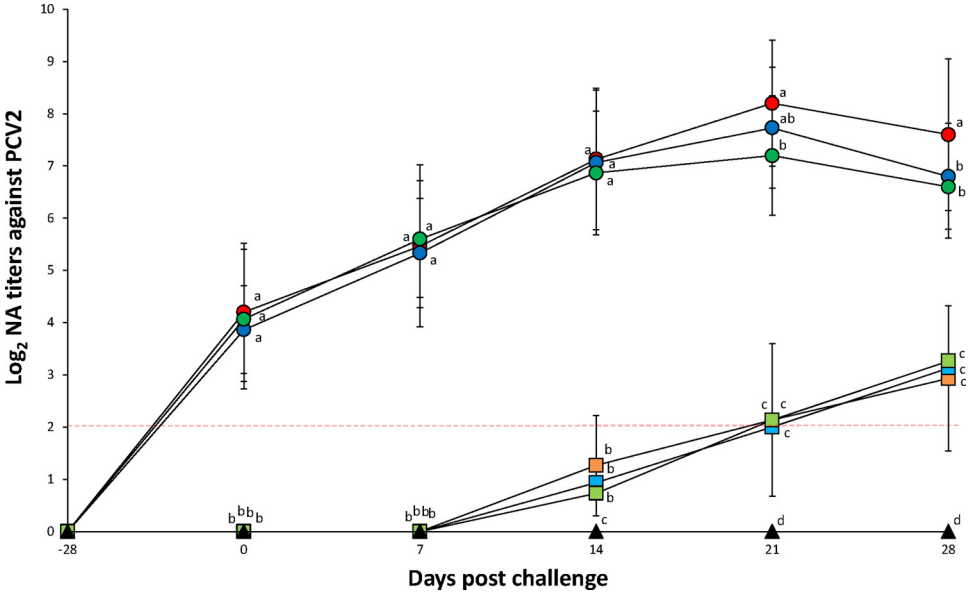

Fig. 3. Neutralizing antibody (NA) titers against PCV2 in serum from Vac/Ch2a (●), Vac/Ch2b (●), Vac/Ch2d (●), UnVac/Ch2a (■), UnVac/Ch2b (■), UnVac/Ch2d (■), and UnVac/UnCh (▲) groups. The detection limit of the assay is 2.0 $\log_2$ (red dotted line). Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 7 groups (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

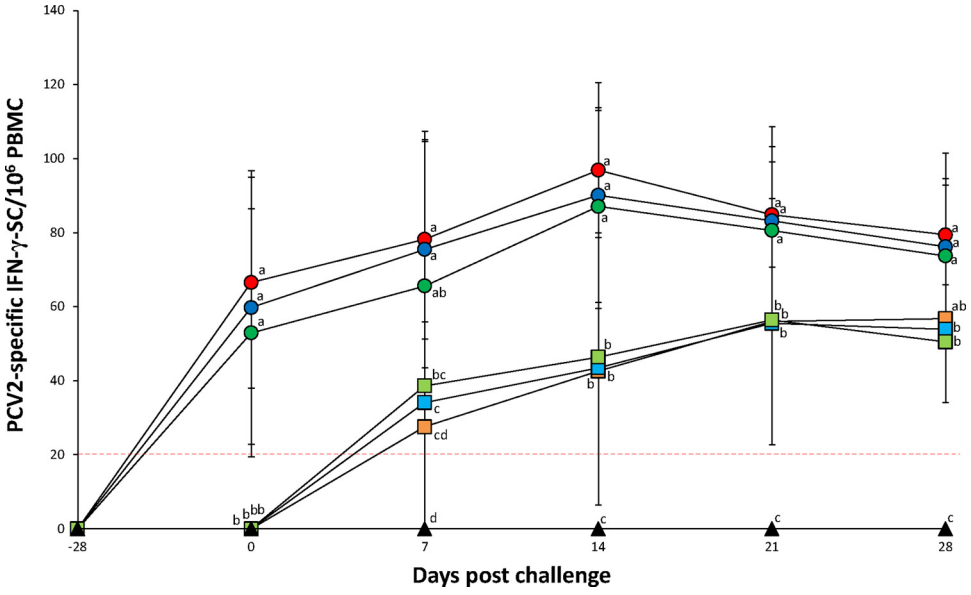

Fig. 4. Frequency of PCV2-specific interferon-γ secreting cells (IFN-γ-SC)/10^6 in peripheral blood mononuclear cells (PBMC) from Vac/Ch2a (●), Vac/Ch2b (●), Vac/Ch2d (●), UnVac/Ch2a (■), UnVac/Ch2b (■), UnVac/Ch2d (■), and UnVac/UnCh (▲) groups. The frequency of PCV2-specific IFN-γ-SC is considered to be positive if the number of PCV2-specific IFN-γ-SC is greater than 20 cells/10^6 PBMC (red dotted line). Variation is expressed as the standard deviation. Different superscripts (a, b, c, and d) indicate significant ($P < 0.05$) difference among 7 groups (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

neutralizing effect and may explain the difference in neutralizing antibody titers against PCV2b and PCV2d genotype in this study.

Despite the possible difference in neutralizing effect against PCV2a, PCV2b or PCV2d, there was no significant difference in reduction of viremia from either genotype. This suggests that perhaps cell-mediated immunity is another layer of immune response contributing to PCV2 clearance. Cell-mediated immunity against PCV2 was evaluated using IFN-γ ELISpot assay. Previous studies with other PCV2 vaccines have also reported induction of IFN-γ-SC, resulting in protection from PCVAD (Fort et al., 2008, 2009; Seo et al., 2014b). In our study, induction of IFN-γ-SC against PCV2a, PCV2b, and PCV2d by the commercial PCV2a vaccine also seems to play an important role in the reduction of PCV2a-, PCV2b-, and PCV2d-viremia. The T cells from PCV2a vaccinated pigs are able to recognize other PCV2 genotypes. In contrast to NA, the overall kinetics of the T cell responses against either of the 3 PCV2 genotypes are not significantly different. These broad cross-reactive T cells are shown to provide cross-protection.

Pathological observation is a very important tool for the evaluation of PCV2 vaccines because pigs with PCVAD have characteristic lymphoid lesions such as lymphoid depletion and histiocytic replacement of follicle with grape-like intracytoplasmic inclusion bodies (Chae, 2004, 2005). In general, PCV2 vaccines prevent or decrease the typical lymphoid lesions observed in PCVAD (Fort et al., 2008, 2009; Shen et al., 2010; Chae, 2012). In our study, since the unvaccinated pigs were only infected with PCV2, we were only able to observe moderate lymphoid depletion of follicle regardless of genotype. Nevertheless, vaccinated pigs had significantly reduced lymphoid lesions compared to unvaccinated pigs. These results suggest that this PCV2a vaccine is able to reduce and prevent lymphoid lesions in vaccinated pigs in regardless of PCV2 genotypes.

Since PCV2d emerged in China and the US, vaccine efficacy has been continuously put into question as cases of PCVAD continue to be reported. Therefore, cross-protection of PCV2a-based vaccines against PCV2d has major clinical implications. The results of this study regarding vaccine efficacy against PCV2b and PCV2d demonstrate that the PCV2a-based subunit vaccine confer cross-protection against PCV2b and PCV2d challenge. Therefore, the PCV2a-based subunit vaccine could help control PCVAD despite the prevalence of PCV2d.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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