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Evaluation of Porcine Circovirus type 2 Vaccine based on Clinical, Virological, Immunological and Pathological Analyses

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바이러스학적, 면역학적, 병리학적 분석을 통한 평가

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Evaluation of Porcine Circovirus type 2 Vaccine based on Clinical, Virological, Immunological and Pathological Analyses

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Abstract

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Porcine circovirus type 2 (PCV2) is one of the most important swine infectious pathogen which cause a variety of swine diseases called porcine circovirus associated diseases (PCVAD) such as postweaning multisystemic wasting syndrome (PMWS), porcine respiratory disease complex (PRDC), porcine dermatitis, nephropathy syndrome (PDNS), and reproductive associated disorder.Vaccination against PCV2 is nowdays the most effective tool for preventing the PCVAD. Since the first PCV2 vaccine was introduced in 2004, the six commercial PCV2 vaccine have been registered worldwide up to date. These vaccines were largely classified in inactivated whole virus vaccine, capsid protein recombinant vaccine, and chimeric PCV1/PCV2 vaccine according to antigen types.

The objectives of these studies were to evaluate the PCV2 vaccines based on clinical, virological,

immunological, and pathological analyses and investigate the difference of efficacy among the vaccines. The experimental challenge study with a reformulated inactivated chimeric PCV1-2 vaccine was perfomed for 42 days to investigate the vaccine efficacy about humoral and cell-mediated immunity. Vaccinated challenged animals had a significantly lower number of genomic copies of PCV2 in the blood than non-vaccinated challenged animals at 14 and 28 days post challenge (dpc; $P < 0.001$). The percentage of viremic pigs was significantly lower in vaccinated challenged animals compared to non-vaccinated challenged animals ($P < 0.05$). The Neutralizing antibody (NA) titers were significantly higher in vaccinated challenged and vaccinated non-challenged animals than in non-vaccinated challenged animals at 0, 14, and 28 dpc. The mean numbers of PCV2-specific IFN-r-SCs were significantly higher in vaccinated challenged and vaccinated non-challenged animals compared to non-vaccinated challenged animals at 0 and 14 dpc($P < 0.05$). The vaccinated animals displayed significantly greater PCV2-specific DTH responses than the non-vaccinated animals ($P < 0.01$). The results of the present study demonstrated the protective immunity induced by the reformulated inactivated chimeric PCV1-2 vaccine correlated with the reduction of PCV2 viremia.

The efficacy of the reformulated inactivated chimeric PCV 1-2 vaccine was evaluated under field conditions. Three farms were selected based on their history of postweaning multisystemic wasting syndrome (PMWS). On each farm, a total of 50 3-week-old pigs were randomly allocated to one of two treatment groups: (i) vaccinated at 3 weeks of age and (ii) non-vaccinated. In case of field trial, it is most important to investigate for vaccine efficacy of economical aspects. Clinical examination indicated that vaccinated animals displayed an improved average daily weight gain (ADWG) (+47.3 grams/day; $P < 0.05$) and a reduced time to market (-6 days; $P < 0.05$). Virological and pathological examinations indicated that vaccinated animals displayed a reduced PCV2 viremia and associated lesion compared to non-vaccinated animals. Immunological examination indicated that vaccinated animals induced PCV2-specific NAs and IFN-r-SCs. The vaccine not only successfully induced the humoral and cell-mediated immune response but also improved the average daily weight gain which is criteria for vaccine efficacy of production improvement.

Studies for comparion of PCV2 vaccines efficacy were performed to investigate different level of

immune responses induced by 4 commercial vaccines. These studies were divided by two experiments. The objective of first experiment was to compare the vaccines without challenge for investigating a tendancy of vaccines such as duration of persistent efficacy and time to indicate the peak level of efficacy. Inactivated chimeric PCV1-2 vaccines induced higher levels of PCV2-specific NAs and IFN-r-SCs in pigs than did the other 3 commercial PCV2 vaccines. The proportions of CD4+ cells were significantly higher in animals vaccinated with inactivated whole virus and chimeric PCV 1-2 vaccines than in animals vaccinated with the 2 subunit vaccines. Maximal induction of cell-mediated immunity is reached at 21 days post vaccination (dpv), whereas maximal induction of humoral immunity is reached at 42 dpv in all four vaccines.

In second experiment, protective efficacy induced by pcv2 vaccines were compared through a challenge of single PCV2. The groups vaccinated with inactivated whole virus and chimeric PCV 1-2 vaccines exhibited significantly higher NA titers, number of IFN-r-SCs, and proportion of CD4+ lymphocytes than the other 2 vaccinated groups ($P < 0.05$). The groups vaccinated with inactivated whole virus and chimeric PCV 1-2 vaccines reduced significantly more viral load in blood than another subunit vaccine group ($P < 0.05$). These studies demonstrated that the different types of antigens in the 4 commercial single-dose PCV2 vaccines induce different levels of protective immune responses.

The effects of PCV2 vaccines on PCV2 virus in experimentally infected boars were evaluated and compared based on virus shedding in semen. In the first experiment, the vaccinated group were immunized with an inactivated PCV2 vaccine at a 3-week interval, and then, 3 weeks after the second vaccination, the boars were intranasally inoculated with PCV2b. Serum and semen samples were collected for 60 dpc. The number of PCV2 genome copies of PCV2 in the serum and semen were significantly lower in vaccinated challenged boars than in non-vaccinated challenged boars at 7 to 60 dpc ($P < 0.05$). In the second experiment, efficacy of three commercial vaccines were compared in boars for 70 dpc. Serum and semen samples from the group vaccinated with chimeric PCV 1-2 vaccine had significantly decreased PCV2 genomic copy numbers compared with the goup vaccinated with subunit vaccine at 21 and 28 dpc ($P < 0.05$). Consequently, the vaccination protocol reduced the

amount of PCV2 DNA shed in the semen. However, there was a significantly different amount of PCV2 DNA shed in semen among the 3 vaccinated groups.

Keywords: Porcine circovirus type 2; Porcine circovirus-associated disease; Postweaning multisystemic wasting syndrome; Commercial porcine circovirus type 2 vaccines; Vaccine efficacy; Semen

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I.

Chapter 1.

Chapter 2.

PART II.

Chapter 1.

Chapter 2.

PART III.

Chapter 1.

Chapter 2.

LIST OF ABBREVIATION

APP	acute phase proteins
CDCD	Cesarean-derived, colostrum-deprived
CMI	Cell mediated immunity
DC	Dendritic cell
dpc	Days post-challenge
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
MDI	Maternally derived immunity
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 circovirus (PCV), a nonenveloped, single-stranded DNA virus with a closed-circular genome of 1.76 kb is a member of the family Circoviridae. PCV was first idenfied as a contaminant in cultures of the porcine kidney cell-line PK15. It was belived to be non-pathogenic agent because the virus did not develop cytopathic effects and associated diseases (Tischer et al., 1986; Allan et al., 1995). But the pathogenic new virus named as porcine circovirus type 2 (PCV2) was identified to induce Postweaning multisystemic wasting syndrome (PMWS) (Harding, 1996, 1998; Clark, 1997; Harding and Clark, 1997), because it was mainly developed in postweaning ages and it showed several wating clinical and histopathological sign. Isolation and identification of PCV antigen and nucleic acid in tissue of pigs with PMWS had led to suggestion that a pathogenic new PCV emerged in the many contries (Allan, 1998).

PCV2 is characterized by a small and simple genomic structure with a genome of approximately 1760 nucleotides (Mankertz et al., 1997). It is the smallest mammalian virus yet known and encode two major open reading frames (ORFs), rep and cap, which perform the main functions of the virus. The genes are mainly arranged in a Positive strand (ORF1) and a negative strand (ORF2) structures resulting in an ambisense organization of the genome (Mankertz et al., 1997).

The replication of PCV2 be done by a rolling circle replication (RCR) strategy (Navidad et al., 2008). The circular, single-stranded DNA makes a double-stranded replicative form (RF) DNA which serves as a templete for viral DNA replication after virus introduction. But the form of the double stranded DNA for starting of replication is still unknown (Navidad et al., 2008). Combination of Rep and Rep' proteins was used as a RCR initiator protein complex (Cheung, 2003).

Currently, three different phylogenetic genotypes for PCV2 are recognized, and a unified nomenclature (PCV2a, PCV2b, PCV2c) has been proposed (Segales et al., 2008). The number of PCV2a genome nucleotide is 1,768, while the genome length of PCV2b is 1,767 nucleotides (Olvera et al., 2007). Homology between the entirely nucleotide sequences of the two genotypes was 91.6 %

(Carmen et al., 2008). Each group has stretches of distinct nucleotide and amino acid sequences, and signature motifs.

PMWS causes considerable economic losses to the swine industry. The disease has a low rate of morbidity but has a high mortality rate in animals that are 5-12 weeks of age (Allan and Ellis, 2000; Chae, 2004). The most prominent clinical signs are wasting, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice (Harding 2004, 2006). Pigs with chronic wasting disease may develop cachexia, with marked muscle wasting and serous atrophy of fat (Chae, 2004; Segales et al., 2004). Microscopic lesions in lymphoid tissues are almost unique. Recent results of many field stdudy suggest that PCV2 may play an important role in the porcine respiratory disease complex (PRDC) (Harms et al., 2002; Kim et al., 2003). PRDC is a condition observed with multiple respiratory pathogens associated with Porcine reproductive and respiratory virus (PRRSV), Swine influenza virus (SIV), and bacteria such as Mycoplasma hyopneumoniae (Chae, 2005; Opriessnig et al., 2007). PDNS is characterized clinically by acute onset of skin lesions, fever, and lethargy (Chae, 2005). Other characteristic macroscopic lesions include enlarged tan, waxy appearing kidneys with petechial hemorrhages. Microscopically, there is systemic vasculitis with dermal and epidermal necrosis and necrotizing and fibrinous glomerulonephritis.

Oral and nasal excretion is considered the most likely and frequent source of PCV2 infection and transmission and, consequently, most experimental studies on PCV2 infection have used this route (Tomás et al., 2008). PCV2 has been detected in all potential routes of shedding in nasal excretions, tonsillar, bronchial and ocular secretions, faeces, saliva, and urine (Shibata et al., 2003; Segalés et al., 2005b) of both naturally PMWS-affected and asymptomatic pigs, and even in sow milk and boar semen (Larochelle et al., 2000; Shibata et al., 2006).

The transmission of PCV2 from sow to the fetus in utero were demonstrated, and transplacental transmission has been identified twice after experimental intranasal infection of pregnant sows three weeks before the expected date of farrowing. PCV2 has been also isolated in semen of naturally and experimentally infected boars, even after the appearance of antibodies in serum (Larochelle et al., 2000; Madson et al., 2009b). PCV2 is frequently detected and isolated in seminal plasma and in the

sperm and non-sperm cell fractions (Kim et al., 2003). Furthermore, it has been demonstrated that PCV2 shed in semen of experimentally virus inoculated boars can be infectious through artificial insemination.

The low levels of neutralizing antibodies could also be correlated to high levels of PCV2 replication (Meerts et al., 2005; Fort et al., 2007). Pigs with low levels of neutralizing antibodies also had low levels of total antibodies to PCV2 indicating that PMWS affected pigs have an impaired humoral response to PCV2 that subsequently results in a higher viral load. These authors also observed that the drop in the viral load in serum was concurrent with the appearance of PCV2-specific IFN-γ-secreting cells (IFN-γ-SC) and NA, suggesting that the viral clearance might be mediated by the development of PCV2-IFN-γ-SC in contribution to the PCV2-specific NA.

Different types of commercial vaccines have used to control PCV2 infection worldwide after first commercial vaccine released in 2004 (Opriessnig et al, 2007; Chae, 2012). There are two recommended vaccination strategies. The one is recommended to be given to sows, relying on the protective effects of passive transfer of maternal immunity to young piglets. The other three vaccines target piglets approximately 3 weeks of age. The use of vaccines in sows and gilts increases PCV2 antibody titres in both serum and colostrum; the transfer of the later one provides protection of piglets against PMWS development (Charreyre et al., 2005; Joisel et al., 2007).piglet vaccines are applied around 3 weeks of age or later, during the waning of maternal antibodies, eliciting PCV2 total and NA responses and reducing or delaying PCV2 infections at weaning or fattening ages (Fort et al., 2008; Opriessnig et al., 2009a).

LITERATURE REVIEW

1. Porcine Circovirus

1.1. Historical background

Porcine circovirus (PCV), a nonenveloped, single-stranded DNA virus with a closed-circular genome of 1.76 kb is a member of the family Circoviridae. PCV was first idenfied as a contaminant in cultures of the porcine kidney cell-line PK15 (Tischer et al., 1974). It was belived to be non-pathogenic agent because the virus did not develop cytopathic effects and associated diseases (Tischer et al., 1986; Allan et al., 1995). In 1991 firstly, and afterwards in 1994, a new mysterious syndrome wasdescribed in Saskatchewan and Alberta (Canada) by Dr. Edward Clark and Dr.John Harding (Harding, 1996; Clark, 1997). Affected pigs were characterized by suffering from wasting, respiratory distress, skin pallor and icterus, and the mortality was about 12-15%. Gross lesions consisted of generalized lymphadenopathy in combination with interstitial pneumonia, hepatitis, renomegaly, splenomegaly, gastric ulcers and intestinal wall oedema. Microscopic lesions were mainly lymphocyte depletion and granulomatous infiltration in lymphoid tissues (Harding and Clark, 1997). Similar wasting syndrome was identified in pigs from california, France, and spain.

The wasting disease was named Postweaning multisystemic wasting syndrome (PMWS) (Harding, 1996, 1998; Clark, 1997; Harding and Clark, 1997) because it was mainly developed in postweaning ages and it showed several wating clinical and histopathological sign. Isolation and identification of PCV antigen and nucleic acid in tissue of pigs with PMWS had led to suggestion that a pathogenic new PCV emerged in the many contries (Allan, 1998). The pathogenic new virus which induced PMWS was named as porcine circovirus type 2 (PCV2) and differenciated the non-pathogenic porcine circovirus type 1 (PCV1) (Meehan et al., 1998). PCV2 was similar nucleotide sequence with PCV1 but sequence identify was to be less than 80% the original virus. Results of retrospective serologic polymerase chain reaction (PCR) with serum from 1962 in Europe (Jacobsen et al., 2009) demonstrated PCV2 infection was already present in the swine livestock. Besides, Identification of PCV2 antigen through immunohistochemistry (IHC) in paraffin embedded lymphoid tissues collected in 1985 in Germany (Jacobsen et al., 2009), 1989 in japan (Mori et al., 2000).

1.2. Genome organization

PCV is characterized by a small and simple genomic structure with a genome of approximately 1760 nucleotides (Mankertz et al., 1997). It is the smallest mammalian virus yet known and encode two major open reading frames (ORFs), rep and cap, which perform the main functions of the virus. The genes are mainly arranged in a Positive strand (ORF1) and a negative strand (ORF2) structures resulting in an ambisense organization of the genome (Mankertz et al., 1997). The rep gene encoded two replicase proteins and the cap gene expressed the structural protein of the virus. The rep gene, a largest ORF of PCV containing around 940 nucleotides, encodes the Rep and Rep' protein. These two rep isoforms bound to stem-loop element of genome which is part of the origin of replication and lead to initiate the replication of virus (Mankertz et al., 2004).

The second largest ORF of PCV is located on the negative sense strand and expressed the capsid protein which make up for virus structure. The protein had a molecular mass of 30 kDa, similar to that detected in purified virus particles and it formed capsid-like particles when visualised by electron microscopy (Nawagitgul et al., 2000). It is generally approved that cap encodes the major structural protein of PCV. Cap is the most important antigenic determinant portion of PCV and contains an highly conserved arginine-rich N-terminus.

ORF3 located within ORF1 is transcribed counter clock wise. Expression of ORF3 was shown in PCV2 infected PK-15 cells and found to play a role in virus induced cell death by aopotosis (Liu et al., 2005). The apoptosis induced by the ORF3 of PCV2 could also have a role in the rapid spread of the virus, leading to high viral load, which is associated with the development of PCV2 associated diseases. Transfection experiments with an ORF3 deficient PCV2 mutant showed that the protein is not essential for viral replication (Karuppannan et al., 2011).

1.3. Replication dynamics

PCV2 genome might be at the interface of Geminivirus and Nanovirus genomes in the way that those viruses contained similar nucleic acid sequences (Niagro et al., 1998). The Rep protein sequence suggests that the PCV2 was orginated form a Nanovirus and Calicivirus recombination (Gibbs et al., 1999). It has been suggested that replication of PCV2 be done by a rolling circle replication (RCR) strategy (Navidad et al., 2008). The circular, single-stranded DNA makes a double-stranded replicative form (RF) DNA which serves as a templete for viral DNA replication after virus introduction. But the form of the double stranded DNA for starting of replication is still unknown (Navidad et al., 2008). Combination of Rep and Rep' proteins was used as a RCR initiator protein complex (Cheung, 2003). The RCR initiator protein cleaves the loop to generate a free 3'OH extremity and covalent binding between the complex and the 5' viral genome extremity. Cellular DNA polymerase initiates viral DNA replication from the free 3'OH end. This leads to the release of a positive circular single-stranded parental DNA molecule and a circular double-stranded DNA molecule composed of the negative parental strand and the newly synthesized positive strand. During this procedure, the single-stranded DNA molecule is encapsidated by capsid protein. The replication origin is a stem-loop structure. The nucleotide sequences of the region are AxTAxTAC which play a role in binding the REP complex for starting and termination of replication (Cheung, 2003; Faurez et al., 2009). In vitro experiments, many valuable information was generated in respect of the mechanism of viral infection and replication in different cell types. PCV2 is consistently found in the cytoplasm of monocytes, macrophages and dendritic cells (DC), but the absence of replicated double stranded DNA intermediates and infectious virus progeny indicate that replication does not take place in these cells (Gilpin et al., 2003). A specific receptor for PCV2 entry into cells through clathrin-mediated endocytosis has not yet been found, but the glucosaminoglycans heparan sulfate and chondroitin sulfate B have been identified as attachment receptors for PCV2 on monocytes (Misinzo et al., 2005, 2006). The infection of monocytic cells by PCV2 has been demonstrated to depend on the acidic environment provided through the endosome-lysosome system acidification (Misinzo et al., 2008b). In epithelial cells, however, cholroquine inhibition of the same mechanism of acidification in

the early stages of infection enhanced the replication of PCV2. In addition, enhanced PCV2 replication was observed when monocytic cells were treated with interferon (IFN)-r, suggested that it is related to an enhanced internalization of the virus (Misinzo et al., 2008a). Consequently, the characterization of PCV2 replication seem dependent on the cell type that it enters, and no specific receptor for viral uptake and entry has yet been identified.

1.4. Classfication of genotype

Several genotyping studies on PCV2 were performed after PCV2 identified (Larochelle et al., 2002; Grierson et al., 2004; Olvera et al., 2007). Although there were attempts to find genetic differences between PCV2 isolates from farms with and without clinical PMWS, no obvious differences were found. In 2006, polymerase chain reaction-restriction fragment length polymorphism (PCV-RFLP) was used for genotyping, which was used to separate the samples into RFLP type 422 and 321 (Carman et al., 2008). Investigation of PCV2 genotype was first attempted with an evaluation of the phylogenetic trees, using the cap and the rep genes as molecular markers, and an assessment of potential PCV2 clonality was conducted (Olvera et al., 2007). In a study of the phylogenetic tree in 2007, PCV2b comprised PCV2 strains from European contries and Asian strains, were included in the PCV2a category (Grau-Roma et al., 2007). In 2007, a Danish case study showed that the PCV2 nucleotide sequence had changed over time and found a new type of PCV2 from serum samples collected in 1980s, which was designated PCV2c (Dupont et al., 2008). Currently, three different phylogenetic genotypes for PCV2 are recognized, and a unified nomenclature (PCV2a, PCV2b, PCV2c) has been proposed (Segales et al., 2008). The number of PCV2a genome nucleotide is 1,768, while the genome length of PCV2b is 1,767 nucleotides (Olvera et al., 2007). Homology between the entirely nucleotide sequences of the two genotypes was 91.6 % (Carmen et al., 2008). Each group has stretches of distinct nucleotide and amino acid sequences, and signature motifs. Analysis of nucleotide sequence for the cap gene of PCV2 strains from PMWS ffected and non-affected herds revealed consistent detection of PCV2b, while the later reported exclusively PCV2a (Grau-Roma et al., 2008). PCV2b was detected in the United States for the first time during a serever outbreak of PMWS during

2005-2006 (Cheung et al., 2007). A shrply increase in PCV2b associated deaths was reported in Canada (Carmen et al., 2008). These observations led to speculation that the more pathogenic PCV2 strains belong to PCV2b. In contrast, other studies could neither identify a direct link between a particular PCV2 genotype associated with severe PMWS outbreaks(de Boisseson et al., 2004; Larochelle et al., 2002). Although there was apparently no link between the PCV2 genotype and geographical area (Olvera et al., 2007), some countries observed a shift form PCV2a to PCV2b (Wiederkehr et al., 2009). While PCV2a and PCV2b viruses have been reported in Europe and Asia, PCV2b viruses were not detected in North America prior to 2004 and 2005 (Dupont et al., 2008). After the shift in 2003, all of the countries submitted primarily PCV2b sequences (Dupont et al., 2008).

2. Porcine circovirus associated diseases (PCVAD)

2.1. Post-weaning multisystemic wasting syndrome (PMWS)

PMWS, one of the PCV2 associated disease (PCVAD), was first recognized by a group of scientists at the University of Saskatchewan in several high health herds in Western Canada in 1998 and subsequently reported worldwide (LeCann et al., 1997; Segales et al., 1997; Kennedy et al., 1998; Choi and Chae, 1999; Allan and Ellis, 2000; Kiss et al., 2000; Wellenberg et al., 2000; Trujano et al., 2001; Celer and carasova, 2002). PMWS causes considerable economic losses to the swine industry. The disease has a low rate of morbidity but has a high mortality rate in animals that are 5-12 weeks of age (Allan and Ellis, 2000; Chae, 2004). The most prominent clinical signs are wasting, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice (Harding 2004, 2006). Bronchopneumonia and gastric ulceration of esophagus are frequently found in PMWS cases, but are not related to the direct effect of PCV2 (Segales and Domingo, 2002). Bronchopneumonia is associated with bacterial infections while gastric ulceration is of multifactorial origin. However, the lesion in the stomach cause severe hemorrhage and it is the cause of death of a number of pigs with PMWS (Segales and Domingo, 2002). Pigs with chronic wasting disease may develop cachexia, with marked muscle wasting and serous atrophy of fat (Chae, 2004; Segales et al., 2004). Microscopic lesions in lymphoid

tissues are almost unique. A variable degree of lymphocyte depletion is systemically present, usually combined with a multifocal to diffuse, slight to very intense histiocytic infiltration with the possible presence of giant cells. Thymus atrophy is microscopically characterized by cortical atrophy due to lymphocyte depletion (Chae, 2004; Opriessnig et al., 2007). PCV2 inclusion bodies can be frequently observed in the cytoplasm of histiocytes of dendritic cells (DC) of lymphoid tissues (Opriessnig et al., 2007). They appear as basophilic, round shaped and with variable sized. Among non-lymphoid tissues, the most common affected ones are lungs, liver and kidneys. Lungs show sub-acute interstitial pneumonia, with alveolar, peribronchial and peribronchiolar mononuclear infiltrates (Allan and Ellis, 2000; Segales et al., 2004).

2.2. Porcine resiratory disease complex (PRDC)

Recent results of many field stdudy suggest that PCV2 may play an important role in the porcine respiratory disease complex (PRDC) (Harms et al., 2002; Kim et al., 2003). PRDC is a condition observed with multiple respiratory pathogens associated with Porcine reproductive and respiratory virus (PRRSV), Swine influenza virus (SIV), and bacteria such as Mycoplasma hyopneumoniae (Chae, 2005; Opriessnig et al., 2007). PRDC is characterized by a decreased rate of growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea. There may be diagnostic overlap between PCV2-associated systemic infection and PCV2-associated pneumonia (Chae, 2005). The presence of prolonged and unusually severe clinical respiratory disease, granulomaous bronchointerstitial pneumonia with bronchiolitis and bronchiolar fibrosis, and abundant PCV2 antigen associated with the lesions is suggestive that PCV2 may play a role in the PRDC problem (Chae, 2005; Opriessnig et al., 2007).

2.3. Porcine dermatitis and nephropathy syndrome (PDNS)

PDNS is characterized clinically by acute onset of skin lesions, fever, and lethargy (Chae, 2005). Other characteristic macroscopic lesions include enlarged tan, waxy appearing kidneys with petechial hemorrhages. Microscopically, there is systemic vasculitis with dermal and epidermal necrosis and

necrotizing and fibrinous glomerulonephritis (Chae, 2005; Opriessnig et al., 2007). The hallmark microscopic lesions of PDNS, generalized vasculitis and glomerulonephritis, are suggestive of a type 3 hypersensitivity reaction, which is characterized by deposition of antigen-antibody aggregates or immune complexes at certain tissue sites (Opriessnig et al., 2007). Several pathogens including viruses (PRRSV) and bacteria (Pasteurella multocida, Streptococcus suis type 1 and 2, Escherichia coli, Proteus sp., Haemophilus parasuis, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Arcanobacterium pyogenes, Staphyloccoccus aureus, or Salmonella sp.) have been incriminated as possible etiologies for PDNS (Thomson et al., 2001; Lainson et al, 2002) .

The association of PCV2 with PDNS was first reported in 2000 (Rosell et al., 2000). Investigation of PDNS cases observed in Northern Ireland in 1990, which at the time was PRRSV free, demonstrated the presence of PCV2 antigen associated with granulomatous lymphadenitis. A recent case-control study investigating PDNS in the Netherlands found that there was a significant association of high anti-PCV2 antibody titers to PCV2 and the development of PDNS (Wellenberg et al., 2004). Some cases were not able to show PCV2 antigen by IHC in all of the PDNS lesions, but they were able to confirm the presence of PCV2 DNA by PCR in all cases of PDNS. Importantly, previous study were able to show that porcine parvovirus or PRRSV nucleic acids were not present in many of the PDNS cases as determined by PCR (Opriessnig et al., 2007). A study comparing PCV2 serum viral load in PMWS and PDNS cases found that PDNS cases had significantly lower numbers of PCV2 DNA in serum compared to healthy, subclinically PCV2-infected pigs (Olvera et al., 2004).

3. PCV2 pathogenesis

3.1. Experimental reproduction

Experimental models are essential for researchers to study the pathogenesis of infectious diseases, host immunological responses, as well as to evaluate the efficacy of vaccines against an etiological agent. However, in the case of PMWS, a large number of unsuccessful (understood as lack of clinical PMWS reproduction) experiments have been reported (Allan et al., 2000; Loizel et al., 2005; Ostanello et al., 2005; Opriessnig et al., 2006; Fernandes et al., 2007), evidencing the lack of a

consistent experimental model to develop the disease. Early experimental models using piglets inoculated with apparently PCV2 alone developed a mild clinical disease with slight histological lesions characteristic of PMWS (Balasch et al., 1999; Kennedy et al., 2000; Magar et al., 2000a). However, as mentioned before, when PCV2 was combined with another infectious agent, such as PPV (Allan et al., 1999; Kennedy et al., 2000; Ha et al., 2008), PRRSV (Allan et al., 2000b; Harms et al., 2001; Opriessnig et al., 2006c) or M. hyopneumoniae (Opriessnig et al., 2004b; Krakowka et al., 2007), severe clinical signs and lesions of PMWS were occasionally reproduced in both conventional and gnotobiotic pigs. It was hypothesized that PCV2 alone was not able to cause disease and needed the help of other infectious agents as triggers. Moreover, modulation of the host immune system with non-infectious factors has also been described as a trigger for PMWS. The effect of immunomodulation has been demonstrated either experimentally using KLH/ICFA (Krakowka et al., 2001, 2007) or under natural conditions using a parapoxvirus immunomodulator combined with a vaccine against M. hyopneumoniae (Kyriakis et al., 2002). Nevertheless, other attempts using also KLH/ICFA (Ladekjaer-Mikkelsen et al., 2002) and GM-CSF (Loizel et al., 2005) or M. hyopneumoniae vaccine (Haruna et al., 2006) failed to confirm such effect. Additionally, previously mentioned commercial inactivated or attenuated vaccines against other pig pathogens (Krakowka et al., 2007; Ha et al., 2009) and the use of mitogens such as concavalin A (Yu et al., 2007) have also been suggested as enhancers of PMWS by up-regulating of PCV2 replication. The picture becomes even more complex with the reports indicating that the use of an immunosuppressor such as cyclosporine A was able to increase PCV2 replication in pigs (Krakowka et al., 2002). On the other hand, few but several authors have described the reproduction of the full spectrum of PMWS clinical and pathological signs using PCV2 alone (Albina et al., 2001; Bolin et al., 2001; Ladekjaer-Mikkelsen et al., 2002). All together, indicate that, among all the suggested factors influencing PMWS development, PCV2 seems to be the only one with an essential role. Due to the difficulty of reproducing the severe clinical expression of PMWS with PCV2 alone and to obtain repeatable results, a meta-analysis on published results of experimental infections was recently performed (Tomás et al., 2008).

3.2. Microscopic lesions and antigen amounts in lymphoid

A percentage of pigs that suffer from PMWS are able to recover from the disease. Thus, although animals show emaciation in this chronic stage, histopathological examination of them reveals only mild or no lesions compatible with PMWS together with minimal amounts of PCV2 in lymphoid tissues (Segalés et al., 2004). Recently, the evolution dynamics of microscopic lesions and amount of PCV2 in lymphoid tissues in experimentally infected pigs have been outlined by Opriessnig et al. (2007). However, the evolution of clinical signs was not mentioned in this description, which would have been interesting for its diagnostic implications.

3.3. PCV2 replication and its target cells

Since PCV2 does not encode its own polymerase, the replication of PCV2 depends on the cell polymerases present in the nucleus of during the S phase of the cell cycle (Tischer et al., 1987). Once there, PCV2 replicates by a rolling cycle mechanism, which is similar to the one used by other circular ssDNA viruses, such as plant viruses from the family Geminiviridae (Cheung, 2004). Thus, a complementary DNA strand is synthesized and the resulting double-stranded DNA (dsDNA) is known as the replicative form (RF) of the virus (Mankertz et al., 2004). The helicase and nickase activities of Rep and Rep´, respectively, facilitate the interaction of the host DNA polymerase with the viral genome, synthesizing the complementary DNA strand of PCV2 (Mankertz and Hillenbrand, 2001). Viral replication, DNA transcription as well as encapsidation of the virus apparently take place in the nucleus (Tischer et al., 1987). The identification of the target cells for PCV2-replication has been the subject for many studies, giving often controversial results. Immunohistochemistry (IHC) and in situ hybridisation (ISH) techniques demonstrated large amounts of PCV2 antigen or nucleic acid in the cytoplasm of macrophages and DC in the depleted follicles of lymphoid tissues (Allan and Ellis, 2000; Sorden, 2000). Moreover, PCV2 has also been detected in the cytoplasm and, occasionally, in the nucleus of fibroblasts, lymphocytes, pancreatic acinar cells, endothelial cells, other macrophage cell types such as alveolar macrophages and Kupffer cells, and several types of

epithelial cells such as renal and respiratory epithelial cells, hepatocytes or enterocytes (Rosell et al., 2000; Kennedy et al., 2000; Chianini et al., 2003). However, the presence of nucleic acid and/or antigen in tissues or cell cultures, even in the cell nucleus, does not necessarily imply PCV2 replication. For this reason, new strategies have been developed to investigate PCV2 replication, including IHC using antibodies against ds and ssDNA (Hamberg et al., 2007), ISH with a probe specific for RF (Pérez-Martín et al., 2007), rolling-cycle amplification (Navidad et al., 2008), and the measurement of Cap messenger RNA (mRNA) and viral DNA synthesis by quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR) assays (Yu et al., 2007). The application of some of these techniques in vivo indicated that PCV2 replication mainly occurs in lymph nodes, lung, tonsil and liver (Pérez-Martín et al., 2007; Yu et al., 2007). Despite the replication of PCV2 in macrophages and lymphocytes was ruled out in some initial in vitro studies (Gilpin et al., 2003), other works indicated the opposite (Meerts et al., 2005; Pérez-Martín et al., 2007; Yu et al., 2007) and even one of them have suggested lymphocytes as the primary site of PCV2 replication. Pérez-Martín et al. (2007) showed that a low proportion of macrophages and lymphocytes may support PCV2 replication. Moreover, these authors indicated that, apparently, the main cells where PCV2 replicates are of epithelial/endothelial origin, suggesting that PCV2 is epitheliotropic (Pérez-Martín et al., 2007). Besides, it has been suggested that persistent infected monocytes and DC might be the potential mechanism of PCV2 dissemination throughout the body (Vincent et al., 2003).

4. Transmission of PCV2

4.1 Horizontal transmission

It is necessary to differentiate the PCV2 transmission from transmission of the disease. In case of the PCV2 infectious state, the fact that almost all pigs have seroconverted against PCV2 at 6 months of age (Larochelle et al., 2003) already suggests that the horizontal transmission of PCV2 between pigs is developed very easily. In experimental infections studies, where control pigs comingled with previously PCV2 infected pigs resulted in transmission of the virus to all contact sentinel pigs (Albina et al., 2001; Bolin et al., 2001). Additionally, a recent study indicated that PCV2 transmission between

pigs allocated within the same pens was more efficient than transmission between pigs in different pens (Andraud et al., 2008).

Oral and nasal excretion is considered the most likely and frequent source of PCV2 infection and transmission and, consequently, most experimental studies on PCV2 infection have used this route (Tomás et al., 2008). PCV2 has been detected in all potential routes of shedding in nasal excretions, tonsillar, bronchial and ocular secretions, faeces, saliva, and urine (Shibata et al., 2003; Segalés et al., 2005b) of both naturally PMWS-affected and asymptomatic pigs, and even in sow milk and boar semen (Larochelle et al., 2000; Shibata et al., 2006). Therefore, this virus can potentially be shed by all excretion and secretion routes. In addition, one recent experimental study has demonstrated the transmission of the virus using uncooked lymphoid tissues, bone marrow and skeletal muscle from PCV2 viremic to naive pigs by the oral route (Opriessnig et al., 2009).

Horizontal transmission of PCV2-associated disease have also been experimentally demonstrated after joining together healthy pigs with pigs suffering from PMWS (Kristensen et al., 2009). Healthy pigs developed PCV2-associated disease 1-2 weeks after joinging them with PMWS affected ones (Kristensen et al.,2009). Although the transmission of the disease was more frequent between animals that had direct contact, it also developed between pigs that had indirect contact and even it was observed in one animal that had no physical contact with the diseased pigs (Kristensen et al., 2009).

4.2 Vertical transmission

In previous studies, the transmission of PCV2 from sow to the fetus in utero were demonstrated, and transplacental transmission has been identified twice after experimental intranasal infection of pregnant sows three weeks before the expected date of farrowing (Park et al., 2005; Ha et al., 2008). One of those studies demonstrated abortion and stillborn in the sows inoculated by PCV2 (Park et al., 2005), while the other described normal farrowings (Ha et al., 2009). Both were able to identify the genome and the proteins of PCV2 in aborted fetus or piglets. On the other hand, PCV2 has been isolated or detected to high amounts within specific myocarditis lesions from aborted fetus and stillborns in cases of reproductive failure (O'Connor et al., 2001; Brunborg et al., 2007).

PCV2 has been also isolated in semen of naturally and experimentally infected boars, even after the appearance of antibodies in serum (Larochelle et al., 2000; Madson et al., 2009b). PCV2 is frequently detected and isolated in seminal plasma and in the sperm and non-sperm cell fractions (Kim et al., 2003). Furthermore, it has been demonstrated that PCV2 shed in semen of experimentally virus inoculated boars can be infectious through artificial insemination (Madson et al., 2009b). This finding was demonstrated by the bioassay test, which consists of the detection of PCV2 seroconversion and viremia in naive piglets after pigs were inoculated intraperitoneally with semen from those PCV2 infected boars. However, when this semen was used to inseminate sows, none of the sows or their offspring got PCV2 infection (Madson et al., 2009b). In addition, another recent experimental infection in PCV2 naive sows reported reproductive failure as well as fetal infection (Madson et al., 2009a). However, this result was achieved by using semen spiked with PCV2. On the other hand, another study reported PCV2 infection and myocarditis lesions in fetus after artificial insemination using semen infected with PCV2. However, these sows had already been infected with PCV2 two months before the insemination (Rose et al., 2007). Therefore, it remains unknown if the amount of PCV2 naturally shed by boars is enough to transmit the virus to the fetus or the sow. It is noteworthy that the frequency by which PCV2 is associated to reproductive alterations under field conditions remains controversial.

5. Immune responses of PCV2

5.1. Humoral immunity to PCV2

Previous studies on naturally and experimentally infected pigs have revealed important information on the development of adaptive antibody mediated immunity to PCV2 and how it may affect the expression of PMWS. Passive immunity through maternal antibodies from colostrums has been shown to protect from PMWS outbreak in a dose-dependent manner (Rose et al., 2007; McKeown et al., 2005). It is not only the supply of colostrum that is of importance, but the PCV2 infection status of the sow may influence the protection of the offspring. Sows with lower levels of PCV2 antibodies had a higher proportion of offspring affected by PMWS (Calsamiglia et al., 2007). Furthermore, presence

of PCV2 antibodies is not necessarily protective because not all antibodies have neutralizing activity for PCV2 infection. Experimentally infected pigs commonly seroconvert to PCV2 between 14 and 28 days post inoculation (Segalés et al., 2005) but PMWS-affected pigs have shown a delayed and weak humoral immune response and neutralizing antibodies have been detected later or not at all in these pigs (Bolin et al., 2001; Meerts et al., 2005, 2006; Fort et al., 2007). It is demonstrated that the low levels of neutralizing antibodies could also be correlated to high levels of PCV2 replication (Meerts et al., 2005; Fort et al., 2007). Pigs with low levels of neutralizing antibodies also had low levels of total antibodies to PCV2 indicating that PMWS affected pigs have an impaired humoral response to PCV2 that subsequently results in a higher viral load (Meerts et al., 2005; Fort et al., 2007). In field conditions, the pigs are generally protected against PCV2 infection by the passive immunity transferred from the sow during the first weeks of life and active seroconversion to PCV2 usually occurs between 7-12 weeks of age (Segalés et al., 2005). If the interval duration between the decline of maternal immunity and onset of active seroconversion where the pigs are not protected is extended due to an impaired humoral response, the risk for developing PMWS following infection with PCV2 may increase. The major immunoactive B-cell epitopes of PCV2 have been detected within the ORF2 capsid protein and several studies report similar locations of these epitopes (Mahe et al., 2000; Truong et al., 2001; Lekcharoensuk et al., 2004). These areas of the capsid protein seem to have a variable sequence of amino acid, and potential differences within epitopes of PCV2 isolates originating from various genetic and clinical backgrounds have been reported (Lefebvre et al., 2008; Timmusk et al., 2008). Few studies have so far been performed in order to characterize the T-cell epitopes of PCV2. According to the previous stduy, these epitopes seem, in contrast to the B-cell epitopes, to be located on the nonstructural proteins of ORF1 and ORF3 (Stevenson et al., 2007).

5.2. Immunosuppression in clinical PMWS

Several studies have searched the failure of immune responses induced by PMWS in affected pigs and the complex correlation between the immune system and PCV2 infection during disease (Segalés et al., 2004a; Darwich et al., 2002; Krakowka et al., 2002). The most remarkable immune response of

PMWS-affected pigs is the severe depletion of lymphocytes in lymphoid tissue and the replacement with histiocytes and macrophages (Krakowka et al., 2002). In PMWS affected pigs, this change in cell proportion is accompanied by leukopenia affecting B lymphocytes as well as helper, cytotoxic and rd TCR-expressing T cells and natural killer (NK) cells but not granulocytes or monocytes (Nielsen et al., 2003; Segales et al., 2001). The depletion of lymphocytes has been suggested to be a result of apoptosis, but the evidence for this theory is still unidentified. PCV2 is generally not recovered from lymphocytes, and interaction with PCV2-infected dendritic cells in fact seemed to increase the survival of the lymphocytes (Vincent et al., 2003). This suggests that PCV2 infection of DC is not responsible for the lymphocyte depletion in severe PMWS-affected animal.

5.3. Protein and cytokine expression in PMWS

PMWS-affected pigs have higher serum levels of the acute phase proteins (APP) haptoglobin, pig-major acute phase protein (pig-MAP), C-reactive protein (CRP), serum amyloid A (SAA) and albumin compared to subclinical PCV2 infected pigs (Parra et al., 2006; Stevenson et al., 2006; Segalés et al., 2004b). Attempts to investigate the expression of cytokines in lymphoid tissues and peripheral blood mononuclear cells (PBMC) from PMWS-affected pigs have so far not generated a common pattern of cytokine production, and the classification of the immune response into a T helper (Th) 1 type has been debated (Sipos et al., 2004; Darwich et al., 2003). Nevertheless, increased expression and production of interleukin (IL)-10 have been found in several independent studies indicating that it may be related in the development of PMWS (Stevenson et al., 2006; Sipos et al., 2004; Darwich et al., 2003). The IL-10 production has increased as mRNA expression level in thymus in association with histopathological lesions (Darwich et al., 2003) and in PBMC (Sipos et al., 2004) of PMWS-affected pigs. In experimentally infected pigs, elevated levels of IL-10 were detected in serum of pigs that subsequently developed clinical PMWS (Stevenson et al., 2006), and viremia correlated with increased expression level of IL-10 during subclinical infection status has been identified (Darwich et al., 2008).

These results indicate a severe immune depression of pigs with clinical PMWS, but the underlying

mechanisms are still poorly elucidated. The expression of cytokines detected in clinically affected pigs may be an effect of the disease rather than the cause of PMWS. This may explain the variety results reported in previous studies attributed to the different status of disease in which the cytokine expression was observed.

5.4. Interaction between PCV2 and immune cells in vitro

PCV2 infects cells of the monocytic lineage such as macrophages and DC, but does not seem to be associated with replicating in these cells (Gilpin et al., 2003; Vincent et al., 2003). Moreover, there is little effect on the viability and function of the cells, and the infection appears silent and persistent for an extended period of time. DC infected with PCV2 in vitro do not change their expression of major histocompatibility complex (MHC) class I and II or cluster of differentiation (CD) 80/86, even after exposure to IFN-α and tumor necrosis factor (TNF)-a, indicating that PCV2 does not induce or interfere with maturation of the DC (Vincent et al., 2003, 2005). Thease studies also showed that PCV2 infection seems to have no effect on the antigen presenting and processing function of DCs as demonstrated by infections with foot and mouth disease virus (FMDV) or classical swine fever virus (CSFV) (Vincent et al., 2005). In addition, the infectivity of PCV2 was not changed and the virus was not transmitted from DCs infected with PCV2 to T lymphocytes even after stimulation of the cell (Vincent et al., 2003). These results indicated a potential mechanism involved for PCV2 to evade the protective immune response of the host, and to be disseminated throughout the body via the circulation of DCs. There are, however, reports about changed function of immune cells from PMWS-affected animals. In response of stimulation to recall antigen (PCV2), PBMC isolated from clinical PMWS pigs produced an increased levels of IL-10 and IFN-r compared to PBMC isolated from infected healthy pigs, and showed an down regulation to produce IL-4, IL-2 and IFN-r upon stimulation with mitotic antigen (Darwich et al., 2003). These results indicate a negative effect on immune cells by PCV2 in late stages of disease, but it does not explain the apparently silent infection found in subclinically infected animals. The immune depression of PCV2 on DCs in vitro has been attributed to the function of viral DNA rather than the whole virion (Vincent et al., 2007), and this

role of viral DNA may be involved with the presence of specific Immunomodulatory DNA motifs in the genome of PCV2.

5.5. Cellular immune responses

It is not demonstrated whether the depletion of lymphocytes in clinical PMWS pigs is due to reduced production in the bone marrow and thymus, reduced proliferation in secondary peripheral lymphoid tissues or increased loss of lymphocytes in the bone marrow, thymus, peripheral blood or secondary lymphoid tissues via virus-induced necrosis or apoptosis (Opriessnig et al., 2007). Among these reports, devated results have been suggested about the importance of apoptosis. Therfore, several authors did not find a higher frequency of apoptosis in lymphoid tissues of PMWS affected pigs than in non-diseased pigs (Mandrioli et al., 2004), others suggested the opposite (Shibahara et al., 2000; Kiupel et al., 2005). In addition, the product of ORF3 was suggested to be involved in PCV2-induced apoptosis (Liu et al., 2005, 2006). Recently, the same research group performed an experiment using a mutant of PCV2 lacking the expression of ORF3 and reported an attenuation of the PCV2 pathogenicity compared with the wild type virus infection (Karuppannan et al., 2009). Authors hypothesized that the apoptosis induced by the ORF3 might be a factor that helps in the spread of the virus in vivo, even though it is not necessary for the replication of the virus (Karuppannan et al., 2009).Naturally PMWS-affected pigs suffer from an altered cytokine mRNA expression profile in the lymphoid tissues (Darwich et al., 2003b). This cytokine imbalance is characterized by an IL-10 over-expression in the thymus (Darwich et al., 2003) and peripheral blood mononuclear cells (PBMC) (Sipos et al., 2004), and decreased IL-2, IL-4 and IL-12 expression in several secondary lymphoid organs, suggesting an impairment of the T cell immune response (Darwich et al., 2003). Accordingly, cytokine profile evaluation of blood samples from PCV2-experimentally inoculated pigs showed an association between plasma levels of IL-10 and development of PMWS (Hasslung et al., 2005; Stevenson et al., 2006). Moreover, PCV2 has been demonstrated to induce IL-10 secretion in in vitro cultured PBMC (Darwich et al., 2003), leading to down-regulation of other cytokines produced during recall antigen responses (Kekarainen et al., 2008a). These data suggest the involvement of IL-10 in

the suppressed Th1 responses observed during the course of PMWS (Kekarainen et al., 2008b). On the other hand, PCV2 was demonstrated to block the induction of IFN-α and other cytokines by natural interferon producing cells (NIPC), a DC subpopulation (Vincent et al., 2007). This effect is presumably induced through the presence of oligodeoxynucleotides (ODN) with central cytosine-phosphateguanine (CpG) motifs within the PCV2 genome, thereby preventing the maturation of both NIPC and other DC subpopulations (Vincent et al., 2007; Kekarainen et al., 2008a). This situation would diminish the ability of these cells to mediate antiviral responses upon the infection and render the host more susceptible to secondary or concomitant microbial infections (Vincent et al., 2007). Thus, the presence of immunomodulatory sequences in the PCV2 genome is a potential mechanism to escape the immune response of the host and it may attribute to the development of clinical disease. However, the interaction between PCV2 and the immune system is highly complex. Thus, regulation of cytokine production appeared to be different depending on the PCV2 viral elements or the cell populations studied (Wikstrom et al., 2007; Kekarainen et al., 2008a). Recently, a subclinical PCV2 experimental infection reported the increase of IL-8 and of IFN-α blood concentration few days after infection, suggesting the capacity of the innate immune response of non-PMWS affected pigs to counteract the inhibitory activity of PCV2 (Fort et al., 2009). Taken together, these results suggest that the balance between the host ability to mount a proper innate antiviral response and the virus ability to dampen it might be determinant for the infection evolution and triggering of the disease (Fort et al., 2009). These authors also observed that the drop in the viral load in serum was concurrent with the appearance of PCV2-specific IFN-γ-secreting cells (IFN-γ-SC) and NA, suggesting that the viral clearance might be mediated by the development of PCV2-IFN-γ-SC in contribution to the PCV2-specific NA.

The haemogram of pigs with PMWS also shows significant alterations (Segalés et al., 2000; Darwich et al., 2002). In diseased pigs, the number of lymphocytes is significantly decreased, and monocytes and neutrophils are increased, with an inversion of the ratio lymphocyte/neutrophil. However, the total number of leukocytes is not altered. Pigs with PMWS usually have normocytic hypochromic anemia with a slight increase of total number of red blood cells (Segalés et al., 2000; Darwich et al.,

2002). In addition, analysis of the acute phase proteins in PMWS affected pigs showed an increase in the serum concentrations of several positive APPs including haptoglobin (HPT), pig-major acute phase protein (pig-MAP), C-reactive protein (CRP) and serum amyloid A (SAA), and a decrease of albumin concentration, which is considered a negative APP (Segalés et al., 2004; Parra et al., 2006). It is known that the increase in hepatic release of positive APP is the consequence of an increase of proinflammatory cytokines, mainly IL-1, IL-6 and tumor necrosis factor-α (TNF- α), which are in turn primary secreted by monocytes in response to infection stimuli (Petersen et al., 2004). Thus, increases in IL-1, IL-6 and TNF-α have also been demonstrated in cases of PMWS (Sipos et al., 2004). However, other authors could not detect such increases in proinflammatory cytokines concentrations (Stevenson et al., 2006). Those controversial results might be explained by the fact that, meanwhile APPs serum concentrations can be elevated during days or weeks, the increase of inflammatory cytokines is usually cleared from the circulation within few hours
(Petersen et al., 2004).

6. PCV2 vaccines

6.1. Commercial vaccines

Different types of commercial vaccines have used to control PCV2 infection worldwide after first commercial vaccine released in 2004 (Opriessnig et al, 2007; Chae, 2012). The first vaccine on the market was Circovac (Merial) was an inactivated, oil-adjuvanted whole virus vaccine for use in sows and gilts 2-4 weeks prior to farrowing. The other three (Ingelvac CIRCOFLEX®, Fostera™ PCV2 and Porcilis PCV®/Circumvent PCV®) are indicated for piglets (Chae, 2012). Ingelvac CIRCOFLEX® contains PCV2a ORF2 capsid antigen expressed in a Baculovirus backbone with poly carbomer adjuvant and administered only one dose (1 ml) intramuscular injection. Porcilis PCV®/Circumvent PCV® is also recombinant vaccine containing ORF2 capsid antigen with tocopherol adjuvant and administered one or two dose (2ml) intramuscular injection (Beach and Meng, 2012; Chae, 2012). Recently, Fostera™ PCV, formerly known as Suvaxyn® PCV2, a reformulated inactivated chimeric PCV1-2 vaccine was re-entered the market (Hemann et al., 2012). It was based on a chimeric PCV1-2

virus containing the genomic backbone of the non-pathogenic PCV1, with the ORF2 capsid gene replaced by that of PCV2 and administered 1 dose (2ml) intramuscular injection.

6.2. Strategies and efficacy of PCV2 vaccines

There are two recommended vaccination strategies. Circovac® (Merial) is recommended to be given to sows, relying on the protective effects of passive transfer of maternal immunity to young piglets. The other three vaccines target piglets approximately 3 weeks of age; primary immunization is recommended at 3-4 weeks of age to induce active immune responses prior to the anticipated expected high risk period. In one study (Desrosiers et al., 2009), mortality rate up to finisher age (~19-22 weeks) were compared among four groups of pigs i.e., i. immunized through sow vaccination (passive immunization) ii. immunized at young age (active immunization) with two different PCV2 vaccines designated A & B and iii. non-immunized controls. There was no difference in mortality rate between non-vaccinated pigs (10.7%) compared to pigs born to vaccinated sows (10.4%). However, piglets vaccinated at a young age had significantly lower mortality 3.9% and 3.1 % for vaccine A and B, respectively, favouring piglet vaccination over sow vaccination.

The use of vaccines in sows and gilts increases PCV2 antibody titres in both serum and colostrum; the transfer of the later one provides protection of piglets against PMWS development (Charreyre et al., 2005; Joisel et al., 2007). In addition, it has been suggested that vaccination of the sow may also contribute to protection during the gestation phase and prevent potential pathogenic effects of PCV2 during this physiological state (Joisel et al., 2007). Besides, piglet vaccines are applied around 3 weeks of age or later, during the waning of maternal antibodies, eliciting PCV2 total and NA responses and reducing or delaying PCV2 infections at weaning or fattening ages (Fort et al., 2008; Opriessnig et al., 2009a).

Excellent results have been reported with all four vaccines either under experimental or field conditions. During field efficacy studies in Germany and France, the use of the vaccine in sows produced a rise in PCV2 antibody titres in the breeder herds and a concurrent decrease in PMWS rates and mortality ratios in the growing pigs originating from those vaccinated breeding herds (Charreyre

et al., 2005; Joisel et al., 2007). When used in Canada, a reduction of mortality was observed after 6 months of use (Plourde and Machell, 2007). Experimental studies assessing the performance of PCV2 vaccines for piglets showed a reduction of PCV2 viremia and/or microscopic lesions, even in the presence of maternal antibodies (Fort et al., 2008; Opriessnig et al., 2008a). In addition, PCV2 vaccination with two doses of vaccine reduced PCV2 viremia and microscopic lesions by 97.1% and 81.8%, respectively, meanwhile such reduction was by 78.5 and 78.7% when piglets were vaccinated with only one dose (Opriessnig et al., 2009a). In experimental challenge study with pigs vaccinated in 5 days or 21 day, it was demonstrated that vaccination of PCV2-naive pigs at day 5 or day 21 resulted in development of a sufficient humoral immune response and provided complete protection against PCV2 viremia and PCV2-associated lesions after triple challenge with PCV2, PPV, and PRRSV (O'Neill et al., 2011). Under field conditions, the application of PCV2 vaccines in 3 or in 6-week-old pigs from PMWS affected farms showed that vaccinated pigs had lower mortality rate and higher average daily gain than unvaccinated pigs (Horlen et al., 2008). Moreover, vaccinated pigs had reduced number of coinfections with PRRSV and Mycoplasma hyorhinis than unvaccinated animals (Kixmöller et al., 2008). In addition, PCV2 piglet vaccination was reported to improve the growth performance of pigs affected by PRDC (Fachinger et al., 2008). On the other hand, autogenous PCV2 vaccines have been sporadically used by some practitioners facing severe losses associated with PMWS. This situation mainly occurred some years ago, when commercial vaccine availability was limitated. These products were prepared from lung or lymphoid tissue homogenates obtained from diseased pigs and inactivated with 2% formaldehyde. Those practitioners generally reported marked reduction of mortality. However, the industry remains concerned about the safety and potential legal ramifications from using killed autogenous viral vaccines (Opriessnig et al., 2007).

7. References

Albina, E., Truong, C., Hutet, E., Blanchard, P., Cariolet, R., L'Hospitalier, R., Mahe, D., Allee, C., Morvan, H., Amenna, N., Le Dimna, M., Madec, F., Jestin, A., 2001. An experimental model

for post-weaning multisystemic wasting syndrome (PMWS) in growing piglets. Journal of Comparative Pathology 125, 292-303.

Allan, G., Meehan, B., Todd, D., Kennedy, S., McNeilly, F., Ellis, J., Clark, E.G., Harding, J., Espuna, E., Botner, A., Charreyre, C., 1998a. Novel porcine circoviruses from pigs with wasting disease syndromes. Veterinary Record 142, 467-468.

Allan, G., Meehan, B., Todd, D., Kennedy, S., McNeilly, F., Ellis, J., Clark, E.G., Harding, J., Espuna, E., Botner, A., Charreyre, C., 1998b. Novel porcine circoviruses from pigs with wasting disease syndromes. Veterinary Record 142, 467-468.

Allan, G.M., Ellis, J.A., 2000a. Porcine circoviruses: a review. Journal of Veterinary Diagnostic Investigation 12, 3-14.

Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. Journal of Comparative Pathology 121, 1-11.

Allan, G.M., Mcneilly, F., Cassidy, J.P., Reilly, G.A.C., Adair, B., Ellis, W.A., Mcnulty, M.S., 1995. Pathogenesis of Porcine Circovirus - Experimental Infections of Colostrum Deprived Piglets and Examination of Pig Fetal Material. Veterinary Microbiology 44, 49-64.

Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000b. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Archives of Virology 145, 2421-2429.

Andraud, M., Grasland, B., Durand, B., Cariolet, R., Jestin, A., Madec, F., Rose, N., 2008. Quantification of porcine circovirus type 2 (PCV-2) within- and between-pen transmission in pigs. Veterinary Research 39.

Balasch, M., Segales, J., Rosell, C., Domingo, M., Mankertz, A., Urniza, A., Plana-Duran, J., 1999. Experimental inoculation of conventional pigs with tissue homogenates from pigs with post-weaning multisystemic wasting syndrome. Journal of Comparative Pathology 121, 139-148.

Beach, N.M., Meng, X.J., 2012. Efficacy and future prospects of commercially available and experimental vaccines against porcine circovirus type 2 (PCV2). Virus Research 164, 33-42.

Bolin, S.R., Stoffregen, W.C., Nayar, G.P.S., Hamel, A.L., 2001. Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-deprived piglets with type 2 porcine circovirus. Journal of Veterinary Diagnostic Investigation 13, 185-194.

Brunborg, I.M., Jonassen, C.M., Moldal, T., Bratberg, B., Lium, B., Koenen, F., Schonheit, J., 2007. Association of myocarditis with high viral load of porcine circovirus type 2 in several tissues in cases of fetal death and high mortality in piglets. A case study. Journal of Veterinary Diagnostic Investigation 19, 368-375.

Calsamiglia, M., Fraile, L., Espinal, A., Cuxart, A., Seminati, C., Martin, M., Mateu, E., Domingo, M., Segales, J., 2007. Sow porcine circovirus type 2 (PCV2) status effect on litter mortality in postweaning multisystemic wasting syndrome (PMWS). Research in Veterinary Science 82, 299-304.

Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lusis, P., Fairles, J., Alexander, H.S., Van Dreumel, T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease - 2004-2006. Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire 72, 259-268.

Celer, V., Carasova, P., 2002. First evidence of porcine circovirus type 2 (PCV-2) infection of pigs in the Czech Republic by semi-nested PCR. Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health 49, 155-159.

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. Veterinary Journal 168, 41-49.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. Veterinary Journal 169, 326-336.

Chae, C., 2012. Commercial porcine circovirus type 2 vaccines: Efficacy and clinical application. Veterinary Journal 194, 151-157.

Charreyre, C., Beseme, S., Brun, A., Bublot, M., Joisel, F., Lapostolle, B., Sierra, P., Vaganay, A., 2005. Vaccination strategies for the control of circoviral diseases in pigs. In: Proceedings of the European Society for Veterinary Virology, International Conference on Animal Circoviruses and Associated Diseases, pp. 26–30.

Cheung, A.K., 2003. The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology 313, 452-459.

Cheung, A.K., 2004. Identification of an octanucleotide motif sequence essential for viral protein, DNA, and progeny virus biosynthesis at the origin of DNA replication of porcine circovirus type 2. Virology 324, 28-36.

Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Archives of Virology 152, 1035-1044.

Chianini, F., Majo, N., Segales, J., Dominguez, J., Domingo, M., 2003. Immunohistochemical characterisation of PCV2 associate lesions in lymphoid and non-lymphoid tissues of pigs with natural postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 94, 63-75.

Choi, C., Chae, C., 1999. In-situ hybridization for the detection of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. Journal of Comparative Pathology 121, 265-270.

Clark, E.G., 1997. Post-weaning multisystemic wasting syndrome. Proceedings of the American Association of Swine Practitioners, 28th Annual Meeting, Quebec City, Canada, pp. 499-501.

Darwich, L., Balasch, M., Plana-Duran, J., Segales, J., Domingo, M., Mateu, E., 2003. Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. Journal of General Virology 84, 3453-3457.

Darwich, L., Segales, J., Resendes, A., Balasch, M., Plana-Duran, J., Mateu, E., 2008. Transient correlation between viremia levels and IL-10 expression in pigs subclinically infected with porcine circovirus type 2 (PCV2). Research in Veterinary Science 84, 194-198.

Darwich, L., Segales, J.S., Domingo, M., Mateu, E., 2002. Changes in CD4+, CD8+, CD4+ CD8+, and immunoglobulin M-positive peripheral blood mononuclear cells of postweaning multisystemic wasting syndrome-affected pigs and age-matched uninfected wasted and healthy pigs correlate with lesions and porcine circovirus type 2 load in lymphoid tissues. Clinical and Diagnostic Laboratory Immunology 9, 236-242.

de Boisseson, C., Beven, V., Bigarre, L., Thiery, R., Rose, N., Eveno, E., Madec, F., Jestin, A., 2004. Molecular characterization of Porcine circovirus type 2 isolates from post-weaning multisystemic wasting syndrome-affected and non-affected pigs. Journal of General Virology 85, 293-304.

Dupont, K., Nielsen, E.O., Baekbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Veterinary Microbiology 128, 56-64.

Fachinger, V., Bischoff, R., Ben Jedidia, S., Saalmuller, A., Elbers, K., 2008. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. Vaccine 26, 1488-1499.

Faurez, F., Dory, D., Grasland, B., Jestin, A., 2009. Replication of porcine circoviruses. Virology Journal 6.

Fernandes, L.T., Mateu, E., Sibila, M., Fort, M., Andaluz, A., McNeilly, F., Allan, G., Sanchez, A., Segales, J., Stevenson, L., 2007. Lack of in vitro and in vivo effects of lipopolysaccharide on porcine circovirus type 2 infection. Viral Immunology 20, 541-552.

Fort, M., Olvera, A., Sibila, M., Segales, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Veterinary Microbiology 125, 244-255.

Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F., Segales, J., 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine 26, 1063-1071.

Fort, M., Sibila, M., Perez-Martin, E., Nofrarias, M., Mateu, E., Segales, J., 2009a. One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine 27, 4031-4037.

Fort, M., Fernandes, L.T., Nofrarias, M., Diaz, I., Sibila, M., Pujols, J., Mateu, E., Segales, J., 2009b. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. Veterinary Immunology and Immunopathology 129, 101-107.

Gibbs, M.J., Weiller, G.F., 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proceedings of the National Academy of Sciences of the United States of America 96, 8022-8027.

Gilpin, D.F., McCullough, K., Meehan, B.M., McNeilly, F., McNair, I., Stevenson, L.S., Foster, J.C., Ellis, J.A., Krakowka, S., Adair, B.M., Allan, G.M., 2003. In vitro studies on the infection and replication of porcine circovirus type 2 in cells of the porcine immune system. Veterinary Immunology and Immunopathology 94, 149-161.

Grau-Roma, L., Crisci, E., Sibila, M., Lopez-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segales, J., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Veterinary Microbiology 128, 23-35.

Grau-Roma, L., Segales, J., 2007. Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. Veterinary Microbiology 119, 144-151.

Grierson, S.S., King, D.P., Wellenberg, G.J., Banks, M., 2004. Genome sequence analysis of 10 Dutch porcine circovirus type 2 (PCV-2) isolates from a PMWS case-control study. Research in Veterinary Science 77, 265-268.

Ha, Y., Ahn, K.K., Kim, B., Cho, K.D., Lee, B.H., Oh, Y.S., Kim, S.H., Chae, C., 2009a. Evidence of shedding of porcine circovirus type 2 in milk from experimentally infected sows. Research in Veterinary Science 86, 108-110.

Ha, Y., Lee, E.M., Lee, Y.H., Kim, C.H., Kim, D., Chae, S., Ahn, K.K., Kim, B., Chae, C., 2009b. Effects of a modified live CSFV vaccine on the development of PMWS in pigs infected experimentally with PCV-2. Veterinary Record 164, 48-51.

Ha, Y., Lee, Y.H., Ahn, K.K., Kim, B., Chae, C., 2008. Reproduction of Postweaning Multisystemic Wasting Syndrome in Pigs by Prenatal Porcine Circovirus 2 Infection and Postnatal Porcine Parvovirus Infection or Immunostimulation. Veterinary Pathology 45, 842-848.

Hamberg, A., Ringler, S., Krakowka, S., 2007. A novel method for the detection of porcine circovirus type 2 replicative double stranded viral DNA and nonreplicative single stranded viral DNA in tissue sections. Journal of Veterinary Diagnostic Investigation 19, 135-141.

Harding, J.C.S., 1996. Post-weaning multisystemic wasting syndrome (PMWS): preliminary epidemiology and clinical presentation. In: Proceedings of the Western Canadian Association of Swine Practitioners, p. 21.

Harding, J.C.S., 2004. The clinical expression and emergence of porcine circovirus 2. Veterinary Microbiology 98, 131-135.

Harding J.C.S., 2006. Different approaches to handling Circovirus. London SwineConference, 35-40.

Harding, J.C.S., Clark, E.G., 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production 5, 201-203.

Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. Journal of Swine Health and Production 10, 27-30.

Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Veterinary Pathology 38, 528-539.

Hasslung, F., Wallgren, P., Ladekjaer-Hansen, A.S., Botner, A., Nielsen, J., Wattrang, E., Allan, G.M., McNeilly, F., Ellis, J., Timmusk, S., Belak, K., Segall, T., Melin, L., Berg, M., Fossum, C., 2005. Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. Veterinary Microbiology 106, 49-60.

Hemann, M., Beach, N.M., Meng, X.J., Halbur, P.G., Opriessnig, T., 2012. Vaccination with inactivated or live-attenuated chimeric PCV1-2 results in decreased viremia in challenge-exposed pigs and may reduce transmission of PCV2. Veterinary Microbiology 158, 180-186.

Horlen, K.P., Dritz, S.S., Nietfeld, J.C., Henry, S.C., Hesse, R.A., Oberst, R., Hays, M., Anderson, J., Rowland, R.R.R., 2008. A field evaluation of mortality rate and growth performance in pigs vaccinated against porcine circovirus type 2. Javma-Journal of the American Veterinary Medical Association 232, 906-912.

Jacobsen, B., Krueger, L., Seeliger, F., Bruegmann, M., Segales, J., Baumgaertner, W., 2009. Retrospective study on the occurrence of porcine circovirus 2 infection and associated entities in Northern Germany. Veterinary Microbiology 138, 27-33.

Joisel, F., Brune, A., Schade, A., Longo, S., Charreyre, C., 2007. Vaccination of sows and gilts against PCV2 diseases with CIRCOVAC® (MERIAL): field experience in Europe. In: Proceedings of

the Annual Meeting of the American Association of Swine Veterinarians, Orlando, Florida, USA, pp. 311–314.

Karuppannan, A.K., Jong, M.H., Lee, S.H., Zhu, Y., Selvaraj, M., Lau, J., Jia, Q., Kwang, J., 2009. Attenuation of porcine circovirus 2 in SPF piglets by abrogation of ORF3 function. Virology 383, 338-347.

Karuppannan, A.K., Kwang, J., 2011. ORF3 of porcine circovirus 2 enhances the in vitro and in vivo spread of the of the virus. Virology 410, 248-256.

Kekarainen, T., Montoya, M., Dominguez, J., Mateu, E., Segales, J., 2008a. Porcine circovirus type 2 (PCV2) viral components immunomodulate recall antigen responses. Veterinary Immunology and Immunopathology 124, 41-49.

Kekarainen, T., Montoya, M., Mateu, E., Segales, J., 2008b. Porcine circovirus type 2-induced interleukin-10 modulates recall antigen responses. Journal of General Virology 89, 760-765.

Kennedy, S., Allan, G., McNeilly, F., Adair, B.M., Hughes, A., Spillane, P., 1998. Porcine circovirus infection in Northern Ireland. Veterinary Record 142, 495-496.

Kennedy, S., Moffett, D., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S., Allan, G.M., 2000. Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination with porcine parvovirus. Journal of Comparative Pathology 122, 9-24.

Kim, J., Chung, H.K., Chae, C., 2003a. Association of porcine circovirus 2 with porcine respiratory disease complex. Veterinary Journal 166, 251-256.

Kim, J., Han, D.U., Choi, C., Chae, C., 2003b. Simultaneous detection and differentiation between porcine circovirus and porcine parvovirus in boar semen by multiplex seminested polymerase chain reaction. Journal of Veterinary Medical Science 65, 741-744.

Kiupel, M., Stevenson, G.W., Galbreath, E.J., North, A., HogenEsch, H., Mittal, S.K., 2005. Porcine Circovirus type 2 (PCV2) causes apoptosis in experimentally inoculated BALB/c mice. BMC Veterinary Research 1, 7.

Kiss, I., Kecskemeti, S., Tuboly, T., Bajmocy, E., Tanyi, J., 2000. New pig disease in Hungary: Postweaning multisystemic wasting syndrome caused by circovirus (short communication). Acta Veterinaria Hungarica 48, 469-475.

Kixmoller, M., Ritzmann, M., Eddicks, M., Saalmueller, A., Elbers, K., Fachinger, V., 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine 26, 3443-3451.

Krakowka, S., Ellis, J., McNeilly, F., Waldner, C., Rings, D.M., Allan, G., 2007. Mycoplasma hyopneumoniae bacterins and porcine circovirus type 2 (PCV2) infection: Induction of postweaning multisystemic wasting syndrome (PMWS) in the gnotobiotic swine model of

PCV2-associated disease. Canadian Veterinary Journal-Revue Veterinaire Canadienne 48, 716-724.

Krakowka, S., Ellis, J.A., McNeilly, F., Gilpin, D., Meehan, B., McCullough, K., Allan, G., 2002. Immunologic features of porcine circovirus type 2 infection. Viral Immunology 15, 567-582.

Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Veterinary Pathology 38, 31-42.

Kristensen, C.S., Baekbo, P., Bille-Hansen, V., Botner, A., Vigre, H., Enoe, C., Larsen, L.E., 2009. Induction of porcine post-weaning multisystemic wasting syndrome (PMWS) in pigs from PMWS unaffected herds following mingling with pigs from PMWS-affected herds. Veterinary Microbiology 138, 244-250.

Kyriakis, S.C., Saoulidis, K., Lekkas, S., Miliotis, C.C., Papoutsis, P.A., Kennedy, S., 2002. The effects of immuno-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. Journal of Comparative Pathology 126, 38-46.

Ladekjaer-Mikkelsen, A.S., Nielsen, J., Stadejek, T., Storgaard, T., Krakowka, S., Ellis, J., McNeilly, F., Allan, G., Botner, A., 2002. Reproduction of postweaning multisystemic wasting syndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old piglets experimentally infected with porcine circovirus type 2 (PCV2). Veterinary Microbiology 89, 97-114.

Lainson, F.A., Aitchison, K.D., Donachie, W., Thomson, J.R., 2002. Typing of Pasteurella multocida isolated from pigs with and without porcine dermatitis and nephropathy syndrome. Journal of Clinical Microbiology 40, 588-593.

Larochelle, R., Bielanski, A., Muller, P., Magar, R., 2000. PCR detection and evidence of shedding of porcine circovirus type 2 in boar semen. Journal of Clinical Microbiology 38, 4629-4632.

Larochelle, R., Magar, R., D'Allaire, S., 2002. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. Virus Research 90, 101-112.

Larochelle, R., Magar, R., D'Allaire, S., 2003. Comparative serologic and virologic study of commercial swine herds with and without postweaning multisystemic wasting syndrome. Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire 67, 114-120.

LeCann, P., Albina, E., Madec, F., Cariolet, R., Jestin, A., 1997. Piglet wasting disease. Veterinary Record 141, 660-660.

Lefebvre, D.J., Costers, S., Van Doorsselaere, J., Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008. Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies. Journal of General Virology 89, 177-187.

Liu, J., Chen, I., Du, Q.Y., Chua, H.K., Kwang, J., 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. Journal of Virology 80, 5065-5073.

Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. Journal of Virology 79, 8262-8274.

Loizel, C., Blanchard, P., Grasland, B., Dory, D., Oger, A., Nignol, A.C., Cariolet, R., Jestin, A., 2005. Effect of granulocyte-macrophage colony-stimulating factor on post-weaning multisystemic wasting syndrome in porcine circovirus type-2-transfected piglets. International Journal of Experimental Pathology 86, 33-43.

Madson, D.M., Patterson, A.R., Ramamoorthy, S., Pal, N., Meng, X.J., Opriessnig, T., 2009. Effect of natural or vaccine-induced porcine circovirus type 2 (PCV2) immunity on fetal infection after artificial insemination with PCV2 spiked semen. Theriogenology 72, 747-754.

Madson, D.M., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X.J., Halbur, P.G., Opriessnig, T., 2009. Infectivity of porcine circovirus type 2 DNA in semen from experimentally-infected boars. Veterinary Research 40.

Mahe, D., Blanchard, P., Truong, C., Arnauld, C., Le Cann, P., Cariolet, R., Madec, F., Albina, E., Jestin, A., 2000. Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. Journal of General Virology 81, 1815-1824.

Mandrioli, L., Sarli, G., Panarese, S., Baldoni, S., Marcato, P.S., 2004. Apoptosis and proliferative activity in lymph node reaction in postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 97, 25-37.

Mankertz, A., Caliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T., Finsterbusch, T., 2004. Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. Veterinary Microbiology 98, 81-88.

Mankertz, A., Hillenbrand, B., 2001. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. Virology 279, 429-438.

Mankertz, A., Persson, F., Mankertz, J., Blaess, G., Buhk, H.J., 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. Journal of Virology 71, 2562-2566.

McKeown, N.E., Opriessnig, T., Thomas, P., Guenette, D.K., Elvinger, F., Fenaux, M., Halbur, P.G., Meng, X.J., 2005. Effects of porcine circovirus type 2 (PCV2) maternal antibodies on experimental infection of piglets with PCV2. Clinical and Diagnostic Laboratory Immunology 12, 1347-1351.

Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. Journal of General Virology 79, 2171-2179.

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Botner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Veterinary Research 2, 6

Meerts, P., Misinzo, G., McNeilly, F., Nauwynck, H.J., 2005. Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, fetal cardiomyocytes and macrophages. Archives of Virology 150, 427-441.

Misinzo, G., Delputte, P.L., Lefebvre, D.J., Nauwynck, H.J., 2008a. Increased yield of porcine circovirus-2 by a combined treatment of PK-15 cells with interferon-gamma and inhibitors of endosomal-lysosomal system acidification. Archives of Virology 153, 337-342.

Misinzo, G., Delputte, P.L., Meerts, P., Lefebvre, D.J., Nauwynck, H.J., 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. Journal of Virology 80, 3487-3494.

Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008b. Inhibition of endosome-lysosome system acidification enhances porcine circovirus 2 infection of porcine epithelial cells. Journal of Virology 82, 1128-1135.

Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingart, H.M., Nauwynck, H.J., 2005. Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. Journal of General Virology 86, 2057-2068.

Mori, M., Sato, K., Akachi, S., Asahi, S., Taniguchi, S., Narita, M., 2000. Retrospective study of porcine circovirus 2 infection in Japan: Seven cases in 1989. Veterinary Pathology 37, 667-669.

Navidad, P.D., Li, H., Mankertz, A., Meehan, B., 2008. Rolling-circle amplification for the detection of active porcine circovirus type 2 DNA replication in vitro. Journal of Virological Methods 152, 112-116.

Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, F.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. Journal of General Virology 81, 2281-2287.

Niagro, F.D., Forsthoefel, A.N., Lawther, R.P., Kamalanathan, L., Ritchie, B.W., Latimer, K.S., Lukert, P.D., 1998. Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminiviruses and plant circoviruses. Archives of Virology 143, 1723-1744.

Nielsen, J., Vincent, I.E., Botner, A., Ladekjaer-Mikkelsen, A.S., Allan, G., Summerfield, A., McCullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 92, 97-111.

O'Connor, B., Gauvreau, H., West, K., Bogdan, J., Ayroud, M., Clark, E.G., Konoby, C., Allan, G., Ellis, J.A., 2001. Multiple porcine circovirus 2-associated abortions and reproductive failure in

a multisite swine production unit. Canadian Veterinary Journal-Revue Veterinaire Canadienne 42, 551-553.

Olvera, A., Cortey, M., Segales, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: Phylogeny and clonality. Virology 357, 175-185.

Olvera, A., Sibila, M., Calsamiglia, M., Segales, J., Domingo, M., 2004. Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. Journal of Virological Methods 117, 75-80.

O'Neill, K.C., Shen, H.G., Lin, K., Hemann, M., Beach, N.M., Meng, X.J., Halbur, P.G., Opriessnig, T., 2011. Studies on Porcine Circovirus Type 2 Vaccination of 5-Day-Old Piglets. Clinical and Vaccine Immunology 18, 1865-1871.

Opriessnig, T., Fenaux, M., Thomas, P., Hoogland, M.J., Rothschild, M.F., Meng, X.J., Halbur, P.G., 2006. Evidence of breed-dependent differences in susceptibility to porcine circovirus type-2-associated disease and lesions. Veterinary Pathology 43, 281-293.

Opriessnig, T., Madson, D.M., Prickett, J.R., Kuhar, D., Lunney, J.K., Elsener, J., Halbur, P.G., 2008. Effect of porcine circovirus type 2 (PCV2) vaccination on porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 coinfection. Veterinary Microbiology 131, 103-114.

Opriessnig, T., McKeown, N.E., Harmon, K.L., Meng, X.J., Halbur, P.G., 2006. Porcine circovirus type 2 infection decreases the efficacy of a modified live porcine reproductive and respiratory syndrome virus vaccine. Clinical and Vaccine Immunology 13, 923-929.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2-associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. Journal of Veterinary Diagnostic Investigation 19, 591-615.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Halbur, P.G., 2009a. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine 27, 1002-1007.

Opriessnig, T., Patterson, A.R., Meng, X.J., Halbur, P.G., 2009b. Porcine circovirus type 2 in muscle and bone marrow is infectious and transmissible to naive pigs by oral consumption. Veterinary Microbiology 133, 54-64.

Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneamoniae and porcine circovirus type 2. Veterinary Pathology 41, 624-640.

Ostanello, F., Caprioli, A., Di Francesco, A., Battilani, M., Sala, G., Sarli, G., Mandrioli, L., McNeilly, F., Allan, G.M., Prosperi, S., 2005. Experimental infection of 3-week-old conventional colostrum-fed pigs with porcine circovirus type 2 and porcine parvovirus. Veterinary Microbiology 108, 179-186.

Park, J.S., Kim, J., Ha, Y., Jung, K., Choi, C., Lim, J.K., Kim, S.H., Chae, C., 2005. Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. Journal of Comparative Pathology 132, 139-144.

Parra, M.D., Fuentes, P., Tecles, F., Martinez-Subiela, S., Martinez, J.S., Munoz, A., Ceron, J.J., 2006. Porcine acute phase protein concentrations in different diseases in field conditions. Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health 53, 488-493.

Perez-Martin, E., Rovira, A., Calsamiglia, M., Mankertz, A., Rodriguez, F., Segales, J., 2007. A new method to identify cell types that support porcine circovirus type 2 replication in formalin-fixed, paraffin-embedded swine tissues. Journal of Virological Methods 146, 86-95.

Petersen, H.H., Nielsen, J.P., Heegaard, P.M.H., 2004. Application of acute phase protein measurements in veterinary clinical chemistry. Veterinary Research 35, 163-187.

Rose, N., Blanchard, P., Cariolet, R., Grasland, B., Amenna, N., Oger, A., Durand, B., Balasch, M., Jestin, A., Madec, F., 2007. Vaccination of porcine circovirus type 2 (PCV2)-infected sows against porcine Parvovirus (PPV) and Erysipelas: effect on post-weaning multisystemic wasting syndrome (PMWS) and on PCV2 genome load in the offspring. J Comp Pathol 136, 133-144.

Rosell, C., Segales, J., Ramos-Vara, J.A., Folch, J.M., Rodriguez-Arrioja, G.M., Duran, C.O., Balasch, M., Plana-Duran, J., Domingo, M., 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Veterinary Record 146, 40-43.

Segales, J., Alonso, F., Rosell, C., Pastor, J., Chianini, F., Campos, E., Lopez-Fuertes, L., Quintana, J., Rodriguez-Arrioja, G., Calsamiglia, M., Pujols, J., Dominguez, J., Domingo, M., 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 81, 37-44.

Segales, J., Calsamiglia, M., Olvera, A., Sibila, M., Badiella, L., Domingo, M., 2005. Quantification of porcine circovirus type 2 (PCV2) DNA in serum and tonsillar, nasal, tracheo-bronchial, urinary and faecal swabs of pigs with and without postweaning multisystemic wasting syndrome (PMWS). Veterinary Microbiology 111, 223-229.

Segales, J., Domingo, M., 2002. Postweaning mulstisystemic wasting syndrome (PMWS) in pigs. A review. Veterinary Quarterly 24, 109-124.

Segales, J., Domingo, M., Chianini, F., Majo, N., Dominguez, J., Darwich, L., Mateu, E., 2004a. Immunosuppression in postweaning multisystemic wasting syndrome affected pigs. Veterinary Microbiology 98, 151-158.

Segales, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynch, H., Dupont, K., McCullough, K., Ellis, J., Krakowka, K., Mankertz, A., Fredholm, M., Fossum, C., Beattie, V., Amstrong, D., Grassland, B., Baekbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. The Veterinary Journal 162, 867-868.

Segales, J., Pastor, J., Cuenca, R., Domingo, M., 2000. Haematological parameters in postweaning multisystemic wasting syndrome-affected pigs. Veterinary Record 146, 675-676.

Segales, J., Rosell, C., Domingo, M., 2004b. Pathological findings associated with naturally acquired porcine circovirus type 2 associated disease. Veterinary Microbiology 98, 137-149.

Segales, J., Sitjar, M., Domingo, M., Dee, S., Del Pozo, M., Noval, R., Sacristan, C., De las Heras, A., Ferro, A., Latimer, K.S., 1997. First report of post-weaning multisystemic wasting syndrome in pigs in Spain. Veterinary Record 141, 600-601.

Shibahara, T., Sato, K., Ishikawa, Y., Kadota, K., 2000. Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. Journal of Veterinary Medical Science 62, 1125-1131.

Shibata, I., Okuda, Y., Kitajima, K., Asai, T., 2006. Shedding of porcine circovirus into colostrum of sows. Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health 53, 278-280.

Shibata, I., Okuda, Y., Yazawa, S., Ono, M., Sasaki, T., Itagaki, M., Nakajima, N., Okabe, Y., Hidejima, I., 2003. PCR detection of Porcine circovirus type 2 DNA in whole blood, serum, oropharyngeal swab, nasal swab, and feces from experimentally infected pigs and field cases. Journal of Veterinary Medical Science 65, 405-408.

Sipos, W., Duvigneau, J.C., Willheim, M., Schilcher, F., Hartl, R.T., Hofbauer, G., Exel, B., Pietschmann, P., Schmoll, F., 2004. Systemic cytokine profile in feeder pigs suffering from natural postweaning multisystemic wasting syndrome (PMWS) as determined by semiquantitative RT-PCR and flow cytometric intracellular cytokine detection. Veterinary Immunology and Immunopathology 99, 63-71.

Sorden, S.D., 2000. Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production 8, 133-136.

Stevenson, L.S., Gilpin, D.F., Douglas, A., Mcneilly, F., Mcnair, I., Adair, B.M., Allan, G.M., 2007. T lymphocyte epitope mapping of porcine circovirus type 2. Viral Immunology 20, 389-397.

Stevenson, L.S., McCullough, K., Vincent, I., Gilpin, D.F., Summerfield, A., Nielsen, J., McNeilly, F., Adair, B.M., Allan, G.M., 2006. Cytokine and C-reactive protein profiles induced by porcine circovirus type 2 experimental infection in 3-week-old piglets. Viral Immunology 19, 189-195.

Thomson, J., Henderson, L., Meikle, C., MacIntyre, N., 2001. Porcine dermatitis and nephropathy syndrome. Veterinary Record 148, 282-283.

Timmusk, S., Wallgren, P., Brunborg, I.M., Wikstrom, F.H., Allan, G., Meehan, B., McMenamy, M., McNeilly, F., Fuxler, L., Belak, K., Podersoo, D., Saar, T., Berg, M., Fossum, C., 2008. Phylogenetic analysis of porcine circovirus type 2 (PCV2) pre- and post-epizootic postweaning multisystemic wasting syndrome (PMWS). Virus Genes 36, 509-520.

Tischer, I., Mields, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on Epidemiology and Pathogenicity of Porcine Circovirus. Archives of Virology 91, 271-276.

Tischer, I., Peters, D., Rasch, R., Pociuli, S., 1987. Replication of Porcine Circovirus - Induction by Glucosamine and Cell-Cycle Dependence. Archives of Virology 96, 39-57.

Tischer, I., Rasch, R., Tochterm.G, 1974. Characterization of Papovavirus-Like and Picornavirus-Like Particles in Permanent Pig Kidney-Cell Lines. Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene Series a-Medical Microbiology Infectious Diseases Virology Parasitology 226, 153-167.

Tomas, A., Fernandes, L.T., Valero, O., Segales, J., 2008. A meta-analysis on experimental infections with porcine circovirus type 2 (PCV2). Veterinary Microbiology 132, 260-273.

Trujano, M., Iglesias, G., Segales, J., Palacios, J.M., 2001. PCV-2 from emaciated pigs in Mexico. Veterinary Record 148, 792-792.

Truong, C., Mahe, D., Blanchard, P., Le Dimna, M., Madec, F., Jestin, A., Albina, E., 2001. Identification of an immunorelevant ORF2 epitope from porcine circovirus type 2 as a serological marker for experimental and natural infection. Archives of Virology 146, 1197-1211.

Vincent, I.E., Balmelli, C., Meehan, B., Allan, G., Summerfield, A., McCullough, K.C., 2007. Silencing of natural interferon producing cell activation by porcine circovirus type 2 DNA. Immunology 120, 47-56.

Vincent, I.E., Carrasco, C.P., Guzylack-Piriou, L., Herrmann, B., McNeilly, F., Allan, G.M., Summerfield, A., McCullough, K.C., 2005. Subset-dependent modulation of dendritic cell activity by circovirus type 2. Immunology 115, 388-398.

Vincent, I.E., Carrasco, C.P., Herrmann, B., Meehan, B.M., Allan, G.M., Summerfield, A., McCullough, K.C., 2003. Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. Journal of Virology 77, 13288-13300.

Wellenberg, G.J., Pesch, S., Berndsen, F.W., Steverink, P.J.G.M., Hunneman, W., Van der Vorst, T.J.K., Peperkamp, N.H.M.T., Ohlinger, V.F., Schippers, R., Van Oirschot, J.T., de Jong, M.F., 2000. Isolation and characterization of porcine circovirus type 2 from pigs showing signs of post-weaning multisystemic wasting syndrome in the Netherlands. Veterinary Quarterly 22, 167-172.

Wellenberg, G.J., Stockhofe-Zurwieden, N., de Jong, M.F., Boersma, W.J.A., Elbers, A.R.W., 2004. Excessive porcine circovirus type 2 antibody titres may trigger the development of porcine dermatitis and nephropathy syndrome: a case-control study. Veterinary Microbiology 99, 203-214.

Wiederkehr, D.D., Sydler, T., Buergi, E., Haessig, M., Zimmermann, D., Pospischil, A., Brugnera, E., Sidler, X., 2009. A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. Veterinary Microbiology 136, 27-35.

Wikstrom, F.H., Meehan, B.M., Berg, M., Timmusk, S., Elving, J., Fuxler, L., Magnusson, M., Allan, G.M., McNeilly, F., Fossum, C., 2007. Structure-dependent modulation of alpha interferon production by porcine circovirus 2 oligodeoxyribonucleotide and CpG DNAs in porcine peripheral blood mononuclear cells. Journal of Virology 81, 4919-4927.

Yu, S., Vincent, A., Opriessnig, T., Carpenter, S., Kitikoon, P., Halburc, P.G., Thacker, E., 2007. Quantification of PCV2 capsid transcript in peripheral blood mononuclear cells (PBMCs) in vitro. Veterinary Microbiology 123, 34-42.

PART I. Evaluation of a reformulated inactivated chimeric PCV1-2 vaccine based on clinical, virological, pathological and immunological examination under experimental and field conditions

Chapter 1. Reduction of porcine circovirus type 2 (PCV2) viremia by a reformulated inactivated chimeric PCV1-2 vaccine-induced humoral and cellular immunity after experimental PCV2 challenge

ABSTRACT

The objective of the present study was to elucidate the humoral and cellular immune response mechanisms by which a reformulated inactivated chimeric PCV1-2 vaccine reduces the PCV2 viremia. Forty PCV2 seronegative 3-week-old pigs were randomly divided into the following four groups: vaccinated challenged (T01), vaccinated non-challenged (T02), non-vaccinated challenged (T03), and non-vaccinated non-challenged (T04) animals. The pigs in groups T01 and T02 were immunized with a reformulated inactivated chimeric PCV1-2 vaccine (Fostera^{TM} PCV; Pfizer Animal Health) administered as a 2.0 ml dose at 21 days of age. At 35 days of age (0 days post-challenge), the pigs in groups T01 and T03 were inoculated intranasally with 2 ml each of PCV2b.

A reduction of PCV2 viremia coincided with the appearance of both PCV2-specific neutralizing antibodies (NA) and interferon-γ-secreting cells (IFN-γ-SCs) in the vaccinated animals. However, the presence of anti-PCV2 IgG antibodies did not correlate with the reduction of PCV2 viremia. Lymphocyte subset analysis indicated that the numbers of $CD3^+$ and $CD4^+$ cells increased in vaccinated animals but the numbers of $CD4^+$ cells decreased transiently in non-vaccinated animals. The observation of a delayed type hypersensitivity response in only the vaccinated animals also supports a $CD4^+$ cell-associated protective cellular immune response induced by the reformulated inactivated chimeric PCV1-2 vaccine.

The induction of PCV2-specific NA and IFN-γ-SCs, and $CD4^+$ cells by the reformulated inactivated chimeric PCV1-2 vaccine is the important protective immune response leading to reduction of the PCV2 viremia and control of the PCV2 infection. To our knowledge this is the first demonstration of protective humoral and cellular immunity induced by the reformulated inactivated chimeric PCV1-2 vaccine and its effect on reduction of PCV2 viremia by vaccination.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is one of the most economically important swine pathogens worldwide. The first commercial PCV2 vaccine was used under special license in France and Germany in 2004, 13 years after postweaning multisystemic wasting syndrome (PMWS) was first identified and reported in western Canada (Chae, 2012). In addition to PMWS, PCV2 is also associated with a number of diseases and syndromes, collectively referred to as porcine circovirus-associated disease (PCVAD) (Chae, 2004, 2005).

Currently, 5 commercial PCV2 vaccines are available worldwide and differ in their antigen (Chae, 2012). One vaccine (Circovac, Merial) is based on the classical approach of an inactivated oil-adjuvanted vaccine. Three subunit vaccines (Circoflex, Boehringer Ingelheim; Circumvent, Intervet/Merck; Porcillis PCV, Schering-Plough/Merck) are based on an open reading frame 2 (ORF2; capsid) protein expressed in the baculovirus system. Another vaccine (Suvaxyn PCV2 One Dose, Pfizer Animal Health/Fort Dodge Animal Health) is based on a chimeric PCV1-2 virus containing the genomic backbone of the non-pathogenic PCV1, with the ORF2 capsid gene replaced by that of PCV2 (Fenaux et al., 2004). In 2008, Pfizer Animal Health temporarily removed the inactivated chimeric PCV1-2 vaccine product from the markets because a chimeric PCV1-2 virus was incidentally detected in the field due to incomplete inactivation of the vaccine (Gagnon et al., 2010). In August 2011, a reformulated version of the chimeric PCV1-2 vaccine under a new brand name (Fostera^{TM} PCV, Pfizer Animal Health) re-entered the market.

PCV2 viremia plays a central role in the development of PMWS. High levels of PCV2 viremia are associated with the development of PCVAD (Meerts et al., 2005, 2006). Reductions in the PCV2 viremia have coincided with the appearance of both PCV2-specific neutralizing antibodies (NA) and interferon (IFN)-γ-secreting cells (IFN-γ-SCs) in PCV2-infected animals (Meerts et al., 2005, 2006; Fort et al., 2007, 2009). Therefore, the induction of PCV2-specific NA and IFN-γ-SCs by commercial PCV2 vaccines is a critical parameter to evaluate the efficacy of PCV2 vaccines in the control of PCV2 infection. It has been reported that subunit and inactivated PCV2 vaccines elicit PCV2-specific NA and IFN-γ-SCs (Fort et al., 2009, Oh et al., 2012). Although the former inactivated chimeric

PCV1-2 vaccine induced PCV2-specific NA (Opriessnig et al., 2009), little is known regarding the protective immunity by which the reformulated inactivated chimeric PCV1-2 vaccine reduces the PCV2 viremia. The objective of present study is to elucidate the mechanisms how humoral and cellular immune response induced by the reformulated inactivated chimeric PCV1-2 vaccine reduces PCV2 viremia under experimental conditions.

MATERIALS AND METHODS

Animals and housing

A total of 40 colostrum-fed, cross-bred, conventional piglets were purchased at 14 days of age from a commercial farm. All piglets were negative for porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* according to routine serological testing. All piglets were negative for PCV1-2a and PCV2 viremia by real-time polymerase chain reaction (PCR), respectively (Gagnon et al., 2008; Shen et al., 2010). All piglets were also seronegative against PCV2 according to commercial ELISA (Synbiotics, Lyon, France). All pigs were housed in an environmentally controlled building as previously described (Kim et al., 2011).

Experimental design

A total of 40 piglets were randomly divided into 4 groups (10 pigs per group). The pigs in groups T01 and T02 were immunized with an inactivated chimeric PCV1-2 vaccine (Fostera^{TM} PCV; Pfizer Animal Health Inc.) administered as a 2.0 ml dose at 21 days of age based on the manufacturer's recommendations. At 35 days of age [0 days post-challenge (dpc)], the pigs in groups T01 and T03 were inoculated intranasally with 2 ml each of a PCV2b [strain SNUVR000463; 5th passage; 1.0 x 10^5 tissue culture infective dose of 50 % (TCID50)/ml]. The pigs in group T04 remained unvaccinated and unchallenged, and served as the negative control group. The pigs in each group were housed separately within the facility. Blood samples and nasal swabs were collected at -14, 0, 14, and 28 dpc. All pigs were euthanized for necropsy at 28 dpc and superficial inguinal lymph nodes were collected for histopathology and immunohistochemistry. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

Quantification of PCV2 DNA in blood and nasal swab

DNA was extracted from serum and nasal samples using the QIAamp DNA mini kit. DNA extracts were used to quantify PCV2 genomic DNA copy numbers by real-time PCR as previously described

by Gagnon et al. (2008). DNA extracts from serum samples were also used to detect PCV1-2a DNA by real-time PCR as previously described by Shen et al. (2010)

Serology

The serum samples were tested using the commercial PCV2 ELISA IgG (Synbiotics, Lyon, France) and serum virus neutralization (SVN) test (Pogranichnyy et al., 2000).

Preparation of PCV2 antigen

The same PCV2 strain used for challenge in the present study, was propagated in PCV-free PK15 cells to a titer of 10^4 TCID50/ml and treated with two freeze-thaw cycles. The PCV2 antigen was prepared by concentrating the virus present in cell culture by ultracentrifugation at 100,000x *g* at 4°C for 3 h. The virus pellet was resuspended with PBS. The concentrated PCV2 was inactivated by exposing to an 8 W germicidal UV lamp at a distance of 15 cm for 1 h. Immunoperoxidase assay was performed to confirm the inactivation of virus as previously described by Rodriguez-Arrioja et al. (2000) (Rodriguez-Arrioja et al., 2000).

Delayed type hypersensitivity

The delayed type hypersensitivity (DTH) test was performed on 40 piglets from 4 groups at 5 weeks of age (2 weeks after vaccination; 0 dpc). Piglets were injected intradermally on the left inguinal area with 250 μg of PCV2 antigen from infected PK15 cells. Phytohemaglutin (PHA; Roche Diagnostics GmbH; 20 μg/ml in 0.1 ml) and saline (0.1 ml) were used as positive and negative controls, respectively. The mean diameters of the induration at the test site were measured with a micrometer 36 h after injection.

Direct immunofluorescence assay

Cryosections (about 7 μm in thickness) were prepared from skin biopsy specimens in DTH test. Sections were transferred to microscope slides treated with poly-L-lysine. The slides were fixed with acetone for 20 min and then air-dried. The slides were then covered with 50 μl of mouse anti-pig

CD4a conjugated with R-phycoerythrin (R-PE) (1:100 dilution; SouthernBiotech, Birmingham, AL, USA) and incubated for 60 min at 37^{o}C in a moisture chamber. After three washing steps with PBS, the slides were mounted in buffered glycerin for observation by fluorescence microscopy.

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) at -14, 0, 14, and 28 dpc as previously described (Williams et al., 1993). Briefly, 100 µl containing 2×10^{6} PBMCs in RPMI 1640 medium supplemented with 10 % fetal bovine serum (HyClone Laboratories, Inc., SelectScience, Bath, UK) were seeded into plates pre-coated 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), phytohemagglutinin (10 µg/ml, Roche Diagnostics GmbH, Mannheim, Germany) as a positive control, or 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, procedure was followed by manufacturer's instructions using a commercial ELISPOT assay kit (MABTECH, Mariemont, OH, USA). 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 million PBMCs.

Flow cytometry

PBMCs were incubated with R-PE- or FITC-conjugated mouse monoclonal antibodies (antiswine CD3 [R-PE] and CD4 [R-PE and FITC]; SouthernBiotech, Birmingham, AL, USA) for 30 min at 4°C in the dark and washed twice with PBS containing 0.1 % sodium azide and 0.1 % bovine serum albumin. Cells stained with conjugated antibodies were resuspended immediately in supplemented RPMI 1640 medium. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) as previously described by Sosa et al. (2009).

Histopathology and immunohistochemistry

For the morphometric analysis of histopathological lesion score and PCV2 antigen score in superficial inguinal lymph nodes were collected at necropsy. In each sample, three sections were randomly selected and examined "blindly" as previously described by Kim et al. (2011). The scores of lesions in lymph nodes ranged from 0 (No lymphoid depletion or granulomatous replacement) to 5 (severe lymphoid depletion and granulomatous replacement), and the PCV2 antigen scores were obtained by counting the number of PCV2 positive cells per unit area ($0.25mm^2$).

Statistical analysis

Continuous data (DTH response, PCV2 DNA, PCV2 serology, PCV2-specific IFN-γ-SCs, and lymphocyte subsets) were analyzed with a one-way analysis of variance (ANOVA). If a one-way ANOVA was significant ($P < 0.05$), pairwise testing using Tukey's adjustment was performed. Discrete data (lymphoid lesion score and PCV2 antigen score, and proportion of viremic pigs) were analyzed by Chi-square and Fisher's exact test.

The Pearson's correlation coefficient was used to assess the relationship among viremia, serum virus neutralization titer, PCV2-specific IFN-γ-SCs, and the Spearman's rank correlation coefficient was used to assess lymphoid lesion score and PCV2 antigen score. A value of $p < 0.05$ was considered significant.

RESULTS

PCV2 DNA in sera and nasal swabs

PCV2 DNA was not detected in serum and nasal samples from any of pigs at 0 dpc. Vaccinated challenged (T01) animals had a significantly lower number of genomic copies of PCV2 in the blood than non-vaccinated challenged (T03) animals at 14 and 28 days post challenge (dpc; $p < 0.001$, Figure 1). The percentage of viremic pigs was significantly lower in vaccinated challenged animals (4/10 at 14 dpc and 2/10 at 28 dpc) compared to non-vaccinated challenged animals (10/10 at 14 and 28 dpc, $p < 0.05$). Vaccinated challenged animals had a significantly lower number of genomic copies of PCV2 in the nasal swab than non-vaccinated challenged animals at 14 and 28 dpc ($p < 0.001$, Figure 1). The percentage of nasal shedders was significantly lower in vaccinated challenged animals (4/10 at 14 and 28 dpc) than in non-vaccinated challenged animals (10/10 at 14 and 28 dpc, $p < 0.05$). The number of genomic copies of PCV2 in the blood correlated with that of PCV2 in the nasal swabs (T01: $r^2 = 0.921$, $p = 0.042$ and T03: $r^2 = 0.972$, $p = 0.002$). No PCV2 DNA was detected in the blood and nasal swabs from vaccinated non-challenged (T02) and non-vaccinated non-challenged (T04) animals throughout the experiment. No PCV1-2a DNA was detected in the blood from vaccinated (T01 and T02) and non-vaccinated (T03 and T04) animals by real-time PCR at -14, 0, 14, and 28 dpc.

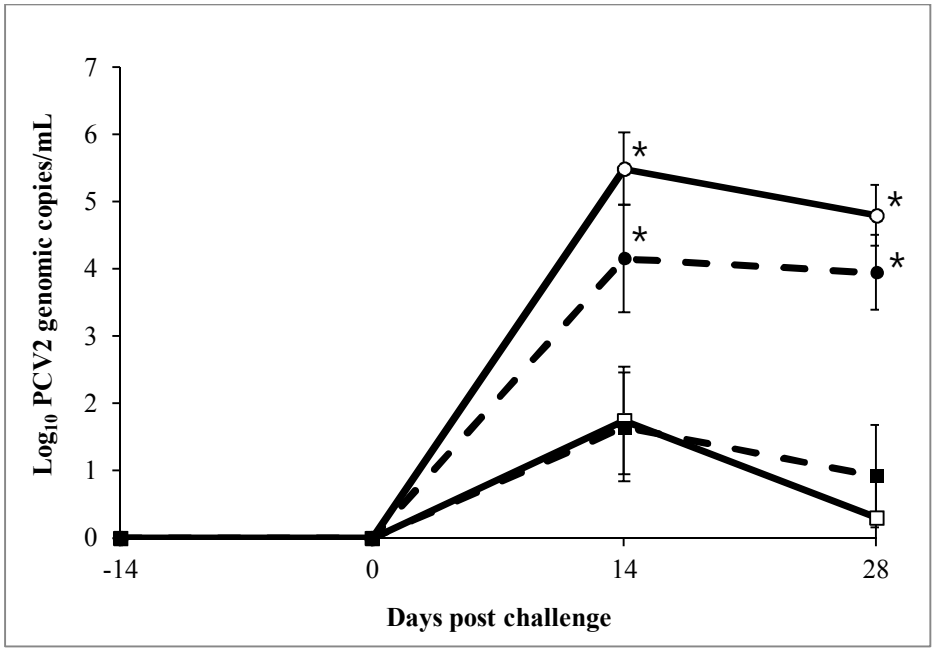


Figure 1. Mean values of the genomic copy number of porcine circovirus type 2 DNA in serum and nasal swabs from vaccinated challenged (T01; □ for serum and ■ for nasal swab) and non-vaccinated challenged (T03; ○ for serum and ● for nasal swab) animals. Variation is expressed as the standard deviation. Significant difference is indicated at p value $<0.001^{*}$.

Anti-PCV2 IgG antibodies

At the time of PCV2 vaccination (3 weeks of age; -14 dpc), pigs in all 4 groups were seronegative against PCV2. Anti-PCV2 IgG antibody titers were significantly higher in vaccinated challenged (T01) and vaccinated non-challenged (T02) animals than in non-vaccinated challenged (T03) animals at 0, 14, and 28 dpc. Anti-PCV2 IgG antibody titers were not detected in non-vaccinated non-challenged (T04) animals throughout the experiment. Anti-PCV2 IgG antibody titers did not correlate with the number of genomic copies of PCV2 in the blood (T01: $r^2 = -0.332$, $p = 0.075$ and T03: $r^2 = -0.105$, $p = 0.126$).

Neutralizing antibodies

At the time of PCV2 vaccination (3 weeks of age, -14 dpc), no significant differences in NA titers were detected among the 4 groups. The NA titers were significantly higher in vaccinated challenged (T01) and vaccinated non-challenged (T02) animals than in non-vaccinated challenged (T03) animals group at 0, 14, and 28 dpc. The NA titers were not detected in non-vaccinated non-challenged (T04) animals throughout the experiment (Figure 2). The NA titers correlated inversely with the number of genomic copies of PCV2 in the blood (T01: $r^2 = -0.712$, $p = 0.012$ and T03: $r^2 = -0.635$, $p = 0.031$).

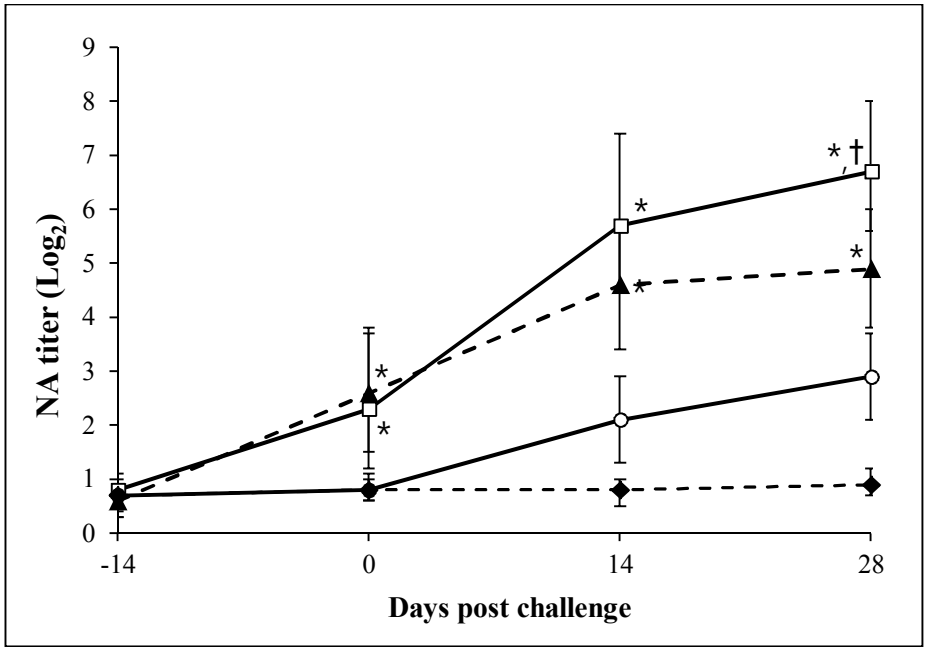


Figure 2. Mean values of the serum neutralizing antibodies (NA) titer in the different groups; vaccinated challenged (T01; □), vaccinated non-challenged (T02; ▲), non-vaccinated challenged (T03; ○), and non-vaccinated non-challenged (T04; ♦) animals. Variation is expressed as the standard deviation. Significant difference (T01 and T02 vs. T03 and T04) is indicated at p value $<0.01^{*}$. Significant difference (T01 vs. T02) is indicated at p value $<0.05^{\dagger}$.

PCV2-specific interferon-γ-secreting cells

PCV2-specific IFN-γ-SCs were not observed in the isolated PBMCs of pigs from the 4 groups at -14 dpc. PCV2-specific IFN-γ-SCs increased sharply in vaccinated challenged (T01) and vaccinated non-challenged (T02) animals at 0 dpc (2 weeks after vaccination). At 0 and 14 dpc, the mean numbers of PCV2-specific IFN-γ-SCs were significantly higher in vaccinated challenged (T01) and vaccinated non-challenged (T02) animals compared to non-vaccinated challenged (T03) animals ($p < 0.05$). At 28 dpc, the mean numbers of PCV2-specific IFN-γ-SCs were significantly higher in vaccinated challenged (T01) than in vaccinated non-challenged (T02) and non-vaccinated challenged (T03) animals ($p < 0.01$). PCV2-specific IFN-γ-SCs were not detected in the PBMCs from non-vaccinated non-challenged (T04) animals throughout the experiment (Figure 3).

PCV2-specific IFN-γ-SCs also correlated inversely with the number of genomic copies of PCV2 in the blood (T01: $r^2 = -0.685$, $p = 0.022$ and T03: $r^2 = -0.625$, $p = 0.028$).

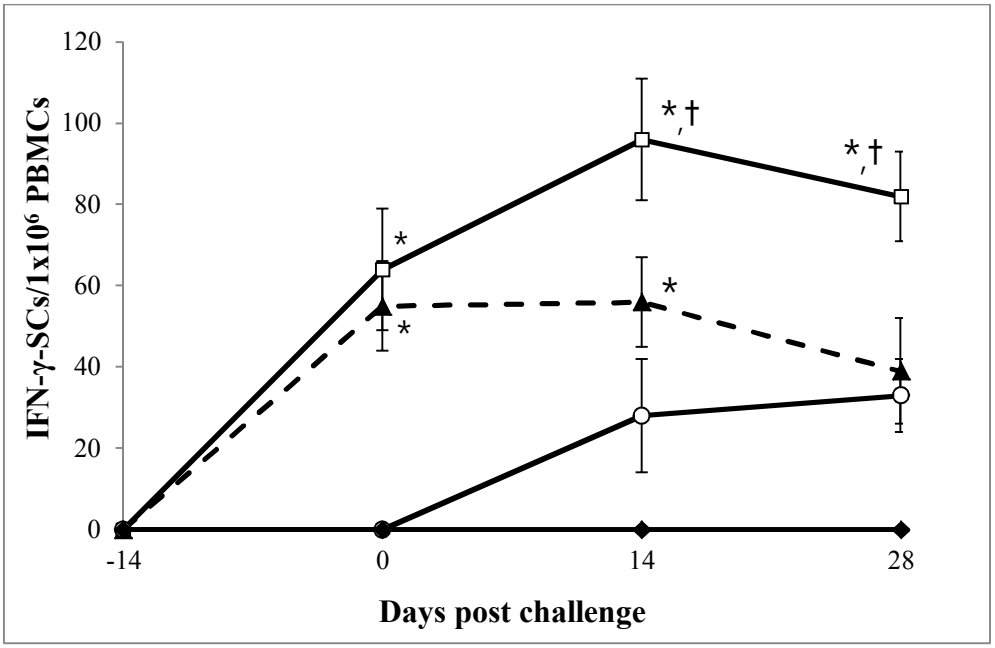


Figure 3. Mean number of porcine circovirus type 2-specific interferon (IFN)-γ-secreting cells (SCs) in vaccinated challenged (T01; □), vaccinated non-challenged (T02; ▲), non-vaccinated challenged (T03; ○), and non-vaccinated non-challenged (T04; ♦) animals. Variation is expressed as the standard deviation. Significant difference (T01 and T02 vs. T03 and T04) is indicated at p value $<0.05^{*}$. Significant difference (T01 vs. T02) is indicated at p value $<0.01^{\dagger}$.

Delayed type hypersensitivity

At 36 h after intradermal injection of the PCV2 antigen, vaccinated animals showed DTH responses consisted of induration and erythematous nodules (Figure 4a). Histopathologically, perivascular infiltrations of lymphocytes were observed in the dermis (Figure 4b). The specific fluorescence signals were observed in cells in perivascular zone (Figure 4c). The positive signals were mainly seen in the cytoplasm of cells.

The skin reaction regressed slowly at 48 h after injection and completely disappeared. DTH responses were not observed in the controls. The vaccinated animals in groups T01 and T02 and non-vaccinated animals in groups T03 and T04 showed DTH responses to the nonspecific mitogen PHA. The PHA DTH response size was not significantly different between vaccinated (T01 and T02) and non-vaccinated (T03 and T04) animals. The vaccinated (T01 and T02) animals displayed significantly greater PCV2-specific DTH responses than the non-vaccinated (T03 and T04) animals ($p < 0.01$, Table 1). DTH responses to saline injection were not observed in any pigs. DTH response correlated with PCV2-specific IFN-γ-SCs (T01: $r^2 = 0.637$, $p = 0.015$ and T03: $r^2 = 0.219$, $p = 0.218$).

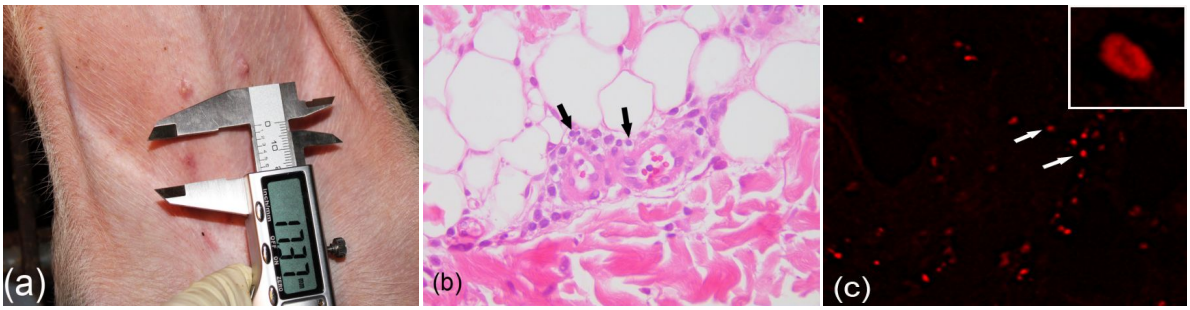


Figure 4. Delayed type hypersensitivity induced by inactivated chimeric PCV1-2 vaccine in vaccinated challenged (T01) animals. (a) erythematous lesion were grossly observed in the skin. (b) Perivascular infiltration of lymphocytes (black arrows) was seen in the dermis. (c) $CD4^+$ cells (white arrows) were seen in the perivascular zone.

Identification of lymphocyte subsets

Vaccinated challenged (T01) and vaccinated non-challenged (T02) animals showed an increase in the relative proportions of $CD3^+$ and $CD4^+$ cells at 0 dpc compared with non-vaccinated challenged (T03) and non-vaccinated non-challenged (T04) animals ($p < 0.05$). Vaccinated challenged (T01) animals showed an increase in the relative proportions of $CD3^+$ and $CD4^+$ cells at 14 dpc, and in the relative proportions of $CD4^+$ cells at 28 dpc compared with non-vaccinated challenged (T03) and non-vaccinated non-challenged (T04) animals ($p < 0.05$). At 14 dpc, the relative proportions of $CD3^+$ cells were significantly higher in vaccinated non-challenged (T02) animals than non-vaccinated challenged (T03) and non-vaccinated non-challenged (T04) animals ($p < 0.05$, Figure 5). The number of $CD4^+$ cells correlated with the number of PCV2-specific IFN-γ-SCs in the blood (T01: $r^2 = 0.624$, $p = 0.025$, T02: $r^2 = 0.589$, $p = 0.047$, and T03: $r^2 = 0.527$, $p = 0.041$). The number of $CD4^+$ cells also correlated with the DTH response (T01: $r^2 = 0.597$, $p = 0.023$ and T03: $r^2 = 0.176$, $p = 0.365$).

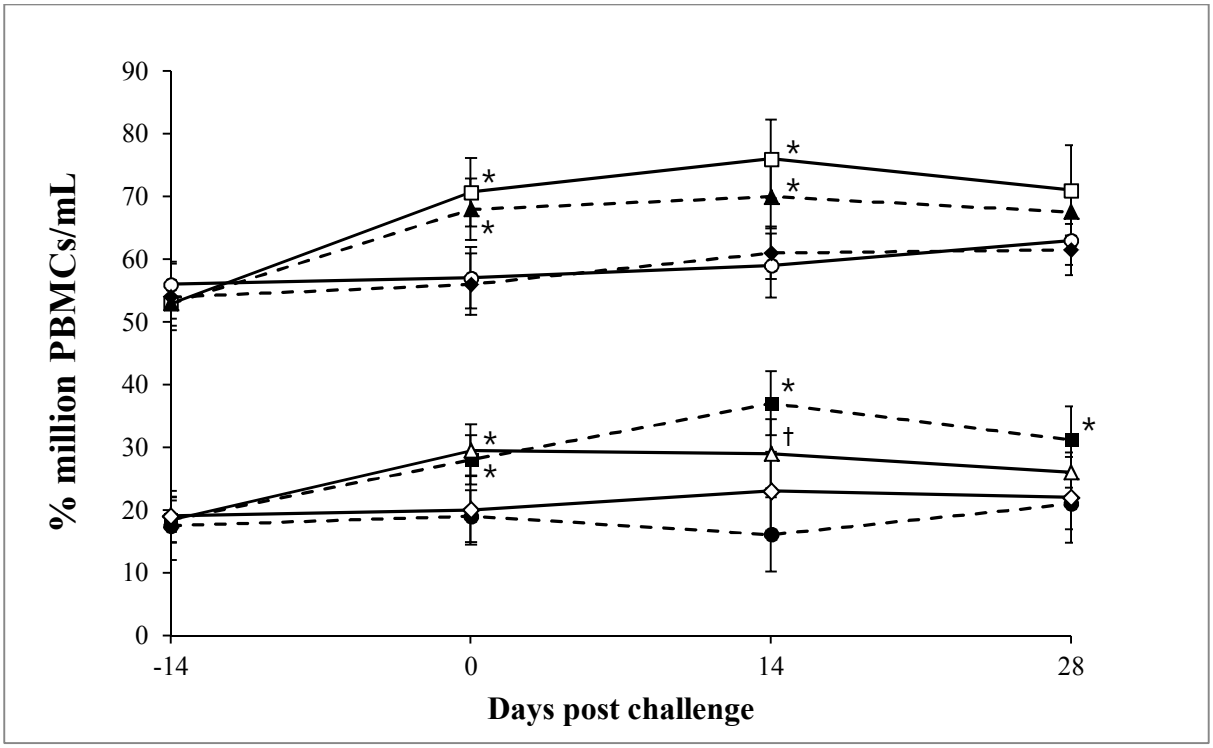


Figure 5. Lymphocyte subsets analysis in the different groups; $CD3^+$ (■) and $CD4^+$(□) from vaccinated challenged (T01) animals, $CD3^+$ (▲) and $CD4^+$(△) from vaccinated non-challenged (T02) animals, $CD3^+$ (●) and $CD4^+$(○) from non-vaccinated challenged (T03) animals, and $CD3^+$ (♦) and $CD4^+$(◇) from non-vaccinated non-challenged (T04) animals. Variation is expressed as the standard deviation. Significant difference (T01 and T02 vs. T03 and T04) is indicated at p value $<0.05^*$. Significant difference (T02 vs. T03) is indicated at p value $<0.05^\dagger$.

Histopathology and immunohistochemistry

The histopathological lymphoid lesion scores were significantly lower in the vaccinated challenged (T01) animals than in the non-vaccinated challenged (T03) animals ($p < 0.05$, Table 1). The histopathological lymphoid lesion scores correlated with the number of genomic copies of PCV2 in the blood (T01: $r^2 = 0.870$, $p = 0.041$ and T03: $r^2 = 0.892$, $p = 0.023$). The mean number of PCV2-positive cells per unit area of lymph node was significantly lower in vaccinated challenged (T01) animals than in non-vaccinated challenged (T03) animals ($p < 0.01$, Table 1). The PCV2 antigen scores correlated with the number of genomic copies of PCV2 in the blood (T01: $r^2 = 0.855$, $p = 0.038$ and T03: $r^2 = 0.872$, $p = 0.047$).

Table 1. Mean number of delayed type hypersensitivity (DTH) response size, microscopic lymphoid lesion score and immunohistochemical PCV2 antigen score in lymph node from vaccinated challenged (T01), vaccinated non-challenged (T02), non-vaccinated challenged (T03), and non-vaccinated non-challenged (T04) animals.

	Groups			
	T01	T02	T03	T04
DTH	15.38* ± 1.87	14.20* ± 4.26	2.19 ± 0.08	1.98 ± 1.09
Lymphoid lesion score	0.40† ± 0.54	-	1.67 ± 0.57	-
PCV2 antigen score	6.18† ± 4.79	-	44.12 ± 9.07	-

*Significant difference (T01 and T02 vs. T03 and T04) is indicated at p value < 0.01. †Significant difference (T01 vs. T03) is indicated at p value < 0.05.

DISCUSSION

Quantitation of the PCV2 viremia could predict PCV2 infection status. Several studies have already shown that PCV2 DNA levels in serum are higher in pig with PMWS than in healthy, subclinically infected pigs (Ladekjær-Mikkelsen et al., 2002; Liu et al., 2000; Rosell et al., 1999). Hence, the reduction of PCV2 viremia by the PCV2 vaccine plays a critical role in controlling PCV2 infection. In the present study, the reformulated inactivated chimeric PCV1-2 vaccine is able to induce PCV2-specific NA and IFN-γ-SCs in vaccinated animals. This protective immunity induced by the reformulated inactivated chimeric PCV1-2 vaccine correlated with the reduction of PCV2 viremia in pigs challenged experimentally with solely PCV2 as induced by other commercial PCV2 vaccines did (Fort et al, 2009; Opriessnig et al., 2010). However, the presence of anti-PCV2 IgG antibodies did not correlate with the reduction of PCV2 viremia. The reformulated inactivated chimeric PCV1-2 vaccine also reduced the PCV2 load in nasal shedding in vaccinated animals, thereby decreasing the risk of transmission to other pigs via a nasal route and decreasing the amount of PCV2 circulating among the pigs.

IFN-γ, which is produced by PCV2-specific IFN-γ-SCs, is a key immunoregulatory cytokine that controls the differentiation of naïve $CD4^+$ into $CD4^+$ cells and mediates cellular immunity against viral infections (Schroder et al., 2004). Our results are further supported by the observation that elevated numbers of $CD4^+$ cells are seen in vaccinated animals only. Lymphocyte subset analysis indicated that the numbers of $CD3^+$ and $CD4^+$ cells increased in vaccinated animals but the numbers of CD4+ cells decreased transiently in non-vaccinated animals. The selective loss of $CD3^+$ and $CD4^+$ cells that is observed in pigs with PMWS (Nielsen et al., 2003; Segales et al., 2001) may impair the immune system in the pigs and result in co-infections with other viral and bacterial pathogens; co-infections are frequently observed in pigs with PMWS under field conditions (Kim et al., 2002; Pallares et al., 2002). Because $CD4^+$ cells promote a DTH response (Sanders et al., 2006), the DTH response observed in only vaccinated animals also supports a $CD4^+$ cell-associated protective cellular immune response that is induced by the reformulated inactivated chimeric PCV1-2 vaccine. PCV2-specific

memory T lymphocytes induced by this chimeric PCV1-2 vaccine mount DTH reactions in response to intradermal injection of the PCV2 antigen. Hence, the induction of PCV2-specific NA and IFN-γ-SCs by the reformulated inactivated chimeric PCV1-2 vaccine is the important protective immune response that leads to reduce the PCV2 viremia and control the PCV2 infection.

Well-controlled experimental studies are necessary to elucidate the protective humoral and cellular immune response induced by the reformulated inactivated chimeric PCV1-2 vaccine to reduce PCV2 viremia because the results can be affected by factors such as environment, feed, pig source, immune status and inoculums. Under field conditions, PCV2 continues to circulate among pigs within the herd and the possibility of exposure and re-exposure to the virus by horizontal transmission occurs once one animal becomes infected. To our knowledge this is the first demonstration of protective humoral and cellular immunity induced by the reformulated inactivated chimeric PCV1-2 vaccine and its effect on reduction of PCV2 viremia by vaccination .

The reformulated inactivated chimeric PCV1-2 vaccine is able to induce PCV2-specific NA and IFN-γ-SCs in vaccinated animals. This protective immunity induced by the reformulated inactivated chimeric PCV1-2 vaccine correlated with the reduction of PCV2 viremia in pigs challenged experimentally with solely PCV2.

REFERENCES

Chae, C., 2012. Commercial porcine circovirus type 2 vaccines: Efficacy and clinical application. The Veterinary Journal 194, 151–157.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. The Veterinary Journal 169, 326–336.

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. The Veterinary Journal 168, 41-49.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. Journal of Virology 2004, 78:6297-6303.

Gagnon, C.A., Music, N., Fontaine, G., Tremblay, D., Harel, J., 2010. Emergence of a new type of porcine circovirus in swine (PCV): A type 1 and type 2 PCV recombinant. Veterinary Microbiology 144, 18-23.

Meerts, P., van Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. Viral Immunology 18, 333-341.

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Botner, A., Kristensen, C.S., Nauwynck, H., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Veterinary Research 2, 6-16.

Fort, M., Olvera, A., Sibila, M., Segalés, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Veterinary Microbiology 125, 244-255.

Fort, M., Fernandes, L.T., Nofrarias, M., Diaz, I., Sibila, M., Pujols, J., Mateu, E., Segales, J., 2009. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-

derived, colostrum-deprived piglets. Veterinary Immunology and Immunopathology 129, 101-107.

Fort, M., Sibila, M., Perez-Martin, E., Nofrarias, M., Mateu, E., Segales, J., 2009. One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine 27, 4031-4037.

Oh, Y., Seo, H.W., Han, K., Park, C., Chae, C., 2012. Protective effect of the maternally derived porcine circovirus type 2(PCV2)-specific cellular immune response in piglets by dam vaccination against PCV2 challenge. Journal of General Virology 93, 1556-1562.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Halbur, P.G., 2009. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2–3-months post vaccination. Vaccine 27, 1002-1007.

Shen, H.G., Beach, N.M., Huang, Y.W., Halbur, P.G., Meng, X.J., Opriessnig, T., 2010. Comparison of commercial and experimental porcine circovirus type 2 (PCV2) vaccines using a triple challenge with PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus (PPV). Vaccine 43, 5960-5966.

Gagnon, C.A., del Castillo, J.R., Music, N., Fontaine, G., Harel, J., Tremblay, D., 2008. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of Porcine circovirus-2 genotypes 2a and 2b in an epidemiological survey. Journal of Veterinary Diagnostic Investigation 20, 545-558.

Kim, D., Kim, C.H., Han, K., Seo, H.W., Oh, Y., Park, C., Kang, I., Chae, C., 2011. Comparative efficacy of commercial Mycoplasma hyopneumoniae and porcine circovirus 2 (PCV2) vaccines in pigs experimentally infected with M. hyopneumoniae and PCV2. Vaccine 29, 3206-3212.

Pogranichnyy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunology 13, 143-153.

Rodriguez-Arrioja, G.M., Segales, J., Balasch, M., Rosell, C., Quintant, J., Folch, J.M., Plana-Duran,

J., Mankertz, A., Domingo, M., 2000. Serum antibodies to porcine circovirus type 1 and type 2 in pigs with and without PMWS. Veterinary Record 146, 762-764.

Williams, P.P., 1993. Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs. Canadian Veterinary Research 57, 1-8.

Sosa, G.A., Quiroga, M.F., Roux, M.E., 2009. Flow cytometric analysis of T-lymphocytes from nasopharynx-associated lymphoid tissue (NALT) in a model of secondary immunodeficiency in Wistar rats. Immunobiology 214, 384-391.

Ladekjær-Mikkelsen, A.S., Nielsen, J., Stadejek, T., Storgaad, T., Krakowka, S., Ellis, J., McNeilly, F., Allan, G., Botner, A., 2002. Reproduction of postweaning multisystemic wasting syndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old piglets experimentally infected with porcine circovirus type 2 (PCV2). Veterinary Microbiology 89, 97-114.

Liu, Q., Wang, L., Willson, P., Babiuk, A., 2000. Quantitative, competitive PCR analysis of porcine circovirus DNA in serum from pigs with postweaning multisystemic wasting syndrome. Journal of Clinical Microbiology 38, 3474-3477.

Rosell, C., Segalés, J., Plana-Duran, J., Balasch, M., Rodriguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. Journal of Comparative Pathology 120, 59-78.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Ramamoorthy, S., Meng, X.J., Halbur, P.G., 2010. Comparison of the effectiveness of passive (dam) versus active (piglet) immunization against porcine circovirus type 2 (PCV2) and impact of passively derived PCV2 vaccine-induced immunity on vaccination. Veterinary Microbiology 142, 177-183.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A., 2004. Interferon-γ: an overview of signals, mechanisms and functions. Journal of Leukocyte Biology 75, 163-189.

Nielsen, J., Vincent, I.E., Bøtner, A., Ladekaer-Mikkelsen, A.S., Allan, G., Summerfield, A., Mc-Cullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and

Immunopathology 92, 97-111.

Segales, J., Alonso, F., Rosell, C., Pastor, J., Chianini, F., Campos, E., Lopez-Fuertes, L., Quintana, J., Rodriquez-Arrioja, G., Calsamiglia, M., Pujols, J., Domingues, J., Domingo, M., 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 81, 37-44.

Kim, J., Chung, H.K., Jung, T., Cho, W.S., Choi, C., Chae, C., 2002. Postweaning multisystemic wasting syndrome of pigs in Korea: prevalence, microscopic lesions and coexisting microorganisms. J Veterinary Medical Science 64, 57-62.

Pallares, F.J., Halbur, P.G., Opriessnig, T., Sorden, S.D., Villar, D., Janke, B.H., Yaeger, M.J., Larson, D.J., Schwartz, K.J., Yoon, K.J., Hoffman, L.J., 2002. Porcine circovirus type 2 (PCV-2) coinfections in US field cases of postweaning multisystemic wasting syndrome (PMWS). Journal of Veterinary Diagnostic Investigation 14, 515-519.

Sanders, V.M., 2006. Epigenetic regulation of Th1 and Th2 cell development. Brain Behav Immun 20, 317-324.

Chapter 2. Efficacy of a reformulated inactivated chimeric PCV1-2 vaccine based on clinical, virological, pathological and immunological examination under field conditions

ABSTRACT

Inactivated chimeric porcine circovirus (PCV) 1-2 vaccine was initially taken off the market due to concerns that the vaccine virus was not killed and thus further replicated and spread in the pig population. In August 2011, a reformulated inactivated chimeric PCV1-2 vaccine re-entered the market. The efficacy of the reformulated inactivated chimeric PCV 1-2 vaccine was evaluated under field conditions for registration as recommended by the Republic of Korea's Animal, Plant & Fisheries Quarantine & Inspection Agency. Three farms were selected based on their history of postweaning multisystemic wasting syndrome (PMWS). On each farm, a total of 50 3-week-old pigs were randomly allocated to one of two treatment groups: (i) vaccinated at 3 weeks of age and (ii) non-vaccinated. Clinical examination indicated that vaccinated animals displayed an improved average daily weight gain (672.2 grams/day vs. 625 grams/day; difference of +47.3 grams/day; $P < 0.05$) and a reduced time to market (177 days vs. 183 days; difference of -6 days; $P < 0.05$). Virological examination indicated that vaccinated animals displayed a reduced PCV2 load in the blood and nasal swabs compared to non-vaccinated animals. Pathological examination indicated that vaccination of pigs against PCV2 effectively reduced the number of PMWS-associated microscopic lesions and the PCV2 load in lymphoid tissues compared to non-vaccinated animals in the 3 herds. Immunological examination indicated that vaccinated animals induced PCV2-specific neutralizing antibodies (NA) and interferon-γ-secreting cells (IFN-γ-SCs). A reduction in the PCV2 load in the blood coincided with the appearance of both PCV2-specific NA and IFN-γ-SCs in the vaccinated animals. The number of $CD4^+$ cells was decreased in non-vaccinated animals compared to vaccinated animals. The reformulated inactivated chimeric PCV1-2 vaccine seems to be very effective in controlling PCV2 infection based on clinical, virological, pathological, and immunological evaluations under field conditions.

INTRODUCTION

Porcine circovirus-associated disease (PCVAD), which is caused by porcine circovirus type 2 (PCV2), has been recognized as one of the most economically important diseases in the global swine industry, including in Korea (Chae, 2004, 2005, 2012). The annual economic loss from severe PCVAD (10-15% mortality) is approximately US $15,000 (exchange rate US $1 = 1,150 Korean won) in 300-sow herds, according to the Korea Rural Economic Institute (http://www.krei.re.kr). Since PCV2 vaccines were introduced into the world market in 2006, vaccination is a major tool for the control of PCV2 infection. Numerous field reports on the efficacy of several commercial PCV2 vaccine products have indicated that vaccination has been a highly efficacious tool for reducing PCVAD in the production system over the last 5 years (Kixmoller et al., 2008; Segales et al., 2009; Martelli et al., 2011; Fraile et al., 2012; Lyoo et al., 2012).

In 2008, a chimeric PCV1-2 isolate was incidentally identified by polymerase chain reaction (PCR) in Canadian pig tissue homogenates (Gagnon et al., 2010). Pfizer Animal Health temporarily removed the inactivated chimeric PCV1-2 vaccine from the market to investigate the possibility that the isolate originated from the vaccine strain. In August 2011, a reformulated inactivated chimeric PCV1-2 vaccine under a new brand name (Fostera™ PCV, formerly known as Suvaxyn® PCV2 One Dose™; Pfizer Animal Health Inc.) re-entered the market. The first objective of the present study is to evaluate the efficacy of the reformulated inactivated chimeric PCV1-2 vaccine based on clinical, virological, pathological, and immunological evaluations under field conditions in accordance with the registration guidelines of the Republic of Korea's Animal, Plant & Fisheries Quarantine & Inspection Agency (QIA, http://www.qia.go.kr). The second objective is to determine the effect of maternally derived antibodies (MDA) on the induction of humoral and cellular immunity by the PCV2 piglet vaccine. The third objective is to elucidate the mechanisms by which humoral and cellular immune responses induced by the reformulated inactivated chimeric PCV1-2 vaccine reduce the PCV2 load in the blood.

MATERIALS AND METHODS

Farms

The clinical field trial was conducted on 3 farms. Farms A, B, and C housed 180-sow, 250-sow, and 430-sow herds, respectively. Farms A and B are one-site production systems. Farm C is a two-site production system with nursery and finishing units. The three farms are all-in/all-out production system. All farms were seropositive for porcine reproductive and respiratory syndrome virus (PRRSV) but did not immunize pigs against PRRSV. All three farms had consistently suffered losses due to postweaning multisystemic wasting syndrome (PMWS) in several recent months. PCV2b but not PCV2a was detected in ten serum samples from 9-week-old pig in each of 3 farms by real-time PCR as previously described (Gagnon et al., 2008). PCV1 but not PCV1-2a was detected in same serum samples from 3 farms by PCR as previously described (Gagnon et al., 2010). The clinical signs first appeared at approximately 7 to 9 weeks of age, and peak mortality (24%, 18%, and 28% for farm A, B, and C, respectively) occurred at approximately 9 to 13 weeks of age.

Clinical field study

The field study was performed at 16 weeks of age based on the experimental design recommended by QIA. QIA guidelines require that 20 piglets be selected and assigned to each group of vaccinated and non-vaccinated animals. In this study, 25 piglets were assigned to each group. This study used a randomized, blinded, weight- and sex-matched, controlled clinical trial design. To minimize sow variation, two 7-day-old piglets were randomly selected from each sow and assigned to the vaccination and non-vaccination groups. The pigs in the vaccination group (T01; n = 25) were injected intramuscularly in the right side of the neck with 2.0 ml of the reformulated inactivated chimeric PCV1-2 vaccine at 21 days of age, and an equal volume of phosphate buffered saline (PBS) (2.0 ml) was injected in the same anatomic location in the control group of pigs (T02; n = 25). The pigs in each group were randomly assigned by weight to pens (8-9 pigs per pen). Five pigs from each group were randomly selected and euthanized for necropsy at 16 weeks of age. All of the methods

were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

Clinical evaluation

The pigs were monitored daily for clinical signs and scored weekly from 0 (normal), 1 (rough haircoat), 2 (rough haircoat and dyspnea), 4 (severe dyspnea and abdominal breathing), and 6 (death) as recommended guideline by QIA. Observers were blinded to vaccination status.

Growth performance

The live weight of each pig was measured at 3, 10, and 16 weeks of age, and immediately before the first batch of animals was sent to the slaughter house. The average daily weight gain (ADWG; gram/pig/day) was analyzed over three time periods: (1) between 3 and 10 weeks of age, (2) between 10 and 16 weeks of age, and (3) between 16 weeks of age and the date of the first shipment to slaughter (S). ADWG during the different production stages was calculated as the difference between the starting and final weight divided by the duration of the stage. Data for dead or removed pigs were included in the calculation.

Mortality rate

For each group, mortality rate was calculated as the number of pigs that died divided by the number of pigs initially assigned to that group within batch. Pigs that died or were culled were collected throughout the study, and those were necropsied.

Serology

Blood samples from each pig were collected by jugular venipuncture at 0, 28, 49, and 91 days post vaccination (dpv). The serum samples were tested using immunoperoxidase monolayer assay (IPMA) (Fort et al., 2009) and serum virus neutralization (SVN) test (Allan et al., 1994; Pogranichnyy et al., 2000).

Quantification of PCV2 DNA in blood and nasal swab

DNA extraction from serum samples collected at 0, 28, 49, and 91 dpv 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). DNA extracts from serum samples collected at 0 and 91 dvp was also performed to detect PCV1-2a DNA by PCR as previously described (Gagnon et al., 2010).

Preparation of PCV2 antigen

The same PCV2 strain as for challenge of the pigs, was propagated in PCV-free PK15 cells 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 present in 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 was inactivated by exposing to an 8 W germicidal UV lamp at a distance of 15 cm for 1 h. Inactivation was confirmed by the absence of virus antigen in PK15 cells as determined by immunoperoxidase assay as previously described (Rodriguez-Arrioja et al., 2000).

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) at 0, 28, 49, and 91 dpv as previously described (Diaz et al., 2005). Briefly, 100 µl containing 2×10^6 PBMCs in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., SelectScience, Bath, UK) were seeded into the pre-coated overnight with anti-porcine IFN-γ monoclonal antibody (5 µg/ml, MABTECH, Mariemont, OH, USA) plates and incubated with 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°C 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.

Flow cytometry

$CD4^+$ and $CD8^+$ cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously described (Williams et al., 1993).

Histopathology and immunohistochemistry

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

Statistical analysis

For clinical field study, continuous data (ADWG, PCV2 DNA, PCV2 serology, PCV2-specific IFN-γ-SCs, and lymphocyte subsets) were analyzed with a generalized linear mixed model and the Student's *t*-test for comparison between groups was used to estimate the difference at each time point. Discrete data (clinical evaluation, lymphoid lesion score, PCV2 antigen score, and proportion of viremic and nasal shedding pigs) were analyzed by Chi-square and/or Fisher's exact test.

The Pearson's correlation coefficient was used to assess the relationship among viremia, serum virus neutralization titer, and PCV2-specific IFN-γ-SCs. A value of $P < 0.05$ was considered significant. A linear regression was performed to determine the correlation between PCV2 antibody titer at the day of vaccination (21 days of age) and the increment of PCV2 antibody titer at 21 dpv (delta value, defined as PCV2 antibody titer at 21 dpv minus PCV2 antibody titer at the day of vaccination in 3 farms.

RESULTS

Clinical evaluation

On farm A, the mean clinical scores were significantly lower in vaccinated animals than in non-vaccinated animals between 35 and 91 dpv ($P < 0.05$). On farm B, the mean clinical scores were significantly lower in vaccinated animals than in controls at 42, 49, 56, 63, and 77 dpv ($P < 0.05$). On farm C, the mean clinical scores were significantly lower in vaccinated animals than in controls at 28, 42, 49, 70, 77, and 91 dpv ($P < 0.05$).

Growth performance

No significant difference in the ADWG was observed between vaccinated and non-vaccinated animals during the 3-10 weeks period. However, during the 10-16 weeks period, the ADWG of vaccinated animals was significantly higher than that of non-vaccinated animals at the 3 farms ($P < 0.05$). The overall growth performance (from 3 weeks of age to S period) of the vaccinated animals was significantly higher than that of the non-vaccinated animals at the 3 farms ($P < 0.05$). The higher ADWG in vaccinated animals resulted in a 5 to 7 day shorter time to market at the 3 farms ($P < 0.05$) (Table 1).

Mortality rate

The overall mortality rate was significantly lower for vaccinated animals (5.3%; 4/75) than non-vaccinated animals (17.3%; 13/75) on all three farms (Table 1). Diagnostic results indicated that mortality was primarily related to PMWS with concomitant infection of *Pasteurella multocida* and *Streptococcus suis*. PMWS was not diagnosed in vaccinated animals from the 3 farms.

PCV2 DNA in sera and nasal swabs

No PCV2b DNA was detected in the blood and nasal swab from vaccinated and non-vaccinated animals at 0 dpv at the 3 farms. A sudden onset of viremia was observed at 7-10 weeks of age (28-49

dpv) at the 3 farms. Peak levels of greater than 90% PCR-positive blood samples from non-vaccinated animals were reached when the animals were 10 weeks of age (49 dpv). A decline in the PCR positivity was detected at 16 weeks of age (91 dpv). Vaccinated animals had a significantly lower number of genomic copies of PCV2b in the blood than non-vaccinated animals at 28, 49, and 91 dpv at the 3 farms ($P < 0.001$). The percentage of viremic pigs was significantly lower among the vaccinated animals compared to non-vaccinated animals on 28, 49, and 91 dpv in all three farms ($P < 0.05$) (Fig. 1).

The vaccinated animals had a significantly lower number of genomic copies of PCV2b in nasal swabs than non-vaccinated animals at 28, 49, and 91 dpv at the 3 farms ($P < 0.001$). The percentage of nasal shedders was significantly lower in vaccinated animals compared to non-vaccinated animals on 28, 49, and 91 dpv at all three farms ($P < 0.05$) (Fig. 2). No PCV2a DNA was detected in the blood and nasal swab from vaccinated and non-vaccinated animals by real-time PCR throughout the experiment at the 3 farms. No PCV1-2 DNA was detected in the blood from vaccinated and non-vaccinated animals by PCR at 0 and 91 dpv in all three farms.

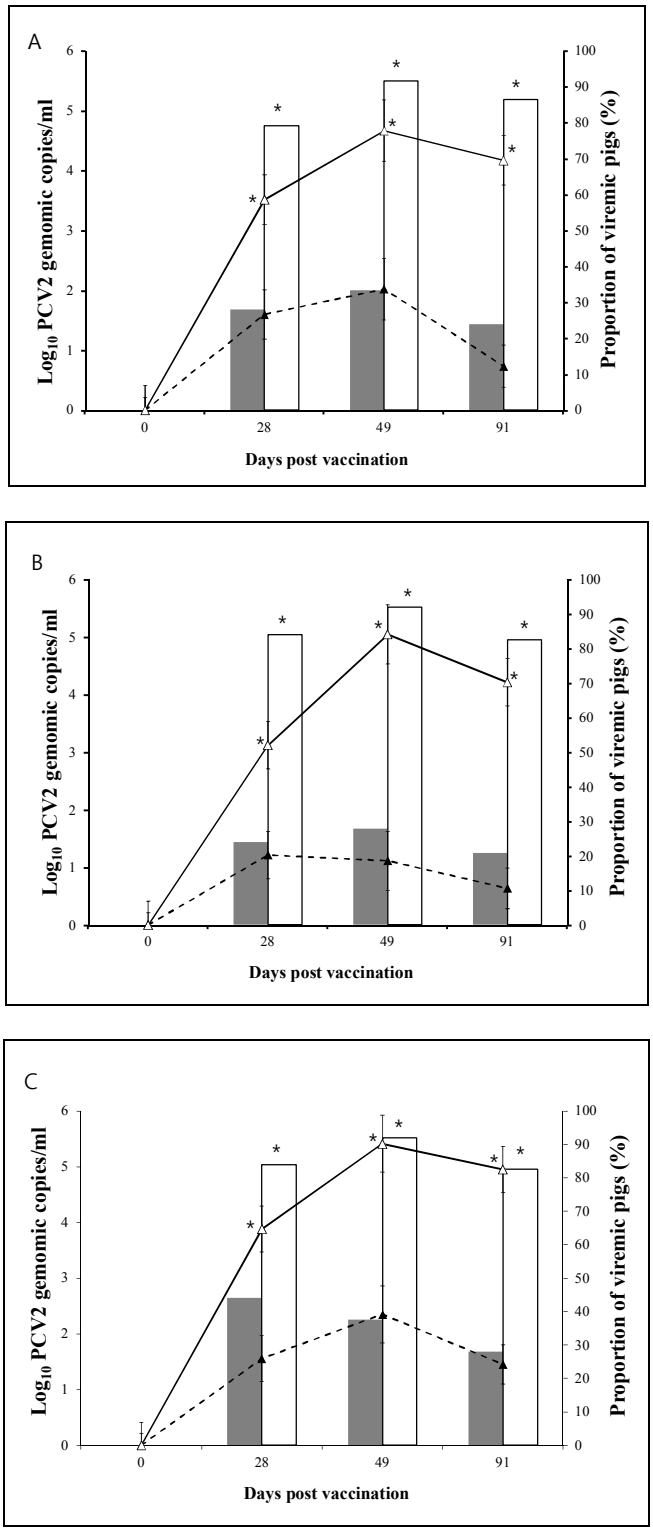


Figure 1. Mean values of the genomic copy number of porcine circovirus type 2b (PCV2b) DNA in serum (vaccinated animals; ▲ and non-vaccinated animals; △) and proportion of viremic pigs (vaccinated animals; ■ and non-vaccinated animals; □) on farm A, B, and C. Variation is expressed as the standard deviation. Significant difference is indicated at *P* value <0.001*.

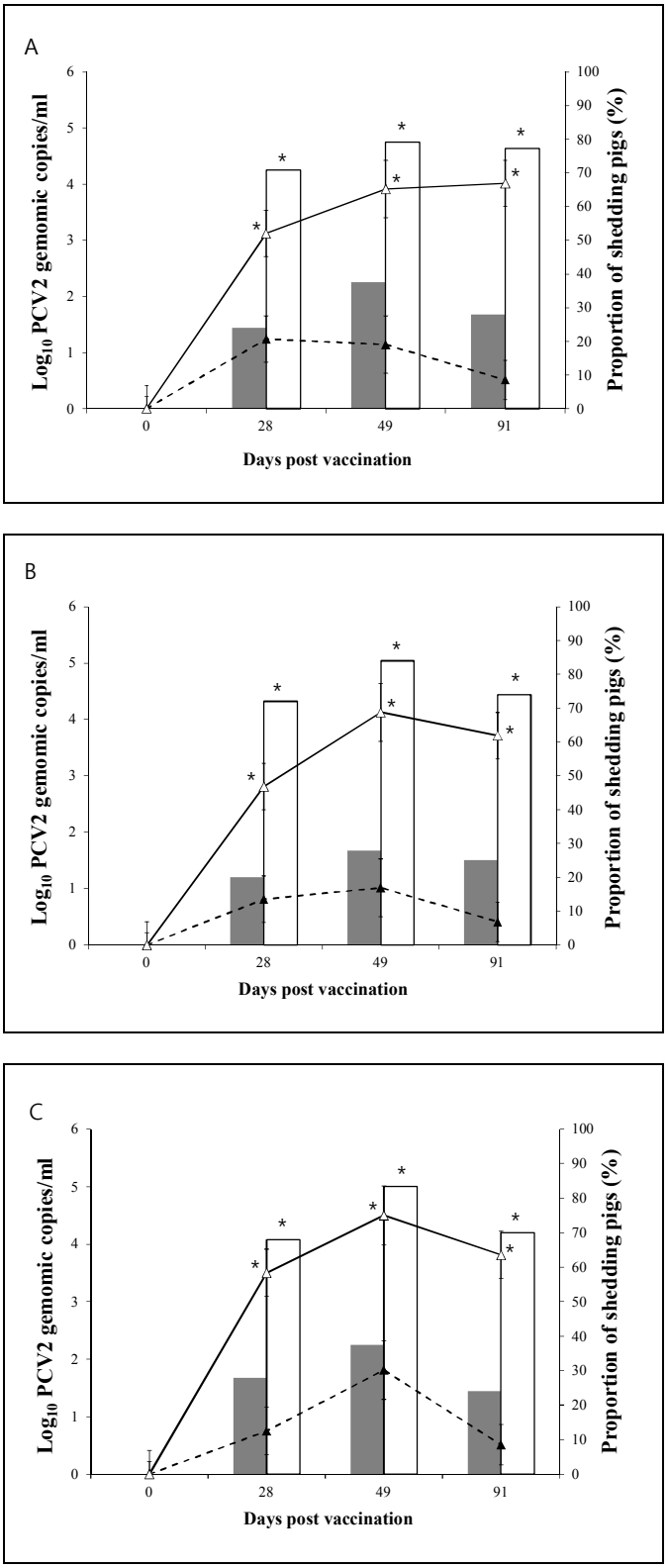


Fig. 2. Mean values of the genomic copy number of porcine circovirus type 2b (PCV2b) DNA in nasal shedding (vaccinated animals; ▲ and non-vaccinated animals; △) and proportion of nasal shedding pigs (vaccinated animals; ■ and non-vaccinated animals; □) on farm A, B, and C. Variation is expressed as the standard deviation. Significant difference is indicated at P value<0.001*.

Immunoperoxidase monolayer assay

At the time of PCV2 vaccination (3 weeks of age; 0 dpv), no significant differences in IPMA titers were detected between the two groups at all 3 farms. At farm A and C, the IPMA titers were significantly higher in the vaccinated groups than in the non-vaccinated group at 28 and 49 dpv ($P < 0.05$). The IPMA titers were significantly higher in the vaccinated groups than in the non-vaccinated group at 28, 49, and 91 dpv at farm B ($P < 0.05$; Fig. 3).

Neutralizing antibodies

At the time of PCV2 vaccination (3 weeks of age; 0 dpv), no significant differences in neutralizing antibodies (NA) titers were detected between the two groups at the 3 farms. The NA titers were significantly higher in the vaccinated groups than the non-vaccinated group at 28, 49, and 91 dpv at all 3 farms (Fig. 3). In vaccinated animals, the NA titers correlated inversely with the number of genomic copies of PCV2 in the blood ($r^2 = 0.682$, $P = 0.023$; $r^2 = 0.825$, $P = 0.011$; $r^2 = 0.652$, $P = 0.042$ for farms A, B, and C, respectively). In non-vaccinated animals, the NA titers correlated inversely with the number of genomic copies of PCV2 in the blood ($r^2 = 0.754$, $P = 0.031$; $r^2 = 0.681$, $P = 0.045$; $r^2 = 0.667$, $P = 0.046$ for farms A, B, and C, respectively).

PCV2-specific interferon- γ-secreting cells

No PCV2-specific IFN-γ-SCs were detected in the PBMCs from either vaccinated or non-vaccinated animals at 0 dpv. PCV2-specific IFN-γ-SCs increased sharply after vaccination and reached a peak at 7 (farm A and B) or 10 (farm C) weeks of age and, then decreased gradually until 16 weeks of age (91 dpv). In contrast, in non-vaccinated animals, PCV2-specific IFN-γ-SCs increased gradually, and reached a peak at 10 weeks of age (49 dpv) and, then decreased gradually until 16 weeks of age (91 dpv). However, the mean number of PCV2-specific IFN-γ-SCs was significantly higher in vaccinated animals at 7 and 10 weeks of age than in non-vaccinated animals ($P < 0.05$; Fig. 3). In vaccinated animals, the number of PCV2-specific IFN-γ-SCs correlated inversely with the number of genomic copies of PCV2 in the blood ($r^2 = 0.721$, $P = 0.012$; $r^2 = 0.625$, $P = 0.031$; $r^2 =$

0.656, P = 0.044 for farms A, B, and C, respectively). In non-vaccinated animals, the number of PCV2-specific IFN-γ-SCs correlated inversely with the number of genomic copies of PCV2 in the blood (r^2 = 0.642, P = 0.022; r^2 = 0.621, P = 0.037 for farms A and C, respectively).

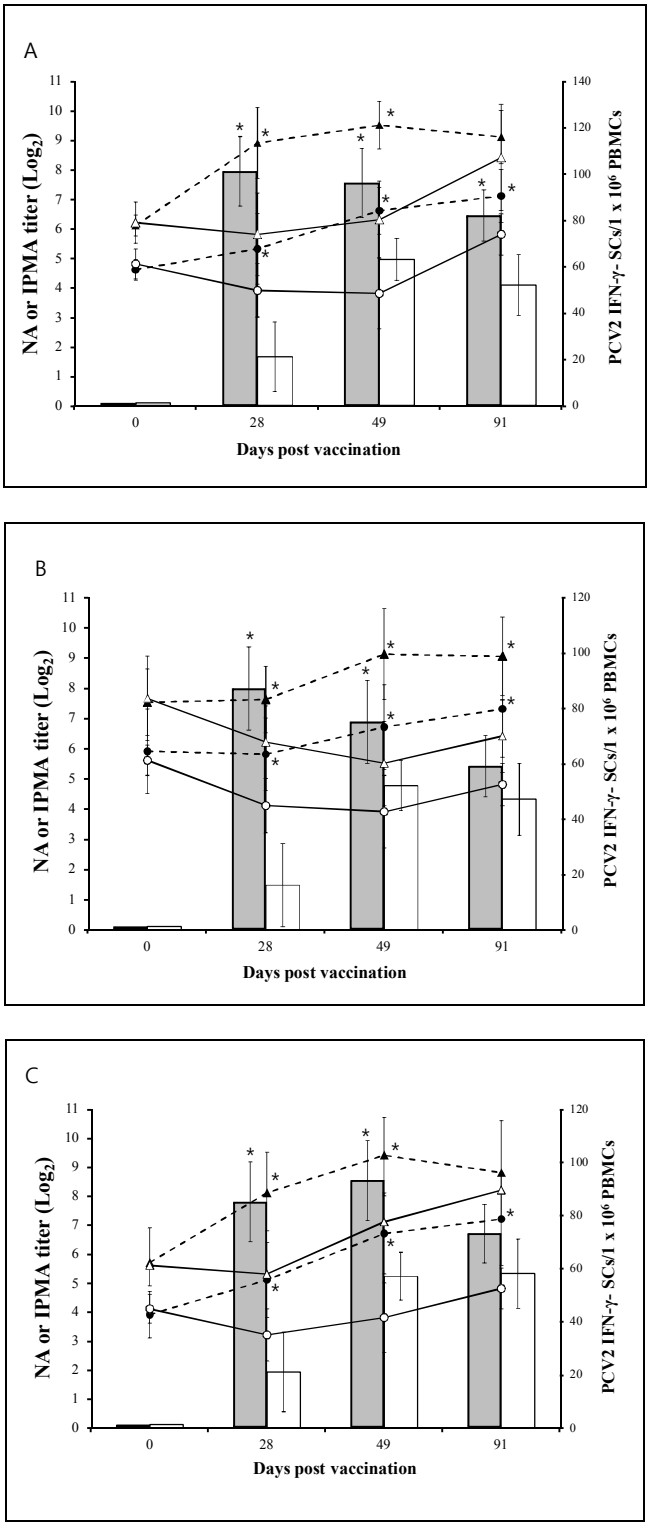


Fig. 3. Mean values of the number of porcine circovirus type 2 (PCV2)-specific interferon-γ-secreting cells (IFN-γ-SCs) in the peripheral blood mononuclear cells (PBMCs), and the titer of neutralizing antibodies (NA) and immunoperoxidase monolayer assay (IPMA) in the different groups. IFN-γ-SCs (■), NA titer (●), and IPMA titer (▲) in vaccinated animals and IFN-γ-SCs (□), NA titer (○), and IPMA titer (△) in non-vaccinated animals on farm A, B, and C. Variation is expressed as the standard deviation. Significant difference is indicated at P value<0.05*.

Identification of lymphocyte subsets

The proportion of $CD4^+$ cells was significantly higher in vaccinated animals at 28 dpv ($P < 0.021$, $P < 0.042$, $P < 0.042$ for farm A, B, and C, respectively) and 49 dpv ($P < 0.035$, $P < 0.027$, $P < 0.031$ for farm A, B, and C, respectively) than in non-vaccinated animals. The proportion of $CD8^+$ cells was not significantly difference between vaccinated and non-vaccinated animals in 3 farms.

In vaccinated animals, the number of $CD4^+$ cells correlated with the number of PCV2-specific IFN-γ-SCs ($r^2 = 0.432$, $P = 0.041$; $r^2 = 0.453$, $P = 0.040$; $r^2 = 0.533$, $P = 0.024$ for farms A, B, and C, respectively). In non-vaccinated animals, the number of $CD4^+$ cells correlated with the number of PCV2-specific IFN-γ-SCs ($r^2 = 0.524$, $P = 0.033$; $r^2 = 0.521$, $P = 0.037$; $r^2 = 0.552$, $P = 0.036$ for farms A, B, and C, respectively).

In vaccinated animals, the number of $CD4^+$ cells correlated inversely with the number of genomic copies of PCV2 in the blood ($r^2 = 0.492$, $P = 0.042$; $r^2 = 0.473$, $P = 0.045$; $r^2 = 0.572$, $P = 0.029$ for farms A, B, and C, respectively). In non-vaccinated animals, the number of $CD4^+$ cells correlated inversely with the number of genomic copies of PCV2 in the blood ($r^2 = 0.625$, $P = 0.031$; $r^2 = 0.595$, $P = 0.039$; $r^2 = 0.594$, $P = 0.022$ for farms A, B, and C, respectively).

Histopathology and immunohistochemistry

The histopathological lymphoid lesion scores were significantly lower in vaccinated animals than in non-vaccinated animals on all 3 farms ($P < 0.05$). The mean number of PCV2-positive cells per unit area of lymph node was significantly higher in non-vaccinated animals than in vaccinated animals on 3 farms A ($P < 0.001$) (Table 1).

Table 1. Average daily weight gain (ADWG), mortality rate, histopathological lymphoid lesion score, and immunohistochemical porcine circovirus type 2 (PCV2)-antigen score between vaccinated and non-vaccinated animals on 3 farms.

	Vaccinated animals			Non-vaccinated animals		
	Farm A	Farm B	Farm C	Farm A	Farm B	Farm C
3-7 weeks ADWG	394 ± 68	340 ± 40	359 ± 39	361 ± 64	320 ± 51	335 ± 40
7-10 weeks ADWG	556 ± 80	545 ± 70	577 ± 65	529 ± 73	499 ± 53	547 ± 52
10-16 weeks ADWG	802 ± 75*	749 ± 85*	721 ± 45*	734 ± 57	670 ± 49	650 ± 52
16 weeks- S†† ADWG	836 ± 58	794 ± 52 *	728 ± 37*	796 ± 41	731 ± 57	682 ± 43
3 weeks-S ADWG	702 ± 44*	671 ± 41*	644 ± 39*	658 ± 34	616 ± 43	601 ± 36
Slaughter age (days)	167†	180†	184†	174	186	189
Mortality rate	1 / 25	1 / 25	2 / 25†	4 / 25	3 / 25	6 / 25
Lymphoid lesion score	0.4±0.55†	0.2±0.45†	1.0±0.70†	2.4±0.54	2.2±0.44	3.0±0.70
PCV2-antigen score	8.76±5.94†	12.7±14.42†	20.48±10.94†	34.3±14.42	42.24±14.3	58.96±18.1

*Indicates significantly higher ADWG in vaccinated animals compared with non-vaccinated animals ($P < 0.05$).

†Indicates significantly lower mortality rate, lymphoid lesion score or PCV2-antigen score, and slaughter age in vaccinated animals than in non-vaccinated animals ($P < 0.05$).

††Date of shipment to slaughter.

Effects of maternally derived antibodies on PCV2 vaccine seroconversion

The results showed a significant and negative correlation of humoral immune response with vaccination of animals from farm A ($r^2 = 0.724$, $P = 0.011$ for IPMA; $r^2 = 0.594$, $P = 0.040$ for NA), farm B ($r^2 = 0.762$, $P = 0.003$ for IPMA; $r^2 = 0.615$, $P = 0.012$ for NA), and farm C ($r^2 = 0.682$, $P = 0.008$ for IPMA; $r^2 = 0.532$, $P = 0.036$ for NA). Thus, the higher the maternal antibody titer at the time of vaccination, the lower the subsequent increment of PCV2 antibody titers.

DISCUSSION

In the present study, the reformulated inactivated chimeric PCV1-2 vaccine reduced the PCV2 load in the blood and nasal swabs under field conditions similar to those of previous experimental studies (Segales et al., 2009). A correlation analysis showed that viremia was associated with nasal shedding. The reduction in the PCV2 nasal shedding is clinically meaningful because a nasal route may be more effective than a fecal and oral route (Patterson et al., 2011). Transmission through nasal secretions has been suggested as a potential mode of horizontal spreading (Patterson et al., 2011); therefore, vaccination would decrease the risk of transmission to other pigs and decrease the amount of PCV2 circulating within the herd.

Pathological evaluation that includes detection of the PCV2 antigen within these lesions is critical to determine the efficacy of the PCV2 vaccines under field conditions. Under experimental conditions, the specific PCV2-associated lesions were not observed in pigs experimentally challenged with solely PCV2, whereas under field conditions, coinfections and other factors induce PMWS-associated lesions. In the present study, the vaccination of pigs against PCV2 effectively reduces PMWS-associated microscopic lesions and the PCV2 load in lymphoid tissues in the 3 herds. Good correlation was observed between the decreased PCV2 load in the serum and decreased PCV2 antigen with low histopathological lesion scores in the lymph nodes.

A clinically important parameter for evaluating the efficacy of the vaccine under field conditions was the comparison of the ADWG between vaccinated and non-vaccinated animals. During the period of study (from 3 weeks of age to slaughter house), vaccinated animals displayed an improved ADWG (672.2 grams/day vs. 625 grams/day; difference of +47.3 grams/day; $P < 0.05$) and a reduced time to market (177 days vs. 183 days; difference of -6 days; $P < 0.05$). These data are comparable to previous field studies in which one-dose vaccination of piglets with other commercial PCV2 vaccines improved the ADWG, which ranged from 16 to 60 gram/day between 3 and 19-26 weeks of age (Kixmoller et al., 2008; Segales et al., 2009; Martelli et al., 2011; Fraile et al., 2012; Lyoo et al., 2012).

In the present study, a reduction in the PCV2 load in the blood coincided with the appearance of

both PCV2-specific NA and IFN-γ-SCs in the vaccinated animals, which is similar to previous studies that have used commercial subunit PCV2 vaccines (Fort et al., 2009; Martelli et al., 2011). Therefore, the induction of PCV2-specific NA and IFN-γ-SCs by the vaccine plays a critical role in the reduction of the PCV2 load in the blood and protects pigs against PCV2 infection. In addition, the number of $CD4^+$ cells was significantly decreased in the non-vaccinated animals compared to vaccinated animals. These results agree with previous findings of reduced $CD4^+$ cells in pigs with PMWS (Segales et al., 2001; Nielsen et al., 2003). A selective loss of $CD4^+$ cells during PCV2 infection may impair the immune system in the pigs and result in co-infections with other viral and bacterial pathogens which are frequently observed in pigs with PMWS in field conditions. Hence, both humoral and cellular immune responses induced by the reformulated inactivated chimeric PCV1-2 vaccine could contribute to PCV2 clearance and control the PCV2 infection under field conditions.

The vaccination of piglets is a popular trend in the swine industry worldwide. In Korea, PCV2 vaccination was administered to approximately 97% of the total piglets farrowed in 2010 and 2011. The increasing use of piglet vaccination may lead to a greater possibility of interference from MDA. In the present study, animals with high IPMA (> 9 $\log_2$) or NA (> 7 $\log_2$) titers seemed to show interference with the development of the humoral immune response after vaccination, similar to previous studies(Fort et al., 2009). However, most of piglets (> 80% in 3 farms) were lower IPMA (< 9 $\log_2$) or NA (< 7 $\log_2$) titers at vaccination (data not shown). Moreover, the reformulated inactivated chimeric PCV1-2 vaccine can elicit PCV2-specific NA and IFN- γ-SCs even in the presence of MDA as other commercial PCV2 vaccines (Fraile et al., 2012; Martelli et al., 2011). Hence, MDA interference does not significantly hamper efficacy of reformulated inactivated chimeric PCV1-2 vaccine as former inactivated chimeric PCV1-2 vaccine (Opriessnig et al., 2008). On the basis of field study, the reformulated inactivated chimeric PCV1-2 vaccine is a choice for swine practitioners and producers to protect pigs from PCV2 infection.

REFERENCES

Allan, G.M., Mackie, D.P., McNair, J., Adair, B.M., McNulty, M.S., 1994. Production, preliminary characterisation and applications of monoclonal antibodies to porcine circovirus. Veterinary Immunology and Immunopathology 43, 357–371.

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. The Veterinary Journal 168, 41–49.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. The Veterinary Journal 169, 326–336.

Chae, C., 2012. Porcine circovirus type 2 and its associated disease in Korea. Virus Research 164,107–113.

Diaz, I., Mateu, E., 2005. Use of ELISPOT and ELISA to evaluate IFN-γ, IL-10 and IL-4 responses in conventional pigs. Veterinary Immunology and Immunopathology 106, 107–112.

Fort, M., Sibila, M., Perez-Martin, E., Nofrarias, M., Mateu, E., Segalés, J., 2009. One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine 27, 4031–4037.

Fraile, L., Grau-Roma, L., Sarasola, P., Sinovas, N., Nofrarias, M., Lopez-Jimenez, R., Huerta, E., Llorens, A., Lopez-Sorea, S., Perez, D., Segales, J., 2012. Inactivated PCV2 one shot vaccine applied in 3-week-old piglets: Improvement of production parameters and interaction with maternally derived immunity. Vaccine 30, 1986–1992.

Gagnon, C.A., Del-Castillo, J.R., Music, N., Fontaine, G., Harel, J., Tremblay, D, 2008. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of *Porcine circovirus-2* genotypes 2a and 2b in an epidemiological survey. Journal of Veterinary Diagnostic Investigation 20, 545–558.

Gagnon, C.A., Music, N., Fontaine, G., Tremblay, D., Harel, J., 2010. Emergence of a new type of porcine circovirus in swine (PCV): A type 1 and type 2 PCV recombinant. Veterinary

Microbiology 144, 18–23.

Kim, J., Chae, C., 2004. Expression of monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 in porcine circovirus 2-induced granulomatous inflammation. Journal of Comparative Pathology 131, 121–126.

Kim, D., Kim, C.H., Han, K., Seo, H.W., Oh, Y., Park, C., Kang, I., Chae, C., 2012. Comparative efficacy of commercial *Mycoplasma hyopneumoniae* and porcine circovirus 2 (PCV2) vaccines in pigs experimentally infected with *M. hyopneumoniae* and PCV2. Vaccine 29, 3206–3212.

Kixmoller, M., Ritzmann, M., Eddicks, M., Saalmuller, A., Elbers, K., Fachinger, V., 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine 26, 3443–3451.

Lyoo, K., Joo, H., Caldwell, B., Kim, H., Davies, P.R., Torrison, J., 2012. Comparative efficacy of three commercial PCV2 vaccines in conventionally reared pigs. The Veterinary Journal 189, 58–62.

Martelli, P., Ferrari, L., Morganti, M., De-Angelis, E., Bonilauri, P., Guazzetti, S., Caleffi, A., Borghetti, P., 2011. One dose of a porcine circovirus 2 subunit vaccine induces humoral and cell-mediated immunity and protects against porcine circovirus-associated disease under field conditions. Veterinary Microbiology 149, 339–351.

Nielsen, J., Vincent, I.E., Bøtner, A., Ladekjær-Mikkelsen, A.-S., Allan, G., Summerfield, A., McCullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 92, 97–111.

Opriessnig, T., Patterson, A.R., Elsener, J., Meng, X.J., Halbur, P.G., 2008. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. Clinical and Vaccine Immunology 15, 397–401.

Patterson, A.R., Ramamoorthy, S., Madson, D.M., Meng, X.J., Halbur, P.G., Opriessnig, T., 2011. Shedding and infection dynamics of porcine circovirus type 2 (PCV2) after experimental

infection. Veterinary Microbiology 149:91–98.

Pogranichnyy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunology 13,143–153.

Rodriguez-Arrioja, G.M., Segalés, J., Balasch, M., Rosell, C., Quintant, J., Folch, J.M., Plana-Duran, J., Mankertz, A., Domingo, M., 2000. Serum antibodies to porcine circovirus type 1 and type 2 in pigs with and without PMWS. Veterinary Record 146, 762–764.

Segalés, J., Alonso, F., Rosell, C., Pastor, J., Chianini, F., Campos, E., Lopez-Fuertes, L., Qnintana, J., Rodriguez-Arrioja, G., Calsamiglia, M., Pujols, J., Dominguez, J., Domingo, M., 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology Immunopathology 81, 37–44.

Segalés, J., Urniza, A., Alegre, A., Bru, T., Crisci, E., Nofrarías, M., Lopez-Soria, S., Balasch, M., Sibila, M., Xu, Z., Chu, H.-J., Fraile, L., Plana-Duran, J., 2009. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) improves clinical, pathological and virological outcomes in postweaning multisystemic wasting syndrome affected farms. Vaccine 27, 7313–7321.

Williams, P.P., 1993. Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs. Canadian Veterinary Research 57, 1–8.

PART II. Comparison of immune responses and protective efficacy induced by porcine circovirus type 2 (PCV2) vaccines

Chapter 1. Antigenic types contribute to elicit different levels of humoral and cell-mediated immune responses among commercial single-dose porcine circovirus type 2 vaccines

ABSTRACT

The objective of this study was to compare the induction of protective humoral and cell-mediated immunity by 4 commercially available single-dose porcine circovirus type 2 (PCV2) vaccines. A total of 50 3-week-old piglets were assigned to 5 groups (10 pigs per group). Four commercial PCV2 vaccines were administered according to the manufacturer's instructions and the piglets were observed for 154 days post vaccination (dpv). Inactivated chimeric PCV1-2 vaccines induced higher levels of PCV2-specific neutralizing antibodies (NAs) and interferon-γ-secreting cells (IFN-γ-SCs) in pigs than did the other 3 commercial PCV2 vaccines. The proportions of $CD4^+$ cells were significantly higher in animals vaccinated with inactivated chimeric PCV1-2 and PCV2 vaccines than in animals vaccinated with the 2 subunit vaccines. To our knowledge, this is the first comparison of the protective humoral and cell-mediated immunity induced by 4 commercial single-dose PCV2 vaccines under the same conditions. The results of this study demonstrated quantitative differences in the induction of humoral and cell-mediated immunity following vaccination among 4 commercial single dose PCV2 vaccines.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is associated with a number of diseases and syndromes, collectively called porcine circovirus-associated disease (PCVAD) (Chae 2004, 2005). PCV2 is considered to be one of the most important viral pathogens, and PCVAD is a major viral swine disease worldwide. Since PCV2 vaccines were introduced to the world market in 2006, PCV2 vaccines have come to account for the largest market share of swine vaccines. Total sales of the PCV2 vaccine are approximately US $ 450 million worldwide (personal communication, Dr. Su-Jin Park, Zoetis).

The protective immunity induced by commercial PCV2 vaccines relies on the development of a humoral and cellular immune response (Chae 2012b). PCV2-specific interferon-γ-secreting cells (IFN-γ-SCs), together with neutralizing antibodies (NAs), are likely to contribute to PCV2 clearance (Fort et al. 2007, 2009a; Meerts et al. 2005, 2006). Therefore, the induction of PCV2-specific NAs and IFN-γ-SCs by commercial PCV2 vaccines is a critical parameter to evaluate in determining the efficacy of PCV2 vaccines in controlling PCV2 infection (Chae 2012). However, induction of PCV2-specific NAs and IFN-γ-SCs in vaccinated pigs has rarely been studied under experimental and field conditions (Fort et al. 2009b; Martelli et al. 2011). Furthermore, most experimental studies have evaluated the efficacy of commercial PCV2 vaccines under a vaccination and challenge model rather than a vaccination and non-challenge model. Only two studies have evaluated the induction of an immune response by commercial PCV2 vaccines under vaccination and non-challenge conditions (Fort et al. 2008, 2009b). The main disadvantage of the vaccination-challenge model is that it does not distinguish the induction of an immune response by the PCV2 vaccine from the induction of an immune response by the post-vaccination PCV2 challenge. Therefore, it is necessary to investigate the induction of a protective immune response by commercial PCV2 vaccines under the same vaccination and non-challenging experimental conditions.

The four single-dose PCV2 vaccines that are currently commercially available have two main antigen types; inactivated and subunit antigen. The two inactivated vaccines are inactivated PCV2 (Circovac) and a chimeric PCV1-2 vaccine (Fostera PCV). The inactivated chimeric PCV1-2 vaccine is based on the genomic backbone of the non-pathogenic PCV1 with the PCV2 ORF2 capsid gene in place of the

PCV1 capsid gene (Fenaux et al. 2004). Two subunit vaccines (Circoflex and Porcilis PCV) are based on an open reading frame 2 protein (ORF2; capsid) expressed in the baculovirus system (Chae 2012b). The 4 currently commercially available single-dose PCV2 vaccines differ in their antigen and adjuvant types (Chae 2012b), which raises the possibility that commercial PCV2 vaccines may induce different levels of protective immune responses. Hence, the objective of the present study was to compare the induction of protective humoral and cell-mediated immunity by 4 commercial single-dose PCV2 vaccines.

MATERIALS AND METHODS

Animals

A total of 50 colostrum-fed, cross-bred, conventional piglets were purchased at 14 days of age from a commercial farm. All piglets were negative for porcine reproductive and respiratory syndrome virus (PRRSV), and *M. hyopneumoniae* according to routine serological testing. All piglets were also negative to PCV2 and its antibodies according to commercial ELISA (Synbiotics, Lyon, France) and serum neutralization test, and real-time PCR as previously described (Gagnon et al. 2008, 2010).

Experimental design

A total of 50 3-week-old piglets were assigned to 5 groups (10 pigs per group). This study used a randomized, blinded, weight- and sex-matched, controlled design. Four commercial PCV2 vaccines were used and administered according to the manufacturer's instructions with regards to timing (3 weeks of age) and route of injection (intramuscularly in the right side of the neck): Fostera PCV (Zoetis, Madison, NJ, USA) was given as one 2.0 ml dose (group T01); Circovac (Merial, Lyon, France) was given as one 0.5 ml dose (group T02); Circoflex (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA) was given as one 1.0 ml dose (group T03); and Porcilis PCV (Merck, Sharp & Dohme Animal Health, Boxmeer, The Netherlands) was given as one 2.0 ml dose (group T04). The pigs in group T05 served as the negative control group.

All pigs were housed throughout the experiment in an environmentally controlled building in pens over totally slatted floors as previously described (Kim et al. 2011). The pigs in each group were housed separately within the facility. Blood samples from each pig were collected by jugular venipuncture at 0, 21, 42, 63, 91, 119, and 154 days post vaccination (dpv). All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

Quantification of PCV2 DNA in blood

DNA extraction from serum samples was performed using the QIAamp DNA mini kit. DNA extracts were used to quantify PCV2 genomic DNA copy numbers by real-time PCR as previously described (Gagnon et al. 2008).

Serology

The serum samples were tested using the commercial PCV2 ELISA IgG (Synbiotics, Lyon, France) and serum virus neutralization (SVN) (Pogranichnyy et al. 2000; Shina et al. 2010). The levels of PCV2 ELISA IgG were expressed as a quantitative titer. Serum samples were considered positive for PCV2 IgG antibody if the titer was greater than 550 according to the manufacturer's instructions.

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) at 0, 21, 42, 63, 91, 119, and 154 dpv as previously described (Segalés et al. 2001). Peripheral blood mononuclear cells (PBMC) were separated from blood by density-gradient centrifugation with Histopaque 1.077 (Sigma-Aldrich, St. Louis, MO, USA). PCV2 antigen was prepared as previously described (Nawagitgul et al. 2002).

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 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., SelectScience, 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°C 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.

Flow cytometry

PBMCs were incubated with R-PE- or FITC-conjugated mouse monoclonal antibodies (antiswine CD4 [R-PE] and CD8 [FITC]; SouthernBiotech, Birmingham, AL, USA) for 30 minutes at 4°C in the dark and washed twice. Cells stained with conjugated antibodies were resuspended immediately in supplemented RPMI 1640 medium. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) as previously described (Sosa et al. 2009).

Statistical analysis

Summary statistics were calculated for all groups to assess the overall quality of the data, including normality. The values of genomic copies of serological data and PCV2 viremia were transformed $\log_2$ and $\log_{10}$, respectively, prior to analysis. Continuous data (PCV2 serology, PCV2-specific IFN-γ-SCs, and lymphocyte subsets) were analyzed with a repeated measures analysis of variance (ANOVA). If the repeated measures ANOVA showed a significant effect, a one-way ANOVA with pairwise testing using Tukey's adjustment was performed at each time point. The Friedman test and the Wilcoxon signed rank test for paired samples were applied to estimate the difference at each time point post vaccination. A value of $P < 0.05$ was considered significant.

RESULTS

Quantification of PCV2 DNA in blood

No PCV2b DNA was detected in the blood samples from the all vaccinated groups and the negative control group throughout the experiment.

Anti-PCV2 IgG antibodies

At the time of PCV2 vaccination (0 dpv), no anti-PCV2 IgG antibodies were detected in vaccinated animals. At 21 dpv, inactivated chimeric PCV1-2 (Fostera PCV), inactivated PCV2 (Circovac) vaccines induced significantly ($P < 0.05$) higher anti-PCV2 IgG antibodies titers than subunit (Circoflex and Porcilis PCV) vaccines (Fig. 1). No anti-PCV2 IgG antibodies were detected in negative control pigs throughout the experiment.

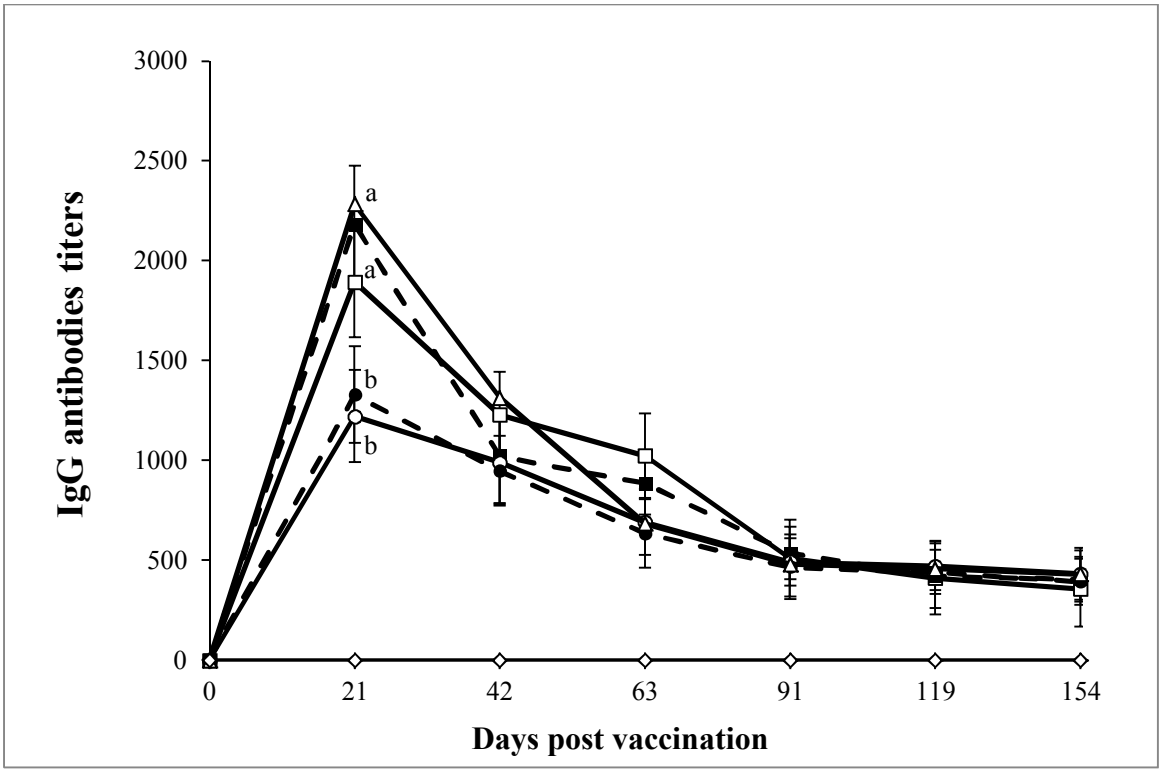


Figure 1. Mean values of titer of serum PCV2 IgG antibodies at different days post vaccination among 4 commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, □); inactivated PCV2 vaccine (Circovac, ■); and subunit PCV2 vaccine (Circoflex, ○ and Porcilis PCV, ●); and non-vaccinated animals (◇). Different letters (a and b) indicate significant ($P < 0.05$) difference among groups.

Neutralizing antibodies

At the time of PCV2 vaccination (0 dpv), no NAs were detected in vaccinated animals. The group mean of the $\log_2$ transformed NA titers reached a peak at 42 dpv and then decreased gradually until 154 dpv (Fig. 2). There was a significant ($P < 0.05$) difference in the log transformed NA titers among individual PCV2 vaccines. At 42 dpv, inactivated chimeric PCV1-2 and PCV2 vaccines induced significantly higher NA titers than two subunit vaccines in pigs ($P < 0.05$). At 63 dpv, inactivated chimeric PCV1-2 vaccine induced significantly higher NA titers than the subunit vaccine (Circoflex) ($P < 0.05$). No NAs were detected in negative control pigs throughout the experiment.

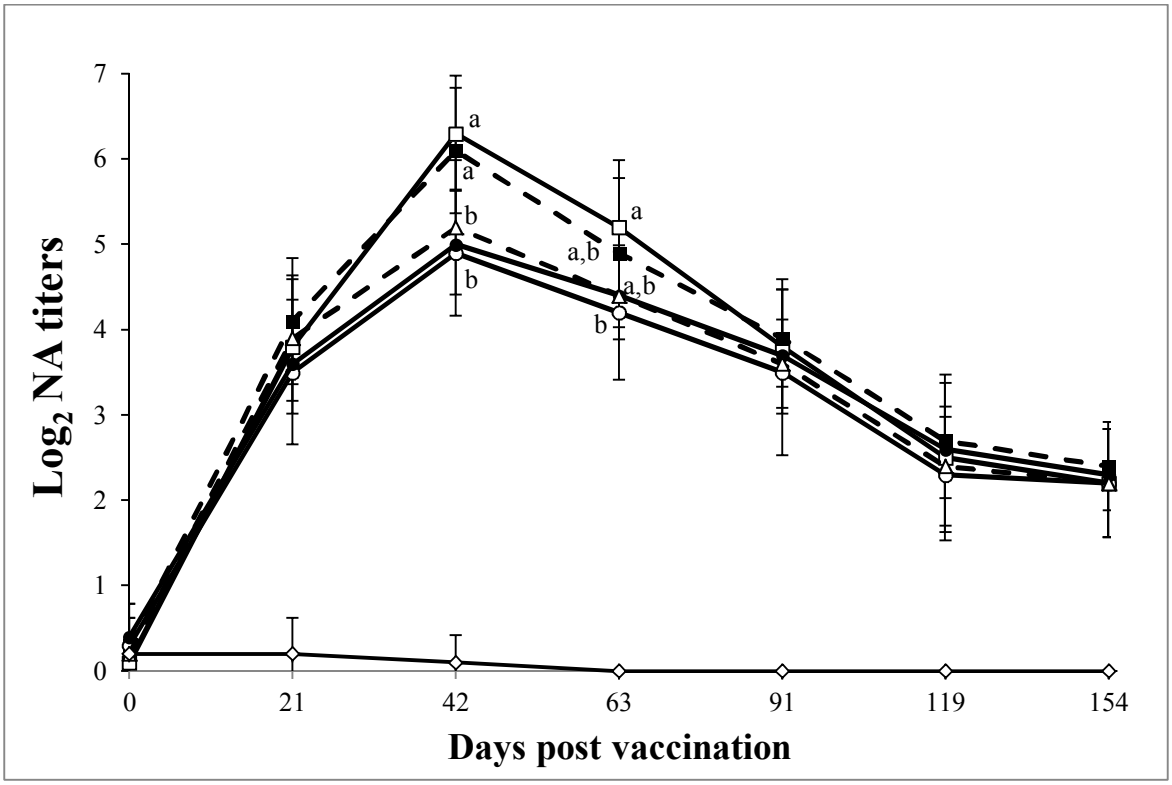


Figure 2. Log transformed group mean and standard deviation for neutralizing antibodies (NAs) response at different days post vaccination among 4 commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, □); inactivated PCV2 vaccine (Circovac, ■); subunit PCV2 vaccine (Circoflex, ○ and Porcilis PCV, ●); and non-vaccinated animals (◇). Different letters (a and b) indicate significant ($P < 0.05$) difference among groups.

PCV2-specific interferon- γ-secreting cells

At the time of PCV2 vaccination (0 dpv), no PCV2-specific IFN-γ-SCs were detected in vaccinated animals. The mean number of PCV2-specific IFN-γ-SCs reached a peak at 21 dpv and then decreased gradually until 154 dpv (Fig. 3). At 21 dpv, inactivated chimeric PCV1-2 vaccines induced significantly higher mean number of PCV2-specific IFN-γ-SCs than two subunit PCV2 vaccines ($P < 0.05$). At 42 dpv, inactivated PCV2 and chimeric PCV1-2 vaccines induced significantly higher mean number of PCV2-specific IFN-γ-SCs than the two subunit vaccines in pigs ($P < 0.05$). No PCV2-specific IFN-γ-SCs were detected in negative control pigs throughout the experiment.

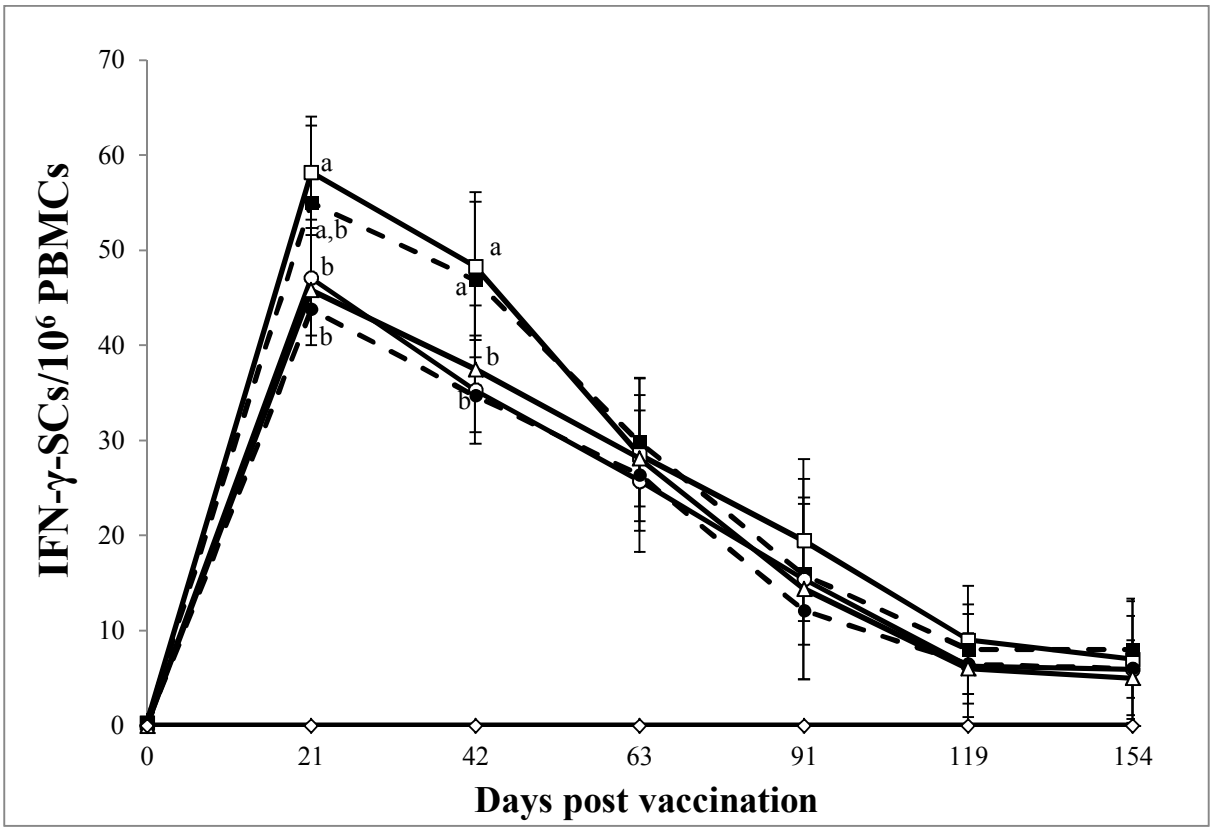
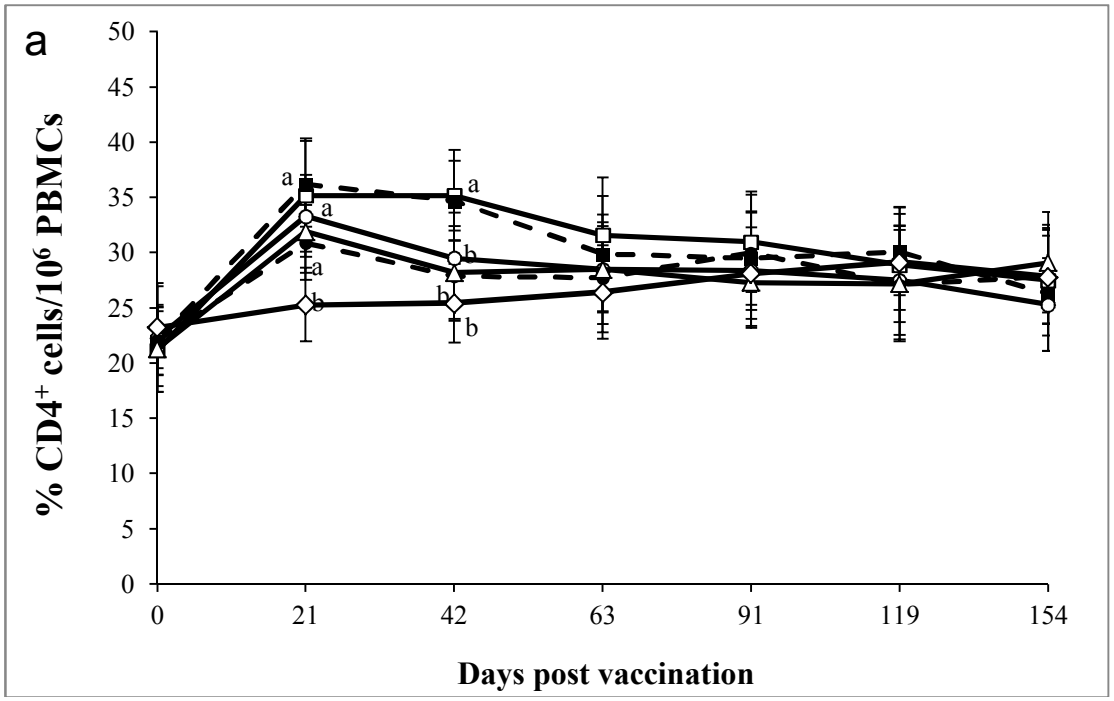


Figure 3. Mean values of the number of PCV2-specific interferon-γ-secreting cells (IFN-γ-SCs) response at different days post vaccination among 4 commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, □); inactivated PCV2 vaccine (Circovac, ■); subunit PCV2 vaccine (Circoflex, ○ and Porcilis PCV, ●); and non-vaccinated animals (◇). Different letters (a and b) indicate significant ($P < 0.05$) difference among groups.

Proportion of lymphocyte subsets

At the time of PCV2 vaccination (0 dpv), no significant differences were observed in the proportions of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ cells among the group receiving the 4 different commercial PCV2 vaccines. The proportion of $CD4^+$ cells was significantly higher in vaccinated animals at 21 dpv ($P < 0.05$) than in non-vaccinated animals (Fig. 4a). At 42 dpv, the proportion of $CD4^+$ cells was significantly higher in animals vaccinated with inactivated chimeric PCV 1-2 and PCV2 vaccines than in animals vaccinated with the two subunit vaccines and in non-vaccinated animals ($P < 0.05$). The proportion of $CD4^+CD8^+$ cells was significantly higher in vaccinated animals at 21 dpv ($P < 0.05$) than in non-vaccinated animals ($P < 0.05$) (Fig. 4b). No significant differences were observed in the proportions of $CD8^+$ among the groups (Fig. 4c).



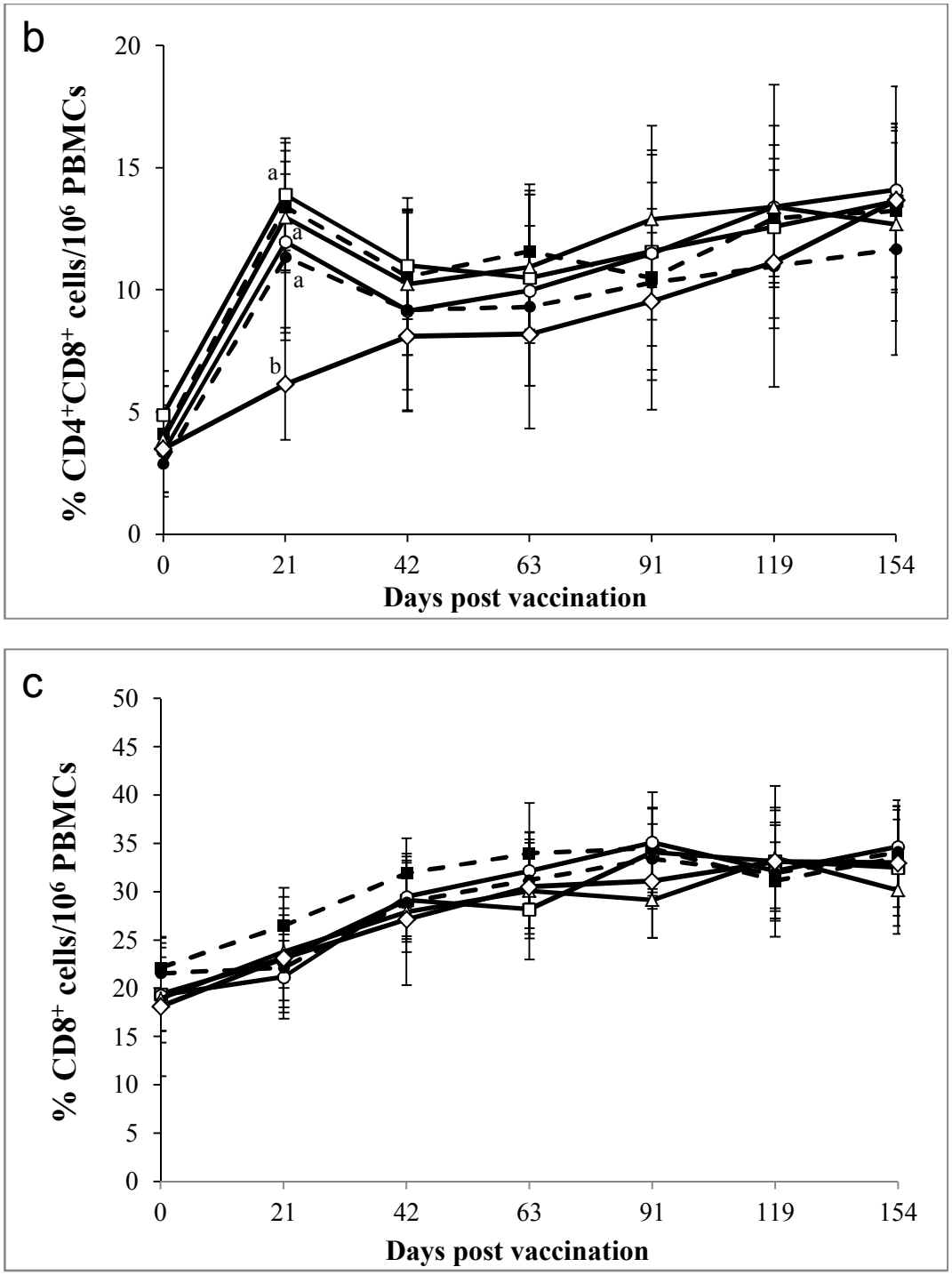


Figure 4. Group mean and standard deviation for the proportion of $CD4^+$ (A), $CD8^+$ (B), and $CD4^+CD8^+$ (C) cells at different days post vaccination among 4 commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, □); inactivated PCV2 vaccine (Circovac, ■); subunit PCV2 vaccine (Circoflex, ○ and Porcilis PCV, ●); and non-vaccinated animals (◇). Different letters (a and b) indicate significant ($P < 0.05$) difference among groups.

DISCUSSION

Although commercial PCV2 vaccines are highly effective in controlling PCV2 infection in pigs (Kixmoller et al. 2008; Segalés et al. 2009; Martelli et al. 2011; Fraile et al. 2012), this study demonstrated a quantitative difference in the induction of humoral and cell-mediated immunity following vaccination with 4 commercial PCV2 vaccines. As PCV2-specific IFN-γ-SCs, together with NAs, are likely to contribute to PCV2 clearance (Fort et al. 2009b; Martelli et al. 2011), it is clinically significant to compare the induction of PCV2-specific IFN-γ-SCs and NAs by commercial PCV2 vaccines as indicators of the control of PCV2 infection in pigs. Inactivated PCV 1-2 vaccine induced higher levels of PCV2-specific IFN-γ-SCs and NAs in pigs compared to subunit vaccines. The differences in immune responses between inactivated and subunit vaccine may be due to differences in antigens, although differences in adjuvants may also affect the immune response. This interpretation is supported by the fact that the two subunit vaccines with different adjuvants elicit similar immune responses throughout the experiment. These results suggest that antigen type is a more critical determinant of the immune response to commercial PCV2 vaccines than adjuvant type. However, since adjuvants from commercial PCV2 vaccine also contribute to elicit the immune responses (Leenaars et al. 1998; Hilgers et al. 1999; Krashias et al, 2010), further studies may be needed to evaluate the immunological role of different adjuvants among single dose PCV2 vaccines.

Lymphocyte subset analysis is also an important parameter to compare the induction of cell-mediated immunity induced by commercial PCV2 vaccines. The relative proportion of $CD4^+$ cells decreased in PCV2-infected pigs (Karuppannan et al. 2008). In addition, significant decreases in the numbers of $CD4^+$ and $CD4^+CD8^+$ cells were also observed in pigs with naturally occurring PMWS (Segalés et al. 2001; Nielsen et al. 2003). These data suggest that selective loss of $CD4^+$ and $CD4^+CD8^+$ cells may impair the pigs' immune system. Although vaccinated-pigs exhibited higher proportions of $CD4^+$ and $CD4^+CD8^+$ cells compared with negative control pigs, significant differences were observed in the relative proportion of $CD4^+$ cells among the 4 commercial PCV2 vaccines.

Inactivated chimeric PCV1-2 vaccine yielded higher relative proportions of $CD4^+$ cells compared to subunit vaccines.

These results show that the development of cell-mediated immunity (i.e., PCV2-specific IFN-γ-SCs) peaks earlier than the development of humoral immunity (i.e., PCV2-specific NAs) in response to all commercial PCV2 vaccines used in the present study. These results agree with previous findings in which subunit vaccines also induced earlier maximal induction of PCV2-specific IFN-γ-SCs compared with PCV2-specific NAs (Fort et al. 2009b). Maximal induction of cell-mediated immunity is reached at 21 days post vaccination, whereas maximal induction of humoral immunity is reached at 42 days post vaccination. These results provided clinically significant meaning to determine the appropriate timing of vaccination. Vaccine efficacy may also be influenced by the interval between vaccination and infection. Ideally, PCV2 vaccination should be administered before pigs become naturally infected. Our results suggested that a 3 to 4 weeks interval between vaccination and infection provides maximal protection against PCV2 infection. As PCVAD most commonly affected pigs between the ages of 5-15 weeks (Chae 2004), the PCV2 vaccine should be administered to pigs between 1 and 11 weeks of ages. Although vaccines are commonly labeled for use at 21 days of age or older, it may be possible to administer the PCV2 vaccine to piglets younger than 3 weeks of age. Experimental challenge studies clearly showed that vaccination of 5 day old pigs resulted in the development of a detectable humoral immune response and reduced PCV2 viremia and PCV2-associated lesions under the PCV2-PPV-PRRSV triple challenge model (O'Neill et al. 2011). Pig practitioners and producers are urged to determine the proper vaccination timing to optimize and maximize the efficacy of vaccination at the time of PCV2 infection in pigs.

One-dose vaccination is less labor intensive and reduces stress to animals. Thus, many producers prefer to vaccinate with a one-dose PCV2 product. Accordingly, the one-dose vaccines for piglets are the most popular in Korea, accounting for 78% of market share (Chae 2012a), and are likely to become more popular among pig producers worldwide. To our knowledge, this is the first comparison of humoral and cell-mediated immunity induced by 4 commercial single-dose PCV2 vaccines under

the same conditions. Our results demonstrated that the different types of antigens in the 4 commercial single-dose PCV2 vaccines induce different levels of protective immune responses.

REFERENCES

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. The Veterinary Journal 168, 41–49.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. The Veterinary Journal 169, 326–336.

Chae, C., 2012a. Porcine circovirus type 2 and its associated disease in Korea. Virus Research 164, 107–113.

Chae, C., 2012b. Commercial porcine circovirus type 2 vaccines: Efficacy and clinical application. The Veterinary Journal 194, 151-157.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. Journal of Virology 78, 6297–6303.

Fort, M., Olvera, A., Sibila, M., Segalés, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS affected pigs. Veterinary Microbiology 125, 244–255.

Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F., Segalés, J., 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine 26, 1063–1071.

Fort, M., Fernandes, L.T., Nofrarias, M., Diaz, I., Sibila, M., Pujols, J., Mateu, E., Segalés, J., 2009a. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in Caesarean-derived, colostrum-deprived piglets. Veterinary Immunology and Immunopathology 129, 101–107.

Fort, M., Sibila, M., Perez-Martin, E., Nofrarias, M., Mateu, E., Segalés, J., 2009b. One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets

elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine 27, 4031–4037.

Fraile, L., Grau-Roma, L., Sarasola, P., Sinovas, N., Nofrarias, M., Lopez-Jimenez, R., Lopez-Soria, S., Silbila, M., Segalés, J., 2012. Inactivated PCV2 one shot vaccine applied in 3-week-old piglets: Improvement of production parameters and interaction with maternally derived immunity. Vaccine 30, 1986–1992.

Gagnon, C.A., Del-Castillo, J.R., Music, N., Fontaine, G., Harel, J., Tremblay, D., 2008. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of *Porcine circovirus-2* genotypes 2a and 2b in an epidemiological survey. Journal of Veterinary Diagnostic Investigation 20, 545–558.

Gagnon, C.A., Music, N., Fontaine, G., Tremblay, D., Harel, J., 2010. Emergence of a new type of porcine circovirus in swine (PCV): A type 1 and type 2 PCV recombinant. Veterinary Microbiology 144, 18–23.

Hilgers, L.A.Th., Lejeune, G., Nicolas, I., Fochesato, M., Boon, B., 1999. Sulfolipo-cyclodextrin in squalane-in-water as a novel and safe vaccine adjuvant. Vaccine 17, 219–228.

Karuppannan, A.K., Jong, M.H., Lee, S.H., Zhu, Y., Selvaraj, M., Lau, J., Jia, Q., Kwang, J., 2008. Attenuation of porcine circovirus 2 in SPF piglets by abrogation of ORF3 function. Virology 383, 338–347.

Kim, D., Kim, C.H., Han, K., Seo, H.W., Oh, Y., Park, C., Kang, I., Chae, C., 2011. Comparative efficacy of commercial *Mycoplasma hyopneumoniae* and porcine circovirus 2 (PCV2) vaccines in pigs experimentally infected with *M. hyopneumoniae* and PCV2. Vaccine 29, 3206–3212.

Kixmoller, M., Ritzmann, M., Eddicks, M., Saalmuller, A., Elbers, K., Fachinger, V., 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine 26, 3443–3451.

Krashias, G., Simon, A.-K., Wegmann, F., Kok, W.-L., Ho, L.-P., Stevens, D., Skehel, J., Heeney, J.L., Moghaddam, A.E., Sattentau, Q.J., 2010. Potent adaptive immune responses induced against HIV-1 gp 140 and influenza virus HA by a polyanionic carbomer. Vaccine 28, 2482–2489.

Leenaars, M., Koedam, M.-A., Hendriksen, C.F.M, Classen, E., 1998. Immune responses and side effects of five different oil-based adjuvants in mice. Veterinary Immunology and Immunopathology 61, 291–304.

Martelli, P., Ferrari, L., Morganti, M., Angelis, D.E., Bonilauri, P., Guazzetti, S., Caleffi, A., Borghetti, P., 2011. One dose of a porcine circovirus 2 subunit vaccine induces humoral and cell-mediated immunity and protects against porcine circovirus-associated disease under field conditions. Veterinary Microbiology 149, 339–351.

Meerts, P., Van-Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. Viral Immunology 18, 333–341.

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Botner, A., Kristensen, C.S., Nauwynck, H., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Veterinary Research 2, 6.

Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., Paul, P.S., 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clinical and Diagnostic Laboratory Immunology 9, 33–40.

Nielsen, J., Vincent, I.E., Bøtner, A., Ladekjær-Mikkelsen, A.-S., Allan, G., Summerfield, A., McCullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 92, 97–111.

O'Neill, K.C., Shen, H.G., Lin, K., Hemann, M., Beach, N.M., Meng, X.J., Halbur, P.G., Opriessnig, T., 2011. Studies on porcine circovirus type 2 vaccination of 5-day-old piglets. Clinical and Vaccine Immunology 18, 1865−1871.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Halbur, P.G., 2009. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine 27, 1002−1007.

Pogranichnyy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunology 13, 143−153.

Segalés, J., Alonso, F., Rosell, C., Pastor, J., Chianini, F., Campos, E., Lopez-Fuertes, L., Quintana, J., Rodriguez-Arrioja, G., Calsamiglia, M., Pujols, J., Dominguez, J., Domingo, M., 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 81, 37−44.

Segalés, J., Urniza, A., Alegre, A., Bru, T., Crisci, E., Nofrarias, M., Lopez-Soria, S., Balasch, M., Silbila, M., Xu, Z., Chu, H.J., Fraile, L., Plana-Duran, J., 2009. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) improves clinical, pathological and virological outcomes in postweaning multisystemic wasting syndrome affected farms. Vaccine 27, 7313−7321.

Shina, A., Shen, H.G., Schalk, S., Beach, N.M., Huang, Y.W., Halbur, P.G., Meng, X.J., Opriessnig, T., 2010. Porcine reproductive and respiratory syndrome virus infection at the time of porcine circovirus type 2 vaccination has no impact on vaccine efficacy. Clinical and Vaccine Immunology 17, 1940-1945.

Sosa, G.A., Quiroga, M.F., Roux, M.E., 2009. Flow cytometric analysis of T-lymphocytes from nasopharynx-associated lymphoid tissue (NALT) in a model of secondary immunodeficiency in Wistar rats. Immunobiology 214, 384−391.

CHAPTER II. Comparison of four commercial one-dose porcine circovirus type 2 vaccines based on clinical, virological, immunological and pathological evaluation

ABSTRACT

The objective of this study was to compare the efficacy of four commercial porcine circovirus type 2 (PCV2) vaccines based on clinical (average daily weight gain; ADWG), virological (PCV2 viremia), immunological (PCV2-specific neutralizing antibodies [NA], interferon-γ-secreting cells [IFN-γ-SCs], and $CD3^+$ and $CD4^+$ subsets), and pathological (lymphoid lesion and PCV2 antigen score) evaluation under the same experimental conditions. A total of 60 3-week-old piglets were assigned to 6 groups (10 pigs per group). Four commercial PCV2 vaccines were administered according to the manufacturer's label instructions. At 4 weeks post-vaccination, the pigs in vaccinated and positive control groups were inoculated intranasally with 2 ml of PCV2b. The ADWG of vaccinated animals was significantly higher than that of non-vaccinated animals during the period 9-16 weeks of age but not thereafter. Significant ($P < 0.05$) differences were observed between vaccinated and positive control groups in log transformed PCV2b DNA in the blood and from nasal swabs, in the log transformed NA titers, in the number of PCV2-specific IFN-γ-SCs at 0, 7, 14, 21, and 42 days post challenge (dpc), and in the proportion of $CD4^+$ cells at 7 and 14 dpc. The histopathological lymphoid lesion scores and PCV2-antigen scores in lymph nodes were significantly ($P < 0.05$) lower in vaccinated groups than in the positive control group. This study demonstrated that the four commercial PCV2 vaccines tested here are highly effective in controlling PCV2 infection.

INTRODUCTION

Porcine circovirus type 2 (PCV2) has been identified as the main etiologic agent of a series of diseases collectively referred to as porcine circovirus-associated disease (PCVAD) (Chae, 2004, 2005). Among these conditions, postweaning multisystemic wasting syndrome (PMWS) is the most important (Chae, 2005). Since PCV2 vaccines were first introduced to the world market in 2006, vaccination has become a major tool for the control of PCVAD. Commercial PCV2 vaccines have been shown to be highly effective in reducing and preventing PCVAD under experimental and field conditions (Fort et al., 2008, 2009b; Fraile et al., 2012; Kixmoller et al., 2008; Martelli et al., 2011; Opriessnig et al., 2009; Segalés et al., 2009; Seo et al., 2012; Shen et al., 2010).

One of the most important criteria for the evaluation of the efficacy of vaccines is the reduction of PCV2 viremia, which plays a central role in the development of PCVAD (Chae, 2012). Although it may vary from laboratory to laboratory based on standards used for quantification, the level of PCV2 viremia is used to categorize PCV2-infected pigs as subclinically infected, suspected and PCVAD-positive, respectively (Brunborg et al., 2004; Liu et al., 2000; Olvera et al., 2004; Segalés et al., 2005). These data suggest that the quantification of PCV2 load could predict PCV2 infection status. Thus, the measurement of PCV2 viremia is an objective method of evaluating the efficacy of PCV2 vaccines. In addition, reduction of PCV2 viremia is correlated with an induction of PCV2-specific neutralizing antibodies (NA) and interferon-γ-secreting cells (IFN-γ-SCs) by commercial PCV2 vaccines (Meerts et al., 2005, 2006; Fort et al., 2007, 2009a). Nevertheless, only one study tested whether commercial PCV2 vaccines induce PCV2-specific IFN-γ-SCs under experimental conditions (Fort et al., 2009b).

Today, four fully licensed commercial one-dose PCV2 vaccines are available in Korea. The commercial PCV2 vaccines include two subunit vaccines based on the PCV2 capsid protein expressed in the baculovirus system and two inactivated vaccines based on PCV2 or chimeric PCV1-2. The fact that these vaccines contain different antigens raises the possibility that commercial PCV2 vaccines induce different degrees of immune responses, reduction of PCV2 viremia, lymphoid lesion and

PCV2 antigen score. Previous studies have compared virological and pathological outcomes only among 3 commercial PCV2 vaccines registered in the USA (Opriessnig et al., 2009; Shen et al., 2010); however, limited data are available comprehensively comparing the efficacy of the four commercially available one-dose PCV2 vaccines. The objective of this study was to compare the efficacy of four PCV2 vaccines based on clinical, virological, immunological, and pathological evaluation.

MATERIALS AND METHODS

Animals and housing

A total of 60 colostrum-fed, cross-bred, conventional piglets were purchased at 14 days of age, derived from PCV2-serongenative gilts (n = 9) in a porcine reproductive and respiratory syndrome virus (PRRSV)-free commercial farm. Gilts and sows had never been vaccinated against PCV2. Clinical signs indicative of PCV2 had not been observed on the farm. All serum samples from the piglets were also negative for PCV2 antibodies and PCV2 virus according to the immunoperoxidase monolayer assay (IPMA) and real-time polymerase chain reaction (PCR) as previously described (Gagnon et al., 2008; Fort et al., 2009b).

Experimental challenge study

At 3-weeks of age the 60 piglets were assigned into 6 groups (10 pigs per group). This study used a randomized, blinded (laboratory personnel), weight- and sex-matched, controlled design. Four commercial PCV2 vaccines were used and administered according to the manufacturer's instructions with regards to timing (3 weeks of age) and route of injection (intramuscularly in the right side of the neck): Fostera PCV/Suvaxyn PCV2 One Dose (Zoetis, Madison, NJ, USA) as one 2.0 mL dose (group T01); Circovac (Merial, Lyon, France) as one 0.5 mL dose (group T02); Circoflex (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA) as one 1.0 mL dose (group T03); Porcilis PCV (Merck, Sharp & Dohme Animal Health, Boxmeer, The Netherlands) as one 2.0 mL dose (group T04); phosphate buffered saline (PBS) was given in a 2.0 mL dose (groups T05 and T06).

At 49 days of age (0 days post challenge [dpc]), the pigs in groups T01, T02, T03, T04, and T05 were inoculated intranasally with 2 mL (1 mL per each nostril) of PCV2b (strain SNUVR000463; 5th passage; 1.0 x 10^5 50% tissue culture infective dose/mL). The pigs in group T06 were exposed in the same manner to uninfected cell culture supernatants and served as the negative control group.

The pigs in each group were housed separately within the facility. Blood samples from each pig were collected by jugular venipuncture and nasal swabs were collected by inserting a flocked sterile

swab (Copan, Brescia, Italy) into each nostril at -28, 0, 7, 14, 21, 42, 70, 98, and 133 dpc. All pigs were tranquilized by an intravenous injection of azaperone (Stresnil, Janssen Pharmaceutica, Beerse, Belgium) and then euthanized by electrocution for necropsy at 133 dpc. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-13021911).

Growth performance

The live weight of each pig was measured at 3, 7, 9, 16, and 25 weeks of age. The average daily weight gain (ADWG; g/pig/day) was analyzed over four time periods: (i) between 3 and 7 weeks of age, (ii) between 7 and 9 weeks of age, (iii) between 9 and 16 weeks of age, and (iv) between 16 and 25 weeks of age. ADWG during the different production stages was calculated as the difference between the starting and final weights divided by the duration of the stage. Data from dead or removed pigs were included in the calculation.

Serology

The serum samples were tested using a commercial PCV2 ELISA IgG (Synbiotics, Lyon, France) and serum virus neutralization (Allan et al., 1994; Pogranichnyy et al., 2000). Serum samples were considered positive for PCV2 IgG antibody if the titer was greater than 550 according to the manufacturer's instructions.

Quantification of PCV2 DNA in blood and nasal swab

DNA extraction from serum and nasal samples was performed using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA, USA). DNA extracts were used to quantify PCV2 genomic DNA copy numbers by real-time PCR as previously described (Gagnon et al., 2008).

Enzyme-linked immunospot (ELISPOT) assay

The numbers of PCV2-specific IFN-γ-SCs were determined in peripheral blood mononuclear cells

(PBMCs) at -28, 0, 7, 14, 21, 42, 70, 98, and 133 dpc as previously described (Diaz et al., 2005; Seo et al., 2012). Whole PCV2b virus (as used for challenge) at multiplicity of infection of 0.01 was used as stimulant of PBMC. Phytohemagglutinin (PHA; 10 μg/mL, Roche Diagnostics GmbH, Mannheim, Germany) and PBS were used as a positive and negative control, respectively. The concentration of PBMCs was adjusted to contain 10^6 PBMCs per well. The number of IFN-γ-SCs was calculated by the number in the virus-stimulated well minus the number in PBS-stimulated well.

Flow cytometry

PBMCs (10^6 cells/mL) were incubated with R-PE (1 μg/mL)- or FITC (5 μg/mL)-conjugated mouse monoclonal antibodies (antiswine CD3 [R-PE] and CD4 [R-PE and FITC]; SouthernBiotech, Birmingham, AL, USA) for 30 min at 4 °C in the dark and washed twice. Cells stained with conjugated antibodies were resuspended immediately in supplemented RPMI 1640 medium. A gate was set to select lymphocytes using forward/side light scatter information. Background staining was compensated using the isotype controls conjugated to FITC or PE. Cells were analyzed using a FACSCalibur flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA) as previously described (Sosa et al., 2009).

Histopathology and immunohistochemistry

For the morphometric analysis of histopathological lesion (Fenaux et al., 2002) and PCV2 antigen (Kim et al., 2009) score in lymph nodes, three superficial inguinal lymph node sections were examined "blindly" as previously described. The lymphoid lesion scores ranged from 0–3; 0 (no lymphoid depletion or granulomatous replacement), 1 (mild lymphoid depletion), 2 (moderate lymphoid depletion), 3 (severe lymphoid depletion and granulomatous replacement). Analyses of the PCV2 antigen scores were performed with the NIH Image J 1.45s Program and the number of PCV2 positive cells per unit area ($0.25mm^2$) was counted.

Statistical analysis

Continuous data (PCV2 DNA, PCV2 serology, PCV2-specific IFN-γ-SCs, lymphocyte subsets, and ADWG) were analyzed with a repeated measures analysis of variance (ANOVA). If the repeated measures ANOVA showed a significant effect, a one-way ANOVA with pairwise testing using Tukey's adjustment was performed at each time point. Discrete data (lymphoid lesion and PCV2 antigen score) were analyzed by Chi-square and Fisher's exact tests. The Pearson's correlation coefficient was used to assess the relationship among viremia, NA titer, and PCV2-specific IFN-γ-SCs. A value of $P < 0.05$ was considered to be significant.

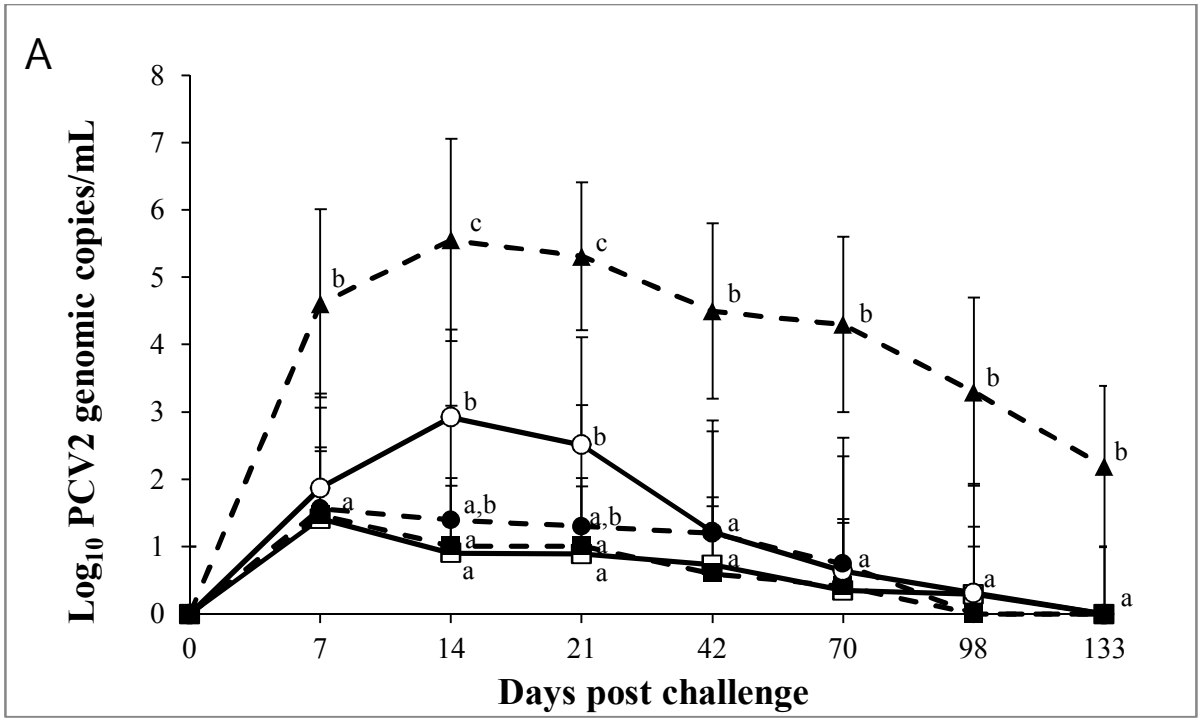
RESULTS

Growth performance

No significant difference was observed between the ADWG results of any groups during the 3-9 weeks period. The ADWG of vaccinated and negative control groups was significantly higher than that of positive group during the 9-16 week period ($P < 0.05$), but not thereafter. The overall growth performance (from 3 to 25 weeks of age) was not significantly different among the 6 groups (Table 1).

PCV2 DNA in sera and nasal swabs

No PCV2b DNA was detected in the blood or nasal swabs from the vaccinated or positive control groups at 0 dpc. Thereafter, there was a significant ($P < 0.05$) difference in log transformed PCV2b DNA in the blood (Fig. 1A) and nasal swabs (Fig. 1B) between the vaccinated and the positive control group throughout the experiment except at 133 dpc. At 14 and 21 dpc, groups vaccinated with Fostera PCV (0.29–1.63 $\log_{10}$ at 14 dpc and 0.25–1.47 $\log_{10}$ at 21 dpc, 95% CI) and Circovac (0.31–1.76 $\log_{10}$ at 14 dpc and 0.35–1.69 $\log_{10}$ at 21 dpc, 95% CI) had a significantly ($P < 0.05$) lower number of genomic copies of PCV2b in the blood than the group vaccinated with Circoflex (1.38–4.35 $\log_{10}$ at 14 dpc and 0.54–4.25 $\log_{10}$ at 21 dpc, 95% CI). The percentage of viremic pigs and nasal shedders was significantly ($P < 0.05$) lower in the groups vaccinated with Fostera PCV, Circovac, and Porcilis PCV than in the positive control group throughout the experiment except at 7 and 133 dpc (Table 1). The percentage of viremic pigs and nasal shedders was significantly ($P < 0.05$) lower in the group vaccinated with Circoflex than in the positive control group at 21, 42 and 70 dpc. No PCV2b DNA was detected in the blood or nasal swabs from the negative control group throughout the experiment.


A
Log10 PCV2 genomic copies/mL
Days post challenge
0
7
14
21
42
70
98
133

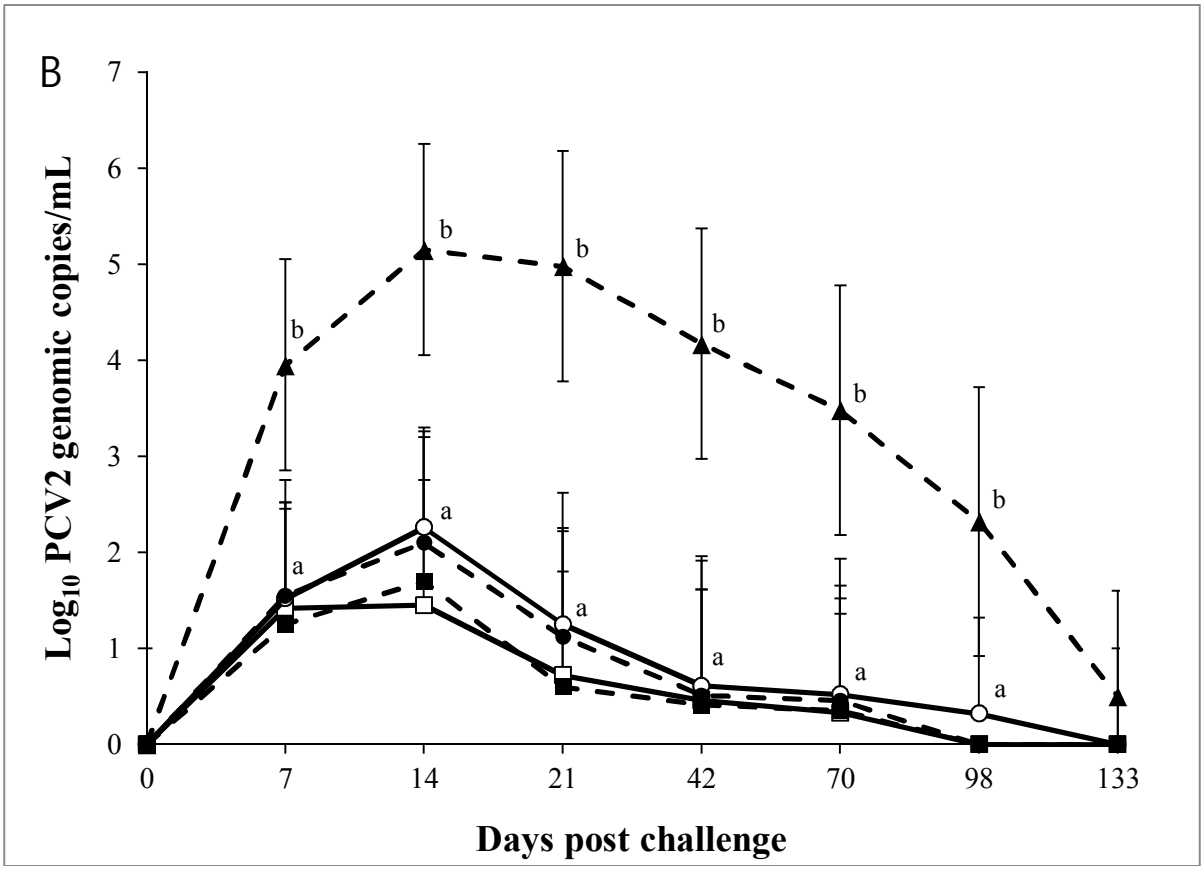

B
Log10 PCV2 genomic copies/mL
Days post challenge
0
7
14
21
42
70
98
133

Figure 1. Mean values of the $\log_{10}$ transformed genomic copy number of porcine circovirus type 2b (PCV2b) DNA in serum (A) and nasal swabs (B) at different days post challenge among four commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, □); inactivated PCV2 vaccine (Circovac, ■); subunit PCV2 vaccine (Circoflex, ○ and Porcilis PCV, ●); and PCV2-infected animals (▲). Variation is expressed as the standard deviation. Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.

Anti-PCV2 IgG antibodies

There was a significant ($P < 0.05$) difference in the titer of anti-PCV2 IgG antibodies between vaccinated and positive control groups at 0 dpc. At 0 dpc, groups vaccinated with Fostera PCV and Circovac exhibited significantly ($P < 0.05$) higher titers of anti-PCV2 IgG antibodies than groups receiving Porcilis PCV and Circoflex (Fig. 2A). No anti-PCV2 IgG antibodies were detected in the negative control group throughout the experiment.

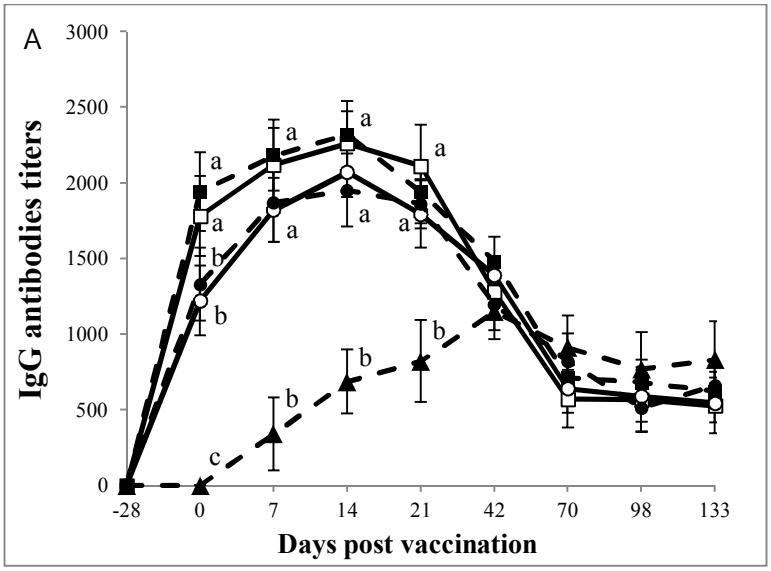
Neutralizing antibodies

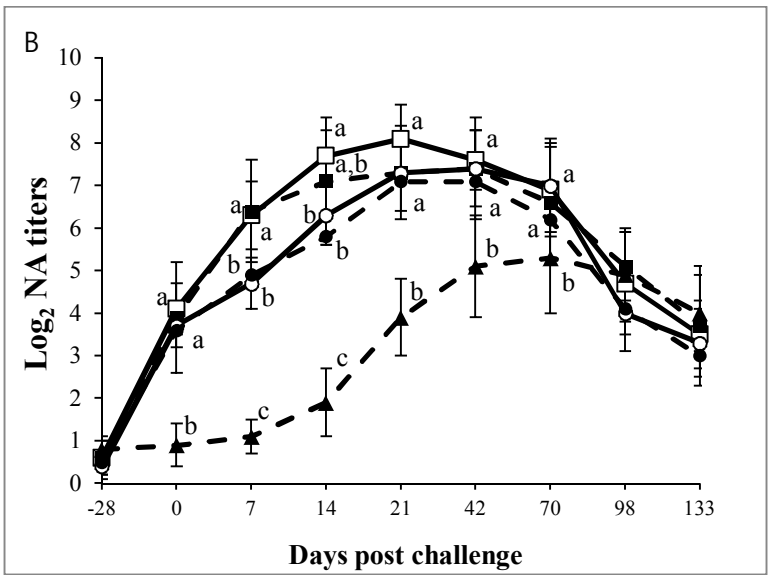
There was a significant ($P < 0.05$) difference in the log transformed NA titers between vaccinated and positive control groups at 0, 7, 14, 21, and 42 dpc, and significant ($P < 0.05$) differences were also noted among different PCV2 vaccines. At 7 dpc, groups vaccinated with Fostera PCV and Circovac exhibited significantly ($P < 0.05$) higher $\log_2$ transformed NA titers than the other 2 vaccinated groups. At 14 dpc, Fostera PCV (6.5–8.4 $\log_2$, 95% confidence interval (CI)) induced significantly ($P < 0.05$) higher log transformed NA titers than the Circoflex (5.3–6.7 $\log_2$, 95% CI) and Porcilis PCV (5.0–6.5 $\log_2$, 95% CI) (Fig. 2B). In vaccinated and positive control groups, the log transformed NA titers correlated inversely with the number of genomic copies of PCV2 in the blood ($r^2 = 0.687$, $P = 0.035$ for Fostera PCV; $r^2 = 0.723$, $P = 0.031$ for Circovac; $r^2 = 0.634$, $P = 0.032$ for Circoflex; $r^2 = 0.711$, $P = 0.024$ for Porcilis PCV; and $r^2 = 0.639$, $P = 0.036$ for positive control).

PCV2-specific interferon- γ-secreting cells

There was a significant ($P < 0.05$) difference in the number of PCV2-specific IFN-γ-SCs between vaccinated and positive control groups at 0, 7, 14, and 21 dpc, and significant ($P < 0.05$) differences were also noted among PCV2 vaccines. At 0 dpc, Fostera PCV (49.5–69.1 cells/10^6 PBMCs, 95% CI) induced a significantly ($P < 0.05$) higher number of PCV2-specific IFN-γ-SCs than did the 3 other vaccines (Circovac, 38.7–52.6 cells/10^6 PBMCs,; Circoflex, 37.5–50.8 cells/10^6 PBMCs; and Porcilis PCV, 31.9–46.1 cells/10^6 PBMCs, 95% CI). At 7 and 14 dpc, Fostera PCV (58.9–6.6 cells/10^6 PBMCs at 7 dpc and 73.9–96.1 cells/10^6 PBMCs at 14 dpc, 95% CI) and Circovac (60.3–82.5

cells/10^6 PBMCs at 7 dpc and 72.9–91.0 cells/10^6 PBMCs at 14 dpc, 95% CI) induced significantly ($P < 0.05$) higher numbers of PCV2-specific IFN-γ-SCs than Circoflex (42.0–63.3 cells/10^6 PBMCs at 7 dpc and 58.2–77.1 cells/10^6 PBMCs at 14 dpc, 95% CI) and Porcilis PCV (37.7–56.4 cells/10^6 PBMCs at 7 dpc and 54.3–72.2 cells/10^6 PBMCs at 14 dpc, 95% CI) (Fig. 2C). In vaccinated and positive control groups, the number of PCV2-specific IFN-γ-SCs correlated inversely with the number of genomic copies of PCV2 in the blood ($r^2 = 0.715$, $P = 0.029$ for Fostera PCV; $r^2 = 0.644$, $P = 0.045$ for Circovac; $r^2 = 0.611$, $P = 0.038$ for Circoflex; $r^2 = 0.732$, $P = 0.021$ for Porcilis PCV; and $r^2 = 0.598$, $P = 0.042$ for the positive control group).


A
IgG antibodies titers
3000
2500
2000
1500
1000
500
0
-28
0
7
14
21
42
70
98
133
Days post vaccination


B
Log2 NA titers
10
9
8
7
6
5
4
3
2
1
0
-28
0
7
14
21
42
70
98
133
Days post challenge

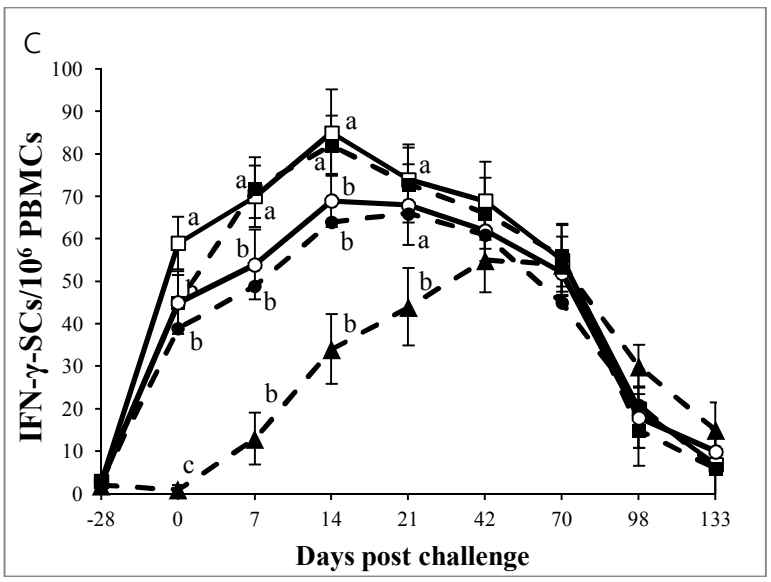
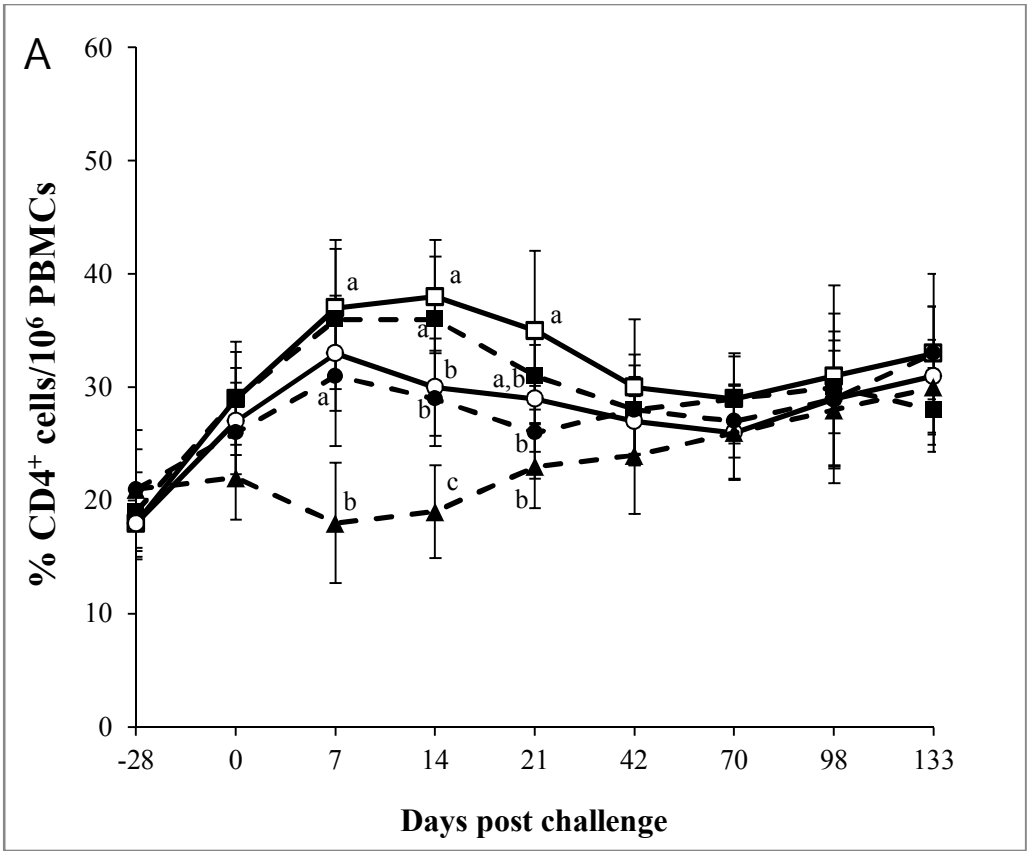

C
IFN-γ-SCs/10^6 PBMCs
100
90
80
70
60
50
40
30
20
10
0
-28
0
7
14
21
42
70
98
133
Days post challenge

Figure 2. Group mean for anti-PCV2-IgG antibodies titers (A), $\log_2$ transformed group means for neutralizing antibodies (NA) titers (B), Mean values of the number of PCV2-specific interferon-γ-secreting cells (IFN-γ-SCs) (C) at different days post challenge among 4 commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, □); inactivated PCV2 vaccine (Circovac, ■); subunit PCV2 vaccine (Circoflex, ○ and Porcilis PCV, ●); and PCV2-infected animals (▲).Variation is expressed as the standard deviation. Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.

Identification of lymphocyte subsets

There was a significant ($P < 0.05$) difference in the proportion of $CD4^+$ cells between vaccinated and positive control groups at 7 and 14 dpc, and significant ($P < 0.05$) differences were also noted among different PCV2 vaccines. At 14 dpc, Fostera PCV and Circovac induced significantly ($P < 0.05$) higher proportion of $CD4^+$ cells than the other vaccines. At 21 dpc, the Fostera PCV group exhibited a significantly ($P < 0.05$) higher proportion of $CD4^+$ cells than the Porcilis PCV group and positive control group (Fig. 3A). There was a significant ($P < 0.05$) difference in the proportion of $CD3^+$ cells between vaccinated and positive control groups at 7 dpc but not thereafter (Fig. 3B).


A
% CD4+ cells/10^6 PBMCs
60
50
40
30
20
10
0
-28
0
7
14
21
42
70
98
133
Days post challenge

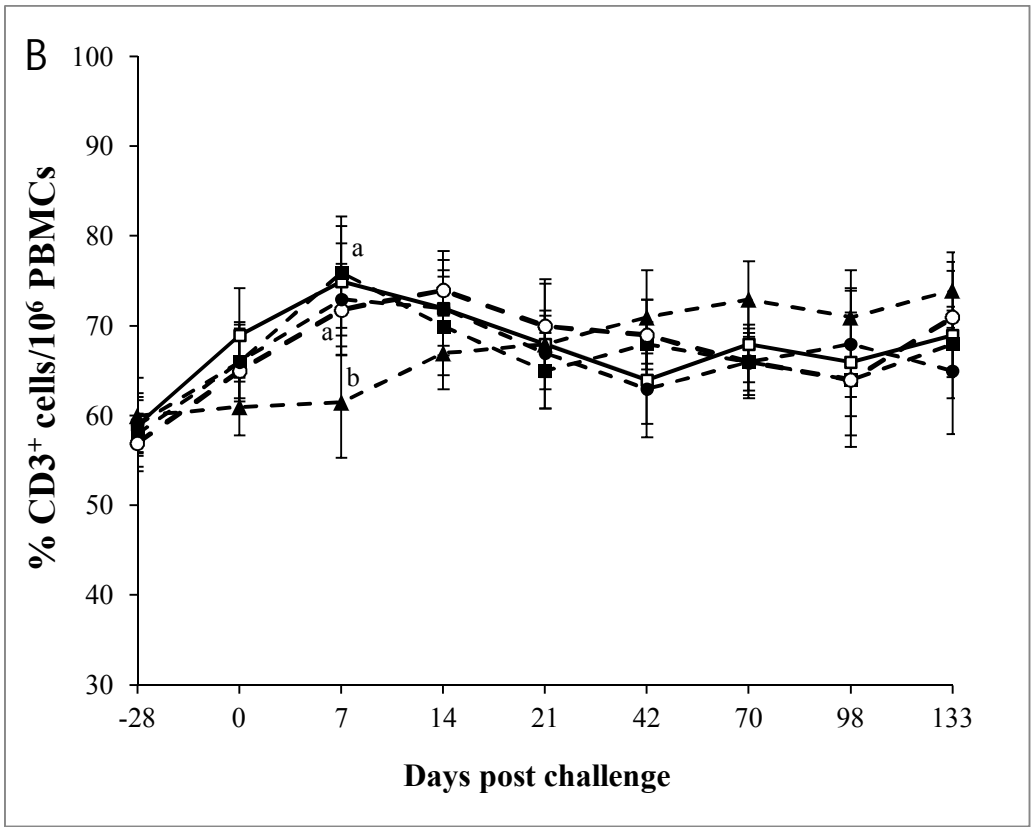

B
% CD3+ cells/10^6 PBMCs
100
90
80
70
60
50
40
30
-28
0
7
14
21
42
70
98
133
Days post challenge

Figure 3. Group mean for the proportion of $CD4^+$ (A) and $CD3^+$ (B) cells at different days post challenge among 4 commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, □); inactivated PCV2 vaccine (Circovac, ■); subunit PCV2 vaccine (Circoflex, ○ and Porcilis PCV, ●); and PCV2-infected animals (▲). Variation is expressed as the standard deviation. Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.

Histopathology and immunohistochemistry

The histopathological lymphoid lesion and PCV2-antigen scores were significantly ($P < 0.05$) lower in the vaccinated groups than in the positive control group (Fig. 4). However, there were no significant differences between histopathological lymphoid lesion and PCV2-antigen scores in lymph nodes among vaccinated groups (Table 1).

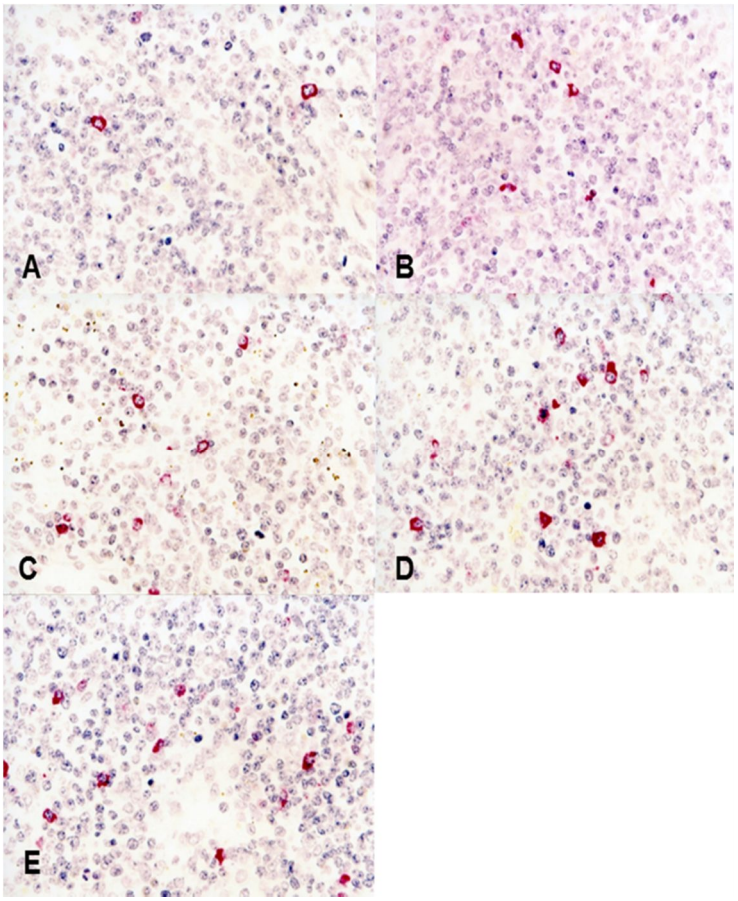


Figure 4. Immunohistochemisty for the detection of porcine circovirus type 2 (PCV2) antigen in lymph node from pigs at 133 days post challenge among 4 commercial vaccines: inactivated chimeric PCV1-2 vaccine (Fostera PCV, A); inactivated PCV2 vaccine (Circovac, B); subunit PCV2 vaccine (Circoflex, C and Porcilis PCV, D); and PCV2-infected animals (E). Positive immunohistochemical signals (red grain) were seen in macrophages.

Table 1. Average daily weight gain (ADWG), proportion of viremic pig and nasal shedder at different days post challenge (dpc), histopathological lymphoid lesion score, immunohistochemical porcine circovirus type 2 (PCV2) antigen score among groups.

		Groups					
		Fostera PCV	Circovac	Circoflex	Porcilis PCV	Positive	Negative
ADWG	3-7	329 ± 25	322± 26	324 ± 22	332 ± 28	326 ± 25	331 ± 31
(weeks)	7-9	706 ± 33	701 ± 41	711 ± 36	701 ± 39	691 ± 35	709 ± 38
	9-16	751 ± 35^{a}	756 ± 28^{a}	749 ± 33^{a}	743 ± 38^{a}	706 ± 27^{b}	748 ± 34^{a}
	16-25	712 ± 41	700 ± 34	704 ± 29	696 ± 38	687 ± 32	706 ± 33
	3-25	654 ± 33	649 ± 37	650 ± 29	645 ± 41	627 ± 35	652 ± 38
Viremic pigs	7	3/10^{a}	3/10^{a}	4/10^{a,b}	3/10^{a}	8/10^{b}	0/10
(dpc)	14	2/10^{a}	3/10^{a,b}	7/10^{b,c}	3/10^{a,b}	10/10^{c}	0/10
	21	2/10^{a}	2/10^{a}	5/10^{a}	3/10^{a}	10/10^{b}	0/10
	42	2/10^{a}	1/10^{a}	3/10^{a}	2/10^{a}	9/10^{b}	0/10
	70	1/10^{a}	2/10^{a}	2/10^{a}	1/10^{a}	9/10^{b}	0/10
	98	1/10^{a}	0/10^{a}	1/10^{a}	0/10^{a}	5/9^{b}	0/10
	133	0/10	0/10	0/10	0/10	3/9	0/10
Nasal shedders	7	3/10	3/10	4/10	3/10	7/10	0/10
(dpc)	14	3/10^{a}	3/10^{a}	6/10^{a}	3/10^{a}	10/10^{b}	0/10
	21	2/10^{a}	2/10^{a}	4/10^{a}	3/10^{a}	10/10^{b}	0/10
	42	2/10^{a}	1/10^{a}	2/10^{a}	1/10^{a}	8/10^{b}	0/10
	70	1/10^{a}	1/10^{a}	2/10^{a}	1/10^{a}	7/10^{b}	0/10
	98	0/10^{a}	0/10^{a}	1/10^{a,b}	0/10^{a}	4/9^{b,*}	0/10
	133	0/10	0/10	0/10	0/10	1/9	0/10
Lymphoid lesion score		0.4 ± 0.5^{a,c}	0.3± 0.48^{a,c}	0.7 ± 0.67^{a}	0.9 ± 0.51^{a}	1.7 ± 0.70^{b}	0.1 ± 0.31^{c}
PCV2-antigen score (95% CI)		2.9 ± 4.31^{a,c} (0.1–5.9)	3.9± 4.19^{a,c} (0.8–6.9)	6.2± 5.85^{a} (2.0–10.4)	7.1 ± 4.65^{a} (3.7–10.5)	23.2 ± 6.76^{b} (18.4–23.2)	0^{c}

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

*Euthanized due to a leg injury.

DISCUSSION

This study demonstrated that four commercial PCV2 vaccines are highly effective in controlling PCV2 infection. Reduction of PCV2 viremia is the most important parameter indicating the efficacy of PCV2 vaccines (Fort et al., 2008, 2009b; Opriessnig et al., 2009; Shen et al., 2010) because high levels of PCV2 viremia are associated with the development of PCVAD (Chae, 2005; Meerts et al., 2006). Most importantly, there are significant differences among the four commercial vaccines in the degree of reduction of PCV2 viremia at 14 and 21 dpc. This difference among commercial vaccines was mainly influenced by the different antigen types. For example, the inactivated chimeric PCV1-2 and PCV2 vaccines yielded significantly lower PCV2 viremia compared to subunit vaccines at 14 and 21 dpc. These results agree with previous findings that the inactivated chimeric PCV 1-2 vaccine results in significantly lower PCV2 viremia at 14 and 21 dpc compared with the subunit vaccine (Circoflex) (O'Neill et al., 2011). However, our results contradict those of another previous study (Opriessnig et al., 2010) in which pigs immunized with a subunit PCV2 vaccine (Circoflex) had significantly lower amounts of PCV2 viremia at 21 dpc compared with pigs immunized with inactivated PCV2 vaccine (Circovac). We have no clear explanation for this discrepancy, but it may be due to different Circovac dosages between the two studies. In the current study, pigs received 0.5 ml of Circovac according to the manufacturer's label instructions, while in the previous study conducted prior to licensure for piglet vaccination, pigs received 2.0 ml of Circovac based on off-label use. The difference in results between the two studies could be due to the fact that 2 ml is not an optimal dosage for the vaccination of 3 week old piglets. Other comparative studies also reported variations in the reduction of PCV2 viremia among three commercial vaccines. For examples, two doses of the inactivated chimeric PCV 1-2 and two-dose subunit vaccine (Circumvent) led to a significantly lower amount of PCV2 viremia at 14 and 21 dpc compared to the subunit vaccine (Circoflex) under a triple challenge model with PCV2-PRRSV-SIV at 12 weeks post vaccination (Opriessnig et al., 2009). However, the same research group also reported no significant differences among the same 3 commercial vaccines in the degree of reduction of PCV2 viremia under a triple

challenge model with PCV2-PRRSV-PPV at 13 weeks post vaccination (Shen et al., 2010).

Immunological evaluation showed that regardless of vaccine type, only vaccinated animals exhibited PCV2-specific NA and IFN-γ-SCs on the day of challenge (4 weeks after vaccination). However, significant differences were observed among individual vaccines in induction of immunity, and these differences were related to antigen type. Inactivated chimeric PCV1-2 and PCV2 vaccines induced higher levels of PCV2-specific NA and IFN-γ-SCs compared to subunit vaccines. Induction of different levels of protective immunity may be reflected as different levels of reduction of PCV2 viremia. Hence, both humoral (PCV2-specific NA) and cell-mediated immunity (PCV2-specific IFN-γ-SCs) induced by PCV2 vaccines could contribute to PCV2 clearance and control PCV2 infection. In addition, IFN-γ, which is produced by PCV2-specific IFN-γ-SCs, is a key immunoregulatory cytokine that controls the differentiation of naïve $CD4^+$ into $CD4^+$ cells (Schroder et al., 2004). These $CD4^+$ cells play an important role in cell-mediated immunity (Saalmüller and Bryant, 1994; Schroder et al., 2004). In field conditions, a decrease of $CD4^+$ cells was observed in pigs with naturally occurring PMWS (Segalés et al., 2001). In the present study, elevated proportion of $CD4^+$ cells are seen in vaccinated animals only. The results suggest that the increased proportion of $CD4^+$ cells induced by PCV2 vaccines mediates cell-mediated immunity against PCV2 infection. However, our results should be interpreted carefully. An increase in the percentage of $CD4^+$ cells within PBMCs does not mean that there is an increase in the absolute number of $CD4^+$ cells within PBMCs.

Pathological evaluation through the detection of PCV2 antigen is another critical criterion in evaluating the efficacy of PCV2 vaccines because PCVAD is defined based on the presence of characteristic lymphoid lesions and the detection of PCV2 antigen within these lesions. Vaccination with four commercial PCV2 vaccines effectively reduces lymphoid lesion and PCV2-antigen in vaccinated and challenged animals, in agreement with previous studies (Fort et al., 2009b; Opriessnig et al., 2009, 2010; Shen et al., 2010). However, PCV2-associated microscopic lesions were not prominent in this study because pigs were challenged with PCV2 alone.

The present study used seronegative and non-viremic pigs to rule out possible interference from maternally derived antibodies or PCV2 infection. The PCV2 vaccines studied here have different

antigen types, adjuvants, and dosages (Chae, 2012). This diversity makes it difficult for swine practitioners and producers to select the appropriate PCV2 vaccine for the optimal control of PCVAD. Thus, the results of the present study may provide swine practitioners and producers with valuable information about commercial PCV2 vaccines.

The PCV2 vaccines studied here have different antigen types, adjuvants, and dosages. Nevertheless, four commercial one-dose PCV2 vaccines are highly effective in controlling PCV2 infection based on clinical, virological, immunological, and pathological parameters. The results of the present study may provide researchers and swine practitioners with valuable information about commercial PCV2 vaccines.

REFERENCES

Allan, G.M., Mackie, D.P., McNair, J., Adair, B.M., McNulty, M.S., 1994. Production, preliminary characterisation and applications of monoclonal antibodies to porcine circovirus. Veterinary Immunology and Immunopathology 43, 357–371.

Brunborg, I.M., Moldal, T., Jonassen, C.M., 2004. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. Journal of Virological Methods 122, 171-178.

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. The Veterinary Journal 168, 41–49.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. The Veterinary Journal 169, 326–336.

Chae, C., 2012. Commercial porcine circovirus type 2 vaccines: Efficacy and clinical application. The Veterinary Journal 194, 151–157

Diaz, I., Mateu, E., 2005. Use of ELISPOT and ELISA to evaluate IFN-γ, IL-10 and IL-4 responses in conventional pigs. Veterinary Immunology and Immunopathology 106, 107–112.

Fenaux, M., Halbur, P.G., Haqshenas, G., Royer, P., Thomas, P., Nawagitgul, P., Gill, M., Toth, T.E., Meng, X.J., 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. Journal of Virology 76, 541–551.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. Journal of Virology 78, 6297–6303.

Fort, M., Olvera, A., Sibila, M., Segalés, J., Mateu, E., 2007. Detection of neutralizing antibodies in

postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Veterinary Microbiology 125, 244–255.

Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F., Segalés, J., 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine 26, 1063–1071.

Fort, M., Fernandes, L.T., Nofrarias, M., Diaz, I., Sibila, M., Pujols, J., Mateu, E., Segalés, J., 2009a. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrums-deprived piglets. Veterinary Immunology Immunopathology 129, 101–107.

Fort, M., Sibila, M., Perez-Martin, E., Nofrarias, M., Mateu, E., Segalés, J., 2009b. One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine 27, 4031–4037.

Fraile, L., Grau-Roma, L., Sarasola, P., Sinovas, N., Nofrarias, M., Lopez-Jimenez, R., Lopez-Soria, S., Sibila, M., Segalés, J., 2012. Inactivated PCV2 one shot vaccine applied in 3-week-old piglets: Improvement of production parameters and interaction with maternally derived immunity. Vaccine 30, 1986–1992.

Gagnon, C.A., del Castillo, J.R., Music, N., Fontaine, G., Harel, J., Tremblay, D., 2008. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of *Porcine circovirus-2* genotypes 2a and 2b in an epidemiological survey. Journal of Veterinary Diagnostic Investigation 20, 545–558.

Kim, D., Ha, Y., Lee, Y.H., Chae, S., Lee, K., Han, K., Kim, J., Lee J.-H., Kim, S.-H., Hwang, K.-K., Chae, C., 2009. Comparative study of *in situ* hybridization and immunohistochemistry for the detection of porcine circovirus 2 in formalin-fixed, paraffin-embedded tissues. Journal of Veterinary Medical Science 71, 1001–1004.

Kixmoller, M., Ritzmann, M., Eddicks, M., Saalmuller, A., Elbers, K., Fachinger, V., 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine 26,

3443–3451.

Liu, Q., Wang, L., Willson, P., Babiuk, A., 2000. Quantitative, competitive PCR analysis of porcine circovirus DNA in serum from pigs with postweaning multisystemic wasting syndrome. Journal of Clinical Microbiology 38, 3474–3477.

Martelli, P., Ferrari, L., Morganti, M., Angelis, D.E., Bonilauri, P., Guazzetti, S., Caleffi, A., Borghetti, P., 2011. One dose of a porcine circovirus 2 subunit vaccine induces humoral and cell-mediated immunity and protects against porcine circovirus-associated disease under field conditions. Veterinary Microbiology 149, 339–351.

Meerts, P., Van-Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. Viral Immunology 18, 333–341.

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Botner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Veterinary Research 2, 6.

Olvera, A., Sibila, M., Calsamiglia, M., Segalés, J., Domingo, M., 2004. Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. Journal of Virological Methods 117, 75–80.

O'Neill, K.C., Shen, H.G., Lin, K., Hemann, M., Beach, N.M., Meng, X.J., Halbur, P.G., Opriessnig, T., 2011. Studies on porcine circovirus type 2 vaccination of 5-day-old piglets. Clinical and Vaccine Immunology 18, 1865–1871.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Halbur, P.G., 2009. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine 27, 1002–1007.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Ramamoorthy, S., Meng, X.J., Halbur, P.G.,

2010. Comparison of the effectiveness of passive (dam) versus active (piglet) immunization against porcine circovirus type 2 (PCV2) and impact of passively derived PCV2 vaccine-induced immunity on vaccination. Veterinary Microbiology 142, 177–183.

Pogranichnyy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunology 13, 143–153.

Saalmüller, A., Bryant, J., 1994. Characteristics of porcine T lymphocytes and T-cell lines. Veterinary Immunology and Immunopathology 43, 45-52.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A., 2004. Interferon-γ: an overview of signals, mechanisms and functions. Journal of Leukocyte Biology 75, 163-189.

Segalés, J., Alonso F., Rosell, C., Pastor J., Chianini, F., Campos, E., Lopez-Fuertes, L., Quintana, J., Rodriguez-Arrioja, G., Calsamiglia, M., Pujols, J., Dominguez, J., Domingo, M., 2001. Changes in peripheral blood leukocytes populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology and Immunopathology 81, 37-44.

Segalés, J., Calsamiglia, M., Olvera, A., Sibila, M., Badiella, L., Domingo, M., 2005. Quantification of porcine circovirus type 2 (PCV2) DNA in serum and tonsillar, nasal, tracheo-bronchial, urinary and faecal swabs of pigs with and without postweaning multisystemic wasting syndrome (PMWS). Veterinary Microbiology 11, 223–229.

Segalés, J., Urniza, A., Alegre, A., Bru, T., Crisci, E., Nofrarias, M., Lopez-Soria, S., Balasch, M., Sibila, M., Xu, Z., Chu H.-J., Fraile, L., Plana-Duran, J., 2009. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) improves clinical, pathological and virological outcomes in postweaning multisystemic wasting syndrome affected farms. Vaccine 27, 7313–7321.

Seo, H.W., Han, K., Oh, Y., Park, C., Chae, C., 2012. Efficacy of a reformulated inactivated chimeric PCV1-2 vaccine based on clinical, virological, pathological and immunological examination under field conditions. Vaccine 30, 6671–6677.

Shen, H.G., Beach, N.M., Huang, Y.W., Halbur, P.G., Meng, X.J., Opriessnig, T., 2010. Comparison

of commercial and experimental porcine circovirus type 2 (PCV2) vaccines using a triple challenge with PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus (PPV). Vaccine 28,5960–5966.

Sosa, G.A., Quiroga, M.F., Roux, M.E., 2009. Flow cytometric analysis of T-lymphocytes from nasopharynx-associated lymphoid tissue (NALT) in a model of secondary immunodeficiency in Wistar rats. Immunobiology 214, 384–391.

PART III. Effects of porcine circovirus type 2 (PCV2) vaccines on PCV2 virus shedding in semen from experimentally infected boars

Chapter I. Effects of an inactivated porcine circovirus type 2 (PCV2) vaccine on PCV2 virus shedding in semen from experimentally infected boars

ABSTRACT

The objective of the present study was to determine the effect of an inactivated porcine circovirus type 2 (PCV2) vaccine on PCV2b virus shedding in the semen of experimentally infected boars by measuring the immunological response and the PCV2b DNA load in blood and semen. Twelve boars were randomly divided into three groups. The boars in group 1 (n = 4) were immunized with an inactivated PCV2 vaccine and were challenged with PCV2b.The boars in group 2 (n = 4) were only challenged with PCV2b.The boars in group 3 (n = 4) served as negative controls. The number of PCV2 genomes copies of PCV2 in the serum and semen were significantly lower in vaccinated challenged boars than in non-vaccinated challenged boars at 7, 10, 14, 21, 32, 35, 42, 49, and 60 days post-inoculation. The number of PCV2b genomes in the semen correlated with the number of PCV2b genomes in the blood in both vaccinated challenged (R = 0.714) and non-vaccinated challenged (R = 0.861) boars. The results of the present study demonstrate that the inactivated PCV2 vaccine significantly decreases the amount of PCV2b DNA shedding in semen from vaccinated boars after experimental infection with PCV2b.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is associated with a number of diseases and syndromes collectively referred to as porcine circovirus-associated disease (PCVAD), which includes postweaning multisystemic wasting disease (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, porcine respiratory disease complex (PRDC), and exudative epidermitis (Chae, 2005).

Several commercial PCV2 vaccines are current available in the global market. All PCV2 vaccines have been administered to the either sows or piglets (Auvigne et al, 2006; Opriessnig et al, 2008a, 2009; Fort et al, 2009). Commercial PCV2 vaccines are able to reduce the PCV2 load in the serum and reduce PCV2 shedding in nasal and fecal samples in conventional pigs (Fort et al, 2009; Opriessnig et al, 2008a, 2008b, 2009, 2010). Recently, it has also been reported that vaccination against PCV2 in naturally infected boars can decrease the duration of viral shedding in semen (Alberti et al., 2010). These data strongly imply that vaccination against PCV2 may reduce the subsequent shedding of PCV2 in the semen of boars. Therefore, the objective of the present study was to determine the effect of PCV2 vaccination on the shedding of the PCV2 virus in the semen of experimentally infected boars.

MATERIALS AND METHODS

Commercial vaccine and PCV2 inoculum

The commercial inactivated tissue homogenate PCV2 vaccine based on PCV2 genotype 2b, CircoPrime (Komipharm International Company Ltd., Shiheung-shi, Kyongki-do, Republic of Korea), was used in this study. It was administered intramuscularly in two 2.0 ml doses separated by 3 weeks.

The PCV2 strain (PCV2 genotype b) isolated from the superficial inguinal lymph node of a field case of PMWS was used as the inoculum. The virus was propagated in PCV-free PK15 cells to a titer of 10^5 $TCID_{50}$/ml.

Experimental design

At 8 months of age, twelve purebred, male, Landrace pigs were purchased from a commercial farm. All boars were negative for PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) according to routine serological testing performed prior to delivery and again on arrival.

All boars were housed individually 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 boars were introduced, and each boar was housed separately within the facility.

The boars were randomly divided into three groups.The boars in group 1 (T01, n = 4) were immunized with two 2.0 mL doses of the inactivated PCV2 vaccine at a 3-week interval, and then, 3 weeks after the second vaccination, the boars were intranasally inoculated with PCV2b (3 mL) with an infectious titer of $10^{4.5}$ $TCID_{50}$ per mL. The boars in group 2 (T02, n = 4) were intranasally inoculated with PCV2b (3 mL) with an infectious titer of $10^{4.5}$ $TCID_{50}$ per mL. The concentration of

PCV2b ($10^{4.5}$ $TCID_{50}$ per mL) for inoculation was similar to that used in a previous study to allow for comparison (25). The boars in group 3 (T03, $n = 4$) served as negative controls.

Serology

Blood samples from each pig were collected by jugular venipuncture at -42, -21, 0, 14, 21, 28, 35, 42, 49, and 60 days post-inoculation (dpi), and the serum samples were tested using a commercial PCV2 ELISA IgG kit (Ingezim Circovirus IgG, Ingenasa, Madrid, Spain).

Quantification of PCV2 DNA

DNA was extracted from semen (raw) and serum samples collected -42, -21, -7, 0, 4, 7, 10, 14, 18, 21, 25, 28, 32, 35, 39, 42, 46, 49, 53, 56, and 60 dpi using a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) as described previously (Opriessnig et al., 2010). DNA extracts were used to quantify the PCV2 genome DNA copy numbers by real-time PCR as described previously (Gagnon et al., 2008).

Standard curve

To construct a standard curve, real-time PCRs were performed in quadruplicate in two different assays: (i) 10-fold serial dilutions of the PCV2 plasmid were used as the standard, with concentrations ranging from 10^{10} to 10^{2} copies/mL, and (ii) 10-fold serial dilutions of PCV2b cultured in PK-15 cells free of PCV1 from $10^{4.5}TCID_{50}$/mL to $10^{-3.5}$ $TCID_{50}$/mL. The PCV2 plasmid was prepared as described previously (Gagnon et al., 2008). Briefly, the PCV2b ORF2 sequence was cloned into the pCR 2.1 plasmid (Invitrogen, Carlsbad, CA, USA). The recombinant plasmid was purified using a Qiagen plasmid Miniprep kit (Qiagen) according to the manufacturer's instructions, and the concentration of the purified plasmid was determined using a spectrophotometer.

Virus isolation

PCV2 was isolated from whole semen as previously described (Kim et al., 2001). Briefly, whole semen (2 ml) was frozen and then thawed, mixed with 20 ml of Hank's balanced salt solution, and

centrifuged at 40,000 x *g* for 1 hour. The supernatant was discarded, and the pellet was resuspended and vortexed in 1 ml of minimal essential medium plus 4% fetal bovine serum. Confluent monolayers of PCV-free PK-15 cells were inoculated with 200 μl of the suspension and then incubated for 5 days. Duplicate cultures were fixed in 80% acetone and tested for PCV1, PCV2a, and PCV2b by differential in situ hybridization as previously described (Kim et al., 2001; Kim et al., 2010).

Statistical analysis

Summary statistics were calculated for all groups to assess the overall quality of the data, including normality. For a single comparison, ANOVA with a post hoc Tukey's test was used to compare the primary variables (PCV2b DNA concentrations in the blood and semen) among groups. Student's t test for paired samples (PCV2b DNA quantification) was used to estimate the difference at each time point. $P < 0.05$ was considered to be significant.

Continuous data for PCV2 serology collected over time were analyzed using a nonparametric Kruskal-Wallis one-way ANOVA at each time point. If the difference was significant ($P < 0.05$), Mann-Whitney U tests were used to assess differences between groups. The Wilcoxon signed rank test for paired samples was used to estimate the difference at each time point.

The Spearman's rank correlation coefficient was used to assess the relationship among serology, viremia, and shedding in semen. The Pearson's correlation coefficient was used to assess the relationship between viremia and shedding in semen. A value of $P < 0.05$ was considered significant.

RESULTS

Serology of PCV2

Anti-PCV2 IgG was not detected in any serum samples at days -42, -21 and 0 days post-inoculation (dpi) in non-vaccinated challenged (T02) boars (Fig. 1). The serum anti-PCV2 IgG level was significantly higher at -21, 0, and 7 dpi but was significantly lower at 35 dpi in vaccinated challenged (T01) boars compared to non-vaccinated challenged boars ($P < 0.05$, Fig. 1). No anti-PCV2 IgG was detected in serum samples from negative control (T03) boars.

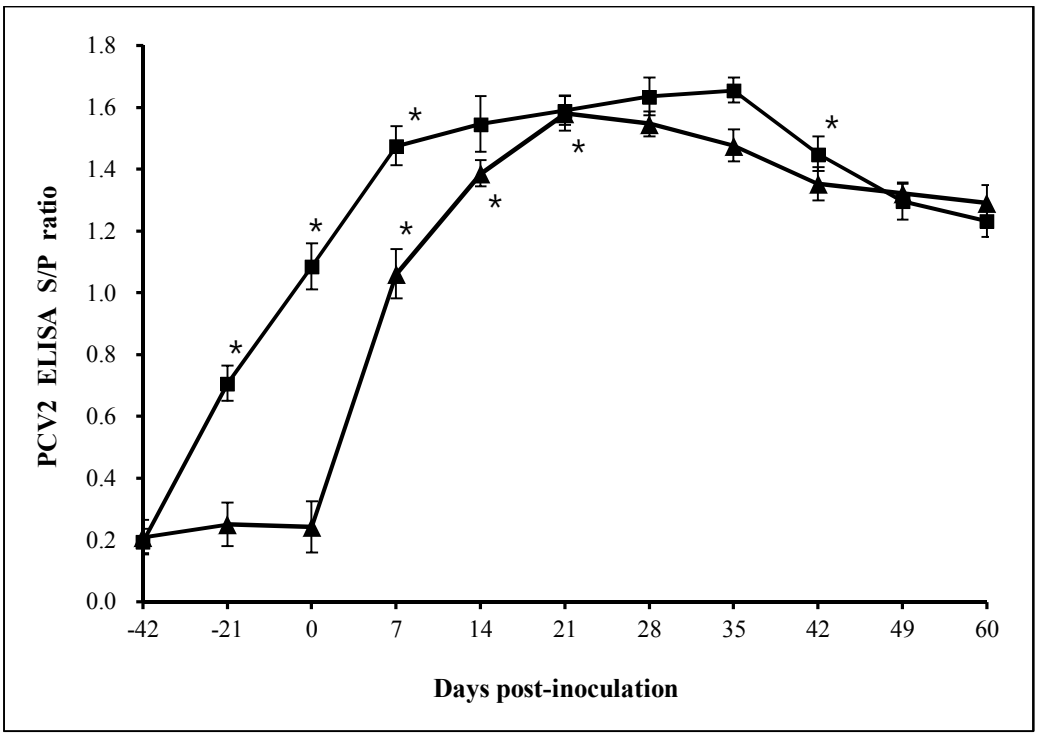
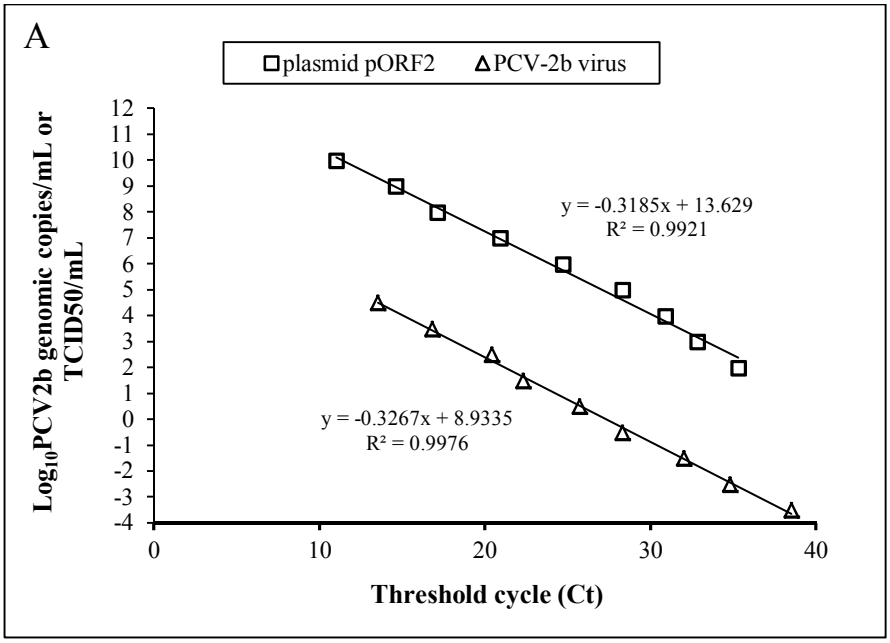


Figure 1. Mean anti-PCV2 IgG antibody response in different treatment groups. Boars were immunized with the PCV2 vaccine and challenged with PCV2b (T01; ■) or were only challenged with PCV2b (T02; ▲). * indicates a significant ($P < 0.05$) difference at each time point vs. the previous time point within a group.

Standard curve

To determine whether there was a correlation between the genome copy number and the $TICD_{50}$/ml, standard curves were constructed with the two different templates (plasmid pORF2 DNA and PCV2b viral DNA). The resulting lines were nearly parallel, with almost the same slope (Fig. 2). Regression analysis revealed a significant correlation ($R^2 > 0.997$).



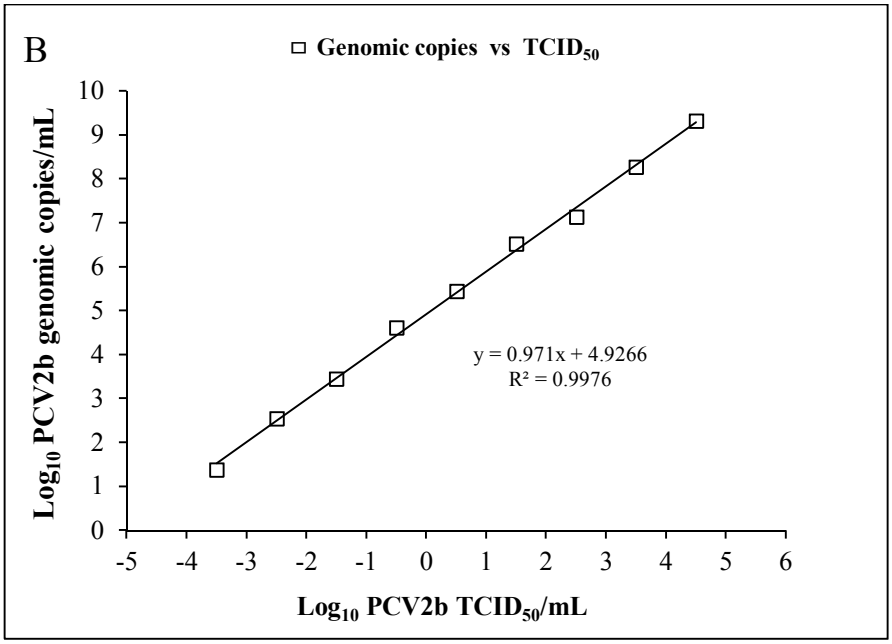


Figure. 2. Standard curves of PCV2b and PCV2b recombinant plasmid DNA measured with realtime PCR (A), and correlation between PCV2b genomic copies and $TCID_{50}$/mL (B). Ten-fold serial dilution of PCV2b and PCV2b recombinant plasmid DNA were used as templates in the reaction. The regression and efficiency analysis revealed a significant value of $R^2 > 0.97$ in all analyses.

Quantification of PCV2 DNA in blood

PCV2b genomes were not detected in any serum samples at -42, -21, -7, and 0 dpi from vaccinated challenged (T01) and non-vaccinated challenged (T02) boars (Fig. 3). No PCV2b genomes were detected in any of the serum samples from negative control (T03) boars throughout the experiment. The number of PCV2b genomes in the serum was significantly lower in vaccinated challenged (T01) boars than in non-vaccinated challenged (T02) boars at 7, 10, 14, 21, 25, 32, 35, 42, 49, and 60 dpi ($P < 0.05$, Fig. 3). The number of PCV2b genomes did not correlate with the serum levels of anti-PCV2 IgG in blood from vaccinated challenged (T01; $R = 0.640$) and non-vaccinated challenged (T02; $R = -0.256$) boars.

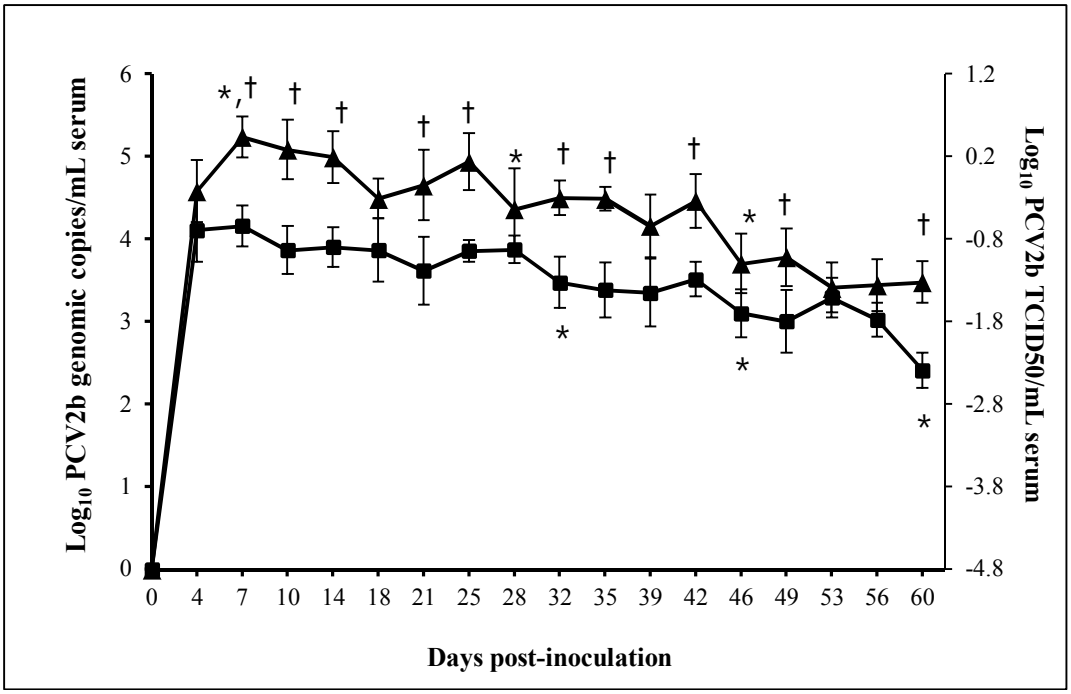


Figure. 3. Mean group $\log_{10}$ PCV2b DNA (or $\log_{10}$ PCV2b $TCID_{50}$/mL) load in blood samples from the different treatment groups. Boars were immunized with the PCV2 vaccine and challenged with PCV2b (T01; ■) or were only challenged with PCV2b (T02; ▲). * indicates a significant ($P < 0.05$) difference at each time point vs. the previous time point, and † indicates a significant ($P < 0.05$) difference between T01 and T02 at the same time point.

Quantification of PCV2 DNA in semen

PCV2b genomes were not detected in any semen samples at -42, -21, -7, and 0 dpi from vaccinated challenged (T01) and non-vaccinated challenged (T02) boars. No PCV2b genomes were detected in any of the semen samples from the negative control (T03) boars throughout the experiment.

The number of PCV2b genomes in semen was significantly lower in vaccinated challenged (T01) boars than in non-vaccinated challenged (T02) boars at 14, 18, 21, 25, 32, 42, 49, and 53 dpi ($P < 0.05$, Fig. 4). The number of PCV2b genomes in the semen did not correlate with the serum levels of anti-PCV2 IgG in either vaccinated challenged ($R = 0.555$) or non-vaccinated challenged boars ($R = -0.007$), but it did correlate with the number of PCV2b genomes in the blood in both vaccinated challenged ($R = 0.714$) and non-vaccinated challenged ($R = 0.861$) boars.

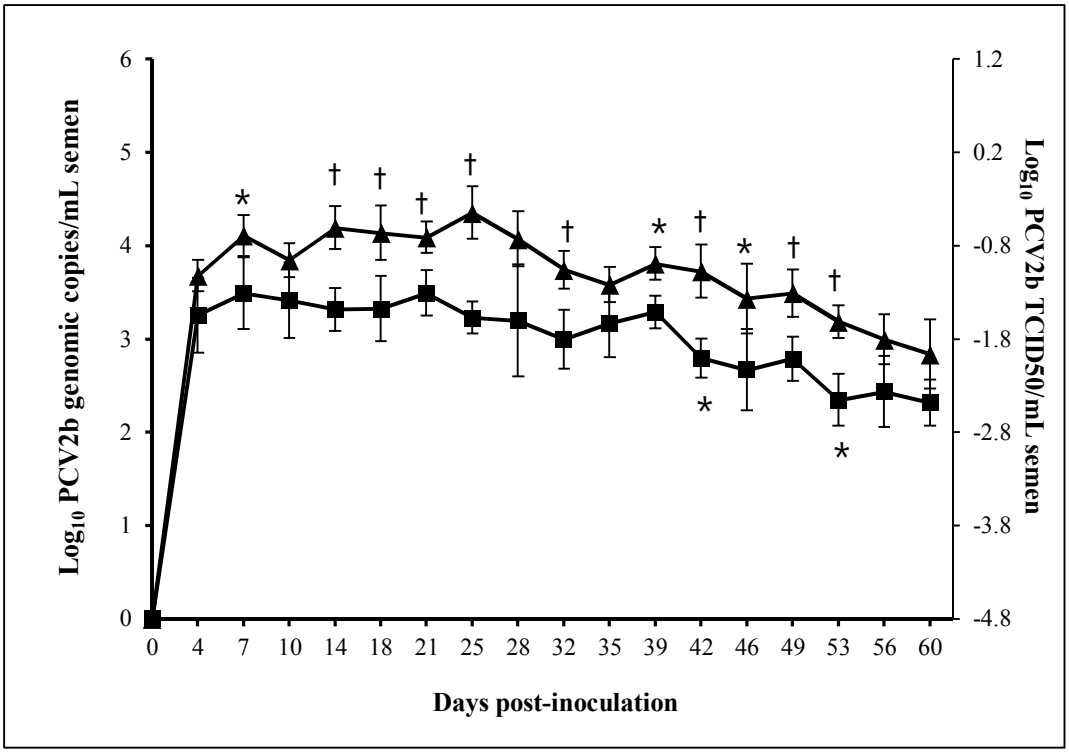


Figure 4. Mean group $\log_{10}$ PCV2b DNA (or $\log_{10}$ PCV2b $TCID_{50}$/mL) load in semen samples from the different treatment groups. Boars were immunized with the PCV2 vaccine and challenged with PCV2b (T01; ■) or were only challenged with PCV2b (T02; ▲). * indicates a significant ($P < 0.05$)

difference at each time point vs. the previous time point, and †indicates a significant ($P < 0.05$) difference between T01 and T02 at the same time point.

Virus isolation

Attempts were made to isolate and identify PCV1, PCV2a, and PCV2b from whole semen in the three groups. In vaccinated challenged boars (T01), PCV2b was isolated, and the presence of its DNA was confirmed in the cytoplasm of infected cells (Fig. 5) using in situ hybridization in two boars (boars 1 and 3) at 14 dpi and in one boar (boar no. 3) at 39 dpi. In the non-vaccinated and challenged group (T02), PCV2b was isolated, and the presence of its DNA was confirmed in the cytoplasm of infected cells using differential in situ hybridization in four boars (boars 1, 2, 3, and 4) at 10 and 14 dpi, in three boars (boar no. 1, no. 3, and no. 4)at 21 dpi, in two boars (boar no. 1 and no. 2) at 32 dpi, and in one boar (boar no. 1) at 42 and 49 dpi. No PCV1, PCV2a or PCV2b was isolated from or identified in the semen of negative control boars.

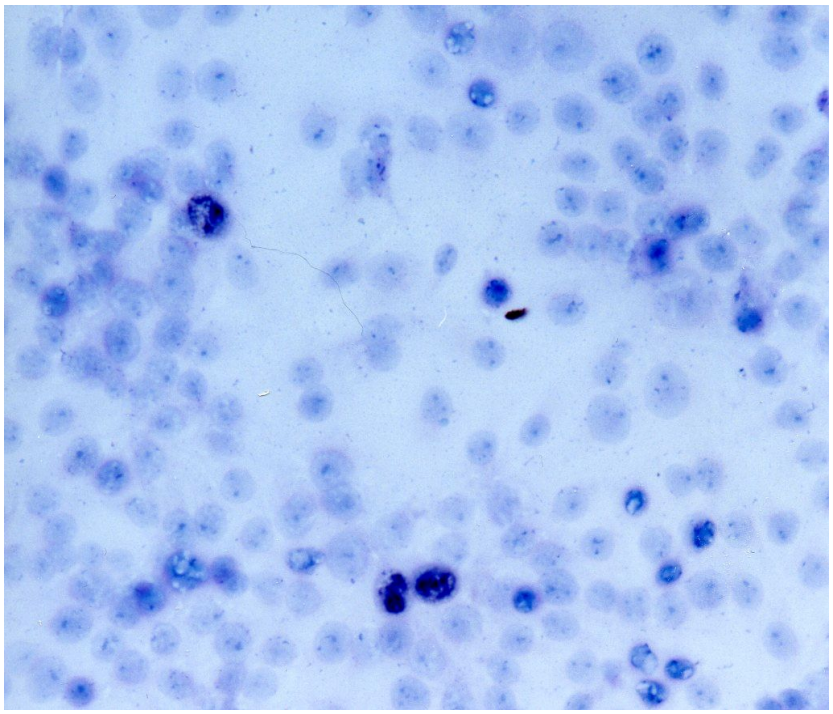


Figure. 5. PCV2b-infected PK-15 cells hybridized with digoxigenin-labeled PCV2b probes. PCV2b DNA (dark brown reaction; arrow) was present in the nucleus and cytoplasm. In situ hybridization with a PCV2b probe.

DISCUSSION

Vaccination of boars was unable to prevent the subsequent shedding of wild-type PCV2 but significantly decreased the amount of PCV2 DNA shed in semen in challenged boars. Vaccinated boars shed PCV2b ($10^{2.4}$ to $10^{3.6}$ PCV2b genome copies per mL, corresponding to 0.002 to 0.02 $TCID_{50}$/ml, respectively) in semen, whereas non-vaccinated boars shed PCV2b ($10^{3.3}$ to $10^{4.3}$ PCV2b genome copies per mL, corresponding to 0.04 to 0.4 $TCID_{50}$/ml, respectively) in semen. Thus, the vaccination of boars reduced shedding of PCV2b by approximately 100% in semen compared to non-vaccinated boars. The reduction in PCV2 DNA shedding is meaningful because the virus titer in the semen plays a major role in the transmissibility of PCV2. For example, PCV2 at $10^{5.6}$-$10^{5.8}$ PCV2 genome copies per mL isolated from the whole semen did not cause reproductive failure, seroconversion, or PCV2 viremia in naive gilts and their offspring when used for artificial insemination, although the PCV2-positive in semen was shown to be infectious in a swine bioassay model (Madson et al., 2009). The amount of PCV2 present in semen from vaccinated boars may minimize the transmission of PCV2 to sows via artificial insemination. Therefore, it is more efficient and economical to vaccinate boars to reduce PCV2 shedding in semen and to minimize the transmission of PCV2 to sows via artificial insemination.

In the present study, the amount PCV2 present in the semen of non-vaccinated boars was less than that in semen from non-vaccinated boars found in the previous study (Madson et al., 2009). We do not know why there is a difference between two studies with respect to the amount of PCV2 in semen from non-vaccinated boars. This difference could be due to differences between breeds of pigs or differences in the virulence and tropism of the PCV2 isolates. The present study used purebred, male Duroc pigs, whereas the previous study used in purebred, male Landrace pigs (Madson et al., 2009). In addition, the PCV2b isolate used in this study was isolated from postweaning 6-week-old pigs with severe PCVAD, whereas the PCV2b isolate used in the previous study was isolated from finishing pigs with severe PCVAD (Madson et al., 2009).

In agreement with previous findings (Kim et al., 2003b; Opriessnig et al., 2010), PCV2 DNA was present in seminal plasma fractions and non-sperm cell fractions, as determined by conventional PCR (data not shown). Because the in vivo tissue and cell tropism of PCV2 in pigs is most likely monocytes/macrophages (Kim et al., 2003a), the potential target cells of PCV2 in the semen include monocytes and macrophages, which could be vehicles for viral shedding in semen. Although the association of PCV2 with semen remains unclear, PCV2 most likely traffics from lymphoid tissue through the peripheral blood to reproductive tissues or directly into the semen.

There was no correlation between the serum levels of anti-PCV2 IgG and the number of PCV2 genomes in a previous study of 3-week-old pigs (Opriessnig et al., 2009). These data suggest that PCV2 viremia in the blood is not reflected in the levels of PCV2 antibodies in the blood. It has been reported that a reduced PCV2 load in the serum is unrelated to the serum antibody response to the PCV2 vaccine (Opriessnig et al., 2008b, 2010). Boars in the vaccinated challenged and non-vaccinated challenged group had detectable serum levels of anti-PCV2 IgG, indicating successful vaccination and/or challenge.

PCV2 DNA may be shed in semen for a considerable period of time, even in vaccinated challenged boars. The presence of PCV2 DNA in the semen of boars indicates that infectious particles exist and that there is a risk for swine (Madson et al., 2009). Artificial insemination has been widely and routinely used in Korean swine farms; >90% of sows are bred by artificial insemination and >80% of swine producers purchase semen for artificial insemination from commercial artificial insemination centers, according to the Korea Institute of Pig Technology (http://www.pigtech.co.kr). Artificial insemination has also become standard practice in nearly 60% of North American swine farms and in greater than 90% of European countries (Gerrits et al, 2005; Singleton, 2001). Although the presence of viral DNA in a clinical sample does not indicate that infectious virus is present, it is strongly recommended that boars be immunized with the PCV2 vaccine regularly to reduce the amount of PCV2 DNA shed in the semen.

The inactivated PCV2 vaccine used in this study is only licensed for the induction of active immunity in piglets. Therefore, the inactivated PCV2 vaccine was used in an extra-label manner to

determine its effect against PCV2 shedding in semen from experimentally infected boars. The reduction in the PCV2 load is one of the criteria used to assess the efficacy of PCV2 vaccines (Opriessnig et al., 2008a; Fort et al, 2009). PCV2 vaccines decrease the PCV2 load in the serum and reduce PCV2 shedding in nasal and fecal samples in conventional pigs (Fort et al, 2009). Commercial vaccines were shown to be able to control two of the multiple disease entities, known as PMWS and PRDC (Auvigne et al., 2006; Fachinger et al., 2008, Fort et al, 2009; Opriessnig et al. 2008b, 2010). To our knowledge, this is the first application of a PCV2 vaccine in boars to determine its effect on PCV2 shedding in the semen. Further studies are needed to evaluate the role of PCV2 vaccination in PCV2-associated reproductive failure and in other clinical entities.

REFERENCES

Alberti, K.A., Estienne, M.J., Meng, X.J., 2010. Effect of vaccination of boars against porcine circovirus type 2 on ejaculate characteristics, serum antibody titers, viremia, and semen virus shedding. Journal of Animal Science doi:10.2527/jas.2010-3388

Auvigne, V., Herin, J.B., Fily, B., Joisel, F., 2006. Evaluation of the first field results of vaccination against PCV2 with Circovac®. Proceeding of 19th International Pig Veterinary Society Congress 07-06.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. The Veterinary Journal 169, 326-336.

Fachinger, V., Bischoff, R., Jedidia, S.B., Saalmuller, A., Elbers, K., 2008. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. Vaccine 26, 1488-1499.

Fort, M., Sibila, M., Perez-Martin, E., Nofrarias, M., Mateu, E., Segales, J., 2009. One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine 27, 4031-4037.

Gagnon, C.A., del Castillo, J.R.E., Music, N., Fontaine, G., Harel, J., Tremblay, D., 2008. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of porcine circovirus-2 genotypes 2a and 2b in an epidemiological survey. Journal of Veterinary Diagnostic Investigation 20, 545-558.

Gerrits, R.J., Lunney, J.K., Johnson, L.A., Pursel, V.G., Kraeling, R.R., Rohrer, G.A., Dobrinsky, J.R., 2005. Perspectives for artificial insemination and genomics to improve global swine populations. Theriogenology 63, 283-299.

Kim, D., Ha, Y, Oh, Y., Han, K., Noh, S.H., Kim, C., Kim, S.-H, Chae, C., 2010. Development of in situ hybridization assay that differentiates between two genotypes of *Porcine circovirus*-2 in

formalin-fixed, paraffin-embedded tissues. Journal of Veterinary Diagnostic Investigation 22, 231-233.

Kim, J., Chae, C., 2001. Differentiation of porcine circovirus 1 and 2 in formalin-fixed, paraffin wax-embedded tissues from pigs with postweaning multisystemic wasting syndrome by in-situ hybridization. Research in Veterinary Science 70, 265-269.

Kim, J., Choi, C., Chae, C., 2003a. Pathogenesis of postweaning multisystemic wasting syndrome reproduced by co-infection with Korean isolates of porcine circovirus 2 and porcine parvovirus. Journal of Comparative Pathology 128, 52-59.

Kim, J., Han, D.U., Choi,C., Chae, C., 2001. Differentiation of porcine circovirus (PCV)-1 and PCV-2 in boar semen using a multiplex nested polymerase chain reaction. Journal of Virological Methods 98, 25-31.

Kim, J., Han, D.U., Choi,C., Chae, C., 2003b. Simultaneous detection and differentiation between porcine circovirus and porcine parvovirus in boar semen by multiplex seminested polymerase chain reaction. Journal of Veterinary Medical Science 65, 741-744.

Madson, D.M., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X.J., Halbur, P.G., Opriessnig, T., 2008. Characterization of shedding patterns of porcine circovirus types 2a and 2b in experimentally inoculated mature boars. Journal of Veterinary Diagnostic Investigation 20, 725-734.

Madson, D.M., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X.J., Halbur, P.G., Opriessnig, T., 2009. Infectivity of porcine circovirus type 2 DNA in semen from experimentally-infected boars. Veterinary Research 40, 10

Opriessnig, T., Madson, D.M., Prickett, J.R., Kuhar, D., Lunney, J.K., Elsener, J., Halbur, P.G., 2008a. Effect of porcine circovirus type 2 (PCV2) vaccination on porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 coinfection. Veterinary Microbiology 131, 103-114.

Opriessnig, T., Patterson, A.R., Elsener, J., Meng, X.J., Halbur, P.G., 2008b. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. Clinical and Vaccine Immunology. 15, 397-401.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Halbur, P.G., 2009. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine 27:1002-7.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Ramamoorthy, S., Meng, X.J., Halbur, P.G., 2010. Comparison of the effectiveness of passive (dam) versus active (piglet) immunization against porcine circovirus type 2 (PCV2) and impact of passively derived PCV2 vaccine-induced immunity on vaccination. Veterinary Microbiology 142, 177-183.

Pal, N., Huang, Y.W., Madson, D.M., Kuster, C, Meng, X.J., Halbur, P.G., Opriessnig, T., 2008. Development and validation of a duplex real-time PCR assay for the simultaneous detection and quantification of porcine circovirus type 2 and an internal control on porcine semen samples. Journal of Virological Methods 149, 217-225.

Singleton, W.L., 2001. State of the art in artificial insemination of pigs in the United States. Theriogenology 56, 1305-1310.

Chapter II. Comparison of three commercial one-dose porcine circovirus type 2 (PCV2) vaccines on PCV2 shedding in semen from experimentally infected boars

ABSTRACT

This study compared the effects of 3 different types of commercial PCV2 vaccines on PCV2 virus shedding in the semen from infected boars. Twenty-five non-PCV2 viremic and seronegative boars were randomly divided into five groups: three vaccinated and challenged groups, a non-vaccinated and challenged group, and a negative control group. The number of genomic copies of PCV2 in serum and semen samples was significantly decreased in vaccinated and challenged boars compared to non-vaccinated and challenged boars from 14 to 70 days post-inoculation (dpi). The number of PCV2 genomic copy in the semen correlated with the number of PCV2b genomic copy in the blood in vaccinated and challenged boars (r^2 = 0.894-0.926, $P < 0.01$), and non-vaccinated and challenged boars (r^2 = 0.903, $P < 0.01$). The vaccination protocol reduced the amount of PCV2 DNA shed in the semen. However, there was a significantly different amount of PCV2 DNA shed in semen among the 3 vaccinated and challenged boar groups.

INTRODUCTION

Porcine circovirus-associated disease (PCVAD), which is caused by porcine circovirus type 2 (PCV2), is economical important disease in the global swine industry (Chae, 2005). Consequently, controlling PCV2 infection is a high international priority. Field reports concerning the efficacy of commercial PCV2 vaccines indicate that vaccination is an effective tool to control and reduce PCVAD under field conditions (Kixmoller et al., 2008; Segalés et al., 2009; Fraile et al., 2012).

Semen is a significant vehicle for PCV2 transmission and plays an important role in disease dissemination from an epidemiological point of view that previously anticipated (Pozzi and Guerin, 2005). The PCV2 has been detected in semen for up to 95 days after intranasal and intramuscular inoculation (Madson et al., 2008). Naturally infected boars sporadically shed PCV2 DNA in semen for up to 27.3 weeks (McIntosh et al., 2006). Transmission of PCV2 to sows through artificial insemination has also been demonstrated with semen spiked with PCV2 (Madson et al., 2009a). Vaccination against PCV2 in naturally and experimentally infected boars can decrease the duration of viral shedding in semen (Alberti et al., 2010; Opriessnig et al., 2011; Seo et al., 2011). These data indicate that vaccination against PCV2 may reduce the subsequent shedding of PCV2 in the semen of boars.

Nowadays, commercial vaccines contain different types of antigen; PCV2, chimeric PCV 1-2 and subunit (Chae, 2012). Different types of antigen raised the possibility that commercial PCV2 vaccines reduce different amount of PCV2 shedding in semen from experimental infected boars. Hence, the objective of the present study was to compare the reduction of PCV2 shedding in infected boars by 3 commercial single-dose PCV2 vaccines which are available in international market.

MATERIALS AND METHODS

Experimental design

At 8 month of age, twenty-five purebred, male, Landrace pigs were purchased from a commercial farm. All boars were negative for PCV2, and porcine reproductive and respiratory syndrome virus (PRRSV) according to routine serological testing for antibody and real-time polymerase chain reaction (PCR) (Gagnon et al., 2008) for PCV2 in serum conducted both prior to delivery and again on arrival. This study used a randomized, blinded, weight-matched and controlled design. The boars were randomly divided into five groups. Three commercial PCV2 vaccines were used and administered intramuscularly in the right side of the neck in this study: Fostera PCV/Suvaxyn PCV2 One Dose (Pfizer Animal Health, New York, NY, USA) given as one 2.0 ml dose (group T01); Circovac (Merial, Lyon, France) given as one 0.5 ml dose (groups T02); Circoflex (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA) given as one 1.0 ml dose (group T03); phosphate buffered saline (PBS) given as 2.0 ml dose (groups T04 and T05). At 3 weeks post vaccination, the boars in groups (T01, T02, T03, and T04) were inoculated intranasally with 3 ml of PCV2b (strain SNUVR000463; 5th passage) solution that had infectious titer of $1 \times 10^{4.5}$ 50% tissue culture infective dose ($TCID_{50}$)/ml. Boars (T05) remained unvaccinated and served as negative control. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

Serology

Blood samples from each pig were collected by jugular venipuncture at days -21, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days post-inoculation (dpi). The serum samples were tested using the commercial ELISA-based Ingezim Circovirus IgG kit (Ingenasa, Madrid, Spain).

Quantification of PCV2 and chimeric PCV1-2 DNA

DNA extraction from raw semen and serum samples collected on 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 dpi was performed using the QIAamp DNA Mini Kit (Qiagen, Crawley, UK) as previously described (Pal et al. 2008). DNA extracts were used to quantify PCV2 and chimeric PCV 1-2 genomic DNA copy numbers by real-time PCR as previously described (Gagnon et al., 2008; Shen et al., 2010). The lower detection limit of this assay was 1.78 $\log_{10}$ PCV2 genomic copies/ml.

Virus isolation and sequence analysis

PCV2 was isolated from whole semen as previously described (Seo et al., 2011). Isolated PCV2 was further analyzed for the sequence as previously described (Dupont et al., 2008).

Statistical analysis

Summary statistics were calculated for all groups to assess the overall quality of the data, including normality. To compare a single variable (PCV2 DNA quantification in blood and semen) among groups, an ANOVA with a *post-hoc* Tukey's test was utilized. Continuous data for PCV2 serology collected over time were analyzed using a nonparametric Kruskal-Wallis one-way ANOVA at each time point. If the difference was significant ($P < 0.05$), Mann-Whitney U tests were used to assess differences between groups. Pearson's correlation coefficient was used to assess the relationship between viremia and shedding in semen. A value of $P < 0.05$ was considered significant.

RESULTS

Clinical signs

All boras in 5 groups were clinically normal in health and rectal temperature (38-39.5^{o}C) throughout the experiment.

Serology

Anti-PCV2 IgG antibody levels were significantly higher in boars in the vaccinated and challenged groups (T01, T02, and T03) compared to non-vaccinated and challenged group (T04) at 0, 7, 14, and 21 dpi ($P < 0.05$). Thereafter, there were no significantly different anti-PCV2 IgG antibody levels throughout the experiment (Fig. 1). No anti-PCV2 IgG antibodies were detected in serum samples from negative control boars (T05).

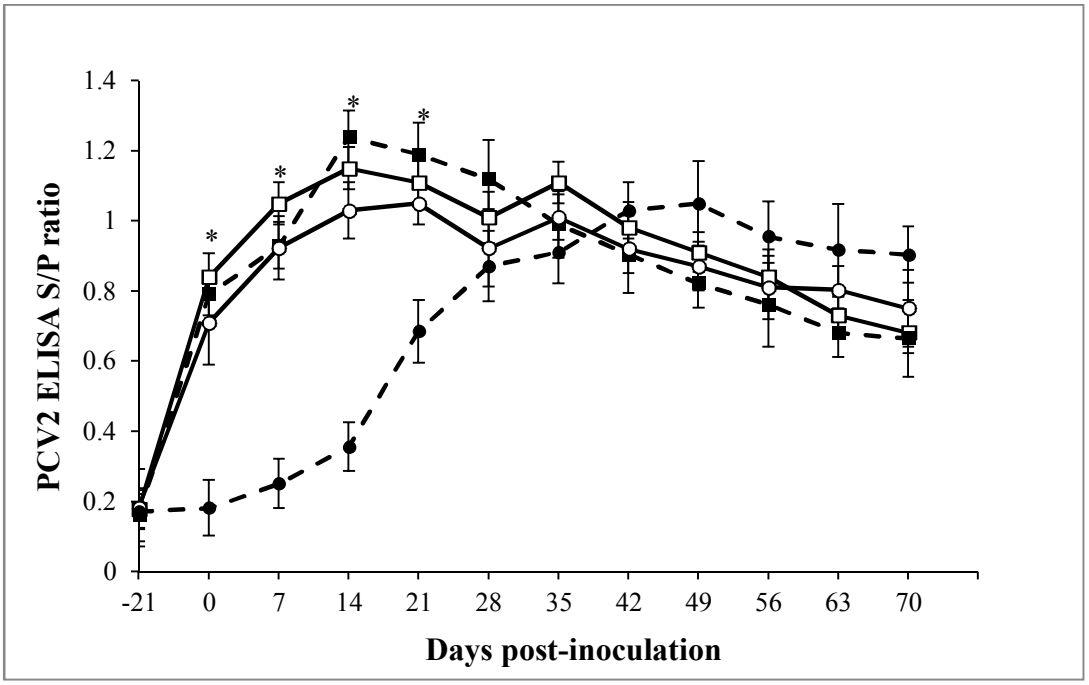


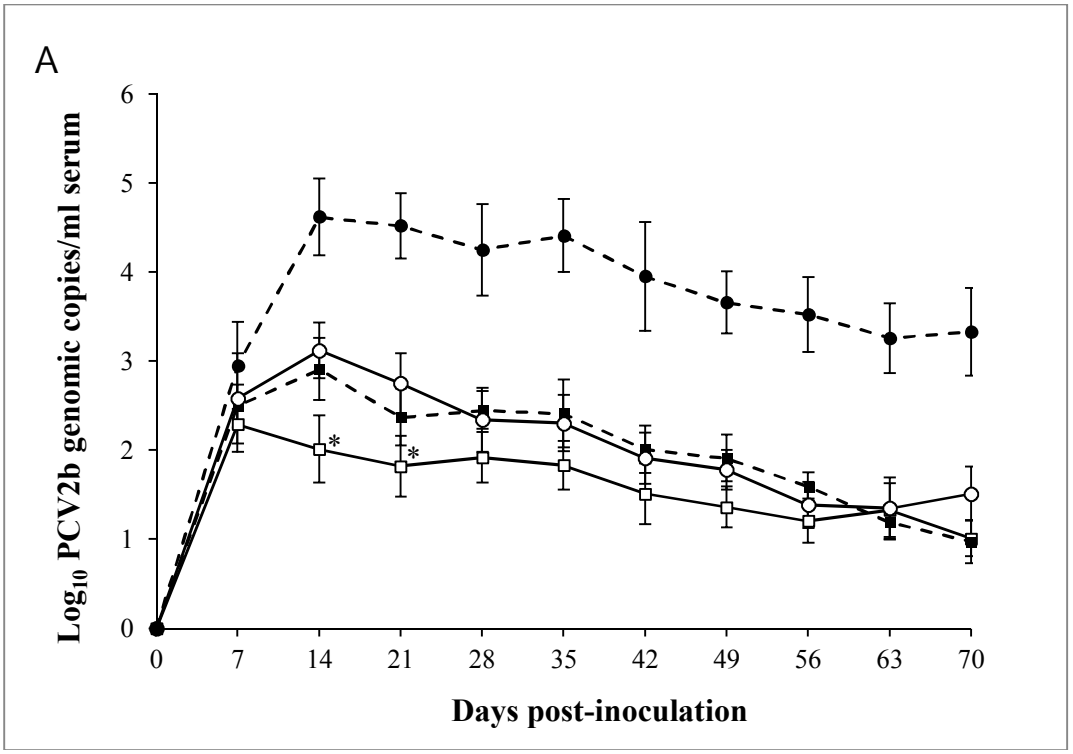
Figure 1. Mean group anti-PCV2-IgG antibody levels at different days post-inoculation (dpi) among 3 commercial vaccines (□, inactivated chimeric PCV1-2 vaccine [Fostera PCV]; ■, inactivated PCV2 vaccine [Circovac]; ○, subunit PCV2 vaccine [Circoflex]; and ●, non-vaccinated and challenged animals). Variation is expressed as the standard deviation. *Significant ($P < 0.05$) difference between vaccinated and non-vaccinated groups.

Quantification of PCV2 and chimeric PCV1-2 DNA in blood

The serum PCV2 genomic copy number was significantly less in vaccinated and challenged boars (T01, T02, and T03) compared to non-vaccinated and challenged boars (T04) from 0 to 70 dpi ($P < 0.01$). Among the vaccinated and challenged boars, serum samples from vaccinated and challenged boars (T01) had significantly ($P < 0.05$) reduced PCV2 genomic copy numbers compared to other vaccinated and challenged boars (T02 and T03) at 14 dpi. Serum samples from vaccinated and challenged boars (T01) had significantly ($P < 0.05$) decreased PCV2 genomic copy numbers compared to other vaccinated and challenged boars (T03) at 21 dpi (Fig. 2A). No genomic copies of chimeric PCV 1-2 were detected in any of the semen samples from vaccinated and challenged boars, non-vaccinated and challenged boars, and negative control boars. No genomic copies of PCV2 were detected in any of the serum samples from negative control boars (T05) throughout the experiment.

Quantification of PCV2 and chimeric PCV1-2 DNA in semen

The PCV2 genomic copy number in semen was significantly less in vaccinated and challenged boars compared to non-vaccinated and challenged boars from 14 to 70 dpi ($P < 0.01$). Vaccinated and challenged boars (T01) had significantly ($P < 0.05$) fewer PCV2 genomic copies in semen compared to other vaccinated and challenged boars (T03) at 21 dpi. Vaccinated and challenged boars (T01) had significantly ($P < 0.05$) reduced numbers PCV2 genomic copies in semen compared to other vaccinated and challenged boars (T02 and T03) at 28 dpi (Fig. 2B). The number of PCV2 genomic copies in the semen samples correlated with the number of PCV2 genomic copies in the blood in vaccinated and challenged boars ($r^2 = 0.908$ for T01, $r^2 = 0.926$ for T02, $r^2 = 0.894$ for T03) ($P < 0.01$) and non-vaccinated challenged boars ($r^2 = 0.903$, $P < 0.01$). No genomic copies of chimeric PCV 1-2 were detected in any of the semen samples from vaccinated and challenged boars, non-vaccinated and challenged boars, and negative control boars. No genomic copies of PCV2 were detected in any of the semen samples from negative control boars throughout the experiment.



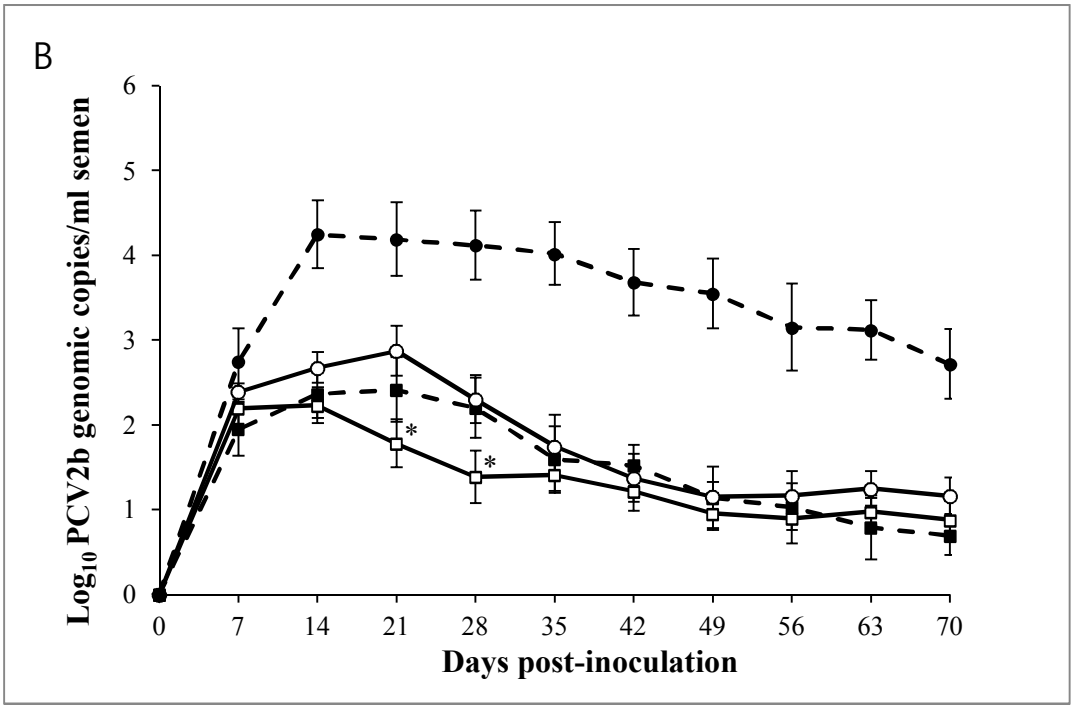


Fig. 2. Mean group $\log_{10}$ PCV2b DNA load in serum (A) and semen (B) samples at different days post-inoculation (dpi) among 3 commercial vaccines (□, inactivated chimeric PCV1-2 vaccine [Fostera PCV]; ■, inactivated PCV2 vaccine [Circovac]; ○, subunit PCV2 vaccine [Circoflex]; and ●, non-vaccinated and challenged animals). Variation is expressed as the standard deviation. *Significant ($P < 0.05$) difference among vaccinated groups.

Virus isolation and sequence analysis

Attempts were made to isolate and identify PCV2b from whole semen samples from each of the five groups. No PCV1, PCV2a was isolated from the semen of boars in T01, T02, T03, and T04 groups. The presence of PCV2b DNA was confirmed in the cytoplasm of infected cells using PCV2b-specific in situ hybridization. In vaccinated and challenged boars in T01 group, PCV2b was isolated in one boar (no. 1) at 14 dpi and in three boars (nos. 1, 2 and 4) at 21 dpi. In vaccinated and challenged boars in T02 group, PCV2b was isolated in two boars (nos. 2 and 4) at 7 dpi and in one boar (no. 3) at 35 dpi. For vaccinated and challenged boars in T03 group, PCV2b was isolated in one boar (no. 5) at 7 dpi, in two boars (nos. 1 and 4) at 21 dpi, and in one boar (no. 5) at 28 dpi. In the non-vaccinated and challenged boars in T04 group, PCV2b was isolated in four boars (nos. 1, 2, 4, and 5) at 7 and 14 dpi, in two boars (nos. 2 and 3) at 21 dpi, in three boars (nos. 1, 3, and 5) at 28 dpi and in two boars (nos. 1 and 3) at 35 dpi. No PCV1, PCV2a or PCV2b was isolated from or identified in the semen of negative control boars. All PCV2b isolates was confirmed to be the same propagating virus in the challenge stock by sequence analysis.

DISCUSSION

The results of present study demonstrated that PCV2 vaccination is able to reduce subsequent PCV2 shedding in the semen of experimental infected boars. In the present study, 3 commercial vaccines were selected on the basis of antigen type, recommended dose, and frequency of use by Korean swine producers. Vaccination protocol (one-dose and different amount of dosage per administration) was followed by piglet vaccination based on manufacture's recommendation because two commercial PCV2 vaccines are licensed for piglets only (Chae, 2012). However, recommendation of vaccination is two doses in adult sows for one inactivated PCV2 vaccine (Chae, 2012). Therefore, one-dose vaccination with 0.5 ml as piglet vaccination may be influenced the results in one vaccinated and challenged group (T02).

Vaccinated and challenged boars shed PCV2 (mean viral load: 0.8 to 2.9 $\log_{10}$ PCV2 genomic copies per ml) in semen, whereas non-vaccinated and challenged boars shed PCV2 (mean viral load: 3.1 to 4.2 $\log_{10}$ PCV2 genomic copies per ml) in semen. These results demonstrated that this experimental challenge model was not able to prevent seminal shedding of PCV2 in the vaccinated and challenged boars; however, the amount of PCV2 load in semen was significantly reduced in vaccinated and challenged boars compared to the non-vaccinated and challenged boars. Although it is not well known how PCV2 vaccination reduces the amount of PCV2 shedding in semen in the present study, it may be related to the reduction of viremia. Commercial PCV2 vaccines are able to reduce the PCV2 load in the serum and reduce PCV2 shedding in nasal and fecal samples in conventional pigs (Fort et al., 2009; Opriessnig et al., 2010). Our data also found a strong correlation between the amount of PCV2 in serum and semen samples. Hence, the reduction of the PCV2 load by PCV2 vaccination in the serum may have resulted in reduced amounts of PCV2 trafficking from the peripheral blood to testes, subsequently reducing the amount of PCV2 shed in semen.

The reduction of PCV2 seminal shedding by commercial vaccines in this study is clinically significant because of the possible transmission of PCV2 via semen. For example, PCV2-positive semen (5.6 to 5.8 $\log_{10}$ PCV2 genomic copies per ml) did not cause reproductive failure,

seroconversion, or PCV2 viremia in naive gilts and their offspring when used for artificial insemination (Madson et al., 2009b). However, PCV2 DNA-negative semen spiked with PCV2 ($10^{4.2}$ $TCID_{50}$/ml) caused reproductive failure in naive sow (Madson et al., 2009a).

Vaccination against PCV2 in boars has no dramatic effect on semen quality (Alberti et al., 2010). Moreover, the absence of clinical signs is important from the epidemiological point of view because acutely PCV2-infected boars can go unnoticed by their caretakers, thus increasing the risk of PCV2 transmission because no control measures will be taken. Further study is needed to determine the effect of repeated vaccination on PCV2 shedding in semen for regular vaccination and surveillance program.

PCV2 vaccination is able to reduce subsequent PCV2 shedding in the semen of infected boars although there was a significantly different amount of PCV2 DNA shed in semen among the 3 vaccinated boar groups.

REFERENCES

Alberti, K.A., Estienne, M.J., Meng, X.J., 2010. Effect of vaccination of boars against porcine circovirus type 2 on ejaculate characteristics, serum antibody titers, viremia, and semen virus shedding. Journal of Animal Science 89, 1581−1587.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. The Veterinary Journal 169, 326−336.

Chae, C., 2012. Porcine circovirus type 2 and its associated disease in Korea. Virus Research 164, 107−113.

Chae, C., 2012. Commercial porcine circovirus type 2 vaccines: Efficacy and clinical application. The Veterinary Journal 194, 151-157.

Dupont, K., Nielsen, E.O., Baekbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Veterinary Microbiology 128, 56−64.

Fort, M., Sibila, M., Perez-Martin, E., Nofrarias, M., Mateu, E., Segalés, J., 2009. One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine 27, 4031−4037.

Fraile, L., Grau-Roma, L., Sarasola, P., Sinovas, N., Nofrarias, M., Lopez-Jimenez, R., Lopez-Soria, S., Sibila, M., Segalés, J., 2012. Inactivated PCV2 one shot vaccine applied in 3-week-old piglets: Improvement of production parameters and interaction with maternally derived immunity. Vaccine 30, 1986−1992.

Gagnon, C.A., Del Castillo, J.R.E., Music, N., Fontaine, G., Harel, J., Tremblay, D., 2008. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of *Porcine circovirus-2* genotypes 2a and 2b in an epidemiological survey. Journal of Veterinary Diagnostic Investigation 20, 545−558.

Kixmoller, M., Ritzmann, M., Eddicks, M., Saalmuller, A., Elbers, K., Fachinger, V., 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine 26, 3443−3451.

Madson, D.M., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X.J., Halbur, P.G., Opriessnig, T., 2008. Characterization of shedding patterns of porcine circovirus types 2a and 2b in experimentally inoculated mature boars. Journal of Veterinary Diagnostic Investigation 20, 725−734.

Madson, D.M., Patterson, A.R., Ramamoorthy, S., Pal, N., Meng, X.J., Opriessnig, T., 2009a. Reproductive failure experimentally induced in sows via artificial insemination with semen spiked with porcine circovirus type 2. Veterinary Pathology 46, 707−716.

Madson, D.M., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X.J., Halbur, P.G., Opriessnig, T., 2009b. Infectivity of porcine circovirus type 2 DNA in semen from experimentally-infected boars. Veterinary Research 40, 10.

McIntosh, K.A., Harding, J.C., Parker, S., Ellis, J.A., Appleyard, G.D., 2006. Nested polymerase chain reaction detection and duration of porcine circovirus type 2 in semen with sperm morphological analysis from naturally infected boars. Journal of Veterinary Diagnostic Investigation 18, 380−384.

Opriessnig, T., Madson D.M., Schalk, S., Brockmeier, S., Shen, H.G., Beach, N.M., Meng, X.J., Baker, R.B., Zanella, E.L., Halbur, P.G., 2011. Porcine circovirus type 2 (PCV2) vaccination is effective in reducing disease and PCV2 shedding in semen of boars concurrently infected with PCV2 and *Mycoplasma hyopneumoniae*. Theriogenology 76, 351−360.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Ramamoorthy, S., Meng, X.J., Halbur, P.G., 2010. Comparison of the effectiveness of passive (dam) versus active (piglet) immunization against porcine circovirus type 2 (PCV2) and impact of passively derived PCV2 vaccine-induced immunity on vaccination. Veterinary Microbiology 142, 177−183.

Pal, N., Huang, Y.W., Madson, D.M., Kuster, C., Meng, X.J., Halbur, P.G., Opriessnig, T., 2008. Development and validation of a duplex real-time PCR assay for the simultaneous detection and quantification of porcine circovirus type 2 and an internal control on porcine semen samples.

Journal of Virological Methods 149, 217−225.

Pozzi, N., Guerin, B., 2005. Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. Theriogenology 63,556−572.

Segalés, J., Urniza, A., Alegre, A., Bru, T., Crisci, E., Nofrarias, M., Lopez-Soria, S., Balasch, M., Sibila, M., Xu, Z., Chu, H.-J., Fraile, L., Plana-Duran, J., 2009. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) improves clinical, pathological and virological outcomes in postweaning multisystemic wasting syndrome affected farms. Vaccine 27, 7313−7321.

Seo, H.W., Han, K., Kim, D., Oh, Y., Kang, I., Park, C., Jang, H., Chae, C., 2011. Effects of an inactivated porcine circovirus type 2 (PCV2) vaccine on PCV2 virus shedding in semen from experimentally infected boars. Clinical and Vaccine Immunology 18, 1091−1096.

Shen, H.G., Beach, N.M., Huang, Y.W., Halbur, P.G., Meng, X.J., Opriessnig, T., 2010. Comparison of commercial and experimental porcine circovirus type 2 (PCV2) vaccines using a triple challenge with PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus (PPV). Vaccine 28, 5960−5966.

국문 논문 초록

돼지 써코 바이러스 2형 백신의 임상학적, 바이러스학적, 면역학적, 병리학적 효능 평가

(지도 교수: 채 찬 희, 수의사, 수의학박사)

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본 실험의 목적은 돼지 써코바이러스 2형 백신의 효능을 임상학적, 바이러스학적, 면역학적, 병리학적 기법을 통하여 분석하고, 백신 종류에 따른 효능 차이를 평가하는 것이다. 돼지 써코바이러스 1형의 ORF1 과 돼지 써코바이러스 2형의 ORF2 부위를 재조합하여 생산된 키메라 백신을 통한 공격접종 실험을 통하여 체액성 면역 및 세포매개성 면역 효능을 평가하였다. 백신 접종 후 공격 접종한 돼지의 경우 공격 접종 후 14일, 28일에 혈중 바이러스양이 양성 대조군에 비하여 감소되었으며, 중화항체와 인터페론 감마 분비세포의 경우 0, 14, 또는 28일에 양성 대조군에 비하여 증가되는 것으로 나타났다. 돼지 써코바이러스 2형 항원 주입을 통한 지연형 과민 반응의 경우에도 백신 접종군에서 유의적인 차이를 보이며 높은 반응을 나타냈다. 공격 접종 실험을 통하여 PCV1/PCV2 키메라 백신에 의해 유도된 면역이 바이러스의 감소와 상관성이 있음이 나타났다.

실험실내 공격접종 실험과는 달리 야외 임상 실험의 경우 폐사율과 증체량 등 경제적인 측면의 효능이 중요하며, 본 실험에서는 백신군이 일일 증체량은 47.3 그램이 증가한 것으로 나타났고, 도축일령의 경우 6일이 감소한 것으로 나타났다. 공격접종 실험과 마찬가지로 혈중 바이러스양과 관련 병리학적 병변이 백신군에서 감소한 것으로 나타났으며, 백신군에서 돼지 써코바이러스에 특이적인 중화항체 및 인터페론 감마 분비세포 유도가 더 높게 나타난 것으로 확인되었다. 야외 임상 실험을 통하여 PCV1/PCV2 키메라 백신이 실제 농장에서도 면역반응을 충분히 유도할 수 있으며, 아울러 경제적인 측면에서도 효능이 입증되었다.

공격접종을 통한 4개 백신의 효능 분석에서도 PCV1/PCV2 키메라 백신 및 불활화 전바이러

스가 재조합 백신보다 세포 매개성 면역과 체액성 면역을 높게 유도하여 혈중 바이러스양 및 관련 병리학적 병변을 감소시키는 것으로 확인되었다. 본 실험들을 통하여 다른 종류의 항원을 함유한 백신들 사이의 면역 반응 유도 수치가 다를 수 있음을 증명하였다.

돼지 써코바이러스 2형은 돼지 정액에서도 배출되며, 감염성도 있는 것으로 알려져 있다. 본 실험에서는 돼지 써코바이러스 2형 백신이 웅돈 정액으로 배출되는 바이러스 양에 미치는 영향을 분석한다. 8개월령 웅돈에 불화화 전바이러스 백신을 3주 간격으로 2회 접종하고 3주 후 돼지 써코바이러스 2형을 공격 접종하여 혈중 및 정액에 포함되어 있는 바이러스양을 측정한 결과, 공격 접종 후 7일부터 60일까지 백신군이 대조군에 비하여 감소한 것으로 확인되었다. 또한 키메라 백신, 불활화 전바이러스 백신 및 재조합 백신 비교실험에서도 모든 백신이 혈중 및 정액 내 바이러스양을 감소시키는 것으로 나타났으며, 백신군 사이에도 감소량에서 차이가 나타났다.

주요어: 돼지 써코바이러스 2형; 돼지 써코바이러스 관련 질병; 이유후 전신소모성 질환; 돼지 써코바이러스 2형 백신; 백신 효능; 정액

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