A Dissertation for the Degree of Doctor of Philosophy

Interaction of Porcine Reproductive and Respiratory Syndrome virus and Porcine Circovirus type 2

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Abstract

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Porcine reproductive and respiratory syndrome (PRRS) has been recognized as one of the most economically important diseases in the swine industry, causing both reproductive failure in pregnant sows (late-term abortions and stillbirths) and respiratory disease (pneumonia) in nursing, growing, and finishing pigs. Because porcine reproductive and respiratory syndrome virus (PRRSV) has emerged from European countries, the virus is composed of 2 genotypes: Type 1 (European genotype)

and Type 2 (North American genotype). The two PRRSV genotypes produce the same disease symptoms, but the severity is very different and there is poor protection against cross-infection in pigs.

Otherwise, porcine circovirus associated disease (PCVAD) has been recognized as one of the most severe swine wasting diseases. From the first report to the current date, two types of porcine circovirus (PCV) have been recognized in the swine industry: the non-pathogenic PCV type 1 (PCV1) and the pathogenic PCV type 2 (PCV2). PCV2 is the causative agent of PCVAD. Phylogenetic analyses have shown that PCV2 isolates can be further divided into two main clusters, now commonly referred to as PCV2a and PCV2b. Actually, from the perspective of virulence, PCV2a and PCV2b are not different. But epidemiological studies indicate that PCV2b is currently the most prevalent in naturally occurring infections.

Many articles have reported a PCV2-specific character in co-infection with other pathogens. The viral load and replication in tissue of co-infected pig is much higher than in a single infection. There are the many types of co-infecting pathogens that enhance PCV2 replication, such as porcine parvovirus, swine influenza virus, *Mycoplasma hyopneumoniae*, and Torque teno sus virus (TTSuV). However, PRRSV is the most important co-infecting pathogen because of its clinical and pathological specificity, which can make the severity of clinical signs worse and directly affect PCV2 replication. The exact mechanisms of how PRRSV co-infection with PCV2 potentiates clinical signs are unknown. Moreover, vaccine efficacy against PRRSV or PCV2 during co-infections has not been evaluated. Because PRRSV and PCV2 are

prevalent in swine farms worldwide, these topics are very important. The goal of this thesis was to investigate how PRRSV or PCV2 genotypes influence each other regarding viral replication during co-infection and to determine which vaccine against PRRSV or PCV2 protects against clinical symptoms during co-infection.

An experimental challenge study with a combination of PCV2a (or 2b) and type 1 (or type 2) PRRSV was performed to compare the virulence of a combination of concurrent infections in terms of PCV2 viremia and PCV2-associated lesions and antigens in co-infected pigs. Pigs challenged with PCV2a (or 2b) and type 1 (or type 2) PRRSV had significantly ($P < 0.05$) higher mean clinical respiratory scores and lower average daily weight gain compared with pigs with PCV2a (or 2b). Co-infection induced significantly lower levels of anti-PCV2 and anti-PRRSV IgG antibodies than infection with one genotype alone, regardless of the genotype of the two viruses. Pigs challenged with PCV2a (or 2b) and type 2 PRRSV had significantly ($P < 0.05$) higher levels of PCV2 viremia, more severe PCV2-associated lesions, and more PCV2 DNA within the lesions compared to pigs with PCV2a (or 2b)/type 1 PRRSV. However, there was no significant difference in these parameters in pigs with PCV2a/type 2 PRRSV or PCV2b/type 2 PRRSV. The results of this study demonstrated that there was a significant difference in the severity of PCVAD, and the difference resulted not from PCV 2 genotypes but from PRRSV genotypes.

A study for the comparison of PCV2 vaccine and PRRSV vaccine efficacy was performed to determine the effects of PCV2 and PRRSV vaccinations on post-weaning multisystemic wasting syndrome (PMWS) by experimental PCV2-PRRSV challenge.

A total of 72 pigs were randomly divided into 9 groups (8 pigs per group): 5 vaccinated and challenged groups, 3 non-vaccinated and challenged groups, and a negative control group. Vaccination against PCV2 induced immunological responses (NAs and PCV2-specific IFN-γ-SCs) and reduced PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the co-infected pigs. However, vaccination against PCV2 did not affect the immunological responses (NAs and PRRSV-specific IFN-γ-SCs) or, PRRSV viremia, PRRSV-induced lesions, or PRRSV antigens in the co-infected pigs. Vaccination against PRRSV did not induce immunological responses (PRRSV-specific IFN-γ-SCs) or reduce PRRSV viremia, PRRSV-induced lesions, or PRRSV antigen in the co- infected pigs. In addition, vaccination against PRRSV increased PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the co-infected pigs. In summary, vaccination against PCV2 reduced PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the dual infected pigs. However, vaccination against PRRSV increased PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the co-infected pigs. Therefore, the PCV2 vaccine decreased the potentiation of PCV2-induced lesions by PRRSV in co-infected pigs. In contrast, the PRRSV vaccine alone did not decrease the potentiation of PCV2-induced lesions by PRRSV in co-infected pigs.

A study of commercial PCV2 vaccine efficacy on pigs experimentally challenged with PCV2 and PRRSV at 17 weeks post vaccination was performed to determine the effects of PCV2 vaccinations on porcine respiratory disease complex (PRDC) in Korean field conditions. A total of 60 pigs were randomly divided into 6 groups (10

pigs per group): 4 vaccinated and challenged groups, a non-vaccinated and challenged group, and a negative control group. Regardless of which commercial PCV2 vaccine was used, vaccination of piglets at 3 weeks of age was efficacious against co-challenge of PCV2 and PRRSV based on growth performance and PCV2-associated lesions. However, the inactivated chimeric PCV2 1-2 and PCV2 vaccine induced higher PCV2-specific neutralizing antibody titers and PCV2-specific interferon-γ-secreting cells and lower PCV2 viremia compared to the two PCV2 subunit vaccines. PCV2 vaccination of piglets at 3 weeks of age was effective in reducing PCV2 viremia and PCV2-associated lesions during the finishing period, which is an age frequently affected by PRDC caused by co-infection with PCV2 and PRRSV in Korean field conditions.

Keywords: Porcine reproductive and respiratory syndrome virus; Porcine circovirus type 2; Genotype; Porcine circovirus-associated disease; Postweaning multisystemic wasting syndrome; Porcine respiratory disease complex; Vaccine efficacy

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LIST OF ABBREVIATION

CDCD	Cesarean-derived, colostrum-deprived
CMI	Cell mediated immunity
DC	Dendritic cell
dpc	Days post-challenge
dpi	Days post-inoculation
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immune-sorbent assay
HE	Haematoxylin and eosin
IFN-γ-SCs	Interferon gamma secreting cells
IHC	Immunohistochemistry
IL	Interleukin
ISH	*in situ* hybridization
MHC	Major histocompatibility complex
NA	Neutralizing antibody
NK cell	Natural killer cell
ORFs	Open reading frames
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCV	Porcine circovirus
PCVAD	Porcine circovirus associated diseases
PDNS	Porcine dermatitis ans nephropathy syndrome
PMWS	Postweaning multisystemic wasting syndrome
PRDC	Porcine respiratory disease complex
PRRS	Porcine reproductive and respiratory syndrome
RCR	Rolling circle replication
Th	Helper T lymphocyte
$TCID_{50}$	Median tissue culture infective dose

GENERAL INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is currently considered the most significant and economically important infectious disease to threaten the swine industry. The virus causes economic losses of approximately US$ 560 million annually in the US and 88% of total economic loss is attributed to respiratory diseases in nursery and growing-finishing pigs (Neumann et al., 2005). A common biological property of this virus is its primary replication in host alveolar macrophages (Wensvoort et al., 1991) and some cells of the monocyte lineage (Voicu et al., 1994), so from lung, the virus may start to influence the immune response of piglets. When a piglet is exposed to PRRSV, as a first clinical sign, it shows respiratory disorder and fever. In the infected piglets, lung lesions develop by 3 days post infection, persist through day 21 and are characterized by alveolar septa thickened by macrophages, alveolar proteinaceous and karyorrhectic debris, alveolar syncytial cells, and multifocal type II pneumocyte hypertrophy.

Because PRRSV can induce damage to alveolar macrophage and change local immunity, it may be important in the pathogenesis of PRRSV-induced increased susceptibility to other pathogens such as PCV2 (Allen et al, 2000), swine influenza virus (SIV) (Pol et al., 1997), CSFV (Albina et al., 2000), *Streptococcus suis* (Galina et al., 1994), *Haemophilus parasuis* (Solano et al., 1997), *Mycoplasma hyopneumoniae* (Thacker et al., 1998), *Actinobacillus pleuropneumoniae*, and *Pasteurella multocida* (Carvalho et al., 1997; Pol et al., 1997). The clinical expression of combined infections

is more pronounced and complicated, which is not easy to diagnose. This complex disease syndrome exposes the swine herds to increased infection.

One of the remarkable characteristics of PRRSV infection is that the virus can enhance the replication of another virus that is also an important pathogen of swine industry, porcine circovirus type 2 (PCV2). Several studies have previously demonstrated the relationship between PCV2 and PRRSV. PRRSV increases the PCV2 DNA load in the sera of co-infected pigs (Rovira et al, 2002; Opriessnig et al, 2008) and increases the levels of PCV2 antigens in tissues (Allen et al, 2000), which results in more severe PCV2-associated lesions (Harms et al, 2001).

An increased PCV2 load causes severe swine wasting syndrome, porcine circovirus associated disease (PCVAD). Some experimental studies have shown that PCVAD has been reproduced in pigs by inoculation with PRRSV (Allan et al., 2000; Harms et al., 2001). The representative examples of PCVAD by PRRSV co-infection are post-weaning multisystemic wasting syndrome (PMWS), which is wasting with or without respiratory signs, diarrhea, paleness of the skin, or icterus (Clark, 1997; Harding and Clark 1997; Ellis et al., 1998; Allan et al., 1999; Choi et al., 2000) in 6-8-week old growing pigs and porcine respiratory disease complex (PRDC), which is characterized by slow growth, decreased feed efficiency, lethargy, anorexia, fever, cough, and dyspnea (Halbur, 1998; Thacker, 2001) in 16-22-week old finishing pigs.

The mechanism of PCV2-enhanced replication by PRRSV infection has three possibilities. First, because the mononuclear cell lineage is a common host cell type, PRRSV and PCV2 can simultaneously infect the same individual cell. The

nucleocapsid protein of PRRSV binds to viral PRA to form part of the virus core and could be transported to the nucleolus of an infected cell. The protein function is not well understood but might affect control of viral genomic replication or expression (Hiscox, J.A., 2003). Therefore, though a few individual cells were co-infected by two viruses, the nucleocapsid protein of PRRSV might stimulate the PCV2 genome to replicate in the cells. However, there is no evidence.

Second, when the pig is infected by PRRSV, the host cannot make proper anti-viral immune responses because of the immune modulation effect by PRRSV. The representative immune modulation effect by PRRSV is inducing IL-10, which is known to be a potent cytokine capable of inhibiting the production of several pro-inflammatory cytokines (Suradhat and Thanawongnuwech 2003). Because pigs co-infected with PCV2 and PRRSV have reduced IFN-γ and increased IL-10 expression in peripheral blood mononuclear cells (PBMC) (Shi et al., 2010), the decreased anti-viral immune activity by PRRSV might enhance PCV2 replication.

Finally, during the early PRRSV infection stage, the pig can induce an inflammatory reaction despite IL-10 expression. After 7 days of infection, the pig immune system can produce non-neutralizing antibodies and increase IL-2 expression. Such an immune reaction may lead to enhance PCV2 replication. Several experiments reported that when immune stimulants used in commercially available swine vaccines were injected with PCV2, they could trigger the progression of PCV2 infection to diseases and lesions characteristic of PMWS (Krakowka et al., 2001, Allan et al, 2007; Opriessnig et al., 2003). Likewise, immune stimulation by PRRSV infection can lead

to enhanced PCV2 replication. Because macrophages, the mononuclear cell line, and lymphocytes are major host cells for PCV2, when the host immune system is stimulated, increased numbers of these cell types means sufficient food for PCV2.

Based on these results, one possible way to minimize the effects of the PRRSV-associated enhancement of the replication of PCV2 and the induction of PMWS may be the use of a PRRSV-based vaccination in preweaned pigs. However, there are no reports in the literature describing the effects of PCV2 and PRRSV challenges on pigs that have been immunized with either the PCV2 or PRRSV vaccines. In the absence of such a study, the PCV2 vaccine-PCV2-PRRSV and PRRSV vaccine-PCV2-PRRSV interactions have not been completely elucidated.

LITERATURE REVIEW

1. Porcine reproductive and respiratory syndrome virus

1.1. Genotype

Porcine reproductive and respiratory syndrome virus (PRRSV) has been recognized as one of the most economically important diseases inducing pathogens in the swine industry, causing both reproductive failure in pregnant sows (late-term abortions and stillbirths) and respiratory disease (pneumonia) in nursery and grower/finishing pigs (Zimmerman et al., 2006). The virus genome is a single-stranded, positive-sense RNA, which is approximately 15.0 kb in length and contains nine open reading frames (ORFs). Its genome structure is very similar to that of other arteriviruses including equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase-elevating virus (LDV). The 5′ end of the genome is methyl-capped, and the 3′ end of the genome is polyadenylated. The arterivirus genome is polycistronic, containing two large open reading frames (ORF1a and b) and a set of six to nine ORFs downstream of the 1b gene. The genome is complex and extremely compact because most structural genes overlap one another at the ends, encoding proteins in two reading frames. Originally the virus was recognized as a clinical entity in North America and Europe by the end of the 1980s, but later studies have suggested the existence of the virus in the pig population since 1979 (Carman et al., 1995). The PRRSV that emerged in North America had limited genetic relationship to the PRRSV that emerged in Europe. Two emerged virus have only a 60-70% nucleotide homology and they are clearly genetically distinguished (Allende et al., 1999). Thus, PRRSV is recognized as

consisting of 2 genotypes: Type 1 (European genotype) and Type 2 (North American genotype) (Snijder et al., 2005). The two PRRSV genotypes produce the same disease symptoms but are antigenically very different, and there is poor protection against cross-infection in pigs (Woensel et al., 1998a,b).

1.2. Variations in virulence of genotypes

Generally in swine farm situation, type 2 PRRSV is more virulent than type 1 PRRSV in terms of its ability to cause respiratory diseases. Many articles reported that type 2 PRRSV-infected pigs exhibited significantly higher loads of type 2 PRRSV than type 1 PRRSV loads exhibited by type 1 PRRSV-infected pigs, in blood and lung tissues (Halbur et al., 1995; Martínez-Lobo et al., 2011; Han et al., 2012). Different amounts of PRRSV in blood and lung between two genotypes may be due to different affinity of the two genotypes to viral receptors on macrophages and monocytes. Different affinity between two genotypes to the scavenger receptor is found in several CD163 expressed recombinant cell lines (Calvert et al., 2007). As difference of affinity, Type 2 PRRSV may have higher tropism and replicates more efficiently in cells of the monocytic lineage when compared to type 1 PRRSV, leading to higher levels of type 2 PRRSV in blood and lung in infected pigs. And also, type 2 PRRSV is better adapted to the pulmonary target cells, such as interstitial and alveolar macrophages, and type II pneumocytes.

Even with difference between heterogeneous strains, type 2 PRRSV has almost a thousand times as much as type 1 PRRSV has in blood of piglet. In experimental study,

Lelystad virus, representative type 1 PRRSV, induced only mild transient pyrexia, dyspnea and tachypnea, but North American isolates included in the study induced laboured respiration, pyrexia, lethargy, anorexia and patchy dermal cyanosis (Halbur et al., 1995).

1.3. Pathogenesis in piglet

Porcine reproductive and respiratory syndrome (PRRS) is currently considered to be the most significant and economically important infectious disease to threaten swine industry. The virus causes economic losses of approximately US$ 560 million annually in the US and 88% of total economic loss is attributed to respiratory diseases in nursery and growing-finishing pigs (Neumann et al., 2005). A common biological property of this virus is their primary replication in host alveolar macrophages (Wensvoort et al., 1991) and some cells of the monocyte lineage (Voicu et al., 1994), so from lung, they may start to influence immune response of piglets. When piglet is exposed by PRRSV, as first clinical sign, it shows respiratory disorder and fever. In the infected piglets, lung lesions develop by 3 day post infection, persist through day 21 and are characterized by alveolar septa thickened by macrophages, alveolar proteinaceous and karyorrhectic debris, alveolar syncytial cells, and multifocal type II pneumocyte hypertrophy.

Additionally, PRRSV can localize in various organ systems (Mengeling et al., 1995,1996; Rossow et al., 1995; Shibata et al., 1997) and produce persistent infections in the absence of viremia (Allende et al., 2000; Van Reeth, 1997; Wills et al., 1997b).

One experiment using gnotobiotic pigs demonstrated that piglets experimentally challenged with PRRSV were viremic at 12 hours post infection and subsequently developed pneumonia, lymphadenopathy, vasculitis, myocarditis, and encephalitis (Halbur et al, 1995).

1.4. Interaction with other pathogens

PRRSV can induce damage to alveolar macrophage and change local immunity. It may be important in the pathogenesis of PRRSV-induced increased susceptibility to other pathogens such as PCV2 (Allen et al, 2000), swine influenza virus (SIV) (Pol et al., 1997), CSFV (Albina et al., 2000), *Streptococcus suis* (Galina et al., 1994), *Haemophilus parasuis* (Solano et al., 1997), *Mycoplasma hyopneumoniae* (Thacker et al., 1998), *Actinobacillus pleuropneumoniae*, and *Pasteurella multocida* (Carvalho et al., 1997; Pol et al., 1997). The clinical expression of combined infections is more pronounced and complicated, which is not easy to be diagnosed. This complex disease syndrome makes the swine herds expose to infection.

With infection of PCV2 in swine farm, change of PRRSV titer in serum is some controversial. One experimental study reported that co-infection with PCV2 could reinforce PRRSV replication in alveolar macrophage and induce more severe lesion by PRRSV (Harms et al, 2001). But other study being associated with same authors has shown there is no evidence of effect of co-infection with PCV2 (Allen et al, 2000). This means PRRSV infection and replication may be dependent on variable conditions of experiments.

1.5. Host Immunity

When PRRSV infect their host, piglet cannot produce proper anti viral immune response because of PRRSV special immunological modulations as viral replication. The unusual characteristics of the immune response to PRRSV suggest that the virus strongly modulates the immune response. First, Pigs infected by PRRSV seldom produce neutralizing antibodies (NAs). For humoral immunity, Circulating antibodies against PRRSV are detectable in pigs by 5 days post infection and all animals have sero-converted by 14 day (Yoon et al., 1992; Yoon et al., 1995). PRRSV-specific immunoglobulin M (IgM) reaches a peak at 14 day and then declines to undetectable levels by 42 days. Concentrations of IgG reach a maximum at 21–49 days (Vezina et al., 1996; Loemba et al., 1996). However, this rapid IgM and IgG response does not correspond to neutralizing antibodies (NAs) (Yoon et al., 1994). These early development of non-NAs may have rather a significant effect on the development of PRRS. It has been shown that non-NAs enhance viral replication in alveolar macrophages, a phenomenon known as antibody-dependent enhancement (ADE) (Yoon et al., 1996; Yoon et al., 1997). Targets for these antibodies are mainly GP5 and N proteins (Yoon et al., 1996). The non-neutralizing humoral response may act as a Trojan horse for PRRSV by coating the virus and enhancing the internalisation of viral particles into macrophages.

NAs may be detected by 28 days post infection or later for both type 1 or 2 strains of PRRSV (Yoon et al., 1994; Meier et al., 2003; Diaz et al., 2005). These NAs are

mainly directed against GP5, which contains the major neutralisation epitope (Nelson et al., 1994; Pirzadeh and Dea, 1997,1998; Gonin et al., 1999). It has been claimed that GP4, M, and N proteins also contain neutralising epitopes (Meulenberg et al., 1997; Gonin et al., 1999; Weiland et al., 1999) However, these proteins seem to be of minor biological significance compared to GP5. The main neutralization epitope of PRRSV is located in the N-terminal ectodomain of GP5 (amino acids 37–44) in both type 1 and 2 strains (Ostrowski et al., 2002; Plagemann et al., 2002; Plagemann, 2004). But this neutralisation epitope is flanked by glycosylation sites.

An additional immunodominant epitope is located in the N-terminal ectodomain of GP5 (amino acids 27 and 31) and has the characteristics of a decoy epitope, similar to that in human immunodeficiency virus type 1 (Ostrowski et al., 2002). The decoy epitope may interfere with the immune response to the main neutralisation epitope, resulting in a delay in the NA response. It suggests that the proximity of the decoy epitope is important in delaying the main NA response.

Second, Cell-mediated immunity (CMI) is also produced slowly and weekly. CMI is extremely important in protection of PRRS infection and viral clearance. However, the virus-specific IFN-γ secreting cells hardly appeared in the third week post infection, fluctuated erratically from 50–100 per million peripheral blood mononuclear cells (PBMCs) for the next ten weeks (Meier et al., 2003). IFN-γ secreting cells were mainly $CD4^+CD8^+$ cells, with a small proportion of $CD4^-/CD8\alpha\beta^+$ cytotoxic T cells.

Early studies showed that PRRSV is highly susceptible to the action of type I IFNs and suggested that the virus was able to inhibit IFN-α responses, since this cytokine

could not be detected in the lungs of pigs in which PRRSV was actively replicating (Albina et al., 1998; Buddaert et al., 1998). IFN-α levels in the lungs of PRRSV-infected pigs were much lower than in the lungs of pigs infected with porcine coronavirus or swine influenza virus (van Reeth et al., 1999).

IL-10 may have an important role in the regulation of the immune response to PRRSV. After infection with either type 1 or 2 strains of PRRSV, levels of IL-10 mRNA were increased in porcine PBMCs (Suradhat and Thanawongnuwech, 2003) and concentrations of IL-10 were increased in bronchoalveolar lavage (BAL) fluid (Thanawongnuwech et al., 2004). Some type 1 strains of PRRSV induce strong IL-10 responses in PBMCs from naïve pigs, suggesting that this is a not a memory feature (Diaz et al., 2006). Pigs vaccinated with IL-10-inducing strains had lower frequencies of PRRSV-specific IFN-γ secreting cells than animals vaccinated with a non-IL-10 inducing strain (Diaz et al., 2006). Monocytes and natural killer cell appear to be the major source of IL-10 in PRRSV infection (Charerntantanakul et al., 2006). This cytokine release seems to make the virus modulate host CMI and replicate enhanced for early infection.

1.6. Vaccine efficacy

Both modified live and inactivated PRRS vaccines have been developed and licensed for commercial use. It is generally considered that modified-live (MLV) PRRSV vaccines confer solid protection against clinical disease induced by homologous infection, but none of the current vaccines is able to completely prevent respiratory

infection, transplacental transmission, as well as pig-to-pig transmission of the virus. For example, one study compared three commercial vaccines in their ability to induce protection against PRRSV strains of high virulence, and found that these vaccines confer protection against clinical disease, but not against infection. On challenge, Pigs had still viremia and antigen in lung lobes (Osorio et al, 1998). It is further evident that the current vaccines, based on a single PRRSV strain, are not or only partially effective in protecting against infections with genetically diverse field strains of PRRSV (Meng, 2000; Murtaugh et al, 2002; Scortti et al, 2007; Okuda et al, 2008). Several experiments show the contribution of vaccination using a modified live vaccine on transmission and persistence of pigs previously infected, a so-called therapeutic vaccine intervention. This vaccination protocol did not eliminate wild-type PRRSV, but it significantly reduced the number of pigs persistently infected with a homologous strain, but not of pigs persistently infected with a heterologous strain (Cano et al, 2007).

Several inactivated PRRSV vaccines have also been developed to induce virus neutralizing antibodies but the vaccines offer only limited protection or even adverse effect upon challenge (Vanhee et al., 2009). The vaccines were considered ineffective or of limited efficacy at best, even against homologous challenge. While PRRSV-neutralizing antibodies are believed to prevent infection and transplacental infection of pregnant sows, a killed vaccine that induced neutralizing antibodies failed to protect pigs against an *in vivo* challenge (Zuckermann et al., 2007). Together, the study suggests the significance role of cell-mediated immunity in PRRSV protection.

Altogether correlates of protection following vaccination have not been clearly

defined. Both neutralizing antibodies and virus-specific IFN-γ producing cells have been examined in this respect Zuckermann et al., 2007; Osorio et al, 2002; Lopez et al, 2004). The levels of PRRSV-neutralizing antibodies and virus-specific IFN-γ producing cells have been reported to correlate with viremia level but many other authors report there is no definite correlation with virus titer and immunity level against it (Murtaugh et al, 2011). Until now, there has been no clear explanation about the vaccines against viral infection.

2. Porcine Circovirus type 2

2.1. Genotype

Porcine circovirus (PCV) is a single-stranded circular DNA virus (Tischer et al.,1982) that belongs to the genus Circovirus of the family Circoviridae (Todd et al., 2005). From first report to current date, two types of PCV have been recognized (Allan et al., 1998; Hamel et al., 1998; Morozov et al., 1998) in pigs: the non-pathogenic PCV type 1 (PCV1) and the pathogenic PCV type 2 (PCV2). PCV2 is the causative agent of porcine circovirus associated disease (PCVAD). Phylogenetic analyses have shown that PCV2 isolates can be further divided into two main clusters (Larochelle et al., 2002; Mankertz et al., 2000) now commonly referred to as PCV2a and PCV2b (Gagnon et al., 2007). Actually, irrespective of virulence, the phylogenetic points to genotypes of PCV2 have shown that PCV2 can be divided to various genotypes not only two types. Besides of ‘a’ and ‘b’, PCV2 cluster has another genotype, ‘c’. Unfortunately, the different nomenclature used by different authors induced confusion within the scientific community. To clarify matters, a PCV2 genotype definition and

nomenclature has been proposed (Grau-Roma et al., 2008; Segales et al., 2008), with three genotypes identified to date (Segales et al., 2008) including: 'a' (PCV2a) and 'b' (PCV2b), the two main phylogenetic groups and a third genotype 'c' (PCV2c) (Dupont et al., 2008). To date, PCV2c has only been found in Denmark.

2.2. Variations in virulence of genotypes

Although initial studies did not find any link between PCV2 genotypes and the occurrence of PCV2-associated disease (PCVAD) (Larochelle et al., 2002; Pogranichniy et al., 2002; Boisseson et al., 2004; Wen et al., 2005), later work suggested an association between occurrence of the diseases and a new variant of PCV2 (Carman et al., 2006; Timmusk et al., 2008).

epidemiological studies indicate that genotype PCV2b is currently most prevalent in naturally occurring infections (Chiarelli-Neto et al., 2009; Dupont et al., 2008; Takahagi et al., 2008; Timmusk et al., 2008; Cortey et al., 2011) and the PCV2b and 2a genotypes have been associated with PCVAD-affected and non-affected farms, respectively (Cheung et al., 2007; Carman et al., 2008; Grau-Roma et al., 2008; Horlen et al., 2008; Wiederkehr et al., 2009). This indicates that PCV2b may be more virulent than PCV2a. Moreover, phylogenetic assessment suggests that PCV2a is older in evolutionary terms, than PCV2b (Grau-Roma et al., 2008) and a shift from the PCV2a to 2b genotype has been implicated in outbreaks of PCVAD (Dupont et al., 2008; Timmusk et al., 2008; Wiederkehr et al., 2009). Interestingly only PCV2a has been identified in Australia, where Postweaning multisystemic wasting syndrome (PMWS)

has not been reported (Dupont et al., 2008). But several experimental challenging studies have shown that PCV2a can cause PCVAD in infected piglets and there are no significant differences on pathogenic lesion between PCV2b and PCV2a infected piglets (Sinha et al., 2011). Based on difference of epidemiological studies and experimental challenging studies, Occurrence of PCVAD may be affected by etiological environment where another agents can infect pigs with PCV2 in swine farm but not variance of PCV2 genotypes.

2.3. Pathogenesis of PCV2 in growing pig

Porcine circovirus 2 (PCV2) is considered to be most important pathogen associated with a number of different syndromes and diseases in pigs. Although PCV2 can infect all age of pigs, it is post-weaning period between 60 and 80 days of age when PCV2 can make most severe systemic disease in pigs (Kim et al., 2002). PMWS is a wasting disease associated with PCV2 and characterized by wasting with or without respiratory signs, diarrhea, paleness of the skin, or icterus (Clark, 1997; Harding, 1997; Ellis et al., 1998; Allan et al., 1999; Choi et al., 2000), and a marked increase in mortality from multiple concurrent bacterial infections (Madec et al., 2000; Kim et al., 2002) in post-weaning and early finishing pigs. Initial symptom can be usually ignored because it takes some time for the virus to infect immune cell and destroy immune system. After that, various diseases symptoms are followed.

Clinically PMWS is characterized as reduce of average daily weight gain of pig. Histopathologically, PMWS has a main characteristic lesion as granulomatous inflammation. This unique lesion is characterized by infiltrates of epithelioid cells and

multinucleated giant cells and is seen in the lymph nodes, liver, spleen, tonsil, thymus, and Peyer's patches but occurs consistently in superficial inguinal lymph nodes (Kim et al., 2002).

Some experimental studies show PMWS has been reproduced in piglets by either inoculation with PCV2 alone or in PCV2-infected swine co-infected with porcine parvovirus (PPV) or porcine reproductive and respiratory syndrome virus (PRRSV). It also occurs when PCV-2-infected piglets are immuno-stimulated by injections of an immunogen emulsified in an oil-based macrophage-targeted adjuvant (Allan et al., 2000; Choi and Chae, 2000; Ellis et al., 1999; Harms et al., 2001; Kennedy et al., 2000; Kim et al., 2003; Krakowka et al., 2001; Kyriakis et al., 2002).

Porcine respiratory disease complex (PRDC) is also a serious health problem in growing and finishing pigs aged around 16–22 weeks. It is characterized by slow growth, decreased feed efficiency, lethargy, anorexia, fever, cough, and dyspnoea (Halbur, 1998; Thacker, 2001). The role PCV2 plays in PRDC always involves interaction or synergism with other respiratory pathogens. Over 55% of cases diagnosed as PRDC had evidence of concurrent infections of both PCV2 and PRRSV. The clinical signs seen in field cases are non-specific and variable. Pathologically, a hallmark of the microscopic lesions of PRDC is bronchointerstitial pneumonia with peribronchial and peribronchiolar fibrosis. Because of considerable diagnostic overlap between PCV2-associated PRDC and PMWS, the diagnosis of PCV2-associated PRDC must meet four criteria: (i) the presence of respiratory signs such as prolonged dyspnoea that are refractory to antibiotic therapy, (ii) the presence of characteristic

pulmonary microscopic lesions, (iii) the presence of PCV2 within these lesions, and (iv) the absence of the characteristic microscopic lesions of PMWS in lymphoid tissues. PCV2-associated PRDC should be differentiated from PMWS clinically and histopathologically. To some up, PMWS is characterized by cachexia, dyspnoea, and occasionally jaundice or pallor in pigs of a younger age group, typically between 8 and 16 weeks (Allan and Ellis, 2000; Harding and Clark, 1997). Histopathologically, the most striking and consistent lesions of PCV2-associated PRDC are bronchointerstitial pneumonia with peribronchial and peribronchiolar fibrosis (Harms et al., 2002). In contrast, PMWS is characterized by widespread granulomatous inflammation, multinucleated giant cells, and variable numbers of intracytoplasmic basophilic viral inclusion bodies within infiltrating histiocytes and macrophages (Allan et al., 1999; Choi and Chae, 1999; Choi et al., 2000; Ellis et al., 1999; Kennedy et al., 2000; Kim et al., 2002; Krakowka et al., 2000).

Besides two representative diseases, there is a another clinically important disease associated PCV2 infection. Porcine dermatitis and nephropathy syndrome (PDNS) is a relatively new and often fatal disease that primarily affects recently weaned and feeder pigs from 1.5 to 4 months of age (Smith et al., 1993; Thibault et al., 1998). Pigs affected with PDNS are older pigs, particularly animals ranging from 12 to 14 weeks of age, but PDNS has also been described in finishing pigs. The first signs were skin lesions that were multifocal, well circumscribed, slight raised, dark red, circular to irregular and 1–20 mm in diameter. In fatal cases, cutaneous lesions consist of severe necrotizing vasculitis affecting the dermis and subcutis, characterized by

leukocytoclastic inflammation involving capillaries, small and medium-sized venules, and arterioles, accompanied by epidermal necrosis and ulceration and dermal haemorrhage (Choi and Chae, 2001; Duran et al., 1997; Smith et al., 1993; Thibault et al., 1998). Histopathologically, PDNS is generalized severe necrotizing vasculitis and fibrinonecrotic glomerulonephritis (Choi and Chae, 2001; Duran et al., 1997; Ramos-Vara et al., 1997).

2.4. Interaction with other pathogens

Many articles reported PCV2 specific character in co-infection with other pathogens. The viral load and replication in tissue of co-infected pig is much higher than in single infection. And severity of clinical sign of PCV2 infection seems worse when pig get infected with other pathogens. There are many kinds of co-infecting pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus, swine influenza virus, *Mycoplasma hyopneumoniae*, and Torque teno sus virus (TTSuV) can make the severity of clinical sign worse and result in increased PCV2 viral load in infected pigs (Opriessnig et al, 2012). The exact mechanisms by which viral or bacterial co-infection with PCV2 potentiates clinical sign are unknown, but some article have suggested that modulation of the host immune system is likely a key event in the pathogenesis of this disease (Gillespie et al., 2009 and Krakowka et al., 2001). PCV2 is the primary causative agent of PCVAD. However, under experimental conditions, PCV2 pathogenic lesions and clinical sign such as PMWS, PCVAD, and PDNS may occur in co-infection with other pathogens. PCV2 single infection often has

no clinical sign. Even though the viral antigens could be found in lymphoid follicles and blood, these amounts might be very small. It seems like truce period. But when a number of pathogens elsewhere in swine farm infect same individual pig with PCV2, the virus could come from lymphoid follicles and demolish the structures and whole immune system.

All these pathogens have something in common with that they can infect macrophage (monocytes, pulmonary macrophages, and monocyte-derived macrophages) like PCV2. It seems that the pathogens may interact with PCV2 in same host cell.

2.5. Host immunity in PCV2 infection

When exposed to PCV2 in vitro, cultured monocytes, pulmonary macrophages, and monocyte-derived macrophages can uptake the virus and PCV2-specific antigen. Evidence of PCV2 replication was detected also in T and B lymphocytes and monocytes from PBMC and in bronchial lymph nodes of infected pigs (Yu et al., 2007, 2009). Internalization of PCV2 has been demonstrated in both conventional blood DCs (Vincent et al., 2005), but the persistence of PCV2 in DCs did not cause loss of viral infectivity, cell death, or alteration of the immune function of DCs (Vincent et al., 2003). PCV2 may simply be internalized by DCs through their phagocytic or endocytic activities rather than through infection (Vincent et al., 2003). Nevertheless, a silent, nonproductive PCV2 infection in DCs may help the virus evade the host immune system and facilitate the transmission of PCV2 in pigs owing to the migratory ability of DCs (Vincent et al., 2003).

PCV2 impairs IFN-α and TNF-α production in natural interferon-producing cells and thus prevents the autocrine and paracrine maturation processes of myeloid DCs (Ramamoorthy et al, 2009; Vincent et al., 2005). A higher level of activation and earlier expression of MHC-II on T and B cells and a higher level of IL-2 receptor CD25 expression were found in pigs with PMWS (Grierson et al, 2007). Also, it appears that the presence of PCV2 in macrophages reduces the phagocytic and microbicidal ability of these cells, thus favoring the survival and spread of PCV2 (91, 92). The PCV2-mediated inhibition of natural IFN-producing cells appears to have a broad spectrum and affect responses induced by Toll-like receptors TLR7 and TLR9 agonists and by several other swine viral pathogens (Vincent et al., 2007), which suggests that PCV2 modulation of the innate immune response may render the pigs more susceptible to secondary or concurrent viral and bacterial infections (Vincent et al., 2007).

As a virus that primarily targets the lymphoid tissues and immune cells, PCV2 interaction with immune cells and with the lymphoid system appears to play important roles in the pathogenesis process of PCV2 (Ramamoorthy et al, 2009), which significantly alters the cytokine responses in infected pigs. The IL-10 and proinflammatory cytokines such as IL-1 and TNF-α are upregulated, and IL-2 and IL-4 expression are downregulated, in PCV2-infected pigs with PCVAD (Ramamoorthy et al, 2009; Darwich et al, 2012). Upregulation of IL-10 gene expression in thymus is associated with thymic depletion and atrophy in infected pigs (Darwich et al, 2003a). Pigs with PCVAD had an increased level of IL-10 gene expression in thymus and IFN-γ in tonsils as well as a decreased level of IL-2 and IL-12p40 gene expressions in the

spleen; IL-4 in tonsils; and IFN-γ, IL-10, IL-12p40, and IL-4 in inguinal lymph nodes (Darwich et al, 2003b). Pigs subclinically infected by PCV2 also developed a transient elevation of IL-10 response during the peak viremic stage (Darwich et al, 2008). PCV2-infected pigs had a significantly decreased number of $CD8^+$ and $CD4^+CD8^+$ double-positive cell subsets compared with PCV2-negative pigs, which further indicates that PCV2 infection impairs the host immune response (Darwich et al, 2002).

PCV2 infection induces the expression of MCP-1 and macrophage inflammatory protein-1 (MIP-1) in pigs (Kim et al, 2004), which suggests a potential role of MCP-1 and MIP-1 in granulomatous inflammation of lymph nodes. Also, in PCV2-infected pigs that developed severe PCVAD, elevated levels of serum C-reactive protein and IL-10 were detected (Stevenson et al, 2006), which further suggests a role of proinflammatory cytokines in PCV2 pathogenesis. In porcine PCV2-infected alveolar macrophages, significant increases in gene expressions for TNF-α and IL-8, alveolar macrophage-derived neutrophil chemotactic factors-II (AMCF-II), G-CSF, and MCP-1 were observed (Chang et al, 2006). PBMC from PCVAD-affected pigs were shown to respond well to recall PCV2 antigen by releasing IL-10 and IFN-γ, but were less responsive or nonresponsive to mitogen or superantigen in producing IL-4, IL-2, or IFN-γ (Darwich et al, 2003a). In addition, PCV2 has been shown to down regulate or suppress IL-4 and IL-2 release from PBMC and to stimulate the production of proinflammatory cytokines such as IL-1β and IL-8 (Darwich et al, 2003a), which suggests that pigs with PCVAD have a diminished ability to perform their immunological functions on viral infection. Overall, it appears that IL-10 and other

proinflammatory cytokines play important roles in PCV2 pathogenesis and PCVAD (Ramamoorthy et al, 2009, Darwich et al, 2012).

2.6. Vaccine efficacy against PCV2 infection

After postweaning multisystemic wasting syndrome (PMWS) was first identified and reported in western Canada in 1991, it took 13 years for the first commercial porcine circovirus type 2 (PCV2) vaccine to be used under special licence in France and Germany in 2004. Before the introduction of the vaccine, the control of PMWS and PCVAD was limited. But now, vaccination is a major and successful tool for the control of PCV2 infection. Commercial PCV2 vaccines were initially developed to control PMWS, but they are now also used against other PCVAD. Commercially available PCV2 vaccines differ in their antigen and adjuvant types, in the recommended animals for use (sow or piglet or both) and in the dosage (one or two doses). And their efficacy is different in some criteria

Regardless of the vaccine used, PCV2 vaccines reduce the proportion of viraemic pigs and the viral load in blood and also shorten the duration of viraemia under experimental and field conditions (Seo et al, 2012a,b). Single dose vaccination with different vaccines reduced viraemia by 42.0–86.1% in pigs experimentally infected with PCV2 alone (Fort et al., 2009b; Opriessnig et al., 2010). Analyzing all types of PCV2 vaccines and challenge models, none of the vaccinated pigs had a viral load $>10^7$ DNA copies/mL (Brunborg et al., 2004; Fort et al., 2009b; Opriessnig et al., 2009, 2010; Shen et al., 2010). Therefore, a well-documented feature of PCV2 vaccination

of piglets under field conditions is the ability to decrease both the proportion of viraemic pigs and the viral load in vaccinated animals compared to non-vaccinated animals (Cline et al., 2008; Fort et al., 2008; Horlen et al., 2008; Kixmoller et al., 2008; Opriessnig et al., 2010; Pejsak et al., 2010; Fraile et al., 2012). Also, PCV2 vaccination reduces nasal and fecal shedding of PCV2 (Fort et al., 2008) too. Single-dose vaccination was able to reduce the PCV2 viral load in nasal secretions under experimental conditions (Fort et al., 2009b), as well as to reduce the PCV2 viral load in fecal shedding under field conditions (Fraile et al., 2012). These studies indicate that the PCV2 vaccine is a useful tool for the control of PCV2 transmission on both individual animal and population scales.

Neutralizing antibodies (NAs) protecting PCV2 replication are induced by commercial PCV2 vaccines (Fort et al., 2009b; Opriessnig et al., 2009, 2010). Among these products, a chimaeric PCV1/2 vaccine induced significantly higher NA titres compared to subunit vaccines (Opriessnig et al., 2009), but there was no significant difference in the NA titres between subunit and inactivated vaccines (Opriessnig et al., 2010).

IFN-γ, which is produced by antigen-stimulated T cells, is a key immunoregulatory cytokine that controls the differentiation of naïve CD4 T cells into Th1 effectors and mediates cell-mediated immunity against viral infections. PCV2-specific Interferon-γ-secreting cell (IFN-γ-SC) was detected in pigs clinically infected with PCV2 between 14 and 21 days post-inoculation, coinciding with the decline in the blood viral load (Fort et al., 2009a). One-dose vaccination against PCV2 can induce PCV2-specific

IFN-γ-SCs 3 weeks after immunization, the same levels being maintained for 6 weeks after immunization (Martelli et al., 2011). Since the commercial PCV2 vaccine induces NAs and IFN-γ-SCs specific for PCV2 (Fort et al., 2009b; Opriessnig et al., 2009,2010; Martelli et al., 2011); cell-mediated immunity, together with NAs, is likely to contribute to PCV2 clearance.

At result of efficient vaccine, field studies show increased productivity such as improvements in ADWGs or reductions in mortality in response to PCV2 vaccination. Regardless of the PCV2 vaccine used, one-dose vaccination of piglets improves ADWGs by 16–69 g/day from 3 to 19–26 weeks of age under field conditions. In addition, one-dose vaccination of piglets decreases mortality by 1.9–9.3% (Fachinger et al., 2008; Kixmoller et al., 2008; Martelli et al., 2011). The different growth performance in several field trials by different PCV2 vaccines may not be due to the different efficacies of vaccines, but to different co-infections, feeding systems and quality, production systems and housing and environmental facilities.

3. Interaction of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2

3.1. Farm situation.

There are many microorganisms on swine farms. The ecosystem on the farms consists of variable bacteria, viruses, fungi, and parasites that live in their host pig. One clinical symptom of an individual pig may be associated with a balance of a number of microorganisms. As previously mentioned, the severity of clinical signs of PCV2 infection appears worse when pigs are infected with other pathogens. Therefore, it is important to understand the farm'sentire microorganism constitution to analyze PCV2 pathologic conditions. In Korea, representative pathogens on swine farms are PCV2, PRRSV, classical swine fever virus, *Actinobacillus pleuropneumoniae* type 2 and 5, *Haemophilus parasuis*, *Pasteurella multocida*, and *Bordetella bronchiseptica*. According to one report performed in 2013, the PCV2 antibody positive ratio is 82.2% of whole Korean swine farms, and the antigen positive ratio is 1.2%. The PRRSV antibody positive ratio is 64.5% and increases from 66.7% to 83% as pigs are growing. The classical swine fever virus antibody positive ratio is 84.6%, and the antigen positive ratio is 0%. The *Actinobacillus pleuropneumoniae* type 2 antibody positive ratio is 63.3%, and the infection identified ratio from these farms is 30.5%, while the type 5 antibodies positive ratio is 72.0%. The *Haemophilus parasuis* antibody positive ratio is 67.5%. The *Pasteurella multocida* antibody positive ratio is 86.5%. The *Bordetella bronchiseptica* antibody positive ratio is 86.5% (Korea pork producer

association, 2013 research on Korean swine farm diseases).

3.2. PCV2 viral replication changes in individual piglets.

A controversial question about PCV2-enhanced replication during co-infection with other pathogens is whether the immunological modulation effect of co-infected pathogens can induce replication changes in PCV2. One experiment suggested that several cytokines may be suspected of PCV2 enhancement. Whether various cytokines (interleukin-1 [IL-1], IL-6, IL-10, tumor necrosis factor-α [TNF- α], interferon- α [IFN- α], and IFN-γ) can influence PCV2 infection *in vitro* was investigated. No changes were observed in IL-1, IL-6, TNF- α, or IL-10-treated cells. However, it was demonstrated that IFN- α and IFN-γ influenced PCV2 infection in porcine kidney cells (PK-15) and porcine monocytic cells (3D4/31). IFN- α added to the culture medium increased the number of PCV2 antigen- positive cells by a maximum of 691% in PK-15 cells and by 706% in 3D4/31 cells. The effect of both IFNs on PCV2 infection was dose dependent. This study suggests that IFN- α and IFN-γ influence access of PCV2 to the host cell (Meerts et al, 2005a). However, the cytokine effect on PCV2 replication *in vivo* is completely different. An *in vivo* experiment by the same author demonstrated that pigs with high IFN-γ mRNA expression levels in PBMC are less susceptible to PCV2 replication (Meerts et al, 2005a,b**).** As a result, these experiments demonstrated that the cytokines could enhance PCV2 replication *in vitro* but the enhancement of PCV2 replication by the cytokines was more complex *in vivo*. (Lefebvre et al, 2008). Other experiments suggested that PCV2 enhancement may be affected by host immune

stimulation. When immune stimulants used in some commercially available swine vaccines were injected with PCV2, they triggered the progression of PCV2 infection to diseases and lesions characteristic of PMWS (Allan et al., 2000,2007; Opriessnig et al., 2003). In contrast, other studies using conventional pigs failed to demonstrate the progression of the disease by immune stimulants (Resendes et al., 2004; Ostanello et al., 2005). Although the mechanism of PCV2 enhancement is not clearly explained, according to these results, host immune stimulation may be involved.

3.3 Interaction of PRRSV and PCV2 in farm conditions

Interestingly, antibodies against PRRSV in serum samples could be detected when animals were approximately 10–15 weeks old, indicating an onset of infection with this virus slightly before the time of onset of respiratory signs and PCV2 viremia (Fachinger et al., 2008). The predisposition of PRRSV-infected animals to PCV2 infections has also been reported by others. In Canadian field observations, five out of seven analyzed farms were co-infected with PRRSV and PCV2, with PRRSV viremia always occurring before PCV2 infection (Larochelle et al., 2003). Typically, the experimental PMWS-reproducing design is to infect pigs with PCV2 and PRRSV simultaneously. However, the time interval between PRRSV and PCV2 infection may not induce PMWS in growing pigs.

When the pig is infected by PRRSV, the host cannot make proper anti-viral immune responses because of the immune modulation effect by PRRSV. The representative immune modulation effect by PRRSV is to induce IL-10, which is known to be a

potent cytokine capable of inhibiting the production of several pro-inflammatory cytokines (Suradhat and Thanawongnuwech 2003). Because pigs co-infected with PCV2 and PRRSV have reduced IFN-γ and increased IL-10 expression in peripheral blood mononuclear cells (PBMC) (Shi et al., 2010), the decreased anti-viral immune activity by PRRSV might enhance PCV2 replication. However, IL-10 expression increased until 7 days during early PRRSV infection (Diaz et al., 2005). Therefore as previously mentioned, the infection time interval between PRRSV and PCV2 may interfere with PCV2 replication enhancement by PRRSV immune modulation. Several studies reported that highly virulent PRRSV strains did not induce the cytokine expression in vitro or in vivo (Darwich et al., 2011; Gimeno et al., 2011; Subramaniam et al., 2011). Therefore, immune modulation by PRRSV may not be the only mechanism for PCV2-enhanced replication.

This modulation was not a one-sided. PBMC from PRRSV and PCV2 co-infected piglets had significantly reduced IL-2, IL-4, IL-6, IL-12p40 and IFN-γ compared to piglets infected with either PRRSV or PCV2 alone. The IL-10 mRNA levels in all virus-infected groups were significantly up-regulated early during infection (Shi et al., 2010).

When PRRSV infects the host, it can stimulate the host immune system. During the early infection stage, PRRSV-infected pigs could induce an inflammatory reaction despite IL-10 expression. However, for this period, the pig immune system could also produce non-neutralizing antibodies and increase IL-2 expression. This immune reaction might enhance PCV2 replication. As previously mentioned, several

experiments reported that when immune stimulants used from some commercially available swine vaccines were injected with PCV2, they triggered the progression of PCV2 infection to diseases and lesions characteristic of PMWS (Krakowka et al., 2001, Allan et al, 2007; Opriessnig et al., 2003). Macrophages, mononuclear cell line, and B lymphocytes are the major host cells of PCV2. When the host immune system was stimulated, these cells could be increased. PCV2 may infect increased numbers of host cells under such conditions, and the PCV2 titer in host tissue could be increased.

Another possibility is that cytokines such as IFNγ, which could allow PCV2 to enter host cells more easily, could be secreted by PRRSV infected macrophages (Meerts et al, 2005a). The macrophage is a common host cell of PCV2 and PRRSV. Local immunity could be one of the factors of PCV2 replication enhancement in co-infection. PRRSV must be persistent in the lymph node of the host. This virus can re-emerge from the lymph node according to the host's immune condition. Although PRRSV might infect a host before PCV2 in swine farms, the persistent infection of the virus can affect host local immunity and PCV2 replication.

Because the mononuclear cell lineage is a common host cell type, PRRSV and PCV2 can simultaneously infect the same individual cell. One experiment found a few alveolar macrophages that were positive for both PCV2 and PRRSV genome. Although a small number (2~5%) of labeled mononuclear cells contained both viral products, these cells had double positive signals (Sirinarumitr et al, 2001). Another study reported that PCV2-specific cytokine expression changes were dependent on PCV2 ORF 1, but not ORF 2, in PRRSV co-infected pulmonary alveolar macrophages (Sinha

et al., 2012). A PCV2 replication enzyme is coded in ORF1 and could be expressed in the nucleus of infected host cells. The nucleocapsid protein of PRRSV binds to viral PRA to form part of the virus core and could be transported to the nucleolus of infected cells. The protein function is not well understood but might affect viral genomic replication or expression (Hiscox, J.A., 2003). Therefore, though a few individual cells were co-infected by the two viruses, the nucleocapsid protein of PRRSV might stimulate the PCV2 genome to replicate in the cells. However, there is no evidence.

3.4. Comparison of interaction by viral genotype.

PRRSV infection influences titer, prolonging, and shedding of PCV2. Although A global shift in the prevalence of the main PCV2 genotype from the previously dominant PCV2 genotype PCV2a to PCV2b, a experimental challenge study reported that pathological and clinical severity of PCVAD induced by PRRSV is not different between PCV2 subtype a and b (Sinha et al., 2011). However, the severity of PCVAD could be different between co-infected PRRSV strains. Unlike PCV2, PRRSV genotypes have each specific character of replication, pathogenesis, and host immune response. Significant differences have been described between type 1 and type 2 PRRSV isolates in several characteristics, including their ability to induce disease at the herd level. Early outbreaks of PRRS in Europe where type 1 PRRSV was predominant have been apparently restricted to sow herds, with few reports of significant respiratory disease due to type 1 virus infections in young and growing pigs. On the contrary obvious clinical signs, and specifically marked respiratory disease, are a common outcome of type 2 PRRSV infection in growing pigs (van der Linden et al.,

2003). Thus the most remarkable difference between type 1 and type 2 isolates is related to their ability to induce respiratory signs in growing pigs. This absence of respiratory signs has been previously described in growing pigs exposed to type 1 isolates (Halbur et al., 1995; van der Linden et al., 2003). On the contrary, infections with type 2 isolates frequently result in evident respiratory disease, which is considered as the hallmark of type 2 PRRSV infection in growing pigs (Halbur et al., 1995,1996; Johnson et al., 2004).

ELISA results also show that type 1 isolates induce later seroconversion and lower S/P values than type 2 viruses. Despite similar viral load among experimental challenged type 1 or 2 pigs, seroconversion timing and antibody levels are various (Martínez-Lobo et al, 2011).

Therefore the key point of pathological and clinical severity of PCVAD might be PRRSV strain co-infected with PCV2. PRRSV which has more pathogenic and faster replication character can induce stronger host immune stimulations or modulations. But how many copies is needed for emerge PMWS is questionable.

3.5. Vaccine efficacy

Vaccine efficacy is important for understanding viral progress. PCV2 vaccine has already proven to be efficient in several experimental and farm studies (Opriessnig et al., 2008, 2009; Seo et al., 2012a,b). PCV2 vaccination was effective at inducing a neutralizing antibody response and significantly reducing PCV2-associated lesions and PCV2 viremia in pigs experimentally co-infected with PCV2 and PRRSV (Opriessnig

et al., 2008). However the vaccine did not influence PRRSV replication, viral titer, and antibodies. PCV2 vaccine and PRRSV vaccine could be affected by individual pig infection status. Because pigs of each farm are easily infected by various pathogens, vaccine efficacy could be changed by modulated host immune system. One experimental challenge study determined that whether the presence of PRRSV viremia has an effect on the efficacy of commercial PCV2 vaccine. As results, PCV2 vaccination, regardless of PRRSV infection status at the time of vaccination, was similarly effective in inducing an anti-PCV2 IgG response in the presence of maternally derived immunity and in protecting the pigs from PCV2 challenge, by reducing in the prevalence and amount of PCV2 antigen in lymphoid tissues (Sinha et al., 2010). On the contrary, another experimental study show that PCV 2 infection decreased the efficacy of a modified live PRRSV Vaccine. After PCV2 infection, PRRSV-vaccinated and PRRSV challenged pigs had significantly more-severe macroscopic lung lesions than did the PRRSV vaccinated and PRRSV challenged pigs that were not exposed to PCV2 prior to PRRSV vaccination (Opriessnig et al., 2006).

As a modified live PRRSV Vaccine is a form of most powerful solutions to control PRRSV infecton, PCV2 infection can interrupt to protect the viral infection and transmission seriously. Meanwhile, in swine farm, two vaccines also can be used to control the viruses simultaneously. Such concurrent vaccination against PCV2 and PRRSV has no interference with the development of the specific humoral and cell-mediated immunity (Martelli et al., 2013).

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PART I. Comparison of porcine circovirus type 2 (PCV2)-associated lesions produced by co-infection between two genotypes of PCV2 and two genotypes of porcine reproductive and respiratory syndrome virus

ABSTRACT

The objective of this study was to compare the virulence and pathogenicity of a combination of concurrent infections of two genotypes of porcine circovirus type 2 (PCV2) and two genotypes of porcine reproductive and respiratory syndrome virus (PRRSV) in terms of PCV2 viremia, and PCV2-associated lesions and antigens in co-infected pigs. Pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV had significantly ($P < 0.05$) higher mean clinical respiratory scores and lower average daily weight gain compared with pigs with PCV2a (or 2b). Co-infection induced significantly lower levels of anti-PCV2 and anti-PRRSV IgG antibodies than infection with one genotype alone, regardless of the genotype of the two viruses. Pigs with PCV2a (or 2b)/type 2 PRRSV had significantly ($P < 0.05$) higher levels of PCV2 viremia, more severe PCV2-associated lesions, and more PCV2 DNA within the lesions compared to pigs with PCV2a (or 2b)/type 1 PRRSV. However, there was no significant difference in these parameters in pigs with PCV2a/type 2 PRRSV or PCV2b/type 2 PRRSV. The results of this study demonstrate significant differences in the virulence and pathogenicity of type 1 and type 2 PRRSV but no significant differences in the virulence and pathogenicity of PCV2a and PCV2b with respect to the production of PCV2-associated lesions.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a non-enveloped, single-stranded, circular DNA virus belonging to the family *Circoviridae* (Mankertz *et al.*, 1997) that can be divided into at least two major genotypes, PCV2a and PCV2b, which are present worldwide (Grau-Roma *et al.*, 2008; Segales *et al.*, 2008). PCV2 has been implicated as the etiological agent for postweaning multisystemic wasting syndrome (PMWS) and other associated diseases, which are collectively called porcine circovirus-associated disease (PCVAD) (Chae, 2005). Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded RNA virus belonging to the family *Arteriviridae* (Cavanagh, 1997) that causes reproductive failure in sows and respiratory disease in grower-finisher pigs (Zimmerman *et al.*, 2012). The type 1 (European) and type 2 (North American) genotypes of PRRSV are two distinct genotypes (Allende *et al.*, 1999; Murtaugh *et al.*, 2010).

A global shift in the prevalence of the main PCV2 genotype from the previously dominant PCV2 genotype PCV2a to PCV2b suggests a link between PCV2b and the occurrence of PMWS (Cheung *et al.*, 2007; Gagnon *et al.*, 2007; Dupont *et al.*, 2008; Wiederkehr *et al.*, 2009). These data suggest that PCV2b may potentially be more pathogenic than PCV2a; however, neither genotype was found to be directly correlated with a more severe disease in controlled

experimental infections (Fort *et al.*, 2008; Opriessnig *et al.*, 2008a).

PCV2 and PRRSV are considered to be important viral pathogens and cause tremendous economic losses. Mixed infection of PRRSV and PCV2 is one of the most common co-infections associated with swine disease under current field conditions. Currently, two genotypes of PCV2 and PRRSV are found in most countries, including Asian countries (Chen *et al.*, 2011; Thanawongnuwech *et al.*, 2004). In a co-infection study, the virulence and pathogenicity of PCV2a/type 2 PRRSV and PCV2b/type 2 PRRSV were not different in conventional specific-pathogen-free pigs (Opriessnig *et al.*, 2012). However, an in-depth comparison of the virulence and pathogenicity of concurrent infections with the two genotypes of the two viruses has yet to be undertaken. Hence, the objective of this study was to evaluate the virulence and pathogenicity of concurrent infection with the two genotypes of the two viruses (PCV2a/type 1 PRRSV, PCV2a/type 2 PRRSV, PCV2b/type 1 PRRSV, and PCV2b/type 2 PRRSV) in terms of PCV2 viremia, and PCV2-associated lesions and antigens in co-infected pigs.

METHODS

Animals and housing.

Ninety conventional crossbred pigs derived from 10 sows known to be free of PCV2, PRRSV, and *Mycoplasma hyopneumoniae* were purchased from commercial PRRSV free farm. On the day of arrival, all pigs were tested and found negative for PCV2, PRRSV, and *M. hyopneumoniae* according to routine serological testing. All piglets were also negative for the presence of PCV2 and PRRSV in the blood by real-time polymerase chain reaction (PCR), respectively (Gagnon *et al.*, 2008; Wasilk *et al.*, 2004). All pigs were housed in an environmentally controlled building as previously described (Kim *et al.*, 2011).

Virus inoculation.

Two different genotypes of PCV2 were used for the inoculation of pigs. PCV2a (SNUVR100032 strain) and PCV2b (SNUVR000463 strain) were isolated in superficial inguinal lymph node from different postweaned pigs with severe PMWS in different herds (Kim *et al.*, 2010; Seo *et al.*, 2012). Inoculum of PCV2a and PCV2b was prepared from tissue culture fluid containing 10^4 tissue culture infective doses 50% ($TCID_{50}$)/ml of each genotype (third passage in PCV-free PK-15 cells).

Two different genotypes of PRRSV were used for the inoculation of pigs. Type 2 PRRSV (SNUVR090851 strain) and type 1 PRRSV (SNUVR090485 strain) were isolated in lung samples from newly weaned pigs and neonatal piglets with severe

dyspnea, respectively, from different herds (Han *et al.*, 2012, 2013a). Inoculum of type 2 PRRSV (third passage in MARC-145 cells) and type 1 PRRSV (third passage in alveolar macrophages) was prepared from tissue culture fluid containing 10^5 $TCID_{50}$/ml of each genotype.

Experimental design.

Ninety pigs were placed into one of the nine following treatment groups (10 pigs in each group) at 2 weeks of age: PCV2a/type 1 PRRSV, PCV2a/type 2 PRRSV, PCV2b/type 1 PRRSV, PCV2b/type 2 PRRSV, PCV2a, PCV2b, type 1 PRRSV, type 2 PRRSV, or negative control. At 6 weeks of age, the pigs were inoculated intranasally as follows: PCV2a and 2b, 3 ml of each PCV2 genotype inoculum; type 1 and type 2 PRRSV, 3 ml of each PRRSV genotype inoculum; PCV2/PRRSV, 3 ml of each PCV2 inoculum and PRRSV inoculums. Blood samples were collected from each pig by jugular venipuncture at 0, 5, 7, 10, 14, 21, and 28 dpi. Five pigs from each group were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 14 and 28 dpi, as previously described (Beaver *et al.*, 2001). Tissues were collected from each pig at necropsy. All of the methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

Clinical observation.

The pigs were scored daily for clinical respiratory disease severity using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur *et al*.,

1995). Observers were blinded to vaccination status. Rectal temperatures were recorded daily at the same time by same personnel.

Growth performance.

The live weight of each pig was measured at 0 dpi (6 weeks of age), 14 dpi (8 weeks of age), and 28 dpi (10 weeks of age). The ADWG (grams/pig/day) was analyzed over two time periods: (i) between 0 and 14 dpi and (ii) between 14 and 28 dpi. The ADWG during the different production stages was calculated as the difference between the starting and final weights divided by the duration of the stage.

Serology.

The serum samples were tested using the commercial PCV2 ELISA (Synbiotics, Lyon, France) and PRRSV ELISA (HerdCheck PRRS 3XR, IDEXX Laboratories Inc., Westbrook, ME, USA).

Quantification of PCV DNA in blood.

DNA extraction from serum samples was performed using the QIAamp DNA mini kit. DNA extracts were used to quantify PCV2a and PCV2b genomic DNA copy numbers by real-time PCR as previously described (Gagnon *et al.*, 2008).

Quantification of PRRSV RNA in blood.

RNA extraction from serum samples was performed as previously described (Wasilk *et al.*, 2004). Real-time PCR for the type 1 and type 2 PRRSVs was used to quantify PRRSV genomic cDNA copy numbers using RNA extractions from serum samples as previously described (Wasilk *et al.*, 2004).

In situ hybridization.

Genotype-specific in situ hybridization (ISH) was used to detect and differentiate PCV2a and PCV2b, respectively, in formalin-fixed, paraffin-embedded tissues (Kim *et al.*, 2010). Genotype-specific ISH was used to detect and differentiate type 1 and type 2 PRRSV, respectively, in formalin-fixed, paraffin-embedded tissues (Han *et al.*, 2013).

Morphometric analysis.

For morphometric analysis of the macroscopic pulmonary lesion score, lung lesions were scored to indicate the percentage of total lung that was consolidated (Halbur *et al.*, 1995). For morphometric analysis of the microscopic pulmonary and lymphoid lesion score, tissue sections were blindly examined. Lung sections were given an estimated score of the severity of the interstitial pneumonia with the scores ranging from 0 (normal) to 4 (severe diffuse) based on 5 areas of lung: one from the dorsomedial part of right anterior lobe, one from the dorsomedial part of right middle lobe, one from the dorsomedial part of right caudal lobe, one from the ventromedial part of right caudal lobe, and one from the dorsomedial part of the accessory lobe (Halbur *et al.*, 1995). Three superficial inguinal lymph node sections were given an estimated scores ranged

from 0 (normal, i.e., no lymphoid depletion or granulomatous replacement) to 5 (severe lymphoid depletion and granulomatous replacement) (Kim & Chae, 2004).

For morphometric analysis of in situ hybridization, three sections were cut from each of three blocks of tissue from one entire right pulmonary lobe and superficial inguinal lymph node of each pig. To obtain quantitative data, slides were analyzed with the NIH Image J 1.43m Program (http://rsb.info.nih.gov/ij). In each slide, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm^2 for lung and 0.25 mm^2 for lymph node) was determined as previously described (Kim *et al.*, 2011; Halbur *et al.*, 1996a). The mean values were also calculated.

Statistical analyses.

The summarized statistics for all of the groups were calculated to assess the overall quality of the data, including the normality. The values for the genomic copies of PCV2 and the PRRSV viremia were transformed to $\log_{10}$ values prior to analysis. Continuous data (genomic copies of PCV2 DNA and of PRRSV RNA) were evaluated using a repeated measures analysis of variance (ANOVA). If the repeated measures ANOVA revealed a significant effect, a one-way ANOVA with pairwise testing using Tukey's adjustment was performed for each time point. If the distribution of the variables was not normal, non-parametric Kruskal-Wallis and Mann–Whitney tests were used to analyze the data. Discrete data (pulmonary and lymphoid lesion score) were analyzed using the chi-squared and/or Fisher's exact test. $P < 0.05$ was considered significant.

RESUTLTS

Clinical observation

Pigs with PCV2a (or 2b)/type 2 PRRSV had significantly ($P < 0.05$) higher mean clinical respiratory scores than pigs with PCV2a (or 2b) from 4 to 23 days post-inoculation (dpi). Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly ($P < 0.05$) higher mean clinical respiratory scores than pigs with PCV2a (or 2b) from 7 to 9 dpi. Pigs with type 2 PRRSV had significantly ($P < 0.05$) higher mean clinical respiratory scores than pigs with PCV2a (or 2b) from 4 to 16 dpi. No respiratory distress was observed in negative control pigs throughout the experiment.

Pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV has significantly ($P < 0.05$) higher mean rectal temperature than pigs on other 8 groups from 2 to 7 dpi. Pigs with PCV2a (or 2b)/type 1 PRRSV and pigs with type 1 PRRSV has significantly ($P < 0.05$) higher mean rectal temperature than pigs with PCV2a (or 2b) and negative control pigs at 3 and 4 dpi (Fig. 1).

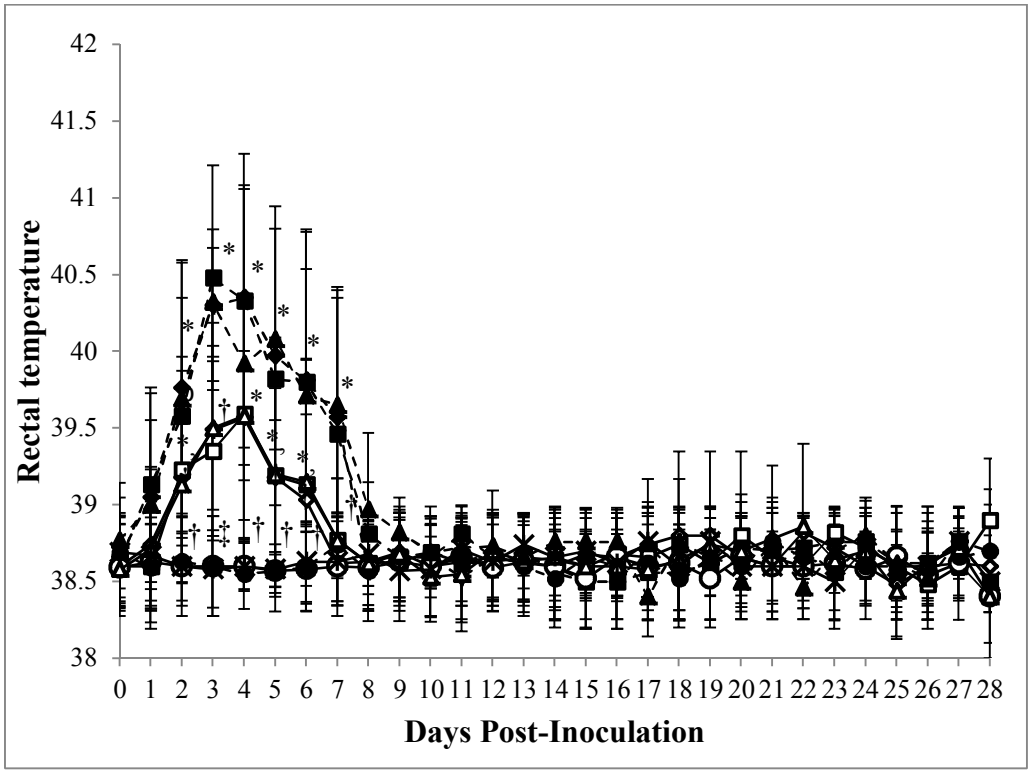


Figure 1. Mean rectal temperature in pigs from 9 groups (PCV2a/type 1 PRRSV, ◇; PCV2a/type 2 PRRSV, ◆; PCV2b/type 1 PRRSV, □; PCV2b/type 2 PRRSV, ■; PCV2a, ○; PCV2b, ●; type 1 PRRSV, △; and type 2 PRRSV, ▲; Negative Control, X). Different letters (*, †, and ‡) indicate statistically significant differences ($P < 0.05$) among groups.

Growth performance

During 0 (6 weeks of age) -14 dpi (8 weeks of age) period, pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV had significantly ($P < 0.05$) lower average daily weight gain (ADWG) than pigs with PCV2a (or 2b), pigs with type 1 PRRSV, and negative control pigs. Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly ($P < 0.05$) lower ADWG than pigs with PCV2a (or 2b) and negative control pigs. During 14 (8 weeks of age) -28 dpi (10 weeks of age) period, pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV had significantly ($P < 0.05$) lower ADWG than pigs with PCV2a (or 2b), pigs with type 1 (or type 2) PRRSV, and negative control pigs (Fig. 2A).

Macroscopic lung lesions

Regardless of the genotype of the viruses, lung lesions was characterized by diffusely tan- to purple, noncollapsed, and rubbery with irregular and indistinct borders in pigs with PCV2/PRRSV. At 14 dpi, pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV had significantly ($P < 0.05$) higher lung lesion scores than pigs with PCV2a/type 1 PRRSV, pigs with PCV2a (or 2b), and pigs with type 1 PRRSV. Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly ($P < 0.05$) higher lung lesion scores than pigs with PCV2a (or 2b), and pigs with type 1 PRRSV (Fig. 2B). No macroscopic lung lesions were noted in negative control pigs throughout the experiment.

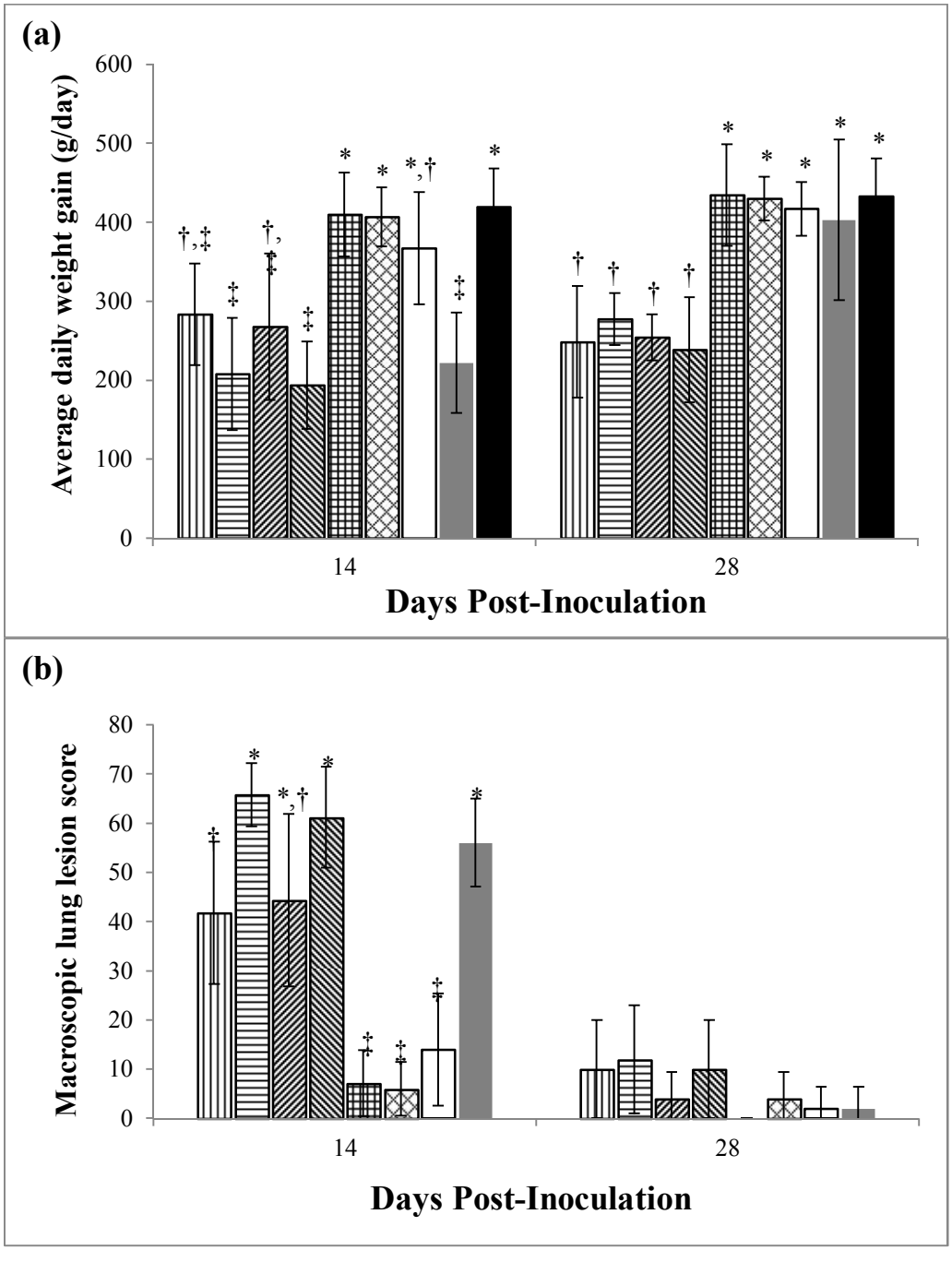

(a)
Average daily weight gain (g/day)
600
500
400
300
200
100
0
14
28
Days Post-Inoculation
(b)
Macroscopic lung lesion score
80
70
60
50
40
30
20
10
0
14
28
Days Post-Inoculation

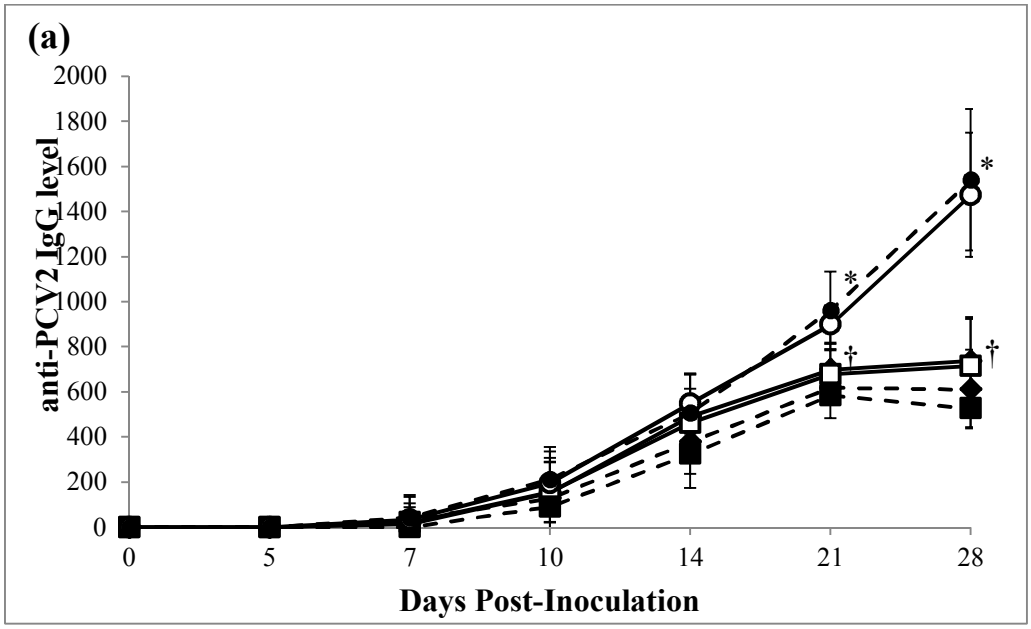
Figure 2. Average daily weight gain (a) and macroscopic lung lesion scores (b) in pigs from 8 groups (PCV2a/type 1 PRRSV, ▥; PCV2a/type 2 PRRSV, ▤; PCV2b/type 1 PRRSV, ▨; PCV2b/type 2 PRRSV, ▧; PCV2a, ▦; PCV2b, ▩; type 1 PRRSV, □; and type 2 PRRSV, ■; Negative Control, ■). Different letters (*, †, and ‡) indicate statistically significant differences ($P < 0.05$) among groups.

Anti-PCV2 IgG antibodies

Regardless of the genotype of PCV2, PCV2-infected pigs were seropositive by ELISA at 10 dpi. Pigs with a PCV2a (or 2b) had significantly ($P < 0.05$) higher anti-PCV2 IgG antibody levels than pigs with a PCV2a (or 2b)/type 1 (or type 2) PRRSV (Fig. 3A). No anti-PCV2 IgG antibodies were detected in negative control pigs.

Anti-PRRSV IgG antibodies

Regardless of the genotype of PRRSV, PRRSV-infected pigs were seropositive by ELISA at 10 dpi. Pigs with type 1 (or type 2) PRRSV had significantly ($P < 0.05$) higher anti-PRRSV IgG antibody levels than pigs with a PCV2a (or 2b)/type 1 (or type 2) PRRSV (Fig. 3B). No anti-PRRSV IgG antibodies were detected in negative control pigs.


(a)
anti-PCV2 IgG level
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
7
10
14
21
28
Days Post-Inoculation
*
*
†
†

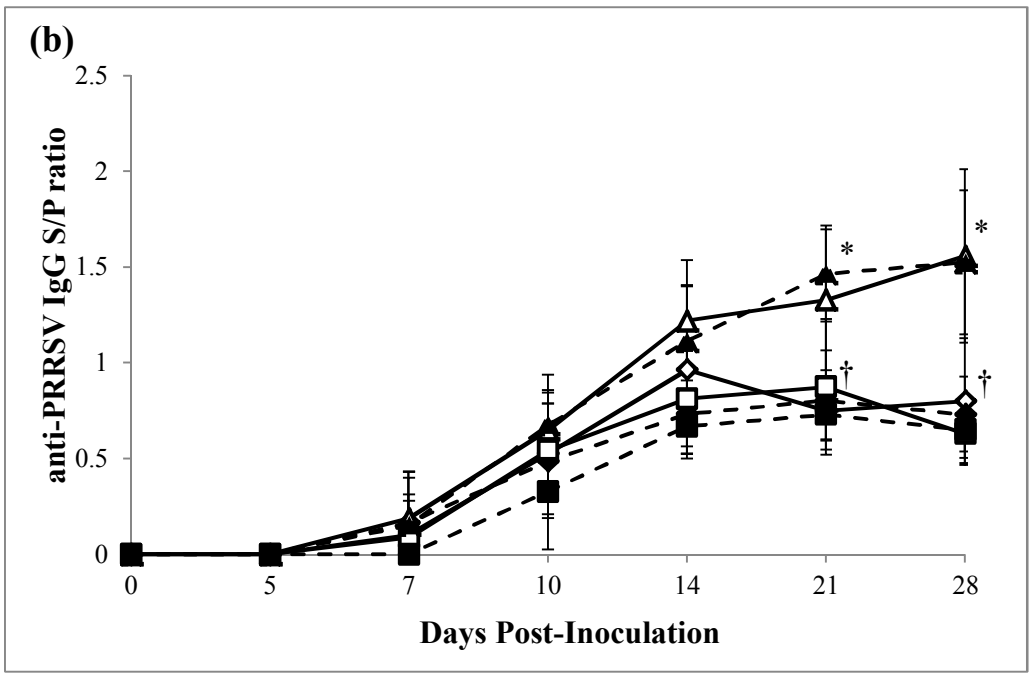

(b)
anti-PRRSV IgG S/P ratio
2.5
2
1.5
1
0.5
0
0
5
7
10
14
21
28
Days Post-Inoculation
*
*
†
†

Figure 3. Humoral immune responses. Anti-PCV2 IgG antibodies (a) and anti-PRRSV IgG antibodies (b) levels in 8 groups (PCV2a/type 1 PRRSV, ◇; PCV2a/type 2 PRRSV, ◆; PCV2b/type 1 PRRSV, □; PCV2b/type 2 PRRSV, ■; PCV2a, ○; PCV2b, ●; type 1 PRRSV, △; and type 2 PRRSV, ▲). Different letters (*, and †) indicate statistically significant differences ($P < 0.05$) among groups.

Quantification of PCV2 DNA in sera

Regarding PCV2a viremia, pigs with PCV2a/type 2 PRRSV had a significantly ($P < 0.05$) higher number of genomic copies of PCV2a in their sera than pigs with PCV2a/type 1 PRRSV or pigs with PCV2a at 10 and 14 dpi. Pigs with PCV2a/type 1 PRRSV had a significantly ($P < 0.05$) higher number of genomic copies of PCV2a in their sera than pigs with PCV2a at 14, 21, and 28 dpi (Fig. 4A). Regarding PCV2b viremia, pigs with PCV2b/type 2 PRRSV had a significantly ($P < 0.05$) higher number of genomic copies of PCV2b in their sera than pigs with PCV2b/type 1 PRRSV or pigs with PCV2b at 10 and 14 dpi. Pigs with PCV2b/type 1 PRRSV had a significantly ($P < 0.05$) higher number of genomic copies of PCV2b in their sera than pigs with PCV2b at 14, 21, and 28 dpi (Fig. 4A). The number of genomic copies of PCV2a and PCV2b in PCV2a-infected pigs and PCV2b-infected pigs was not significantly different. No PCV2a or PCV2b was detected in the blood of negative control pigs.

Quantification of PRRSV RNA in sera

The number of genomic copies of type 2 PRRSV in pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV was significantly ($P < 0.05$) higher than that of type 1 PRRSV in pigs with PCV2a (or 2b)/type 1 PRRSV and pigs with type 1 PRRSV at 5, 7, 10, 14, and 21 dpi. The number of genomic copies of type 2 PRRSV in pigs with PCV2a/type 2 PRRSV and pigs with PCV2b/type 2 PRRSV was not significantly different (Fig. 4B). The number of genomic copies of type 1 PRRSV in pigs with PCV2a/type 1 PRRSV and pigs with PCV2b/type 1 PRRSV was not significantly

different. No type 1 or type 2 PRRSV was detected in the blood of negative control pigs.

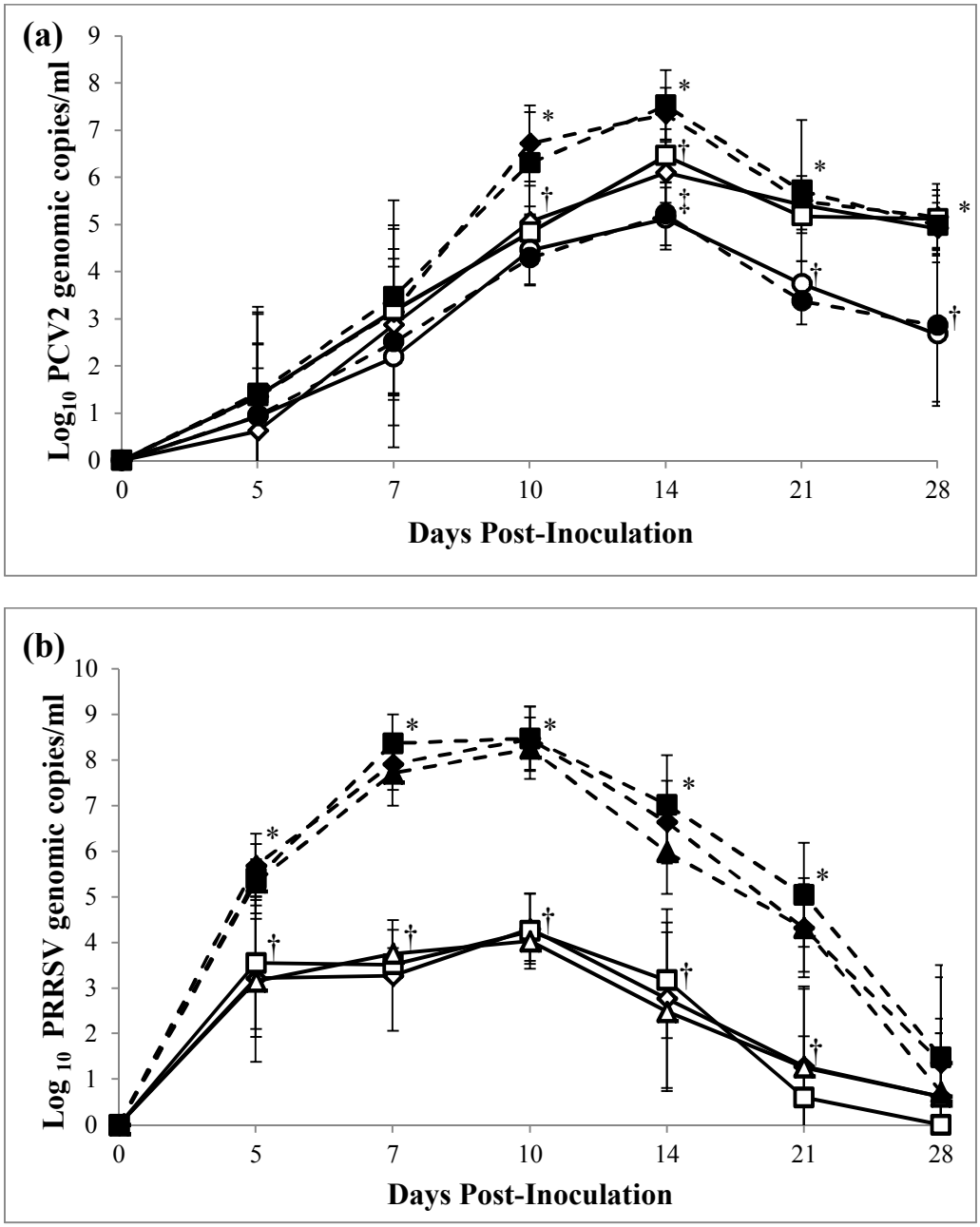


Figure 4. Mean values of the genomic copy number of PCV2 DNA (a) and PRRSV RNA (b) in serum from 8 groups (PCV2a/type 1 PRRSV, ◇; PCV2a/type 2 PRRSV, ◆;

PCV2b/type 1 PRRSV, □; PCV2b/type 2 PRRSV, ■; PCV2a, ○; PCV2b, ●; type 1 PRRSV, △; and type 2 PRRSV, ▲). Different letters (*, †, and ‡) indicate statistically significant differences ($P < 0.05$) among groups.

Microscopic lesions

Regardless of the genotype of the viruses, a typical granulomatous inflammatory reaction and lymphoid depletion consistent with histopathological lesions of PMWS were observed in the lymph nodes of pigs with PCV2/PRRSV. At 14 dpi, pigs with PCV2a (or 2b)/type 2 PRRSV (Fig. 5A and 5B) had significantly ($P < 0.05$) higher lymphoid lesion scores than pigs with PCV2a (or 2b)/type 1 PRRSV (Fig. 5C and 5D), pigs with PCV2a (or 2b), and pigs with type 1 (or type 2) PRRSV. Pigs with PCV2a (or 2b)/type 1 PRRSV had a significantly ($P < 0.05$) higher lymphoid lesion scores than pigs with PCV2a (or 2b) and pigs with type 1 (or type 2) PRRSV. Pigs with PCV2a (or 2b) had a significantly ($P < 0.05$) higher lymphoid lesion scores than pigs with type 1 (or type 2) PRRSV. At 28 dpi, pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV had a significantly ($P < 0.05$) higher lymphoid lesion scores than pigs with PCV2a (or 2b) and pigs with type 1 (or type 2) PRRSV. Pigs with PCV2a (or 2b) had significantly ($P < 0.05$) higher lymphoid lesion scores than pigs with type 1 (or type 2) PRRSV (Fig. 6A).

Microscopic lung lesions in pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV were characterized by thickened alveolar septae, and increased numbers of interstitial macrophages and lymphocytes. At 14 dpi, pigs with PCV2a (or 2b)/type 2 PRRSV had a significantly ($P < 0.05$) higher scores for interstitial pneumonia lesions than pigs with type 1 PRRSV and pigs with PCV2a (or 2b). Pigs with PCV2a (or 2b)/type 1 PRRSV had a significantly ($P < 0.05$) higher scores for interstitial pneumonia lesions than pigs with type 1 PRRSV or pigs with PCV2a (or 2b). Pigs with

type 1 PRRSV had a significantly ($P < 0.05$) higher scores for interstitial pneumonia lesions than pigs with PCV2a (or 2b). The lymph nodes and lungs of negative control pigs were normal (Fig. 6B).

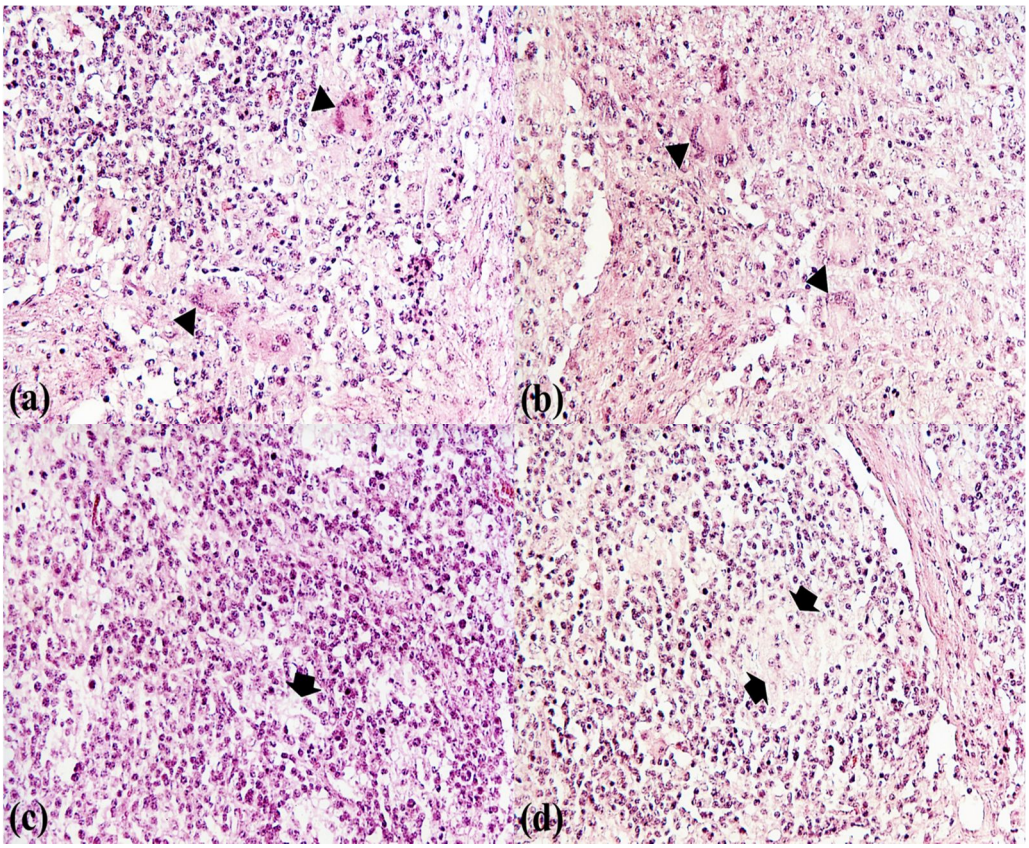
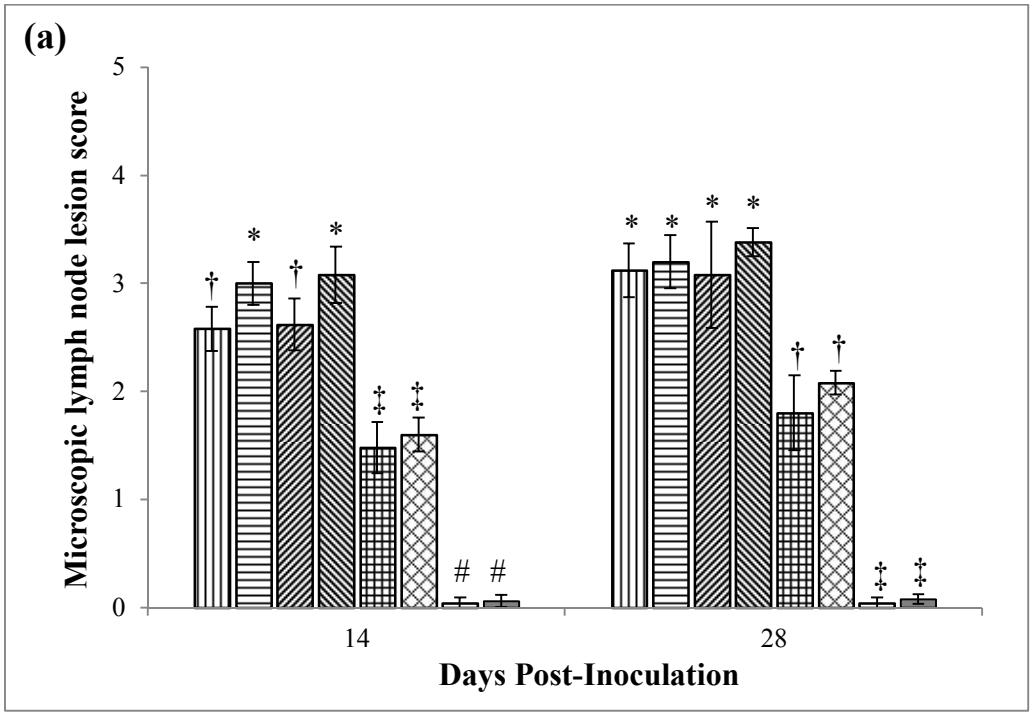


Figure 5. Microscopic lymph node lesions from pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV at 14 dpi. Severe granulomatous inflammation with multinucleated giant cells (arrowheads) from pigs with PCV2a/type 2 PRRSV (a) and PCV2b/type 2 PRRSV (b), and mild to moderate granulomatous inflammation (arrows) from pigs with PCV2a/type 1 PRRSV (c) and PCV2b/type 1 PRRSV (d).


(a)
Microscopic lymph node lesion score
5
4
3
2
1
0
14
28
Days Post-Inoculation

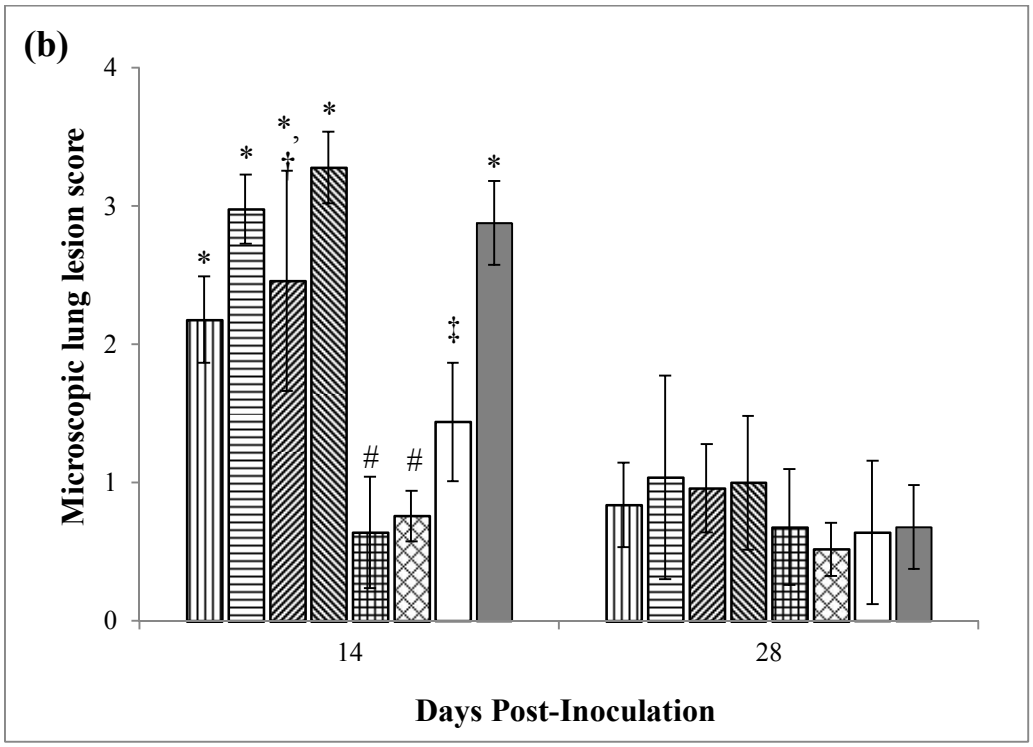
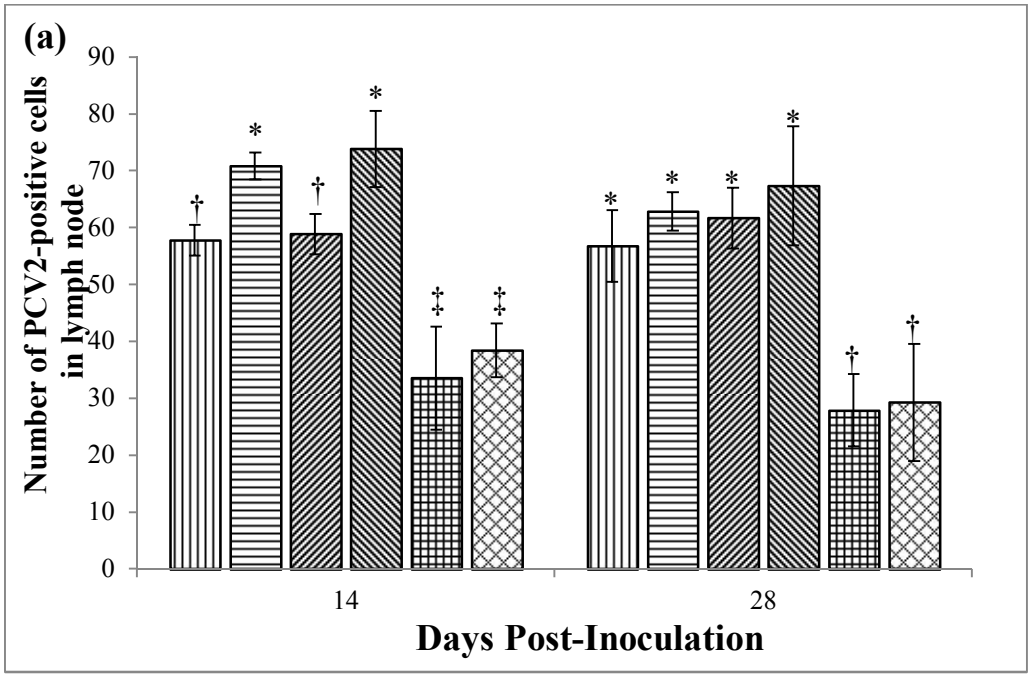

(b)
Microscopic lung lesion score
4
3
2
1
0
14
28
Days Post-Inoculation

Figure 6. Microscopic lymph node (a) and lung (b) lesion scores in pigs from in pigs from 8 groups (PCV2a/type 1 PRRSV, ▥; PCV2a/type 2 PRRSV, ▤; PCV2b/type 1 PRRSV, ▨; PCV2b/type 2 PRRSV, ▧; PCV2a, ▦; PCV2b, ▩; type 1 PRRSV, □; and type 2 PRRSV, ■). Different letters (*, †, ‡, and #) indicate statistically significant differences ($P < 0.05$) among groups.

In situ hybridization of PCV2

PCV2 was detected in mononuclear cells of the lymph nodes and lungs. At 14 dpi, pigs with PCV2a (or 2b)/type 2 PRRSV had a significantly ($P < 0.05$) higher number of PCV2-positive cells per unit in their lymph nodes and lungs than pigs with PCV2a (or 2b)/type 1 PRRSV or pigs with PCV2a (or 2b). Pigs with PCV2a (or 2b)/type 1 PRRSV had a significantly ($P < 0.05$) higher number of PCV2-positive cells per unit in their lymph nodes and lungs than pigs with PCV2a (or 2b). At 28 dpi, pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV had a significantly ($P < 0.05$) higher number of PCV2-positive cells per unit in their lymph nodes and lungs than pigs with PCV2a (or 2b). No PCV2a or PCV2b DNA was detected in the lymph nodes or lungs of negative control pigs (Fig. 7).


(a)
Number of PCV2-positive cells in lymph node
90
80
70
60
50
40
30
20
10
0
14
28
Days Post-Inoculation

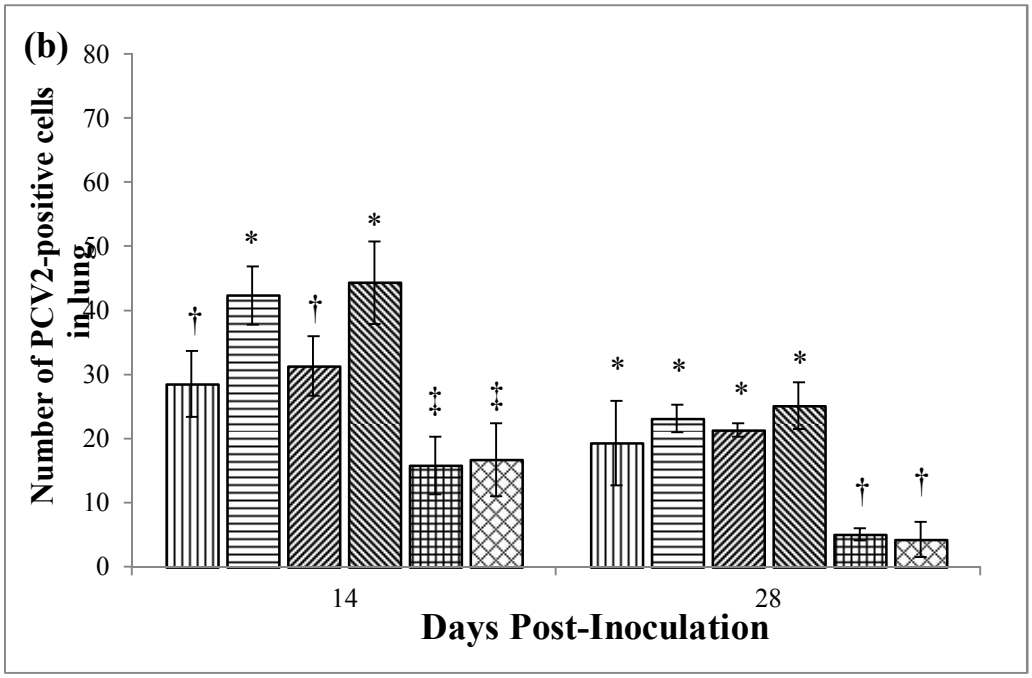

(b)
Number of PCV2-positive cells in lung
80
70
60
50
40
30
20
10
0
14
28
Days Post-Inoculation

Figure 7. Number of PCV2-positive cells per unit in lymph node (a) and lung (b) of pigs from 6 groups (PCV2a/type 1 PRRSV, ▥; PCV2a/type 2 PRRSV, ▤; PCV2b/type 1 PRRSV, ▨; PCV2b/type 2 PRRSV, ▧; PCV2a, ▦; PCV2b, ▩). Different letters (*, †, and ‡) indicate statistically significant differences ($P < 0.05$) among groups.

In situ hybridization of PRRSV

PRRSV was detected in macrophages of the lymph nodes and lungs. At 14 dpi, pigs with PCV2a (or 2b)/type 2 PRRSV had a significantly ($P < 0.05$) higher number of PRRSV-positive cells per unit in their lungs than pigs with PCV2a (or 2b)/type 1 PRRSV or pigs with type 1 PRRSV. No type 1 and type 2 PRRSV RNA was detected in the lymph nodes or lungs of negative control pigs (Fig. 8).

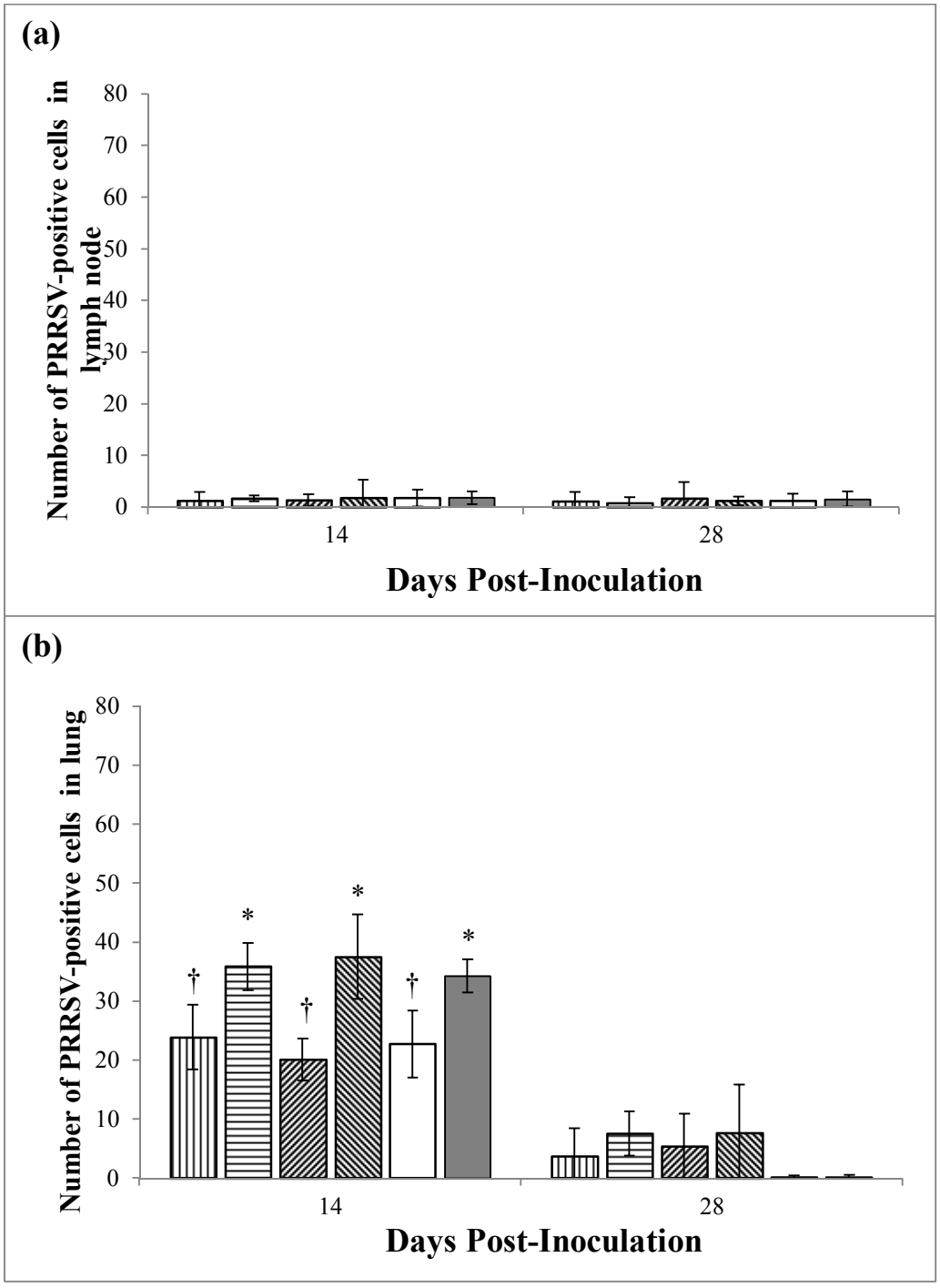

(a)
Number of PRRSV-positive cells in lymph node
80
70
60
50
40
30
20
10
0
14
28
Days Post-Inoculation
(b)
Number of PRRSV-positive cells in lung
80
70
60
50
40
30
20
10
0
†
*
†
*
†
*
14
28
Days Post-Inoculation

Figure 8. Number of PRRSV-positive cells per unit in lymph node (a) and lung (b) of pigs from 6 groups (PCV2a/type 1 PRRSV, ▥; PCV2a/type 2 PRRSV, ▤; PCV2b/type 1 PRRSV, ▨; PCV2b/type 2 PRRSV, ▧; type 1 PRRSV, □; and type 2 PRRSV, ■). Different letters (*, and †) indicate statistically significant differences ($P < 0.05$) among groups.

DISCUSSION

This study demonstrates that co-infection with PCV2a (or 2b)/type 2 PRRSV can result in a more serious disease than co-infection with PCV2a (or 2b)/type 1 PRRSV or infection with PCV2a (or 2b) alone. There were no differences in the virulence and pathogenicity of PCV2a/type 2 PRRSV and PCV2b/type 2 PRRSV or PCV2a/type 1 PRRSV and PCV2b/type 1 PRRSV. These results agree with previous findings that demonstrated no significant differences in the pathogenicity of PCV2a/type 2 PRRSV and PCV2b/type 2 PRRSV (Opriessnig *et al.*, 2012). Our well-controlled experimental study also does not support major differences in the virulence or pathogenicity of PCV2a and PCV2b. These observations are in contrast with those of field studies reporting differences in the open reading frame 2 sequences of PCV2 obtained from animals from PMWS-affected farms (majority PCV2b) and animals from non-PMWS-affected farms (all PCV2a) (Grau-Roma *et al.*, 2008). In addition, epidemiological studies have suggested a link between PCV2b and the occurrence of PMWS and a genotype shift from PCV2a to PCV2b (Cheung *et al.*, 2007; Dupont *et al.*, 2008; Wiederkehr *et al.*, 2009). We have no clear explanation for this discrepancy. Both

genotypes of the PCV2 isolates used in this study originated from postweaned pigs with severe PMWS, and thus both genotypes were expected to be of similar virulence. Another explanation to consider based on this study is that a global shift of the PCV2 genotype, from PCV2a to PCV2b, coincidentally occurred at the same time that the PCVAD outbreak occurred worldwide.

Significant differences in the virulence and pathogenicity of PCV2a (or 2b)/type 2 PRRSV and PCV2a (or 2b)/type 1 PRRSV are reasonable because type 2 PRRSV induces a more severe respiratory disease than type 1 PRRSV (Halbur *et al.*, 1996a; Han *et al.*, 2013a; Martínez-Lobo *et al.*, 2011). Moreover, pigs with PCV2a (or 2b)/type 2 PRRSV had a higher PCV2 load in their blood than pigs with PCV2a (or 2b)/type 1 PRRSV. PCV2 viremia plays a central role in the development of PMWS. High levels of PCV2 viremia are associated with development of PCVAD (Meerts *et al.*, 2005, 2006). The more virulent type 2 PRRSV replicates faster than the less virulent type 1 PRRSV (Han *et al.*, 2013a). Notably, type 2 PRRSV appears to reach higher a level of viremia than type 1 PRRSV, as previous described (Han *et al.*, 2013a). These results suggest that the enhancement of PCV2 replication may be explained by a quantitative difference in the viremia of type 1 and type 2 PRRSV.

In the present study, there were no significant differences in the levels of PRRSV viremia in pigs with PCV2a/type 2 (or type 1) PRRSV and pigs with PCV2b/type 2 (or type 1) PRRSV. The results of this study suggest that PCV2 does not enhance PRRSV viremia. In contrast, another experimental study indicated that a highly pathogenic PRRSV infection followed by a PCV2 infection enhanced the replication of both

viruses in pigs (Fan *et al.*, 2013). These synergistic effects may have resulted from the high virulence of highly pathogenic PRRSV or the use of different (concurrent vs. sequential) co-infection models. Further studies are needed to compare different co-infection models and the virulence of the strains of the same PRRSV genotype.

The diagnosis of PMWS must include characteristic lesions in the lymphoid tissues and the detection of a PCV2 antigen or PCV2 DNA within these lesions (Chae, 2004). Hence, pathological evaluation and detection of a PCV2 antigen within the lesion are the most important criteria for assessing virulence and pathogenicity in co-infected pigs. A hallmark of the pathological lesions in lymphoid tissues is granulomatous inflammation with lymphoid depletion (Chae, 2004). In the present study, typical histopathological changes were observed in pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with PCV2a (2b)/type 1 PRRSV. However, pigs with PCV2a (or 2b)/type 2 PRRSV had more severe PCV2-associated lesions and more PCV2 DNA within the lesions than pigs with PCV2a (or 2b)/type 1 PRRSV. These results suggest that type 2 PRRSV enhances the replication of PCV2 and can induce more severe lymphoid lesions than type 1 PRRSV. Interestingly, the co-infected pigs produced significantly lower levels of anti-PCV2 and anti-PRRSV IgG antibodies than singularly infected pigs, regardless of the genotype of the two viruses. Pigs that were co-infected with PCV2 and PRRSV were found to have more severe lymphoid depletion and enhanced PCV2 replication in previous studies (Allan *et al.*, 2000; Harms *et al.*, 2001; Rovira *et al.*, 2002) and in the present study. Selective loss of $CD3^+$ and $CD4^+$ cells is observed in pigs with PMWS (Nielsen *et al.*, 2003; Segales *et al.*, 2001). Therefore, the

lymphoid depletion induced in pigs by co-infection with PCV2 and PRRSV may render them unable to mount an effective immune response.

Our results should be interpreted carefully because this study has been conducted with only one strain for each genotype of both viruses. Different results could have been obtained from an experiment conducted with a different strain of the same genotype. Variation in the virulence of different PCV2 and PRRSV isolates with the same genotypes in pigs has been documented (Halbur *et al.*, 1996b; Han *et al*., 2013b; Opriessnig *et al*., 2008b). Future works are needed to compare the virulence and pathogenicity of a combination of concurrent infections of each genotype of both viruses using different strains of the same genotype.

Genetically modified PCV2 demonstrated that both the *rep* gene and the origin of replication (Ori) of PCV1 enhance replication of the PCV2-based chimeric viruses *in vitro* (Beach *et al*., 2010). Although the molecular mechanisms of PRRSV-enhanced PCV2 replication and pathogenesis remain poorly understood, interaction between PRRSV and two genes (*rep* and Ori genes) of PCV2 may activate PCV2 replication and exacerbate the clinical outcome of infection. Mixed infection with PRRSV and PCV2 is currently one of the most common co-infections associated with swine disease under field conditions. Hence, the four different combinations of concurrent infections involving the two genotypes of the two viruses in this study will provide swine practitioners and producers with clinical information. Regular surveillance of the two genotypes of the two viruses in sick and dead pigs should be undertaken in herds in which both genotypes coexist.

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PART II. The protective effects induced by PCV2 vaccines in PCV2 and PRRSV co-infected pig.

Chapter I. Comparative effects of vaccination against PCV2 or PRRSV in a co- infected challenge models.

ABSTRACT

The objective of the present study was to determine the effects of the porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) vaccinations in an experimental PCV2-PRRSV challenge model that was based on virological (viremia), immunological (neutralizing antibodies [NAs], interferon-γ-secreting cells [IFN-γ-SCs], and $CD4^{+}CD8^{+}$ double positive cells), and pathological (lesions and antigens in lymph nodes and lungs) evaluations. A total of 72 pigs were randomly divided into 9 groups (8 pigs per group): 5 vaccinated and challenged groups, 3 non-vaccinated and challenged groups, and a negative control group. Vaccination against PCV2 induced immunological responses (NAs and PCV2-specific IFN-γ-SCs) and reduced PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the dual infected pigs. However, vaccination against PCV2 did not affect the immunological responses (NAs and PRRSV-specific IFN-γ-SCs), and PRRSV viremia, PRRSV-induced lesions, and PRRSV antigens in the dual infected pigs. Vaccination against PRRSV did not induce immunological responses (PRRSV-specific IFN-γ-SCs) and reduce PRRSV viremia, PRRSV-induced lesions, and PRRSV antigen in the dual infected pigs. In addition, vaccination against PRRSV increased PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the dual infected pigs. In summary, vaccination against PCV2 reduced PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the dual infected pigs. However, vaccination against PRRSV increased PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the dual infected pigs. Therefore, PCV2 vaccine decreased the potentiation of PCV2-induced

lesions by PRRSV in dual infected pigs. In contrary, PRRSV vaccine alone did not decrease the potentiation of PCV2-induced lesions by PRRSV in dual infected pigs.

INTRODUCTION

Porcine circovirus type 2 (PCV2), which is a single-stranded circular DNA virus within the family Circoviridae, is now recognized as the primary etiological agent of porcine circovirus-associated diseases (PCVAD), such as postweaning multisystemic wasting syndrome (PMWS), porcine respiratory disease complex (PRDC), and other clinical diseases (Chae, 2004; 2005). Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV), which is the causative agent of PRRS, is a single-stranded, positive-sense, enveloped RNA virus in the family *Arteriviridae* and order *Nidovirales* (Cavanagh, 1997). PRRSV infection in swine is characterized by late-term abortion in gilts and sows and severe respiratory diseases in neonatal and nursing pigs (Zimmerman et al, 2012). PRDC is a serious problem in the pig industry. PRRSV and PCV2 are the most commonly associated primary pathogens in PRDC. Epidemiological analyses have revealed that co-infection with PCV2 and PRRSV is most commonly observed in field cases (Kim et al, 2002; 2003; Pallarés et al, 2002).

Several studies have previously demonstrated the relationship between PCV2 and PRRSV. PCV2 does not affect PRRSV replication or lesions, but PRRSV increases the PCV2 DNA load in the sera of co-infected pigs (Rovira et al, 2002; Opriessnig et al, 2008) and increases the levels of PCV2 antigens in tissues (Allan et al, 2000), which results in more severe PCV2-associated lesions (Harms et al, 2001). Based on these results, one possible way to minimize the effects of the PRRSV-associated enhancement of the replication of PCV2 and the induction of PMWS may be the use of a PRRSV-based vaccination in preweaned pigs. However, there are no reports in the

literature describing the effects of PCV2 and PRRSV challenges on pigs that have been immunized with either the PCV2 or PRRSV vaccines. In the absence of such a study, the PCV2 vaccine-PCV2-PRRSV and PRRSV vaccine-PCV2-PRRSV interactions have not been completely elucidated. Therefore, the objective of the present study was to determine the effects of PCV2 and PRRSV vaccinations in an experimental PCV2-PRRSV challenge model based on virological (viremia), immunological (neutralizing antibodies [NAs], interferon-γ-secreting cells [IFN- γ-SCs], and $CD4^+CD8^+$ double positive cells), and pathological (lesions and antigens in lymph nodes and lungs) evaluations.

METHODS

Commercial vaccine.

The inactivated chimeric PCV1-2 vaccine (Fostera PCV™ vaccine, Pfizer Animal Health New York, NY, USA) and modified-live PRRS vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Animal Health, St. Joseph, MO, USA) were used in this study. The inactivated chimeric PCV1-2 vaccine contains the genomic backbone of the non-pathogenic PCV1 with the PCV2 ORF2 capsid gene in placed of the PCV1 capsid gene (Fenaux et al, 2007). Modified-live PRRS vaccine (Ingelvac® PRRS MLV) originated from the American isolate ATCC VR-2332 and was attenuated by serial passage in cell culture. The vaccine contained at least 1 x $10^{4.9}$ tissue culture infective doses 50% ($TCID_{50}$) in 2 ml. Forty pigs were vaccinated with 2.0 ml doses of either the PCV2 or PRRSV vaccines or both intramuscularly at 3 weeks of age (Table 1). All of the

vaccines that were used in this study were administered according to the manufacturer's construction (1 dose, via intramuscular).

Animals and housing.

A total of 72 colostrum-fed, cross-bred, conventional piglets were weaned and purchased at 18 days of age from a PRRSV-free commercial farm. They were all negative for PCV2, PRRSV, swine influenza virus and *Mycoplasma hyopneumoniae* according to routine serological testing. PCV2 and PRRSV were not detected in the serum samples by the real-time polymerase chain reaction (PCR) (Gagnon et al, 2008; Wasilk et al, 2004). Individual piglets from 7 days of age were uniquely identified by their ear notches.

All of the pigs were housed in an environmentally controlled building with pens over completely slatted floors throughout the experiment. To avoid environmental contamination, the building was completely emptied, cleaned three times with hot (>95°C) water, and disinfected with a 2% potassium peroxymonosulfate and sodium chloride-based product (Virkon S, Antec International, Sudbury, Suffolk, UK) for 3 days. The building was emptied for an additional 21 days before the pigs were introduced and each was housed separately within the facility.

Experimental design.

A total of 72 pigs were randomly divided into 9 groups (8 pigs per group): 5 vaccinated and challenged (VC) groups (1, 2, 3, 4, and 5), 3 non-vaccinated and

challenged (NVC) groups (6, 7, and 8), and a negative control group (9).

At 21 days of age (-28 days post challenge, dpc), pigs in groups 1 and 2 were immunized with the PCV2 vaccine, pigs in groups 3 and 4 were immunized with the PRRSV vaccine, pigs in group 5 were immunized with both the PCV2 and PRRSV vaccine. At 49 days of age (0 dpc), pigs in the VC and NVC groups were intratracheally administered an intranasal 3 ml dose of PCV2b (strain SNUVR000463; 5th passage) containing 1.2 x 10^5 $TCID_{50}$/ml and/or 3 ml of PRRSV (strain SNUVR090851; North American genotype; 5th passage) containing doses of 1 x 10^5 $TCID_{50}$/ml. Pigs in groups 1 and 6 were challenged with PCV2. Pigs in groups 3 and 7 were challenged with PRRSV. Pigs in groups (2, 4, 5, and 8) were challenged with both PCV2 and PRRSV (Table 1).

Table 1. Study design with vaccination and challenge statuses of porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) at different days post challenge (dpc).

Group	*n*	Vaccination at -28 dpc		Challenge at 0 dpc		Necropsy at 21 dpc
		PCV2	PRRSV	PCV2	PRRSV	
1	8	+	-	+	-	+
2	8	+	-	+	+	+
3	8	-	+	-	+	+
4	8	-	+	+	+	+
5	8	+	+	+	+	+
6	8	-	-	+	-	+
7	8	-	-	-	+	+
8	8	-	-	+	+	+
9	8	-	-	-	-	+

Serology.

Blood samples from each pig were collected by jugular venipuncture at -28, 0, 10, and 21 dpc, and the sera were stored at -20°C. The serum samples were tested using the commercial PRRSV ELISA (HerdCheck PRRS 2XR, IDEXX Laboratories Inc., Westbrook, ME, USA) and PCV2 ELISA (Synbiotics, Lyon, France). Serum virus neutralization (SVN) test for PCV2 was performed as previously described (Pogranichniy et al, 2000). NAs titers were expressed as the reciprocal of the highest serum dilution that completely blocked the infection in the PK15 cells compared with the virus control. SVN test for PRRSV was also performed as previously described (Zuckermann et al, 2007).

Quantification of PCV2 DNA in blood.

DNA was extracted from the serum samples at -28, 0, 10, and 21 dpc using the QIAamp DNA Mini Kit(QIAGEN Ltd, Crawley, UK). The DNA extracts were used to quantify the PCV2 genomic DNA copy numbers by real-time PCR as previously described (Gagnon et al, 2008).

Quantification of PRRSV RNA in blood.

RNA was extracted from the serum samples at -28, 0, 10, and 21 dpi from all of the pigs that were used in this study as previously described (Wasilk et al, 2004; Han et al, 2011). Real-time PCR for the PRRSV and vaccine strains were used to quantify the PRRSV genomic cDNA copy numbers following the RNA extractions of the serum

samples, which were performed as previously described (Wasilk et al, 2004; Han et al, 2011).

Preparation of PCV2 and PRRSV antigens.

The same PCV2 strain (or PRRSV strain) that was used for the challenge in the pigs was propagated in PCV-free PK15 cells (or MARC-145 cells for the PRRSV strain) to a titer of 10^4 $TCID_{50}$/ml and treated with two freeze-thaw cycles. The PCV2 antigen was prepared by concentrating the virus that was present in the cell culture by ultracentrifugation at 100,000 x g at 4°C for 3 h. The virus pellet was resuspended with PBS. The concentrated PCV2 (or PRRSV) was inactivated by exposure to an 8 W germicidal UV lamp at a distance of 15 cm for 1 h. Inactivation was confirmed by the absence of the virus antigen from the PK15 cells (or MARC-145 cells for the PRRSV strain) as determined by an immunoperoxidase assay as previously described (Bautista and Molitor, 1997; Rodríguez-Arrioja et al, 2000).

Enzyme-linked immunospot (ELISPOT) assay.

The numbers of PCV2- or PRRSV-specific interferon-γ-secreting cells (IFN-γ-SCs) were determined in peripheral blood mononuclear cells (PBMCs) at -28, 0, 10 and 21 dpc as previously described (Diaz and Mateu, 2005). Briefly, 100 µl containing 2×10^6 PBMCs in RPMI 1640 medium that was supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., SelectScience, Bath, UK) was seeded into plates that were precoated overnight with anti-porcine IFN-γ monoclonal antibody (5 µg/ml,

MABTECH, Mariemont, OH, USA) and incubated with 100 μl of PCV2 antigen (20 μg/ml), PRRSV antigen (20 μg/ml), and phytohemagglutinin (10 μg/ml, Roche Diagnostics GmbH, Mannheim, Germany) as positive controls or with PBS as a negative control for 40 h at 37 °C in a 5% humidified CO_2 atmosphere. Then, the wells were washed five times with PBS (200 μl per well). Thereafter, the procedure was conducted according to the manufacturer's instructions using the commercial ELISPOT Assay Kit (MABTECH, Mariemont, OH, USA). The spots on the membranes were read by an automated ELISpot Reader (AID ELISpot Reader, AID GmbH, Strassberg, Germany). The results were expressed as the numbers of IFN-γ-SCs per million PBMCs.

Flow cytometry.

The phenotypic analyses of the $CD4^+CD8^+$ double positive cells in the PBMCs were performed using flow cytometry with the following two monoclonal antibodies: CD4a [R-PE] and CD8a [FITC] (SouthernBiotech, Birmingham, AL, USA) with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously described (Oh et al, 2012).

Immunohistochemistry.

Superficial inguinal lymph nodes that had previously shown consistent and intense labeling for PCV2 and typical granulomatous lesions (Kim et al, 2003) were collected at necropsy for immunohistochemical analyses. The rabbit polyclonal anti-PCV2

antibody (West et al, 1999) (1:200 in PBS containing 0.1% Tween 20) and SR30 monoclonal antibody (Rural Technologies Inc., Brookings, SD, USA) (1:10,000 in PBS containing 0.1% Tween 20) were used as the primary antibodies. Immunohistochemical analyses for PCV2 and PRRSV were performed as previously described (Han et al, 2012; Kim and Chae, 2004).

Morphometric analyses.

For the morphometric analyses of the microscopic pulmonary and lymph lesion scores, lung sections were blindly examined and assigned estimated scores ranging from 1 (mild interstitial pneumonia) to 4 (severe interstitial pneumonia) as previously described (Halbur et al, 1995). Superficial inguinal lymph node sections were also examined blindly, and their scores ranged from 0 (normal; i.e., no lymphoid depletion or granulomatous replacement) to 5 (severe lymphoid depletion and granulomatous replacement) as previously described (Kim and Chae, 2004).

For the immunohistochemistry morphometric analyses, 3 sections were cut from each of three blocks of tissue from one entire pulmonary lobe and from the superficial inguinal lymph nodes of each pig. The slides were analyzed using the NIH Image J 1.43m program (http://rsb.info.nih.gov/ij) to obtain the quantitative data. In each slide, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm^2) was determined as previously described (Kim et al, 2003, Halbur et al, 1996). The mean values were also calculated.

Statistical analyses.

Summary statistics were calculated for all of the groups to assess the overall quality of the data, including normality. For single comparisons, an ANOVA with a post-hoc Tukey's test was used to compare the primary variables (immunohistochemical scores) among groups. The continuous data for the PCV2 and PRRSV serology, PCV2 DNA and PRRSV cDNA quantifications were analyzed using an ANOVA for each time point. When a one-way ANOVA revealed a significance of $P < 0.05$, the Tukey's Honestly Significant Difference test was used to determine the significance of individual between group differences. Discrete data (histopathologic lung and lymphoid lesion scores) were analyzed by the Chi-square and Fisher's exact tests. A value of $P < 0.05$ was considered to be significant.

RESULTS

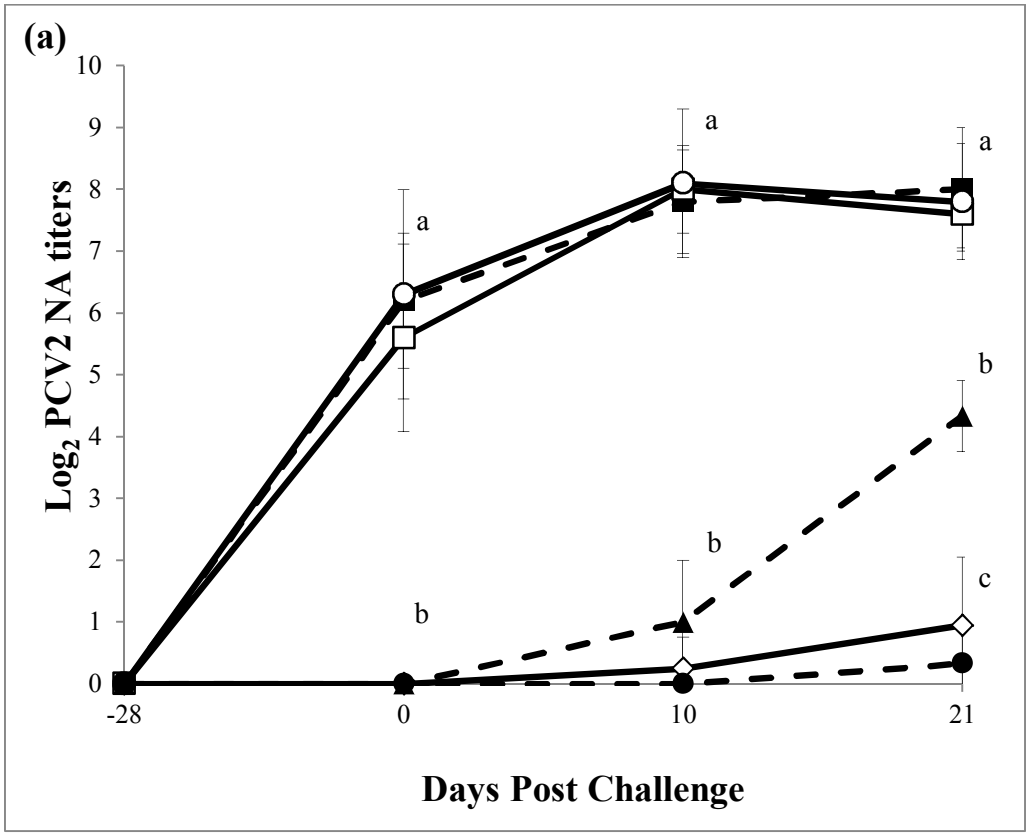
Serology of PCV2.

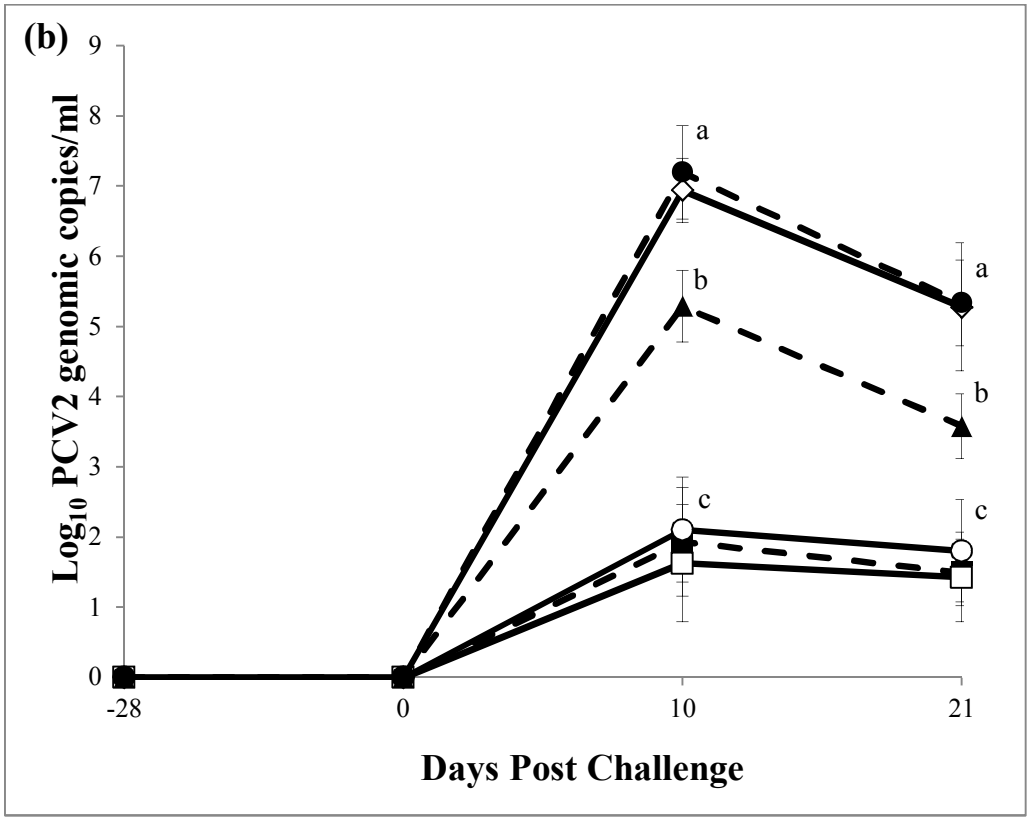
At challenge, all pigs vaccinated against PCV2 were seropositive and all non-vaccinated pigs were negative by ELISA and SVN tests. Pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1), pigs which received PCV2 vaccine followed by dual challenge (group 2), and pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5) had significantly higher anti-PCV2 IgG antibody levels ($P < 0.001$) and NAs titers ($P < 0.01$; Fig. 1A) than pigs which received PRRSV vaccine followed by dual challenge (group 4), pigs which were challenged with PCV2 only (group 6), and pigs which were challenged with both PCV2 and PRRSV (group 8) at 10 and 21 dpc. Pigs in group 6 had significantly higher anti-PCV2 IgG antibody levels ($P < 0.01$) than pigs in groups 4 and 8 at 21 dpc.

Serology of PRRSV.

At challenge, all pigs vaccinated against PRRSV were seropositive and all non-vaccinated pigs were negative by ELISA. Pigs which received PRRSV vaccine followed by PRRSV challenge only (group 3), pigs which received PRRSV vaccine followed by dual challenge (group 4), pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5) had significantly higher anti-PRRSV IgG antibody levels ($P < 0.001$) than pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which were challenged with PRRSV only (group 7), and pigs which were challenged with both PCV2 and PRRSV (group 8) at 10 dpc. Pigs

which received PRRSV followed by PRRSV challenge only (group 3) and pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5) had significantly higher anti-PRRSV IgG antibody levels ($P < 0.05$) than the pigs in other 4 groups (2, 4, 7, and 8) at 21 dpc (Fig. 2A). Low levels of PRRSV NAs titers (< 2 $\log_2$) were detected in all 8 groups throughout the experiment.


(a)
Log2 PCV2 NA titers
10
9
8
7
6
5
4
3
2
1
0
-28
0
10
21
Days Post Challenge
a
a
a
b
b
b
c


(b)
Log10 PCV2 genomic copies/ml
9
8
7
6
5
4
3
2
1
0
-28
0
10
21
Days Post Challenge
a
a
b
b
c
c

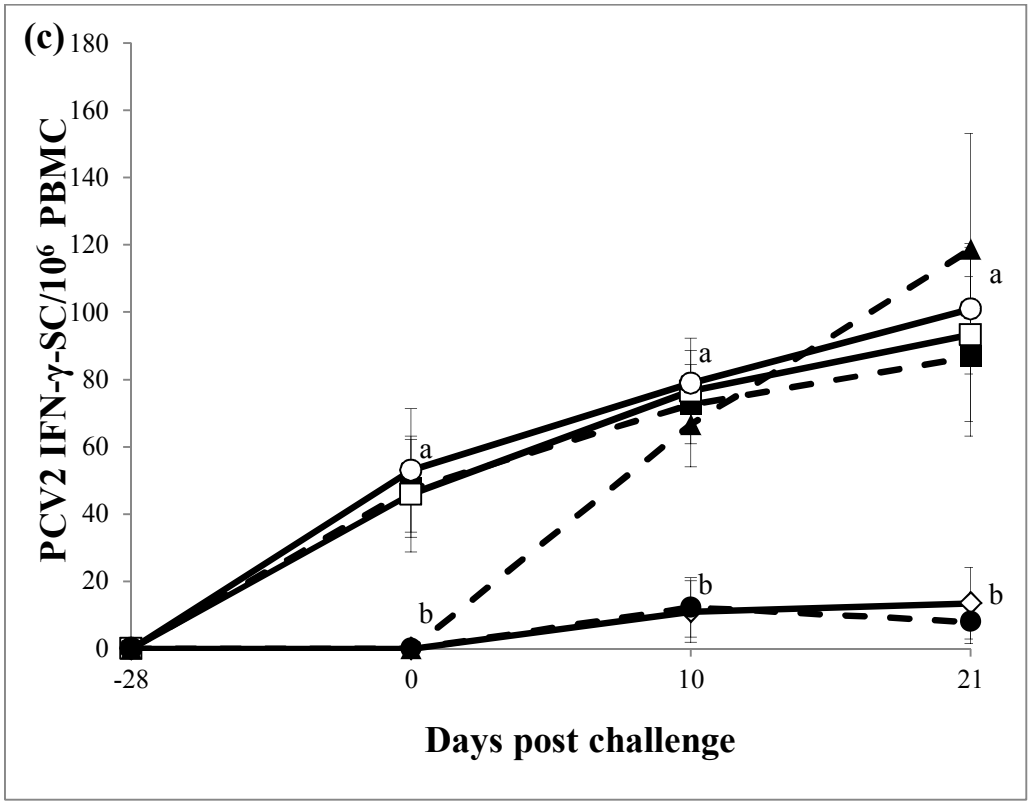
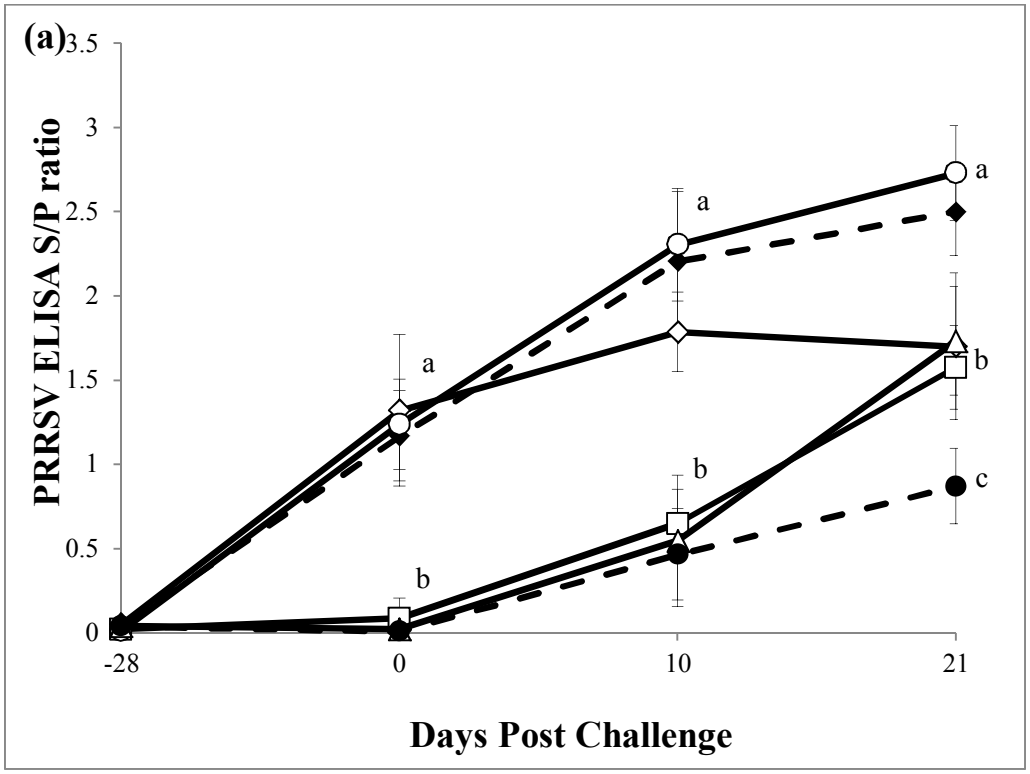
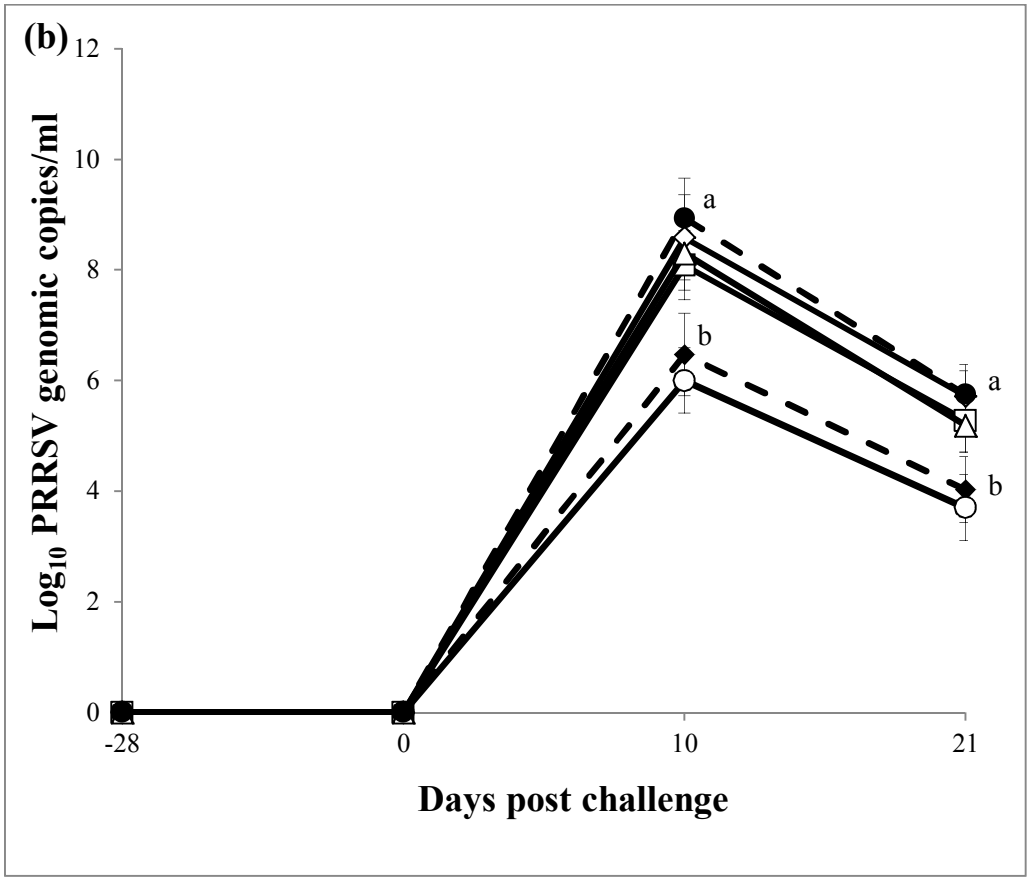


Figure 1. (a). Mean values of the serum neutralizing antibodies (NAs) titers against porcine circovirus type 2 (PCV2). (b). Mean values of the genomic copy numbers of PCV2 DNA in the serum samples. (c). Mean number of PCV2-specific interferon (IFN)-γ-secreting cells (SCs) in the peripheral blood mononuclear cells (PBMCs) in the different groups; pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1, ■), pigs which received PCV2 vaccine followed by dual challenge (group 2, □), pigs which received PRRSV vaccine followed by dual challenge (group 4 ◇), pigs which received PCV2 and PRRSV vaccine followd by dual challenge (group 5, ○), pigs which were challenged with PCV2 (group 6, ▲), and pigs which

were challenged with PCV2 and PRRSV (group 8, ●). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.


(a)
PRRSV ELISA S/P ratio
3.5
3
2.5
2
1.5
1
0.5
0
-28
0
10
21
Days Post Challenge
a
a
b
b
b
c


(b)
12
10
8
6
4
2
0
Log10 PRRSV genomic copies/ml
a
b
a
b
-28
0
10
21
Days post challenge

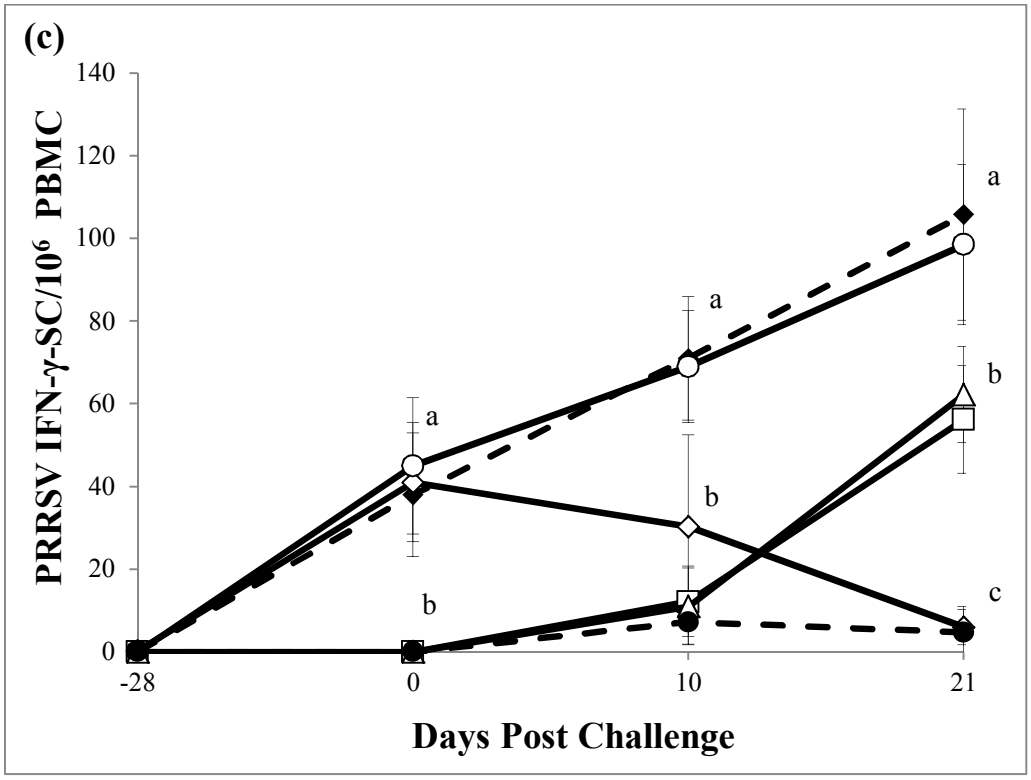


Figure 2. (a). Mean values of the anti porcine reproductive and respiratory syndrome virus (PRRSV) IgG antibody levels in the serum samples. (b). Mean values of the genomic copy numbers of PRRSV DNA in the serum samples. (c). Mean number of PRRSV-specific interferon (IFN)-γ-secreting cells (SCs) in the peripheral blood mononuclear cells (PBMCs) in the different groups; pigs which received PCV2 vaccine followed by dual challenge (group 2, □), pigs which received PRRSV vaccine followed by PRRSV challenge (group 3, ◆), pigs which received PRRSV vaccine followed by dual challenge (group 4, ◇), pigs which received PCV2 and PRRSV vaccines followed by dual challenge (group 5, ○), pigs which were challenged with PRRSV (group 7, △), and pigs which were challenged with PCV2 and PRRSV (group

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

Quantification of PCV2 DNA in blood.

At challenge, no genomic copies of PCV2 were detected in any of the serum samples from all 8 groups. Pigs which received PRRSV vaccine followed by dual challenge (group 4) and pigs which were challenged with both PRRSV and PCV2 (group 8) had significantly higher number of genomic copies of PCV2 in the serum than pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1), pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5), and pigs which were challenged with PCV2 only (group 6) at 10 and 21 dpc ($P < 0.05$). Pigs which were challenged with PCV2 only (group 6) had significantly higher number of genomic copies of PCV2 in the serum than pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1), pigs which received PCV2 vaccine followed by dual challenge (group 2), and pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5) at 10 and 21 dpc ($P < 0.05$; Fig. 1B). No genomic copies of PCV2 were detected in any of the serum samples from non-challenged pigs (groups 3, 7, and 9) throughout the experiment.

Quantification of PRRSV cDNA in blood.

At challenge, no genomic copies of PRRSV were detected in any of the serum samples from all 8 groups. Pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which received PRRSV vaccine followed by dual challenge (group 4), pigs which were challenged with PRRSV only (group 7), and pigs which

were challenged with both PCV2 and PRRSV (group 8) had significantly higher number of genomic copies of PCV2 in the serum than pigs which received PRRSV vaccine followed by PRRSV infection only (group 3) and pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5) at 10 and 21 dpc ($P < 0.05$; Fig. 2B). No genomic copies of PRRSV were detected in any of the serum samples from non-challenged pigs (groups 1, 6, and 9) throughout the experiment.

PCV2-specific interferon-γ-secreting cells.

No PCV2-specific IFN-γ-SCs were detected in the PBMCs at -28 dpc in any of the pigs. PCV2-specific IFN-γ-SCs were detected in the PBMCs at 0 dpc in the PCV2-vaccinated pigs (groups 1, 2, and 5). Pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1), pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5), and pigs which were challenged with PCV2 only (group 6) had significantly higher number of PCV2-specific IFN-γ-SCs than pigs which received PRRSV vaccine followed by dual challenge (group 4) and pigs which were challenged with both PCV2 and PRRSV (group 8) at 10 and 21 dpc ($P < 0.01$; Fig. 1C).

PRRSV-specific interferon-γ-secreting cells.

No PRRSV-specific IFN-γ-SCs were detected in the PBMCs at -28 dpc in any of the pigs. PRRSV-specific IFN-γ-SCs were detected in the PBMCs at 0 dpc in the PRRSV-vaccinated pigs (groups 3, 4, and 5). Pigs which received PRRSV vaccine followed by

PRRSV infection only (group 3) and by dual challenge (group 5) had significantly higher number of PRRSV-specific IFN-γ-SCs than pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which received PRRSV vaccine followed by dual challenge (group 4), pigs which were challenged with PRRSV only (group 7), and pigs which were challenged with both PCV2 and PRRSV (group 8) at 10 and 21 dpc ($P < 0.01$). Pigs which received PCV2 vaccine followed by dual challenge (group 2) and pigs which were challenged with PRRSV only (group 7) had significantly higher number of PRRSV-specific IFN-γ-SCs than pigs in 2 groups (group 4 and 8) at 21 dpc ($P < 0.01$; Fig. 2C).

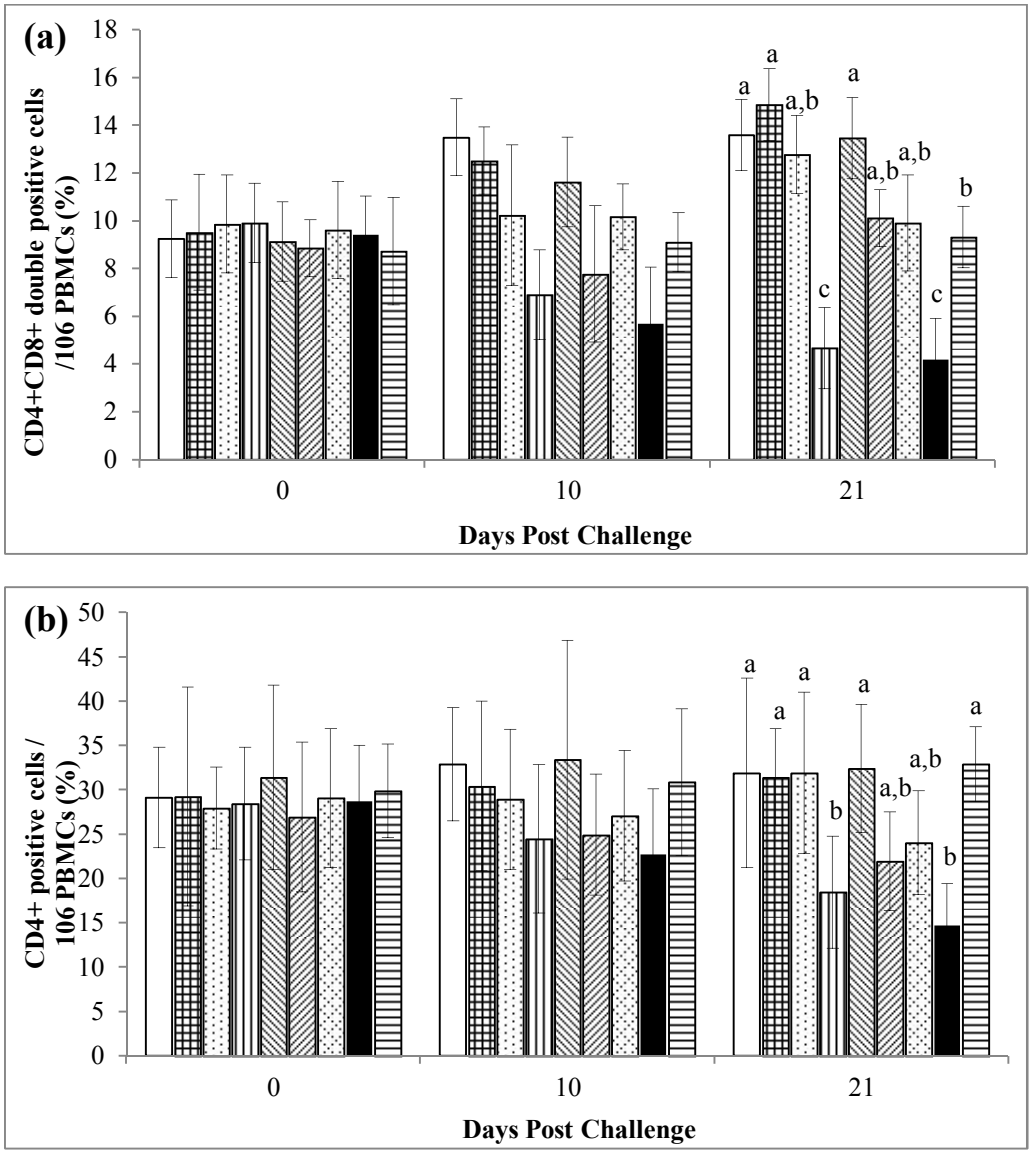


Figure 3. (a) Analysis of $CD4^{+}CD8^{+}$ double positive cell and (b) Analysis of $CD4^{+}CD8^{-}$ cell in the peripheral blood mononuclear cells (PBMCs) in the different groups; pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1, □), pigs which received PCV2 vaccine followed by dual challenge (group 2, ▦), pigs which

received PRRSV vaccine followed by PRRSV challenge (group 3, ▒), pigs which received PRRSV vaccine followed by dual challenge (group 4 ▥), pigs which received PCV2 and PRRSV vaccine followed by dual challenge (group 5, ▧), pigs which were challenged with PCV2 (group 6, ▨), pigs which were challenged with PRRSV (group 7, ▩), pigs which were challenged with PCV2 and PRRSV (group 8, ■), and negative control pigs (group 9, ▤). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.

Identification of $CD4^+CD8^+$ double positive cells.

Pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1), pigs which received PCV2 vaccine followed by dual challenge (group 2), and pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5) had significantly higher proportion of $CD4^+CD8^+$ double positive cells than pigs which received PRRSV vaccine followed by dual challenge (group 4), pigs which were challenged with both PCV2 and PRRSV (group 8), negative control pigs (group 9) at 21 dpc ($P < 0.05$). Negative control pigs had significantly higher proportion of $CD4^+CD8^+$ double positive cells than pigs which received PRRSV vaccine followed by dual challenge (group 4) and pigs which were challenged with both PCV2 and PRRSV (group 8) at 21 dpc ($P < 0.05$; Fig. 3A).

Pigs which received PCV2 vaccine followed by PCV2 challenged only (group 1) and pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs

which received PRRSV vaccine followed by PRRSV infection only (group 3), pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5), and negative control pigs (group 9) had significantly higher proportion of $CD4^+$ cells than pigs which received PRRSV vaccine followed by dual challenge (group 4) and pigs which were challenged with both PCV2 and PRRSV (group 8) at 21 dpc ($P < 0.03$; Fig. 3B).

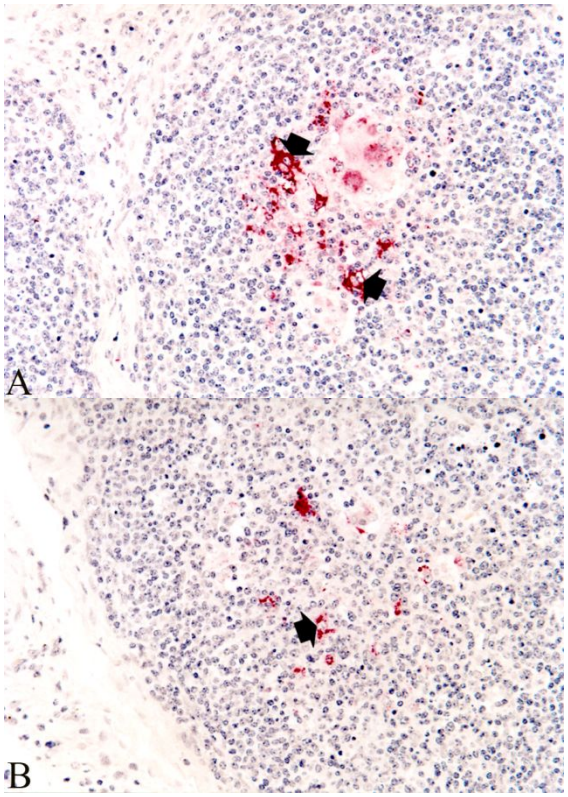


Figure 4. Immunohistochemistry for the detection of porcine circovirus type 2 (PCV2) antigen in lymph node. (a). PCV2 antigens (arrows) were detected in granulomatous inflammation of lymph node from pigs which received PRRSV vaccine followed by dual challenge (group 4) at 21 days post challenge (dpc). (b). PCV2 antigens (arrow) were occasionally detected in macrophages of lymph nodes from pigs which were challenged with PCV2 (group 6) at 21 dpc.

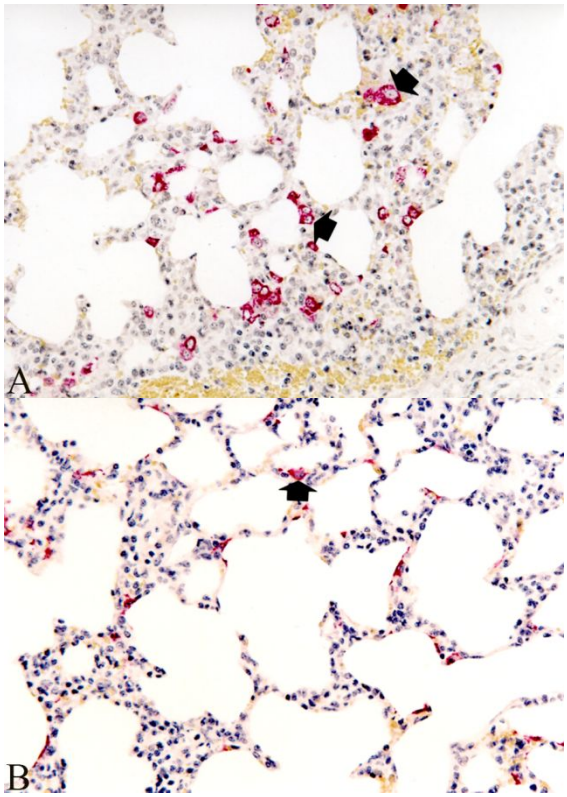


Figure 5. Immunohistochemistry for the detection of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in lung. (a). PRRSV antigens (arrows) were detected in macrophages in severe thickened alveolar septa of lung from pigs which received PRRSV vaccine followed by dual challenge (group 4) at 21 days post challenge (dpc). (b). PRRSV antigens (arrow) were detected occasionally in macrophages in mild thickened alveolar septa of lung from pigs which received PRRSV vaccine followed by PRRSV challenge (group 3) at 21dpc.

Table 2. Histopathologic lesions and antigens of porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) in different groups at 21 days post challenge (dpc).

Groups	Histopathology		PCV2 antigen		PRRSV antigen	
	Lung	Lymph node	Lung	Lymph node	Lung	Lymph node
1	0.5±0.53^{b}	0.37±0.51$_{c}$	7±5.09^{b}	17±6.05^{c}	-	-
2	2.12±0.64^{a}	0.62±0.74$_{c}$	6.5±5.44$_{b}$	20±6.87^{c}	26.5±5.44^{b}	4.5±2.03
3	0.87±0.64^{b}	0.5±0.53^{c}	-	-	14.75±4.03^{c}	5.5±1.29
4	2.75±0.7^{a}	3.87±0.83$_{a}$	44±5.29^{a}	61±8.2^{a}	40.5±3.69^{a}	6.5±2.08
5	0.4±0.23^{b}	0.45±0.42$_{c}$	8±6.91^{b}	22±10.28^{c}	16.5±4.38^{c}	4±2.63
6	0.75±0.7^{b}	2.12±0.64$_{b}$	13.5±5^{b}	43.5±8.69^{b}	-	-
7	2.25±0.7^{a}	0.5±0.53^{c}	-	-	27.75±3.77^{b}	8.5±2.64
8	2.87±0.64^{a}	4.25±0.7^{a}	53±4.24^{a}	67.5±8.38^{a}	41.75±5.9^{a}	7.5±2.64
9	0.25±0.46^{b}	0.25±0.46$_{c}$	-	-	-	-

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

Histopathologic lesion score.

The typical granulomatous inflammatory reaction and lymphoid depletion that is associated with PCV2 infection in pigs, which is consistent with histopathologic lesions in PCVAD, was observed in the lymph nodes from pigs which received

PRRSV vaccine followed by dual challenge (group 4; Fig. 4A) and pigs which were challenged with both PCV2 and PRRSV (group 8). Pigs in these groups (4 and 8) had significantly higher lymphoid lesion scores than pigs in the other 6 groups (1, 2, 3, 5, 6 [Fig. 4B], and 7) ($P < 0.03$). Pigs which were challenged with PCV2 only (group 6) had significantly higher lymphoid lesion scores than pigs in the other 5 groups (1, 2, 3, 5, and 7; $P < 0.03$). No histopathologic lymph node lesions were observed in the negative control pigs (group 9).

The pulmonary histopathologic lesions were characterized by alveolar proteinaceous and karyorrhectic debris that were interspersed with macrophages. The alveolar septa in the pigs which were challenged with both PCV2 and PRRSV (group 8) were thickened with increased numbers of interstitial macrophages and lymphocytes. Pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which received PRRSV vaccine followed by dual challenge (group 4; Fig. 5A), pigs which were challenged with PRRSV only (group 7), and pigs which were challenged with both PCV2 and PRRSV (group 8) had significantly higher pulmonary lesion scores than pigs in the other 4 groups (1, 3 [Fig. 5B], 5, and 6; $P < 0.05$) at 21 dpc (Table 2). The lungs of the negative control pigs (group 9) were normal.

Immunohistochemical score.

Pigs which received PRRSV vaccine followed by dual challenge (group 4; Fig. 4A) and pigs which were challenged with both PCV2 and PRRSV (group 8) had significantly higher number of PCV2-positive cells per unit area in the lymph node and

lung than pigs which received PCV2 vaccine followed by PCV2 challenge only (group 1), pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5), and pigs which were challenged with PCV2 only (group 6 [Fig. 4B]; $P < 0.05$). Pigs in group 6 had significantly higher number of PCV2-positive cells per unit area in the lymph node and lung than pigs in groups 1, 2, and 5 ($P < 0.05$).

Pigs which received PRRSV vaccine followed by dual challenge (group 4) and pigs which were challenged with both PCV2 and PRRSV (group 8) had significantly higher number of PRRSV-positive cells per unit area in the lung than pigs which received PCV2 vaccine followed by dual challenge (group 2), pigs which received PRRSV vaccine followed by PRRSV challenge only (group 3; Fig. 5B), pigs which received both PCV2 and PRRSV vaccines followed by dual challenge (group 5), and pigs which were challenged with PRRSV only (group 7; $P < 0.02$).

DISCUSSION

The goal of the present study was to determine the effects of the PCV2 and PRRSV vaccinations in pigs that were challenged with PCV2 or PRRSV or both. Our study focused on the PRRSV vaccine-PCV2-PRRSV and PCV2 vaccine-PCV2-PRRSV interactions. Under the single challenge model, the PCV2 and PRRSV vaccines reduced PCV2- and PRRSV- viremia in pigs, respectively. However, it was surprising that the reduction of those two viruses in the blood by each of the vaccines was different in the dual challenge model than in the single challenge model. Theoretically, one way to minimize the effect of the PRRSV-associated enhancement of the replication of PCV2 and the induction of PMWS may be the PRRSV-based vaccination of preweaned pigs in PCV2/PRRSV-co-infected herds. Interestingly, pigs which received PRRSV vaccine followed by dual challenge increased significantly PCV2-viremia, PCV2-induced lesion, and PCV2 antigen than pigs which challenged with PCV2 only but did not significantly differ from pigs which challenged with both PCV2 and PRRSV. Hence, PRRSV vaccine-PCV2-PRRSV model suggests that the PRRSV vaccine and/or by PRRSV itself could potentially result in the enhancement of PCV2 replication rather than in its control.

Pigs have a prominent $CD4^+CD8^+$ double positive cells (reportedly up to 60% of total lymphocytes) population in the peripheral blood (Saalmuller et al, 1987). These $CD4^+CD8^+$ double positive cells are memory and effector cells, and have the capacity to produce large amount of IFN-γ. Hence, these double positive cells might play a role in protective immunity against viral diseases (Rodriques-Carreno et al, 2002).

Although depletion of $CD4^+CD8^+$ double positive cells by PCV2 was not demonstrated directly, enhancement of PCV2 replication may impair the immune system. Once the replication of PCV2 reaches high levels, the numbers of $CD4^+CD8^+$ double positive cells and PRRSV-specific IFN-γ-SCs were significantly reduced in pigs which received PRRSV vaccine and followed by dual challenge at 10 dpc. The decreased numbers of $CD4^+CD8^+$ double positive cells and PRRSV-specific IFN-γ-SCs are significant because PRRSV-specific IFN-γ-SCs were predominantly $CD4^+CD8^+$ double positive cells (Mateu and Diaz, 2008) and played key roles in the protective cell-mediated immunity against PRRSV infection (Zuckermann et al, 2007; Meier et al, 2003).

In the present study, pigs which received PRRSV vaccine followed by dual challenge increased significantly PRRSV viremia, PRRSV-associated lesions and PRRSV antigens than pigs which challenged with PRRSV only. The impairment of the protective cell-mediated immunity against PRRSV by PCV2 in the PRRSV vaccine-PCV2-PRRSV model may allow for the increase in PRRSV viremia, PRRSV-associated lesions, and PRRSV antigens rather than their reduction following the PRRSV vaccine. Because these 3 parameters were significantly reduced in pigs which received PRRSV vaccine and followed by PRRSV challenge only compared with pigs which challenged with PRRSV only, our results ruled out its possible limited efficacy against a heterologous virus. Regarding the efficacy, it is clear that the modified-live PRRS vaccine offers protection against the re-infection of homologous and in some cases heterologous viruses (Lunney et al, 2010).

Regardless of the PRRSV challenge, PCV2 vaccination induces high levels of NA and PCV2-specific IFN-γ-SCs, which are important protective immune parameters for the control of PCV2 infection (Mateu and Diaz, 2008; Chae, 2012; Fort et al, 2009; Opriessnig et al, 2009). The induction of protective immunity significantly reduces PCV2 viremia, PCV2-induced lesions, and PCV2 antigens in both pig which received PCV2 vaccine and followed by PCV2 challenge only and pigs which challenged with both PCV2 and PRRSV compared with pigs which challenged with PCV2 only. Our results suggest that the PCV2 vaccination is able to reduce the levels of PCV2 viremia in pigs regardless of the PRRSV infection status as was reported in a previous study (Opriessnig et al, 2008). Our results may explain the reason why the PCV2 vaccine is effective under field conditions where PCV2 and PRRSV are widespread in the pig population, and, accordingly, it is likely that most herds in which PCV2 vaccines are being used are infected with PRRSV (Fachinger et al, 2008). In contrast, the levels of PRRSV viremia are significantly higher in pigs which received PRRSV vaccine followed by dual challenge compared with pigs which were challenged with PRRSV only. These results suggest that the reduction of PRRSV viremia by the PRRSV vaccine is affected by the pig's PCV2 infection status. Our results agree with those of previous similar studies, in which PRRSV infection at the time of PCV2 vaccination had no impact on the efficacy of the vaccine, but PCV2 infection at the time of PRRSV vaccination did affect its efficacy (Opriessnig et al, 2006; Sinha et al, 2010). Our results are clinically meaningful, indicating that swine practitioners should check PCV2 infection statuses in pigs before PRRSV vaccines are used to control PRRSV

infection in swine herds. Therefore, the control of PCV2 infections by the PCV2 vaccine may positively affect the full induction of immunity by the PRRSV vaccine in herds that are suffering from a co-infection with PCV2 and PRRSV.

In summary, vaccination against PCV2 reduced PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the dual infected pigs. However, vaccination against PRRSV increased PCV2 viremia, PCV2-induced lesions, and PCV2-antigens in the dual infected pigs. Therefore, PCV2 vaccine decreased the potentiation of PCV2-induced lesions by PRRSV in dual infected pigs. In contrary, PRRSV vaccine alone did not decrease the potentiation of PCV2-induced lesions by PRRSV in dual infected pigs.

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Chapter II. Comparison of four commercial PCV2 vaccines on pigs challenged with PCV2 and PRRSV at 17 weeks post vaccination to control porcine respiratory disease complex in the Korean field conditions

ABSTRACT

In Korean field conditions, coinfection with porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) is most commonly observed in porcine respiratory disease complex (PRDC). Despite the wide use of PCV2 vaccination, PRDC remains a serious respiratory problem. Thus, the objective of this study was to determine and compare the efficacy of 4 one-dose PCV2 vaccines on 3-week-old pigs with an experimental PCV2-PRRSV challenge at 17 weeks post vaccination. Regardless of which commercial PCV2 vaccine was used, vaccination of piglets at 3 weeks of age was efficacious against co-challenge of PCV2 and PRRSV based on growth performance and PCV2-associated lesions. However, the inactivated chimeric PCV2 1-2 and PCV2 vaccine induced higher PCV2-specific neutralizing antibody titers and PCV2-specific interferon-γ-secreting cells and lower PCV2 viremia compared to the two PCV2 subunit vaccines. PCV2 vaccination of piglets at 3 weeks of age was effective in reducing PCV2 viremia and PCV2-associated lesions during the finishing period, which is an age frequently affected by PRDC caused by co infection with PCV2 and PRRSV in Korean field conditions.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is associated with a number of diseases and syndromes that are collectively referred to as porcine circovirus-associated diseases (PCVAD).Among them, post weaning multisystemic wasting syndrome (PMWS) and porcine respiratory disease complex (PRDC) are the most important(Chae, 2004; 2005). Porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive failure in gilts and sows, and severe respiratory disease in nursery and growing-finishing pigs (Zimmerman et al, 2012).

In current Korean fields, PRDC is an important economic problem in growing and finishing pigs typically around 16 to 22 weeks of age. Co-infection with PCV2 and PRRSV is most commonly observed in field cases (Kim et al, 2003). PCV2b and North American PRRSV are the most commonly circulating genotype in herds (Kim et al, 2003; Lee et al, 2010). Despite the wide use of PCV2 vaccination, the incidence of PRDC remains high. In a European field study, vaccination against PCV2 alone can significantly improve the overall growth performance in herds that are suffering from PRDC caused by a co-infection with PCV2 and PRRSV (Fachinger et al, 2008). Hence, it is necessary to determine whether vaccination against PCV2 alone can control PRDC, which is caused by coinfection with PCV2 and PRRSV at finishing period. This is important because PCV2 vaccination was administered to approximately 95.5% of all piglets farrowed in last 3 years after implementation of the federal government subsidiary program (Chae, 2012a).

Currently, 4 commercial one-dose PCV2 vaccines are available in the Korean market (Chae, 2012a). As these vaccines differ in their antigens: whole PCV2, chimeric PCV1-2, and a baculovirus-expressed subunit based on open reading frame 2 of PCV2 (Chae, 2012b), the objective of this study was to determine and compare the efficacy of 4 one-dose PCV2 vaccines on pigs with an experimental PCV2-PRRSV challenge at 17 weeks post vaccination to mimic Korean field conditions.

METHODS

Experimental design.

A total of 60 colostrum-fed, cross-bred, conventional piglets were purchased at 14 days of age from a PRRSV-free commercial farm which was positive for PCV2 and *Mycoplasma hyopneumoniae*. Selected piglets were negative for PCV2, PRRSV, and *M.hyopneumoniae*by routine serological testing and for PCV1-2 and PCV2 by real-time polymerase chain reaction (PCR), as previously described (Shen et al, 2010; Gagnon et al, 2008). This study used a randomized, blinded, weight- and sex-matched, controlled design. Sixty pigs were randomly assigned into 6 groups (10 pigs per group; Table 1). Four commercial PCV2 vaccines were administered at different dosages at 3 weeks of age intramuscularly in the right side of the neck according to the manufacturer's instructions: 2.0 ml of inactivated chimeric PCV1-2 vaccine (Fostera PCV, Zoetis, Madison, NJ, group 1); 0.5 ml of inactivated PCV2 vaccine (Circovac, Merial, Lyon, France, group 2); 1.0 ml of PCV2 subunit A (Circoflex, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, group 3) and 2.0 ml of PCV2 subunit B vaccine (Porcilis PCV, MSD Animal Health, Boxmeer, The Netherlands, group 4). Phosphate buffered saline (PBS)was also given in a 2.0 ml dose at 3 weeks of age in positive (group 5)and negative (group 6) control groups.

At 17 weeks post vaccination (0 days post challenge[dpc]), the pigs in vaccinated (groups 1, 2, 3, and 4) and positive control (group 5) groups were inoculated intranasally with 3 ml each of PCV2b (strain SNUVR000463; 5th passage; 1.0 x $10^5$50% tissue culture infective dose [$TCID_{50}$]/ml) and North American PRRSV (strain

SNUVR090851; 5th passage; 1.0 x 10^5 $TCID_{50}$/ml)(Table 1).

The pigs in each group were housed separately within the facility as previously described (Kim et al, 2011). Clinical respiratory scores and rectal body temperature were recorded daily from 0 to 35 dpc as previously described (Halbur et al, 1995). Blood samples from each pig were collected by jugular venipuncture at -119, -98, -77, -56, -28, 0, 7, 14, 21, and 35dpc. The pigs were tranquilized by an intravenous injection of azaperon (Stresnil, Janssen Pharmaceutica, Beerse, Belgium) and then euthanized by electrocution for necropsy at 35dpc. All methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

Growth performance.

The live weight of each pig was measured at 3, 20, and 25 weeks of age. The average daily weight gain (ADWG; gram/pig/day) was analyzed over two time periods: (i) between 3 and 20 weeks of age and (ii) between 20 and 25 weeks of age. The ADWG during the different production stages was calculated as the difference between the starting and final weights divided by the duration of the stage.

Quantification of PCV1-2, PCV2, and PRRSV in blood.

DNA extraction from serum samples was performed using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA, USA). DNA extracts were used to quantify PCV1-2 and PCV2 genomic DNA copy numbers by real-time PCR as previously described (Shen et al, 2010; Gagnon et al, 2008). RNA was extracted from serum samples from all pigs used in this study as previously described (Wasilk et al, 2004). Real-time PCR for

PRRSV was used to quantify PRRSV genomic RNA copy numbers using RNA extracted from serum samples as previously described (Wasilk et al, 2004).

Serology.

Serum samples were tested using a commercial PCV2 IgG ELISA kit (Synbiotics, Lyon, France) and considered positive for PCV2 antibody if the titer was greater than 550, according to the manufacturer's instructions. Serum virus neutralization tests were performed using challenging PCV2b strain as previously described (Pogranichnyy et al, 2000). Neutralizing antibody (NA) titerswere expressed as the reciprocal of the highest serum dilution that completely blocked the infection in the PK15 cells compared with the virus control.

Enzyme-linked immunospot (ELISPOT) assay.

The numbers of PCV2-specific interferon-γ-secreting cells (IFN-γ-SCs) were determined in peripheral blood mononuclear cells (PBMCs) by enzyme-linked immunospot (ELISPOT) assay as previously described (Seo et al, 2012). Briefly, 96-well plates were coated with 0.5 μg/ml of mouse anti-porcine IFN-γ monoclonal antibody (5 μg/ml, MABTECH, Mariemont, OH, USA) diluted in PBS and stored at 4°C. The plates were washed five times with 200 μl of PBS. After washing, 100 μl containing 2×10^6 PBMCs in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Select Science, Bath, UK) were dispensed per well and stimulated with either 100 μl of PCV2 antigen (20 μg/ml), phytohemagglutinin (10

μg/ml, Roche Diagnostics GmbH, Mannheim, Germany) as a positive control, or PBS as a negative control for 40 h at 37℃ in a 5% humidified CO_2 atmosphere. Then, the wells were washed five times with PBS (200 μl per well). Thereafter, procedure was followed by manufacturer's instructions using commercial ELISPOT assay kit (MABTECH). The spots on the membranes were read by an automated ELISpot reader (AID ELISpot Reader, AID GmbH, Strassberg, Germany). The results were expressed as the number of responding cells per a million PBMCs.

Histopathology and immunohistochemistry.

For the morphometric analysis of histopathological lesion scores in lymph nodes and lung, three sections of each of the superficial inguinal lymph nodes and lungs were examined in a blinded manner as previously described (Kim et al, 2011, 2004; Halbur et al, 1995).Superficial inguinal lymph node sections were also examined blindly, and their scores ranged from 0 (normal; i.e., no lymphoid depletion or granulomatous replacement) to 5 (severe lymphoid depletion and granulomatous replacement) (Kim et al, 2004). Lung section were also scored for interstitial pneumonia ranging from 0 (normal) to 6 (severe diffuse) as previously described (Halbur et al, 1995).

For morphometric analyses to determine PCV2 and PRRSV antigen scores, 3 sections were cut from each of three blocks of tissue from lung and inguinal lymph nodes of each pig. The slides were analyzed using the NIH Image J 1.43m program (http://rsb.info.nih.gov/ij) to obtain the quantitative data. In each slide, 10 fields were

randomly selected and the number of PCV2-positive cells per unit area (0.25 mm^2) was determined as previously described (Halbur et al, 1995; Kim and Chae 2004).

Statistical analysis.

Prior to statistical analysis, real-time PCR and NAs data were transformed to $\log_{10}$ and $\log_2$ values, respectively. Normality of the distribution of the examined variables was evaluated by the Shapiro-Wilk test. Continuous data (rectal body temperature, PCV2 DNA, PCV2 serology, PCV2-specific IFN-γ-SCs, and PCV2 antigen score) were subjected to a one-way analysis of variance (ANOVA). If the one-way ANOVA was significant (P<0.05), pairwise testing using Tukey's adjustment was then performed. Discrete data (clinical respiratory score, and lymphoid and pulmonary lesion score) were analyzed using Chi-square and Fisher's exact tests. The Pearson's correlation coefficient was used to assess the relationship betweenPCV2 viremia, and PCV2-specific NA and IFN-γ-SCs at time points. A value of P< 0.05 was considered to be significant.

RESULTS

Growth performance.

No significant difference was observed inthe ADWG between vaccinated and negative control groups during the 3-20 week period. The ADWG of vaccinated and negative control groups was significantly higher($P< 0.05$) than that of the positive control group during the 20-25 week period. The overall growth rate (from 3 to 25 weeks of age) was not significantly different among the 6 groups (Table 1).

Table 1. Average daily weight gain (ADWG; g/pig/day), proportion of viremic pig and nasal shedder at different days post challenge (dpc), histopathological lymphoid and pulmonary lesion score, immunohistochemical porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) antigen score among groups.

		Groups					
		Fostera PCV	Circovac	Circoflex	Porcilis PCV	Positive Control	Negative Control
ADWG	3-20	609±48	609±44	604±52	609±43	601±21	610±24
(weeks)	20-25	709±26^{b}	707±38^{b}	676±28^{b}	683±37^{b}	633±28^{a}	718±31^{b}
	3-25	632±38	631±33	621±43	626±30	608±21	634±23
PCV2 viremic	7	3/10^{a}	3/10^{a}	4/10^{a,b}	3/10^{a}	8/10^{b}	0/10^{a}
pigs (dpc)	14	3/10^{a}	3/10^{a}	8/10^{b,c}	4/10^{a,b}	10/10^{c}	0/10^{a}
	21	2/10^{a,b}	2/10^{a,b}	6/10^{b,c}	3/10^{a,b}	10/10^{c}	0/10^{a}
	35	2/10^{a}	2/10^{a}	2/10^{a}	2/10^{a}	9/10^{b}	0/10^{a}
Lymphoid lesion score		0.9±0.99^{a}	1±0.94^{a}	1.8±0.91^{a}	1.1±0.99^{a}	3.3±0.82^{b}	0.4±0.51^{a}
Pulmonary lesion score		0.7±0.67^{a}	0.9±0.87^{a}	1.1±0.99^{a}	1.2±1.13^{a}	2.9±0.99^{b}	0.4±0.64^{a}
PCV2-antigen score	LN*	10.5±4.85^{b}	11.4±5.23^{b}	19.6±8.04^{b}	10.5±4.85^{b}	39.5±16.25^{c}	0^{a}
	Lung	4.4±3.62^{b}	5.5±3.59^{b}	6.1±3.47^{b}	6.1±3.47^{b}	16.9±6.29^{c}	0^{a}
PRRS-antigen score	LN*	1.9±2.18^{b}	1.6±1.71^{b}	2.4±2.27^{b}	2.3±1.7^{b}	2.7±2.21^{b}	0^{a}
	Lung	9.4±6.02^{b}	11.1±5.87^{b}	10.9±7.72^{b}	10.6±4.5^{b}	14±8.7^{b}	0^{a}

*LN = Lymph node

Different letters (a, b, c) indicate that the groups are significantly ($P< 0.05$) different from each other.

Clinical signs.

Clinical respiratory disease was not observed in the 4 vaccinated groups after challenge with PCV2 and PRRSV, whereas moderate to severe respiratory disease characterized by sneezing and increased respiratory rates was observed in the positive control group. In the positive control group, tachypnea and pronounced abdominal breathing were first observed at 5 dpc in 5 of 10 pigs. In addition, 7 pigs developed severe dyspnea and depression between 7 and 10 dpc. Mean clinical respiratory scores increased steadily in the pigs from the positive control group from 8 to 35dpc and were significantly higher ($P< 0.05$) than those of the vaccinated and negative control groups (Fig. 1A). Although pigs from the positive control group had slightly elevated rectal temperatures(ranging from 39.5 to 40^{o}C at 4 and 5 dpc), no significant differences in mean rectal temperature was observed in pigs among vaccinated, positive control, and negative control groups throughout the experiment (Fig. 1B).No dead pigs were found in any groups.

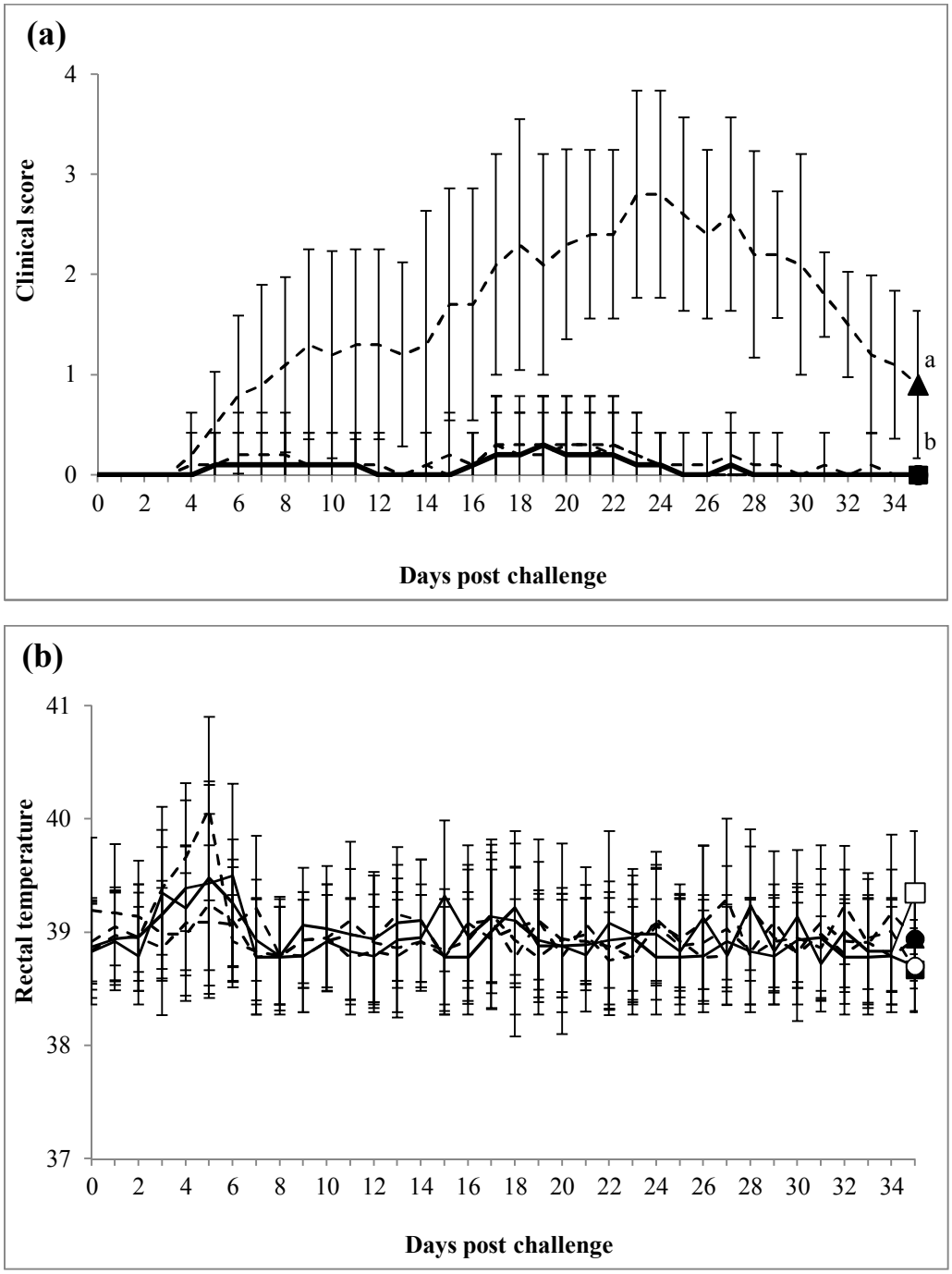


Figure 1. Mean clinical respiratory scores (a). Mean rectal body temperature (b). □,

pigs which received the inactivated chimeric PCV1-2 vaccine followed by dual challenge; ■, pigs which received the inactivated PCV2 vaccine followed by dual challenge; ○, pigs which received the PCV2 subunit A vaccine followed by dual challenge; ●, pigs which received the PCV2 subunit B vaccine followed by dual challenge; ▲, pigs which were challenged with PCV2 and PRRSV. Different letters (a and b) indicate that the groups are significantly ($P< 0.05$) different from each other on that day.

PCV2 DNA in sera.

No PCV2b DNA was detected in the blood of vaccinated or positive control groups on the day of challenge. There was a significant ($P < 0.001$) difference in log-transformed PCV2b DNA in the blood between the vaccinated and positive control groups throughout the experiment and significant ($P< 0.05$) differences were also noted among vaccinated groups (Fig. 2A). At 14 and 21 dpc, pigs vaccinated with the inactivated chimeric PCV1-2 and PCV2 vaccines had a significantly lower number of genomic copies of PCV2b($P< 0.05$) in their blood than the pigs vaccinated with the PCV2 subunit A vaccine(Fig. 2A). No PCV2b DNA was detected in the blood of the negative control group throughout the experiment. No PCV1-2or PCV2a was detected in the blood of any of the 6 groups throughout the experiment.

PRRSV RNA in sera.

PRRSV RNA was not detected in the blood of pigs from vaccinated or positive control groups on the day of challenge but was detected in the blood of the same pigs at 7 dpc. The number of genomic copies of PRRSV was gradually increased until 14 dpc and thereafter, decreased until 35 dpc. However, no significant difference in log transformed PRRSV RNA was observed in the blood between the vaccinated and positive control groups throughout the experiment (Fig.2B). No PRRSV RNA was detected in the blood of the negative control group throughout the experiment.

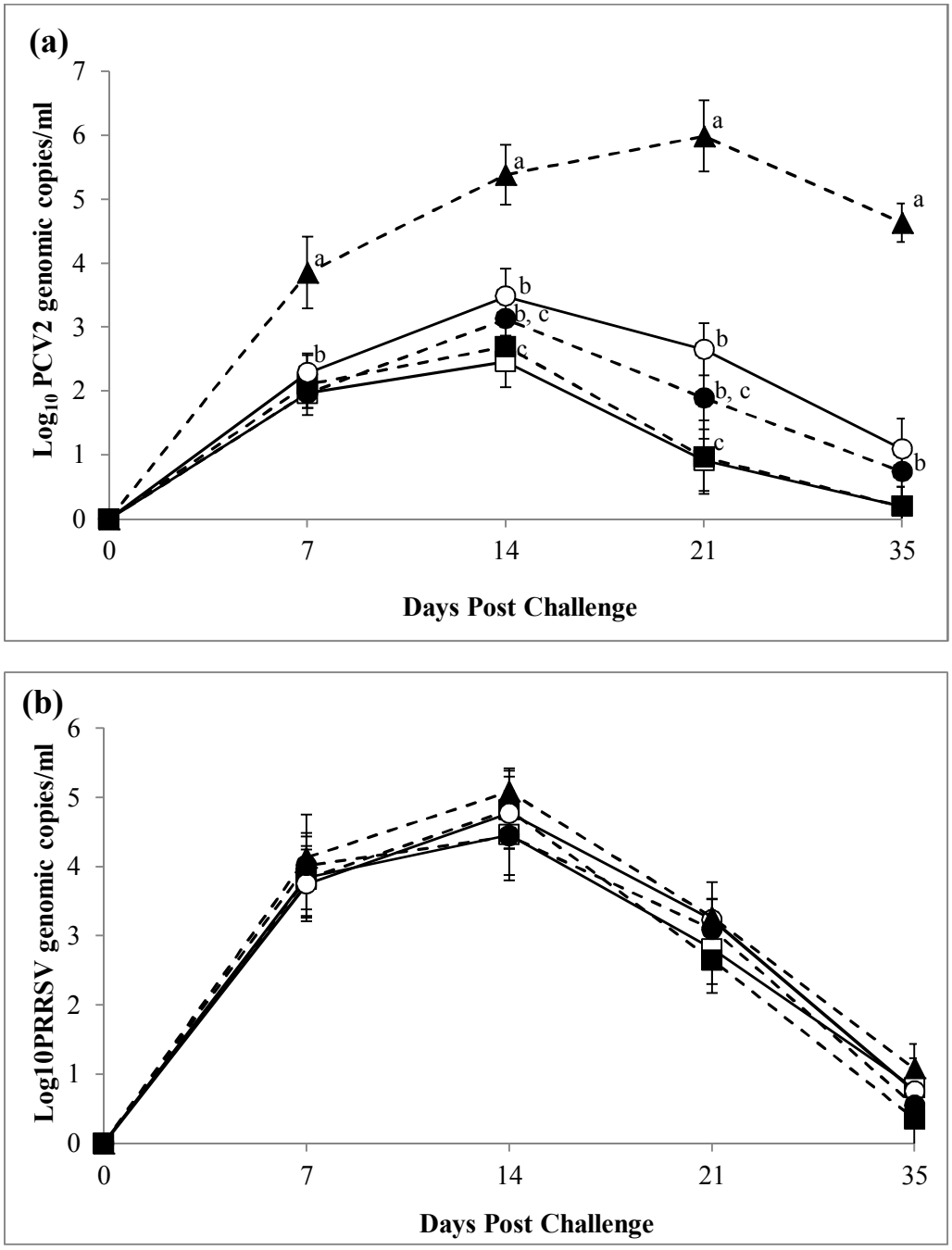


Figure 2. Mean genomic copy numbers of PCV2 DNA (a). Mean genomic copy numbers of PRRSV RNA (b). □, pigs which received the inactivated chimeric PCV1-2

vaccine followed by dual challenge; ■, pigs which received the inactivated PCV2 vaccine followed by dual challenge; ○, pigs which received the PCV2 subunit A vaccine followed by dual challenge; ●, pigs which received the PCV2 subunit B vaccine followed by dual challenge; ▲, pigs which were challenged with PCV2 and PRRSV. Different letters (a, b, c) indicate that the groups are significantly ($P< 0.05$) different from each other on that day.

Anti-PCV2 IgG antibodies.

At 21 days post vaccination, pigs vaccinated with inactivated PCV1-2 and PCV2 vaccines exhibited significantly higher levels of anti-PCV2 IgG antibodies than pigs receiving PCV2subunit A and B vaccines($P< 0.05$). At 7 and 14 dpc, pigs vaccinated with the inactivated chimeric PCV1-2 and PCV2vaccinesexhibited significantly higher levels of anti-PCV2 IgG antibodies than pigs receiving the PCV2 subunit A vaccine ($P< 0.045$). There was a significant ($P< 0.001$) difference in anti-PCV2 IgG antibodies between vaccinated and positive control groups from -119 to 21 dpc (Fig. 3A). No anti-PCV2 IgG antibodies were detected in the negative control group throughout the experiment.

PCV2-specific neutralizing antibodies.

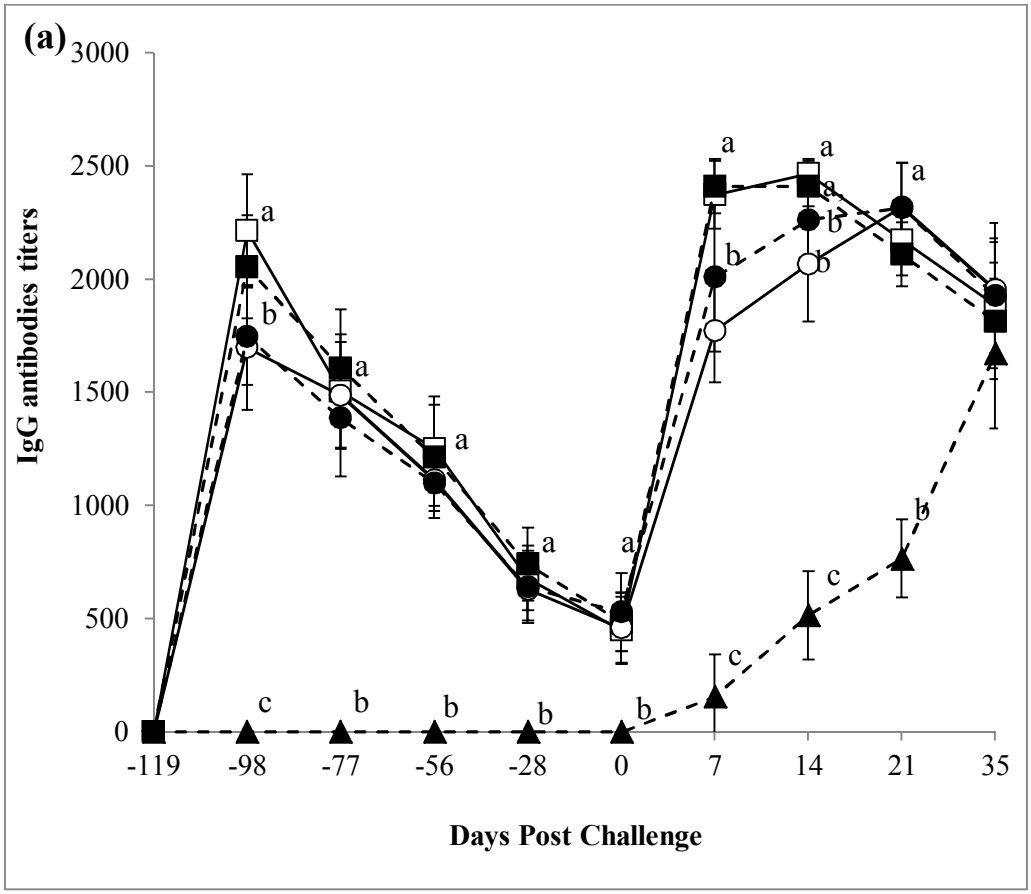
There was a significant($P< 0.001$) difference in the $\log_2$ transformed group mean NA titers between vaccinated and positive control groups throughout the experiment, except at 0dpc.Significant ($P< 0.05$) differences were also noted among different PCV2 vaccines. At -77 and 7 dpc, pigs vaccinated with inactivated PCV1-2 and PCV2 vaccines exhibited significantly higher log transformed group mean NA titers than pigs vaccinated with the PCV2 subunit A or B vaccine ($P< 0.05$). At 14 dpc, the inactivated chimeric PCV1-2 and PCV2 vaccines induced significantly higher log transformed group mean NA titers ($P< 0.05$) than the PCV2 subunit A vaccine (Fig.3B).In vaccinated and control groups, the log transformed group mean NA titers inversely correlated with the number of genomic copies of PCV2 in the blood (21dpc: r = -0.945,

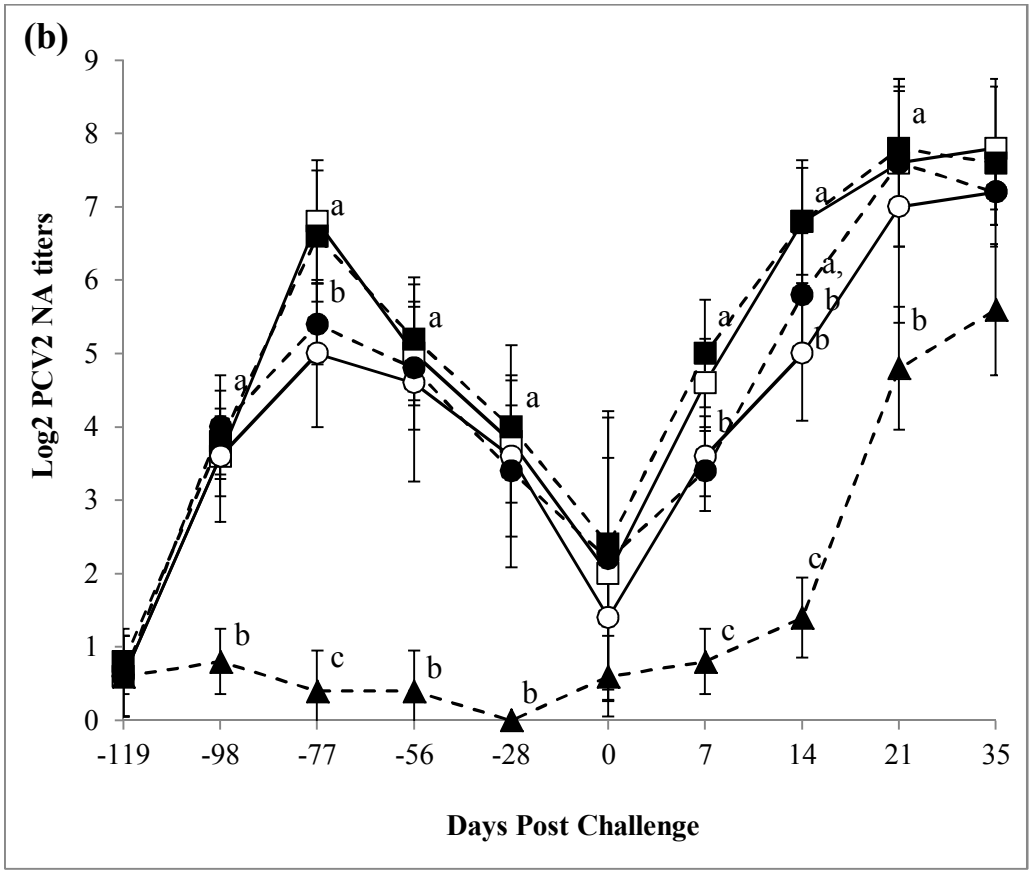
P = 0.015, 35dpc: r= -0.928, *P* = 0.023 for inactivated chimeric PCV1-2 vaccine; 21dpc: r= -0.944, *P* = 0.016, 35dpc: r = -0.928, *P* = 0.023 for inactivated PCV2 vaccine; 21dpc: r = -0.894, *P* = 0.041, 35dpc: r= -0.943,*P* = 0.016 for PCV2 subunit A vaccine;21dpc: r= -0.924, *P* = 0.025, 35dpc : r= -0.922, *P* = 0.026 for PCV2 subunit B vaccine and 35dpc: r = -0.890, *P* = 0.018 for positive control).

PCV2-specificinterferon-γ-secreting cells.

There was a significant(*P*< 0.001) difference in the group mean number of PCV2-specific IFN-γ-SCs between vaccinated and positive control groups throughout the experiment except at -119, -28, 0, and 35dpc.Significant (*P*< 0.05) differences were also noted among PCV2 vaccines. At -98dpc, the inactivated chimeric PCV1-2 vaccine induced a significantly higher number of PCV2-specific IFN-γ-SCs than the PCV2subunit A and B vaccines. At 7dpc, the inactivated chimeric PCV1-2 vaccine induced a significantly higher number of PCV2-specific IFN-γ-SCs than the other 3 vaccinated groups (*P*< 0.05). At -77 and 14 dpc, the inactivated chimeric PCV1-2 and PCV2 vaccines induced significantly higher numbers of PCV2-specific IFN-γ-SCs(*P*< 0.05) than the other 2 vaccines (Fig. 3C). In vaccinated and control groups, the group mean number of PCV2-specific IFN-γ-SCs correlated inversely with the number of genomic copies of PCV2 in the blood (21dpc: r = -0.633, *P* = 0.050 for inactivated chimeric PCV1-2 vaccine; 21dpc : r = -0.684, *P* = 0.029 for inactivated PCV2 vaccine; 21dpc: r = -0.826, *P* = 0.003 for PCV2 subunit A vaccine; 21dpc : r = -0.784, *P* =

0.007 for PCV2 subunit B vaccine and 35dpc: r = -0.740, P = 0.023 for positive control).


(a)
IgG antibodies titers
3000
2500
2000
1500
1000
500
0
-119
-98
-77
-56
-28
0
7
14
21
35
Days Post Challenge


(b)
Log2 PCV2 NA titers
Days Post Challenge
-119
-98
-77
-56
-28
0
7
14
21
35
0
1
2
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4
5
6
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9

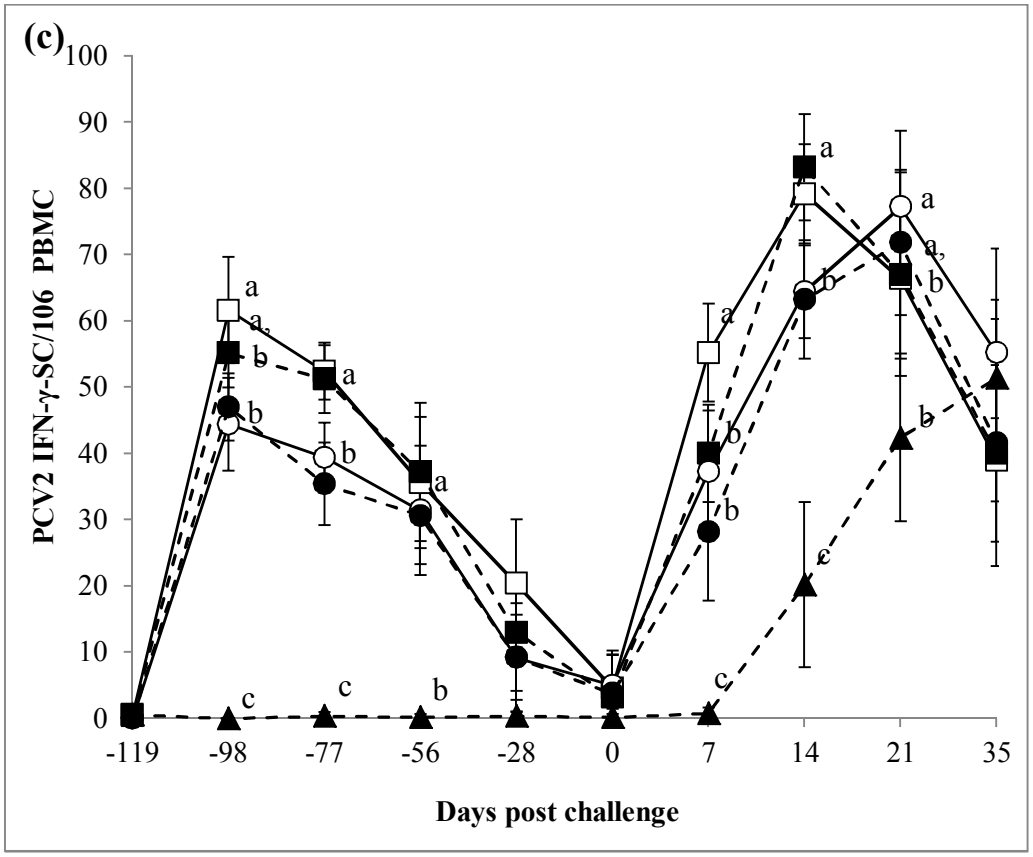

Figure 3. Mean anti PCV2 IgG antibodies titers (a). Mean serum NA titers against PCV2 in serum samples (b). Mean numbers of PCV2-specific IFN-γ-SC in PBMC (c). □, pigs which received the inactivated chimeric PCV1-2 vaccine followed by dual challenge; ■, pigs which received the inactivated PCV2 vaccine followed by dual challenge; ○, pigs which received the PCV2 subunit A vaccine followed by dual challenge; ●, pigs which received the PCV2 subunit B vaccine followed by dual challenge; ▲, pigs which were challenged with PCV2 and PRRSV. Different letters (a, b, c) indicate that the groups are significantly ($P< 0.05$) different from each other on that day.

Histopathology and immunohistochemistry.

The histopathological lymphoid and pulmonary lesion scores induced by coinfectionwithPCV2 and PRRSV were significantly lower ($P< 0.05$) in the vaccinated groups than in the positive control group (Table 1). There were no histopathological lymphoid or pulmonary lesions in the negative control pigs. PCV2 and PRRSV antigens were detected in macrophages in the lymph node and lung. There were significantly different PCV2-antigen scores($P< 0.001$) between vaccinated and positive control groups (Table 1). There were no significantly different PRRSV-antigen scores between vaccinated and positive control groups. No PCV2- and PRRSV-antigens were detected in the lung and lymph nodes of the negative control group.

DISCUSSION

Swine producers are interested in controlling PCV2-associated PRDC to reduce the fattening period from birth to slaughter for economical benefit. Additionally, the longer the fattening period, the greater the chance of developing PRDC in herds. Therefore, the most critical parameter for evaluating the efficacy of PCV2 vaccines under field conditions is the comparison of growth performance. Regardless of which commercial PCV2 vaccine was used, PCV2 vaccination of piglets at 3 weeks of age was effective in improving growth performance (as measured by ADWG) during the finishing period after co-challenge with PCV2 and PRRSV. We do not know why some swine herds still have PCV2-associated PRDC even when PCV2 vaccines are administered into same aged pigs. Since PRDC is multifactorial disease, efficacy of the PCV2 vaccine in the fields can be affected by many factors including: environment, feed, pig source, farm facility, management, and production system (i.e., all-in/all-out, continuous production, and multiple site production).

Currently, all commercial PCV2 vaccines used worldwide are based on the PCV2a genotype (Chae, 2012b). Vaccination with 4 single-dose commercial PCV2a-based vaccines used in this study was able to reduce PCV2 viremia and PCV2-associated lymphoid lesions in experimental PCV2b challenge situations. These results are further supported by the cross-protection of PCV2a-based vaccines against a PCV2b challenge (Shen et al, 2010; Opriessnig et al, 2009; Fort et al, 2008; Seo et al, 2012). This cross-protection is clinically significant because PCV2b is the predominant genotype

circulated in most Korean herds (Kim et al, 2011).

In the challenge model, coinfection with PCV2 and PRRSV induces prolonged and unusually severe clinical respiratory disease, which is similar to typical PRDC. The most striking and consistent microscopic lesions were severe interstitial pneumonia with some degree of peribronchial and peribronchiolar fibrosis as previously described (Kim et al, 2003). PCV2 vaccination of piglets at 3 weeks of age was effective in reducing PCV2 viremia and PCV2-associated lesions during the finishing period, an age of frequent outbreaks of PRDC caused by co-infection with PCV2 and PRRSV in Korean field conditions. These results extend previous findings in which the administration of either inactivated chimeric PCV1-2 or PCV2 subunit A vaccine into pigs was effective in reducing PCV2 viremia during the growth period following a triple challenge with PCV2-PRRSV-porcine parvovirus or PCV2-PRRSV-swine influenza virus at 12-13 weeks post vaccination (Shen et al, 2010; Opriessnig et al, 2009). In contrast, vaccination against PCV2 alone did not reduce PRRSV viremia in dually challenged pigs in the present and previous studies (Park et al, 2012). Although no significant differences in growth performance were observed among 4 vaccinated groups, the present study demonstrates a quantitative difference in the reduction of PCV2 viremia among the 4 vaccinated groups. The inactivated chimeric PCV1-2 and PCV2 vaccines yielded significantly lower PCV2 viremia compared to the two PCV2 subunit vaccines.

In the present study, we show that the 4 commercial single-dose PCV2 vaccines provide enough prolonged active protective immunity to control PCV2-associated

PRDC throughout the finishing period. The induction of PCV2-specific NA and IFN-γ-SCs by the PCV2 vaccine is an important protective immune response that leads to the reduction of PCV2 viremia (Fort et al, 2009; Martelli et al, 2011). Induction of PCV2-specific NAs and IFN-γ-SCs were also observed in this study; however, the 4 commercial PCV2 vaccines elicited different levels of protective immunity. The inactivated chimeric PCV2 1-2 and PCV2 vaccines induced higher PCV2-specific NA titers and PCV2-specific IFN-γ-SCs when compared to the two PCV2 subunit vaccines. We have no clear explanation for these differences in the induction of protective immunity and reduction of PCV2 viremia, however it may be due to different types of antigen and adjuvant among the 4 PCV2 vaccines (Chae, 2012b). Nevertheless, the 4 commercial PCV2 vaccines used in this study were shown to be efficacious in controlling PCV2 infection under experimental conditions (Shen et al, 2010; Fort et al, 2008, 2009; Seo et al, 2012; Opriessnig et al, 2010) as well as PCVAD under field conditions (Seo et al, 2012; Martelli et al, 2011; Fraile et al, 2012; Kixmoller et al, 2008). Hence, further studies may be needed to determine the clinical significance of different levels of PCV2 viremia in pigs vaccinated with different commercial PCV2 vaccines in the finishing period.

To the author's knowledge, this is the first comparative study for 4 single-dose PCV2 vaccines based on clinical, virological, immunological, and pathological evaluation. The conditions in this experiment were designed as to mirror those seen in the Korean fields. However, this experiment included only piglets negative for PCV2 specific antibodies and therefore did not represent the true field conditionswherePCV2

infection is common. In addition, sero-negative piglets selected for the experiments were from a PCV2 positive farm and these piglets had been fed with colostrums. Thus, some piglets may have had maternally derived antibodies (MDA) against PCV2 when immunized with the PCV2 vaccines leading to a potential interference from MDA. However, piglets with high immunoperoxidase monolayer assay (> 9 $\log_2$) or NA (> 7 $\log_2$) titers only seemed to show interference with the development of the humoral immune response after PCV2 vaccination (Seo et al, 2012; Fort et al, 2009). Hence, the results in this study were not likely to be affected by presence of MDA in the piglets. The economic impact of PRDC is important in growing-finishing pigs and concurrent PCV2 and PRRSV infection is one of the most frequent combinations found in the field. Present data provide swine practitioners and producers with useful clinical information on how to select a proper PCV2 vaccine for the control of PCV2-associated PRDC.

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국문 논문 초록

돼지 생식기 호흡기 증후군 바이러스와 돼지 2형 써코 바이러스간의 상호작용 연구

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돼지 생식기 호흡기 증후군 바이러스는 현재 양돈 농가에서 잠재하는 가장 심각한 위협 요소이다. 이 바이러스는 자돈에서 감염 초기 빠르게 증식하여 폐렴을 유발하지만 병변의 소멸과 함께 최대 130일 이상 면역 기관에 잠복해 있는 것으로 알려져 있다. 하지만 폐렴 외에도 이 바이러스의 진정한 위협요인은 써코 바이러스와의 동시 감염 시 유발하는 소모성 증후군이라 할 수 있다. 돼지 생식기 호흡기 증후군 바이러스와 돼지 써코 2형 바이러스가 동시에 감염된 경우, 돼지 써코 2형 바이러스의 증식이 현저하게 강화되는 것이 이미 확인되었다. 증가한 써코 2형 바이러스는 돼지의 수지상 세포 및 림프구와 대식세포 간세포 등에 침투하여 발열, 황달, 피부질환, 호흡기 질환, 체중 감소, 혹은 일당 증체량의 감소, 그리고 사망에 이르게 하는 것으로 알려져 있다.

본 실험의 목적은 이러한 돼지 생식기 호흡기 증후군 바이러스와 돼지 써코 2

형 바이러스 간의 상호 작용을 연구하고 더 나아가 이들이 유발하는 임상증세를 예방하기 위한 백신을 평가 선정하는 것이었다. 첫 번째 실험에서 돼지 생식기 호흡기 증후군 바이러스와 돼지 써코 2형 바이러스의 유전자형의 차이가 서로의 개체 내 증식에 영향을 미치는 지를 확인하였다. 1형과 2형으로 구분되는 돼지 생식기 호흡기 증후군 바이러스의 유전자형과 a형과 b형으로 구분되는 돼지 써코 2형 바이러스의 유전자형을 각각 4가지 조합으로 동시에 돼지에 감염시켰다. 또 대조군으로 각각의 유전자형을 단독으로 돼지에 접종한 그룹을 준비하였다. 실험 결과, 단독으로 써코 바이러스를 감염시킨 경우보다 돼지 생식기 호흡기 증후군 바이러스와 함께 돼지 써코 바이러스를 감염시킨 경우 더 많은 항원량이 혈청 및 림프 조직에서 확인되었고 병변 역시 유의적으로 더 심하였다. 그러나 동시에 두 바이러스를 감염시킨 경우에도 접종 이후 14일 경의 돼지 써코바이러스의 혈중 및 림프 조직의 항원은 2형 돼지 생식기 호흡기 증후군 바이러스와 동시에 감염시킨 경우가 1형 바이러스를 동시에 감염시킨 경우보다 유의적으로 더 많았고 더 심하였다. 반면 돼지 써코 바이러스의 감염이 돼지 생식기 호흡기 증후군 바이러스의 증식에는 영향을 미치지는 않은 것으로 확인되었다. 2형 돼지 생식기 호흡기 증후군 바이러스의 경우 1형 바이러스보다 조직 내에서 더 많은 항원량을 보이고 더 심한 간질성 폐렴을 유발하는 것으로 알려져 있다. 따라서 결론적으로 돼지 생식기 호흡기 증후군 바이러스의 균주의 종류에 따라 돼지 써코 바이러스의 증폭량이 영향을 받는 것으로 확인하였다. 그러나 역은 성립하지 않았다. 또한 돼지 생식기 호흡기 증후군 바이러스가 돼지 써코 2형 바이러스의 증폭을 유도하기 위해서는 최소한 10의 4승 이상의 혈중 바이러스 수치를 유발하여야 한다는 것을 확인하였다.

두번째 실험에서 돼지 생식시 호흡기 증후군 바이러스와 돼지 써코 2형 바이러스가 이유 자돈에 감염되었을 때 일으키는 대표적인 증상인 이유 자돈 전신 소모성 질환을 예방하기 위한 백신을 평가 하였다. 이론상 돼지 생식시 호흡기 증후군 바이러스가 돼지 써코 2형 바이러스를 증폭시키고 증폭된 이 바이러스로 인해 임상증상이 발현되므로 돼지 생식시 호흡기 증후군 바이러스를 백신으로 통제하는 것이 이유 자돈 전신 소모성 질환을 예방하는 방책이 될 수 있다, 실험 결과 돼지 써코 2형 바이러스 백신은 안정적으로 두 바이러스가 동시 감염 시에도 돼지 써코 2형

바이러스의 항원을 조직과 혈액 모두에서 낮추었고 임상 증세와 조직 병변 모두에서 질환을 예방하는 것이 확인되었다. 그러나 돼지 생식시 호흡기 증후군 바이러스 백신을 접종한 경우 두 바이러스가 동시에 감염되면 돼지 생식시 호흡기 증후군 바이러스와 돼지 써코 바이러스의 수치 모두가 증가할 뿐 아니라 증폭된 돼지 써코 바이러스에 의해 돼지 생식시 호흡기 증후군 바이러스에 대항하는 체액성 항체 반응 및 세포성 면역 반응 수치가 현저하게 떨어진 것으로 확인되었다. 이는 백신의 부족한 방어 효능과 밀접한 연관이 있었다. 돼지 생식시 호흡기 증후군 바이러스 백신은 혈중 바이러스의 수치를 써코 바이러스를 증폭시킬 수 있는 최소치인 10의 4승 이하로 낮추지 못하였고 증폭된 써코바이러스로 인해 기존의 돼지 생식시 호흡기 증후군 바이러스에 대항하는 효능마저 감소한 것으로 확인하였다.

세번째 실험으로 비육 말기 육성돈에서 발생하는 돼지 호흡기 복합질병을 예방하긴 위한 상용호된 돼지 써보 바이러스 백신의 효능을 비교 평가하였다. 돼지 호흡기 복합질병은 농가에서 다양한 병원체가 비육말기 돼지에서 항체가가 떨어지는 시점에 재감염되어 유발되는 질병으로 돼지 써코바이러스가 주요 원인체로 알려져 있다. 또 돼지 생식시 호흡기 증후군 바이러스 역시 돼지 써코 바이러스를 증폭시키는 인자로서 이 때 작용하는 것으로 알려져 있다. 앞선 실험을 통해 확인된 바와 같이 써코바이러스의 질환을 예방하기 위해서는 써코바이러스 백신을 사용해야만 하였다. 실제 백신 접종 시점인 3주령 자돈을 그 뒤 17주 뒤인 20주령에 공격접종하여 백신의 방어효능이 이유기부터 비육 말기까지 유지되는 지를 평가하였다. 실험결과 현재 사용화된 4가지 백신 제품 모두 17주간 방어 효능을 제공하는 것으로 확인되었다. 항체가 및 세포성 면역 반응 수치가 모두 유지 되었고 공격 접종에 대응하는 안정적인 반응을 보였다 그러나 상용화된 백신 간의 효능에는 증가한 면역학적인 수치와 감소한 바이러스 항원량에 있어서 유의적인 차이가 있는 것으로 확인되었다.

결론적으로 돼지 생식시 호흡기 증후군 바이러스에 의한 돼지 써코바이러스의 증폭은 생식시 호흡기 증후군 바이러스 균주의 증식과 밀접한 관련이 있다. 더 빠르고 강하게 증식하는 균주가 써코바이러스의 더 높은 증폭을 유발하는 것으로 확인하였다. 또 이 두 바이러스의 상호작용으로 인한 질환을 예방하기 위해서는 생식

시 호흡기 증후군 바이러스 백신이 아닌 써코 바이러스 백신을 사용해야 하며 이는 생식시 호흡기 증후군 바이러스 백신의 부족한 효능 때문인 것으로 확인하였다. 써코 바이러스 백신은 이유기부터 비육 말기까지 안정적인 방어 효능을 개체에 제공해주며 임상증세 개선 및 항원량 감소 그리고 면역 반응을 유지하는 가장 확실한 대응책으로 평가하였다.

주요어: 돼지 생식기 호흡기 증후군 바이러스; 돼지 써코바이러스 2형; 돼지 써코바이러스 관련 질병; 이유후 전신소모성 질환; 돼지 호흡기 복합질병; 돼지 써코바이러스 2형 백신; 백신 효능

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