수의학박사학위논문

Evaluation of Porcine Circovirus Type 2 and *Mycoplasma hyopneumoniae* Vaccines based on Microbiological, Immunological and Pathological Analyses

돼지 써코바이러스 2형, 마이코플라즈마 백신의 미생물학적, 면역학적, 병리학적 분석을 통한 평가

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Evaluation of Porcine Circovirus Type 2 and *Mycoplasma hyopneumoniae* Vaccines based on Microbiological, Immunological and Pathological Analyses

By

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A dissertation submitted in partial fulfillment of
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Abstract

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Porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* are two worldwide economically important dominating pathogens. PCV2, a member of the family *Circoviridae*, is a common virus of pigs and contributed to cause porcine circovirus-associated diseases (PCVAD), including postweaning multisystemic

wasting syndrome (PMWS), porcine respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS). *M. hyopneumoniae* is prevalent and highly contagious in the majority of swine herds. Co-infection with PCV2 and *M. hyopneumoniae* leads to decreased average daily weight gain and an increased number of days to market weight, both of which result in significant economic losses. Vaccination for PCV2 and *M. hyopneumoniae* is one of the most effective strategies in the control of both pathogens. The purpose of this experimental study was to compare the efficacy of combined vaccines.

Studies for the comparison of PCV2 – *M. hyopneumoniae* combined vaccine efficacy were performed to investigate different levels of immune responses. Part I study was to compare the efficacy of 2 different PCV2 – *M. hyopneumoniae* bivalent vaccines with 2 sets of different PCV2 and *M. hyopneumoniae* monovalent vaccines against a dual *M. hyopneumoniae* and PCV2d challenge in swine. Vaccination and challenge improved growth performance and increased the immunologic responses, such as *M. hyopneumoniae*- and PCV2- specific antibodies and interferon-γ-secreting cells (IFN-γ-SCs), when compared to pigs in unvaccinated/challenged groups. All vaccinated groups showed higher growth performance than unvaccinated groups. Vaccinated groups with either a monovalent or bivalent vaccine based on inactivated chimeric PCV1-2a treatment and challenge indicated a larger amount of *M. hyopneumoniae*- and PCV2d- specific IFN-γ-SCs within the pigs and simultaneously reduced the nasal shedding of *M. hyopneumoniae* and PCV2d viremia compared with groups vaccinated with either a monovalent or bivalent vaccine based on inactivated subunit PCV2a treatment and challenge.

Part II study was to evaluate the experimental efficacy of a trivalent vaccine containing PCV2a/b and *M. hyopneumoniae* against PCV2d and *M. hyopneumoniae* challenges. Pigs were administered the vaccine intramuscularly as either at 3 and 24 days of age with 1.0 ml or at 21 days of age with 2.0 ml according to the manufacturer's recommendations. The pigs were challenged at 42 days of age with either PCV2d or *M. hyopneumoniae*, or both. Pigs in vaccinated/challenged and unvaccinated/unchallenged groups exhibited significantly better growth performance when compared with those pigs in the unvaccinated/challenged group in both dosage experiments. Vaccinated pigs in both dosage experiments reduced the amount of PCV2d loads in the blood and *M. hyopneumoniae* load in the larynx when compared with unvaccinated/challenged pigs. Trivalent vaccine elicited protective immunity, such as neutralizing antibody and IFN-γ-SC, and it leads to reduce the severity of lymphoid lesions and the amount of PCV2 antigen within the lymphoid lesions. Trivalent vaccine also able to elicited protective cell-mediated immunity against *M. hyopneumoniae* and this reduced the severity of mycoplasmal pneumonia lesions.

The single challenge did not improve the growth performance between vaccinated/singularly-challenged groups and unvaccinated/singularly-challenged groups in either dosage experiment. This indicated that a single infection with PCV2 cannot produce the full manifestation of clinical PCVAD, even though PCV2 is the primary causative agent of PCVAD.

Both combined vaccine effectiveness was evaluated using microbiological (PCV2

viremia and *M. hyopneumonia* nasal and larynx shedding), immunological (neutralizing antibodies and IFN-γ-SC), and pathological (gross lung lesions, histopathologic pulmonary and lymphoid lesions, and presence of PCV2 antigens within the lesions) analyses. Antigen interference has always been a concern for combined vaccines, but in this study, both combined vaccines produce similar generation of protective immunity compared with monovalent vaccines. Regardless of vaccine type, vaccinated animals with combined vaccine had a significantly greater performance compared to unvaccinated animals. The results of this experimental study demonstrate a strategic method and efficient vaccine regimes against co-infection with PCV2 and *M. hyopneumoniae* for swine producers and practitioners.

Keyword : Porcine Circovirus Type 2; *Mycoplasma hyopneumoniae*, Porcine respiratory disease complex, Co-infection; Combined vaccine, Vaccine efficacy

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PART I

PART II

LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
dpc	Days post-challenge
ELISA	Enzyme-linked immune sorbent assay
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
LAMP	Lipid associated membrane proteins
NA	Neutralizing antibody
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCV	Porcine circovirus
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PI	Post inoculation
PMWS	Post-weaning multisystemic wasting syndrome
PRDC	Porcine respiratory disease complex
PRRS	Porcine reproductive and respiratory syndrome
Th	Helper T lymphocyte
TLR	Toll-like receptors
VNTR	Variable number of tandem repeats

GENERAL INTRODUCTION

Porcine circovirus (PCV), a member of the family *Circoviridae,* is composed of small, non-enveloped virus with a closed-circular, single-stranded DNA genome of 1.76 kb. It was considered a non-pathogenic agent, since the virus did not produce cytopathic effects and any associated disease in pigs under experimental condition (Tischer et al., 1986). Post-weaning multisystemic wasting syndrome (PMWS) were characterized by weight loss, respiratory distress, skin pallor and icterus since 1991. The pathogenic new virus associated with PMWS was designated as porcine circovirus type 2 (PCV2) and the non-pathogenic one as porcine circovirus type 1 (PCV1) (Meehan et al., 1998). PCV2 strains have been classified into at least eight PCV2 genotypes (PCV2a-PCV2h) based on phylogenetic analyses of genomes and ORF2 sequences (Franzo et al., 2018). A recent molecular epidemiological study has shown that PCV2d is currently predominant, followed by PCV2b and PCV2a (Kwon et al., 2017a).

PCV2 has caused a wide variety of syndromes that are collectively termed porcine circovirus associated disease (PCVAD) (Chae, 2005). PCVAD manifests as PMWS, porcine dermatitis and nephropathy syndrome (PDNS), congenital tremors (CT), Porcine respiratory disease complex (PRDC) and reproductive disorders.

PCV2 infection has known to induce immunosuppression in pigs. Following the onset of PCV2 antibody development, the viral titer usually decreases and ends up with the disappear of viremia (Meerts et al., 2005; Opriessnig et al., 2008). Decrease in the humoral responses, particularly the lack of PCV2-specific neutralizing antibodies (NA), are associated with an increased viral replication, resulting in severe lymphoid lesions and significant alterations in the immune system (Meerts et

al., 2005). There is a correlation between the PCV2-spicific NA and protection against virus replication in vitro and development of PCVAD in vivo (Meerts et al., 2006, Song et al., 2007). Interferon-γ-secreting cells (IFN-γ-SCs) contribute to develop specifically in immune response to PCV2 infection and may lead to virus clearance in pigs, and CD4+ CD8+ T cells depletion has been shown to weaken the virus-specific IFN-γ responses. These studies indicated that the viral clearance is likely to be mediated by the production of PCV2- IFN-γ-SCs which contributes to PCV2-specifid NA.

Mycoplasma hyopneumoniae is one of the smallest known bacteria and is the causative agent of enzootic pneumonia and one of the primary agents involved in the PRDC. The clinical presentation of the disease is usually evident in grow-finishing states. In general, the sizes of the genomes are as small as 580-1300 kb, and there are 528 to 691 protein-encoding genes (Leal et al., 2020). Due to the absence of a cell wall, lipid associated membrane proteins (LAMP) play a key role in infection and are important factors in the inflammation process. It is well known that numerous mycoplasma species can invade host cells (Le et al., 2006, Marois et al., 2007). Releasing extracellular DNA allows the organisms to form biofilms on host surfaces and it makes pathogens resistant to antimicrobial agents and host immune responses (Raymond et al., 2018). *M. hyopneumoniae* enchances the severity of PCV2-associated pulmonary and lymphoid lesions and increase the amount and prolongs the presence of PCV2-antigens (Opriessnig et al., 2004).

Immunopathological lesions associated with porcine mycoplasma pneumonia are characterized by peribronchial and perivascular infiltration of mononuclear leukocytes (Wu et al., 2008). *M. hyopneumoniae*-specific IgG levels in serum are not correlated with the severity of lung lesions, that indicates systemic antibodies

induced by vaccination play a minor role in protective immunity (Djordjevic et al., 1997). In previous study indicated a higher level of IFN-γ secreting blood lymphocytes in vaccinated pigs compared to non-vaccinated pigs before and after experimental infection and the vaccinated group showed a significant reduction of lung lesions (Thacker et al., 2000).

Safe and effective vaccination is traditionally considered the most effective way to prevent viral diseases. Pathological examination indicated that PCV2 vaccination effectively reduces the PMWS-associated microscopic lesions and the number of PCV2 load in lymphoid tissues. Immunological examination indicated that vaccinated animals induced PCV2-specific NA and IFN-γ-SCs.

Vaccination is still regarded as the most effective way to control *M. hyopneumoniae* infections. The worldwide used vaccines are inactivated vaccines, live vaccines and a few attenuated vaccines. Vaccinated animals had reduced clinical symptoms and lung lesions, improved performance and reduced numbers of microorganisms in the respiratory tract (Meyns et al., 2006; Sibila et al., 2007; Tao et al., 2019). *M. hyopneumoniae* vaccination induced local, mucosal, humoral, and cellular immune responses.

Due to the variability of swine disease and increased mortality because of cross-infection with pathogens, combined vaccines have gained attention, as they prevent multiple diseases at the same time. There were several studies to evaluate the efficacy of a new bivalent vaccine of PCV2 and *M. hyopneumoniae*. A bivalent vaccine of PCV2 and *M. hyopneumoniae* is able to protect pigs against either PCV2 or *M. hoypneumoniae* challenge or both (Park et al., 2016).

This thesis was designed to investigate the efficacies of bivalent (Part I) and trivalent (Part II) vaccines against PCV2 and *M. hyopneumoniae* challenge in

growing pigs based on clinical, microbiological, immunological and pathological analyses.

LITERATURE REVIEW

1. Porcine circovirus

1-1 Historical background

Porcine circovirus, a member of the family *Circoviridae,* is composed of small, non-enveloped virus with a closed-circular, single-stranded DNA genome of 1.76 kb. Three strains of PCV are known as of 2018: porcine circovirus type 1 (PCV1), porcine circovirus type 2 (PCV2) and porcine circovirus type 3 (PCV3). PCV was first recognized in 1974 as a contaminant in cultures of the porcine kidney cell line (PK 15) (Tischer et al., 1974). Since the virus did not produce cytopathic effects and any associated disease in pigs under experimental condition, it was considered a non-pathogenic agent (Tischer et al., 1986). In 1991, a new mysterious syndrome, which characterized by weight loss, respiratory distress, skin pallor and icterus, etc., was reported from Saskatchewan, Canada (Harding, 1996; Clark, 1997). The syndrome was called as post-weaning multisystemic wasting syndrome (PMWS) because it affects nursery and/or fattening pigs and most affected herds have suffered significant losses due to increased mortality. The pathogenic new virus associated with PMWS was designated as PCV2 and the non-pathogenic one as PCV1 (Meehan et al., 1998). PCV2 is now a ubiquitous virus in both countries with and without porcine circovirus disease (Allan and Ellis, 2000). PCV1 is also considered to be globally distributed, but its exact prevalence would be lower than that of PCV2 (Calsamiglia et al., 2002). In 2016, a novel PCV3 was first identified in the USA associated with porcine dermatitis and nephropathy syndrome (PDNS), multi-systemic inflammation and acute myocarditis (Phan et al., 2016; Palinski et al., 2017).

PCV3 was also identified in pigs without clinical signs of infection or disease state (Stadejek et al., 2017; Kwon et al., 2017b).

1-2 Classification

PCV is small, isosahedral viruses containing single-stranded negative-sense circular DNA genome structure with a genome of approximately 1760 nucleotides (PCV1: 1759, PCV2: 1767-1768. PCV3: 2000) (Mankertz et al., 1997). PCV1 and PCV2 contains eleven predicted open reading frames (ORF). ORF1 and ORF2 are major open reading frames which are oriented in a positive strand and a negative strand, respectively. ORF1 encodes the nonstructural replication proteins Rep and Rep', which are responsible for viral replication. ORF2 encodes for capsid protein Cap, which is having immunodominant antigenic epitopes. ORF1 of PCV1 and PCV2 share 83% nucleotide identity and 86% amino acid identity whereas ORF2 of PCV1 and PCV2 share 67% nucleotide and 65%amino acid sequence identity (Saikumar et al., 2019). The rep protein of PCV3 shared 48% identity to rep protein of PCV2, and the cap protein of PCV3 shared 24% amino acid identity to PCV1 and 26% to PCV2 (Phan et al., 2016).

PCV2 strains have been classified into at least eight PCV2 genotypes have been described in swine based on the open reading frame 2 (ORF2) capsid protein sequence and designated consecutively based on the time of first identification with lower case letters, PCV2a-PCV2h (Franzo et al., 2018). PCV2a was subdivided in four clusters (2A to 2D) and PCV2b into three clusters (1A to 1C) (Olvera et al., 2007). A study has reported novel viral sequences that clustered separately from

existing viruses in phylogenetic analysis. This new virus is tentatively classified as genotype PCV2f (Bao et al., 2018). PCV2a, PCV2b, PCV2d are predominant genotype in pig population worldwide. PCV2a was the initial predominant genotype until the early 2000s, a genotype shift to PCV2b in around 2002 (An et al., 2007). The continuous worldwide circulation of classical PCV2a and PCV2b strains has led to the emergence of recombinant PCV2 stains via intergenotypic recombination within ORF1 (Kim et al., 2009). A new recombinant genotype PCV1/2a, having ORF1 of PCV1 and ORF2 of PCV2, has been reported in Canada in 2008 (Gagnon et al., 2008). A recent molecular epidemiological study has shown that a second genotype shift to PCV2d occurred nationwide before 2012 and PCV2d is currently predominant, followed by PCV2b and PCV2a (Kwon et al., 2017a).

PCV3 is genetically distinct from PCV2, with only 48% amino acid identity in the Rep protein and 26% amino acid identity in the Cap protein (Phan et al., 2016). PCV3 could be classified into three clades by mutations in amino acids 24 and 27 of the Cap protein: PCV3a, PCV3b and PCV3c (Fu et al., 2018). PCV3 has also been reported in worldwide (Stadejek et al., 2017; Kwon et al., 2017b; Shen et al., 2018).

1-3 Porcine circovirus associated disease (PCVAD)

Since its identification in the 1970s, PCV2 has caused a wide variety of syndromes that are collectively termed porcine circovirus associated disease (PCVAD) (Chae, 2005). PCVAD manifests as PMWS, porcine dermatitis and nephropathy syndrome (PNDS), congenital tremors (CT), Porcine respiratory disease complex (PRDC) and reproductive disorders.

PMWS, which was first identified and reported in 1996 (Clark, 1997; Harding et

al., 1997), is now endemic in global swine producing countries and considered to induce severe economic losses on domestic swine production. PCV2 infection is necessary for the development of PMWS, but most studies show that PCV2 requires one or more cofactors for PMWS to develop into a serious and fatal disease (Baekbo et al., 2012). PMWS can be transmitted from infected pigs to healthy pigs after mingling, especially at relatively close contact (pen mates or neighbor pens) (Jaros et al., 2006). In experimental condition, airborne transmission has been shown and these results of studies emphasize the importance of optimal internal as well as external bio-security to reduce transmission of PMWS (Madec et al., 2008).

PRDC is a disease observed with variable number of respiratory pathogens which infect the weaned pigs when maternal antibody declines. Individual pathogens involved include porcine reproductive and respiratory syndrome virus (PRRSV), porcine respiratory corona virus (PRCV), swine influenza (SI) virus, aujeszky's disease virus and bacteria such as *Actinobacillus plueropneumoniae* and *Mycoplasma hyopneumoniae*. PRDC is characterized by a retardation of growth, decreased feed efficiency, anorexia, fever, cough and dyspnea. A variable proportion of affected piglets recover completely, but some remain week and may grow slowly until finishing (Chae, 2005). PRDC can be triggered by entry to a particular pen or occur in every house, and the atmosphere and temperature may contribute to the condition.

PDNS is a vascular disease affecting weaners and growing-finishing pigs. Pathogens involved in PDNS include virus (PRRSV) and bacteria (*Pasteurella multocida, Streptococcus suis* type 1 and 2, *Escherichia coli, Proteus* sp., *Haemophilus parasuis, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Arcanobacterium pyogenes, Staphylococcus aureus,* or *Salmonella*

sp.). These have been known as possible causative pathogens for PDNS (Thibault et al., 1998; Lainson et al., 2002). The most obvious sign is the development of skin lesions that are characterized by round to irregular, red to purple macules and papules that occasionally coalesce to form large, irregular patches and plaques (Drolet et al., 1999). Enlarged tan, waxy looking kidneys with petechial hemorrhages can be observed in macroscopic lesions. Microscopic lesions consist mainly of a systemic necrotizing vasculitis involving small-diameter vessels and occasionally medium-sized vessels (Segales et al., 1998).

PCV has been associated with a condition reported in 1986 (Tischer et al., 1986; Ellis et al., 1998). PCV was found in 14 of 25 PDNS-affected pigs by in situ hybridization and immunohistochemistry (IHC) techniques (Segales et al., 1998). The PCV2 antigens were not seen in some cases by IHC, but the presence of PCV2 DNA were detected in all cases of PDNS by PCR. In case of acute and chronic PDNS, PRRSV antigen has been detected in perivascular macrophages of skin and kidney tissue by using a monoclonal antibody-based immunohistochemical procedures (Thibault et al., 1998). The coinfection of PCV and PRRSV may play an important role in the pathogenesis in PDNS.

2. *Mycoplasma hyopneumoniae*

2-1 Background

Mycoplasma hyopneumoniae is one of the smallest known bacteria and is the causative agent of enzootic pneumonia and one of the primary agents involved in the PRDC. *M. hyopneumoniae* has a small genome, lacks a cell wall and is pleomorphic. Mycoplasmal pnuemoniae of swine is characteristic as highly infectious and highly contagious, causing coughing, asthma, anorexia, and many other symptoms among herds, with reduced daily weight gain and prominent lung lesions in the experiment (Tao et al., 2019). *M. hyopneumoniae* infects the epithetlial cells lining the respiratory cells by adhering to the cilia of the epithelial cells in the host respiratory tract. It proliferates in large numbers and gobbles up the cilia. The cilia became increasingly shorter and fall off over large area. It results in ciliostasis, clumping, damage and loss of the cilia and bronchial goblet cells and finally, the protective ability of the respiratory system was lowered and a series of inflammatory reactions were induced.

There are no clear signs of susceptibility to age, but the clinical presentation of the disease is usually evident in grow-finishing states. *M. hyopneumoniae* is typically infected in close contact between infected and susceptible pigs, and less frequently, through airborne transmission over short distances. Piglets are considered free of *M. hyopneumoniae* at birth, as intrauterine transmission has not been documented. The first exposure event occurs during the lactation period, when piglets come into contact with the dams shedding the microorganism (Calsamiglia et al., 2000; Nathues et al., 2013).

2-2 Virulence factors

The genomes of *M. hyopneumoniae* starins (the pathogenic strains 232 and 7448) were first sequenced in 2004 (Minion et al., 2004; Vasconcelos et al., 2005) and since then, 23 entirely sequence *M. hyopneumoniae* genomes are available now. In general, the sizes of the genomes are as small as 580-1300 kb, and there are 528 to 691 protein-encoding genes (Leal et al., 2020). Many regions of the *M. hyopneumoniae* genome involved in host attachment contain a variable number of tandem repeats (VNTRs). These regions are prone to recombination events and slipped strand mispairing, so it can be possible to lead to express the different sized protein (Torres-Cruz et al., 2003). Despite the small genome size, up to 30% of the gene content is still unknown in function (Felde et al., 2018). The mean GC content is low (28.54%) compared to other bacterial species, and it gives *M. hyopneumoniae* a complex transcriptional organization, unique intrinsic terminator stem-loop formation (Fritsch et al., 2015). With the basic genomes for survival, they derive most of nutrients from host cells.

At least 35 *M. hyopneumoniae* protein have been involved in cell adhesion, including some related to the P97/P102 paralog families (Maes et al., 2018). Because of more than 290 proteins in *M. hyopneumoniae* surface and many uncharacterized surface displayed proteins, the number of *M. hyopneumoniae* adhesins can be much higher. Different adhesins may differ in abundance on the cell surface between strains (Raymond et al., 2015). Adhesion servers as the starting point of infection. Lipid associated membrane proteins (LAMP) have also been implicated in mycoplasma pathogenicity. Due to the absence of a cell wall, LAMPs play a key role in infection and are important factors in the inflammation process. They interact with

the host immune system through Toll-like receptors (TLRs), such as TLR2 (Zuo et al., 2009). LAMPs not only mediate immune responses but also induce either necrosis or apoptosis in monocytes and macrophages (Bai et al., 2015).

It is well known that numerous mycoplasma species can invade host cells, and *M. hyopneumoniae* has been cultured from the liver, spleen, kidneys and bronchial lymph nodes of pig even it has been characterized as a strict extracellular pathogen (Le Carrou et al., 2006; Marois et al., 2007). *M. hyopneumoniae* is capable of releasing extracellular DNA that allows the organisms to form biofilms on host surfaces. This formation makes pathogens resistant to antimicrobial agents and host immune responses (Raymond et al., 2018).

3. Immune response

3-1 Immunopathogenesis of PCV2

PCV2 infection has known to induce immunosuppression in pigs. The events occur at early stages of infection still unknown and target cells of early replication of PCV2 have not yet been specified. PCV2 viremia is first detected around 7 days post inoculation (PI), and the peak of viral titers is between days 14 and 21 PI (Opriessnig et al., 2008). The highest viral loads in lymphoid tissues, but PCV2 can be present in several organs. A specific immune response to PCV2 occur between the second and third week PI (Pogranichnyy et al., 2000). The ability of a pig to elicit an appropriate adaptive immune response has been proposed as a determinant to prevent the progression of PCV2 infection to PMWS. Therefore, following the onset of PCV2 antibody development, the viral titer usually decreases and ends up with the

disappear of viremia (Meerts et al., 2005; Opriessnig et al., 2008). These sub-clinically affected pigs have low levels of PCV2 in tissues and recover with little or no change in the immune system. Conversely, a decrease in the humoral responses, particularly the lack of PCV2-specific neutralizing antibodies (NA), are associated with an increased viral replication, resulting in severe lymphoid lesions and significant alterations in the immune system (Meerts et al., 2005). Pigs undergoing this process had characteristic of the immunosuppressive state of PMWS.

PCV2-specific NA develop between days 10 to 28 PI under experimental studies (Meerts et al., 2005; Maria et al., 2007). The presence of PCV2 antibodies did not necessarily protect against PCV2 infection, as not all antibodies exert neutralizing activity against PCV2 infection. There is a correlation between the PCV2-spicific NA and protection against virus replication in vitro and development of PCVAD in vivo (Meerts et al., 2006; Song et al., 2007). In the field, total anti-PCV2 antibodies seroconversion take place in both sub-clinically and PMWS-affected pigs (Rodriguez-Arrioja et al., 2002). Pigs are usually protected from PCV2 infection by the passive immunity that is inherited from female pigs during their first weeks of life, and active seroconversion to PCV2 generally occurs between 7-12 weeks of age (Segales, 2005). Impaired humoral responses may increase the risk of developing PMWS after PCV2 infection if the period between maternal immunity and the onset of unprotected active seroconversion in pigs is increased.

The role of adaptive cellular immune responses in the control of PCV2 infection and disease has not been studied in detail. The most prominent immune response in PMWS-affected pig is the severe depletion of lymphocytes in lymphoid tissue and their replacement with histiocytes and macrophages (Krakowka et al., 2002). PMWS-affected pigs have impaired T cell responses (Nielsen et al., 2003).

Interleukin (IL)-10 over expression in the thymus and peripheral blood mononuclear cells (PBMC), and decreased IL-2, IL-4, and IL-12 expression in secondary lymphoid organs suggest an impairment of T cell immune responses (Darwich et al., 2003; Sipos et al., 2004). PCV2 has been demonstrated to induce secretion of IL-10 in cultured PBMC in in vitro, that lead to down regulation of other cytokines (Kekarainen et al., 2008).

PBMC from PCVAD-affected pigs have been shown to respond well to recall PCV2 antigen by releasing IL-10 and IFN-γ, but less responsive or nonresponsive to mitogen or superantigen in production of IL-4, IL-2, or IFN-γ (Vincent et al., 2007) IFN-γ-SCs contribute to develop specifically in immune response to PCV2 infection and may lead to virus clearance in pigs, and CD4+ CD8+ T cells depletion has been shown to weaken the virus-specific IFN-γ responses. Overall, it appears that IL-10, IFN-γ and other proinflammatory cytokines important roles in PCV2 pathogenesis. Impaired immune cells and cytokine imbalances may be responsible for several long-term diseases associated with PCV2 infection (Ramamoorthy et al., 2009; Darwich et al., 2012).

Lymphoid depletion and histiocyte replacement of follicles in lymphoid tissues are characteristic microscopic lesions associated with PCV2-infection. The lymphocellular depletion affects lymphoid follicles and parafollicular zone. Syncytial cells could be seen in lymph nodes, Peyer's patehes, and lamina propria of the intestinal villi. Sarply demarcated, spherical, basophilic cytoplasmic inclusion bodies could be seen in macrophages in affected lymphoid tissues (Rosell et al., 1999). Necrotizing lymphadenitis also can be seen in field cases. Follicular necrosis in the middle of prominent lymphoid follicles was the main lesion. PCV2 was detected in lymph nodes with necrotic foci but was not detected in other lymph nodes

(Kim et al., 2005). Previous experimentally infection study indicated that viral antigen was also associated with obliterated blood vessels in areas of granulomatous and necrotizing lymphadenitis (Opriessnig et al., 2006).

3-2 Immunopathogenesis of *Mycoplasma hyopneumoniae*

Immunopathological lesions associated with porcine mycoplasma pneumonia are characterized by peribronchial and perivascular infiltration of mononuclear leukocytes (Wu et al., 2008). *M. hyopneumoniae* stimulates the immune response by inducing macrophages to produce multiple pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, IL-8, and IL-18, and immunoregulatory cytokines like IL-10 (Muneta et al., 2008; Woolley et al., 2013). This excessive inflammatory response has been implicated in lymphatic proliferation and is considered to be a major cause of lung lesions (Lorenzo et al., 2006). TLR2 and TLR6 have been shown to be important for the recognition of *M. hyopneumoniae* by porcine alveolar macrophages (Muneta et al., 2003). Blocking TLR2 and TLR6 reduces TNF-α production by macrophages. This demonstrates that alveolar macrophages are involved in inflammation and innate immune response during *M. hyopneumoniae* infection (Okusawa et al., 2004).

Due to the lack of a cell wall, LAMPs on the mycoplasma surface play an important role in mycoplasma and host interactions (Razin et al., 1998). LAMPs mediate immune responses and induce necrosis or apoptosis in monocytes and macrophages (Bai et al., 2013). A sequence of distinct biochemical and molecular events causes apoptosis. Nitric oxide (NO) is a multifunctional molecule involved in a variety of physiological and pathological processes. Low concentration of NO can protect cells

from apoptosis, but excessive production of NO leads various cell types to apoptosis. Various mycoplasma species trigger apoptosis by inducing excessive NO (Dusanic et al., 2012; Obara et al., 2010). LAMPs of *M. hyopneumoniae* inhibit cell growth, induced the production of NO, which induced apoptosis in PBMC in vitro experiment (Bai et al., 2015).

M. hyopneumoniae-specific IgG antibodies are detected 3-5 weeks PI, peak after 11-12 weeks and decrease very gradually in experimental infection (Kobisch et al., 1993). A highly virulent strain produces earlier seroconversion than a low virulent strain (Villarreal et al., 2011). *M. hyopneumoniae*-specific IgM and IgA can be detected as early as 9 days PI in serum and 6 days PI in nasal swab, respectively (Chae et al., 2020). *M. hyopneumoniae*-specific IgG levels in serum are not correlated with the severity of lung lesions, that indicates systemic antibodies induced by vaccination play a minor role in protective immunity (Djordjevic et al., 1997). Some studies demonstrated that the *M. hyopneumoniae*-specific IgA contribute to prevent adhesion of microorganism to the ciliated cells of the respiratory tract (Thacker et al., 2000; Woolley et al., 2014). In addition, specific IgG that diffuses from the blood into lung tissue or produced locally in the bronchus-associated lymphoid tissue could opsonize *M. hyopneumoniae*, and leads to phagocytosis by macrophages and neutrophils (Marchioro et al., 2013).

T cells are key to regulate the immune response and have a significant impoact on the pathogenesis of mycoplasma-induced pneumonia (Dobbs et al., 2009). In previous study indicated a higher level of IFN-γ secreting blood lymphocytes in vaccinated pigs compared to non-vaccinated pigs before and after experimental infection and the vaccinated group showed a significant reduction of lung lesions (Thacker et al., 2000). CD8+ cells are characterized as killing infected cells (Jones

et al., 2003). Studies performed with the *M. pulmonis* mouse model indicated that CD8+ T cells could weaken the pro-inflammatory Th cell responses that cause lung damage and clinical disease (Dobbs et al., 2009). T helper 1 (Th1), Th17 and CD8+ T cell responses are responsible for protection against Mycoplasma disease. T helper 1 responses cause IFN-γ mediated activation of macrophage killing and that could be a significant role in protection against Mycoplasma infection. It is well-known that Th17 immune responses are important to protect mucosal surfaces, to promote epithelial cell regeneration, production of mucus and antibacterial protein and release of neutrophil recruitment (Abbas et al., 2016). Th17 cells attract other immune cells for clearance of pathogen and elevate secretory IgA levels in the airway lumen (Liang et al., 2007; Jaffar et al., 2009).

3-3 Interaction between PCV2 and *Mycoplasma hyopneumoniae*

Both PCV2 and *M. hyopneumoniae* impair the host defences by targeting the host's immune cells, resulting in a significant increase in the expression of IFN-γ, IL-1β, IL-8, Chemokine ligand 5 and Chemokine ligand 10, and downregulated of IL-13 and IFN-α significantly (Zhang et al., 2011). Previous study indicated that *M. hyopneumoniae* could enhance the levels of PCV2 viremia, but PCV2 could not enhance the levels of mycoplasmal nasal shadding in co-infected pigs, comparing with singly *M. hyopneumoniae* or PCV20-infected pigs (Seo et al., 2014). The synergistic effect occurs in the process of continuous infection of *M. hyopneumoniae* and PCV2, and *M. hyopneumoniae* infection in pigs is generally just before of around PCV2 infection in the field conditions (Larochelle et al., 2003; Fachinger et al., 2008; Chae, 2012).

The porcine lymph nodes were swollen compared to normal size, the lungs had a dark purple consolidation, and co-infection cases with *M. hyopneumoniae* and PCV2 release high levels of PCV2 DNA in semen (Opriessnig et al., 2011). *M. hyopneumoniae* enchances the severity of PCV2-associated pulmonary and lymphoid lesions and increase the amount and prolongs the presence of PCV2-antigens (Opriessnig et al., 2004).

3-4 Pathological lesions

The most prominent lesions on necropsy are non-collapsed lung and lymph node enlargement. However, the pigs that suffered from PMWS are able to recover, these lesions are not always present (Rosell et al., 1999). Lyph nodes could have the presence of multiple lesion areas of macroscopic necrosis. Pigs infected with PMWS have bronchopneumonia and esophageal gastric ulcers that are not directly related to the effects of PCV2. Some PMWS affected animals may have atrophic, discoloured livers, and multifocal white foci in the kidney's cortices (Segales et al., 2002).

The granulomatous inflammation and the presence of intracytoplasmic inclusion bodies are the two characteristic microscopic lesions of PMWS affected pigs. This microscopic lesion is usually combined with a multinucleate giant cell infiltration. Intracytoplasmic inclusion bodies are large, multiple, basophilic or amphophilic grape-like structures may seen in the cytoplasm of histiocytic cells (Chae, 2004). Large histiocytic and multinucleate giant cells are present within the thickened interalveolar walls and alveoli. Bronchiolitis fibrosa obliterans also be present in chronic cases (Clark, 1997).

The pulmonary changes of pic infected with *M. hyopneumoniae* consist of

decreased number of cilia in the bronchial epithelial cells, inflammatory exudate in the bronchial lumen, lymphoid hyperplasia of bronchus-associated lymphoid tissue, and enlarged alveolar septa (Kwon et al., 2002; Sarradell et al., 2003; Lorenzo et al., 2006). *M. hyopneumoniae* causes a well-differentiated bronchointerstitial pneumonia. In the early stage of infection, pneumocyte type Ⅱ hyperplasia, perivascular and peribronchiolar lyphoplasmacytic hyperplasia are observed. As the disease progresses, these lesions exacerbate in the peribroncial and perivascular lypmphoid follicles (Sibila et al., 2007), with an increased number of goblet cells and hyperplasia of submucosal glands (Thacker et al., 2012). The macrophages, stimulated by the *M. hyopneumoniae* antigens, are expected to increase the secretion of inflammatory cytokines and excacerbate the inflammatory response (Kwon et al., 2002; Sarradell et al., 2003; Thanawongnuwech et al., 2003; Rodrıguez et al., 2004).

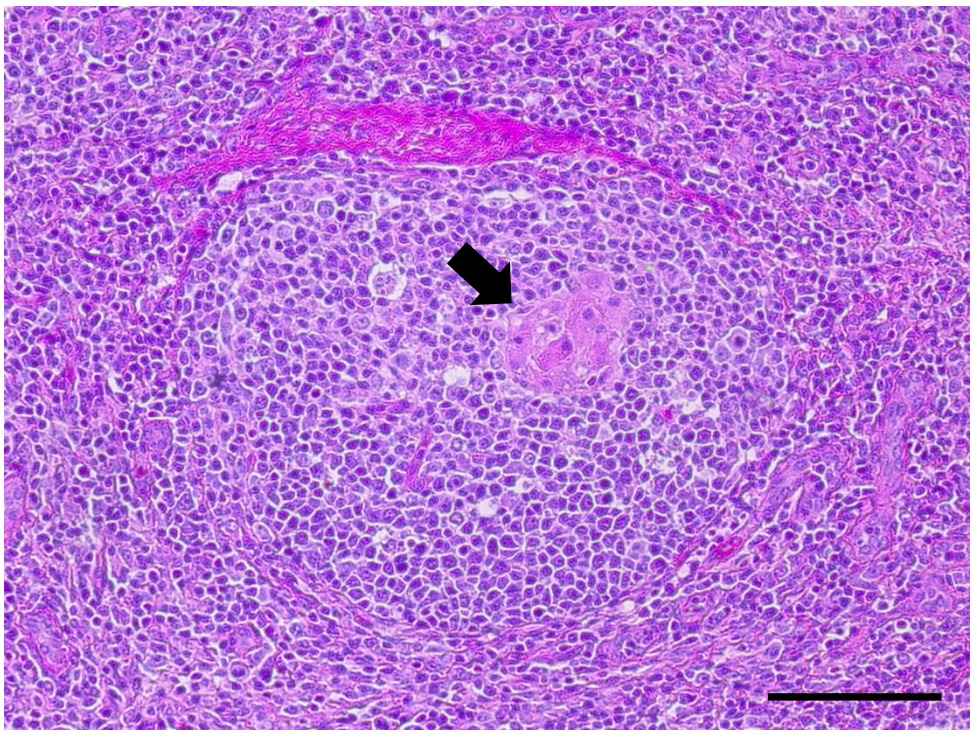

Figure 1. Multinucleated giant cells (arrow) in lymph node tissue of PCV2-infected pig. H&E. Bar = 100 μm.

4. Strategies and efficacy of Vaccines

4-1 PCV2 Vaccines

Safe and effective vaccination is traditionally considered the most effective way to prevent viral diseases. Available commercial PCV2 vaccines have shown effectiveness in alleviating clinical disease severity and improving production parameters, but the duration of protection is limited and complete eradication of virus has not been achieved (Afolabi et al., 2017).

Conventional, inactivated vaccines are whole virus preparations and PCV2 recombinant vaccines target the immunogenic capsid protein of the virus encoded by the ORF2 (Beach et al., 2010). At least five commercial PCV2 vaccines are available in the U.S. Circovac® from Merial, Fostera™ PCV from Pfizer and Suvaxyn® PCV2 One Dose™ from Zoetis Inc are introduced. Circovac is used in both breeding piglets and sows, and Fostera PCV consists of an inactivated PCV1-2 chimeric virus formulation is used for three weeks old piglets. The recombinant vaccines, which are subunit vaccines containing baculovirus expressed PCV2 capsid protein include Porcilis® PCV (Schering-Plough/Merck), Circumvent® (Intervet/Merk), and Ingelvac CircoFLEX® (Boehringer Ingelheim), are for use in three week or older piglets.

Previous meta-analysis indicated that estimate of cost saving due to PCV2 vaccination are £ 19.2/pig in british study and $ 6.00/pig in field studies (Gillespie et al., 2006; Alarcon et al., 2013). Pathological examination indicated that PCV2 vaccination effectively reduce the PMWS-associated microscopic lesions and the number of PCV2 load in lymphoid tissues. Immunological examination indicated that vaccinated animals induced PCV2-specific NA and IFN-γ-SCs. Non-vaccinated

animals showed the decreased number of CD4+ cells (Seo et al., 2012). The PCV2 antibody titers in serum and colostrum are increased in sows and gilts by using vaccines. Vaccination of breeding boar decreases viremia, systemic viral load and subsequently shedding of virus in semen, and it helps controlling vertical transmission of virus through semen. The use of vaccines in the sow may contribute to protection during the gestation phase and prevent pathogenic effects of PCV2 during physiological state (Joisel et al., 2007). Vaccination of breeding sow reduce the amount of virus in sow, reduce quantity of virus transmitted to progeny during pregnancy and pre-weaning period and increase neutralizing antibodies against PCV2 in colostrum. In addition, passive immunization in the form of maternal antibodies reduce pre-weaning mortality and improve the average daily weight gain (ADWG) in the offspring. Vaccination to growing pigs has been shown to reduce viral load and mortality and improve growth performances (Fort et al., 2008; Beach et al., 2012).

The ADWG is considered as the most important parameter for assessing vaccine performance. Other parameters include feed conversion ratio, percentage mortality and cull rates. The previous meta-analyses, the mean differential ADWG between vaccinated and unvaccinated pigs from wean to finish for 4 commercial vaccines was 22.87g. They also found that PRRSV significantly influenced the ADWG in field condition (da Silva et al., 2014). Other meta-analysis of field studies showed a 50% reduction on wean-to-finish mortality and an ADWG of 680g between 3 and 22 weeks of age (Coll et al., 2010; Diaz et al., 2010).

Vaccination of pigs with PCV2a vaccines brings the emergence of the vaccine escape strain PCV2b with severe outbreaks of clinical disease worldwide (Carman et al., 2008). Cross protection between PCV2a and PCV2b is observed, but vaccines

based on genotype PCV2b is more effective against PCV2b than those based on PCV2a (Opriessnig et al., 2013; 2014a). Recently, PCV2d is currently predominant genotype, followed by PCV2b and PCV2a. After the emergence of PCV2d in 2010 and 2012 in China and the U.S., about 37% of the U.S herds are positive for PCV2d (Xiao et al., 2015). Experimental reports consistently concerned that PCV2d is more virulent (Guo et al., 2012; Opriessnig et al., 2014b). When pigs naturally infected with PCV2b were vaccinated with 3 PCV2a vaccines and challenged with PCV2d, showed a reduction in both PCV2b and PCV2d viremia. It has also been suggested that vaccines based on PCV2a can confer effective cross protection against clinical disease with PCV2d genotype (Opriessnig et al., 2017). Similarly, vaccination with commercial PCV2a vaccine in a herd naturally infected with PCV2d and PCV2b, showed in reduction of PCV2d viremia and elimination of PCV2b viremia. They also showed reduction of lesions scores and improvement of ADWG in vaccinated animals compared to unvaccinated controls (Jeong et al., 2015).

4-2 *Mycoplasma hyopnuemoniae* Vaccines

Management and prevention of *M. hyopneumoniae* are based on optimization of control conditions, vaccination, and treatment with antibiotics. Vaccination is still regarded as the most effective way to control *M. hyopneumoniae* infections. Gilt replacement procedures against *M. hyopneumoniae* in positive farms in Europe and North America indicated that vaccination is the key strategy to avoid enzootic pneumonia (Betlach et al., 2019). Recently, there are at least 26 vaccines commercially available worldwide to prevent *M. hyopneumoniae* infection (Vranckx et al., 2012). They consist mainly of inactivated, adjuvanted whole-cell preparations

that are administered intramuscularly (Maes et al., 2008).

The worldwide used vaccines are inactivated vaccines, live vaccines and a few attenuated vaccines. Some study indicated that bacterins could not stimulate the swine immune system to produce significant amount of antibodies against antigens of *M. hyopneumoniae* (Fisch et al., 2016). Another study found that an *M hyopneumoniae*-168 attenuated vaccine could neither induce increasing of lymphokine nor inflammatory cytokines, which demonstrate an increased IL-10 lever and decreased IFN-γ (Shen et al., 2017). This causes a lack of cellular immunity. Recombinant vaccine has been reported to be able to fill the shortage of traditional vaccines by providing several multivalent vaccines against major pathogens. Recent study of vaccine is based on adhesins, such as P97, P95, P46, P42 and P36 delivered as recombinant vectors or recombinant subunits (Martelli et al., 2006; Virginio et al., 2014).

Vaccinated animals had reduced clinical symptoms and lung lesions, improved performance and reduced numbers of microorganisms in the respiratory tract (Meyns et al., 2006; Sibila et al., 2007; Tao et al., 2019). *M. hyopneumoniae* vaccination induced local, mucosal, humoral, and cellular immune responses. Although the exact protection mechanisms required to avoid *M. hyopneumoniae* infection are not fully understood, ongoing efforts are being invested in developing new vaccines that may provide better protection. Despite several efforts to control *M. hyopneumoniae* infection, there is still a significant economic loss in pig production by enzootic pneumonia.

4-3 Combined Vaccines

PCVAD is a multifactorial disease, effective management of the disease requires vaccination along with other interventional strategies. Recently, due to the variability of swine disease and increased mortality because of cross-infection with pathogens, combined vaccines have gained attention, as they prevent multiple diseases at the same time. Vaccination against *M. hyopneumoniae* alone could not decrease the potentiation of PCV2-associated lesions by *M. hyopneumoniae* (Park et al., 2014). There were several studies to evaluate the efficacy of a new bivalent vaccine of PCV2 and *M. hyopneumoniae*. A bivalent vaccine of PCV2 and *M. hyopneumoniae* is able to protect pigs against either PCV2 or *M. hyopneumoniae* challenge or both (Park et al., 2016). They found the efficacy in all tested index in both experimental conditions and field conditions tests (Park et al., 2016). Another study compared ready-to-use PCV2 and *M. hyopneumoniae* combined vaccine to a *M. hyopneumoniae* bacterin, found that combined vaccine is more effective in improvement of ADWG and helps in reduction of the negative effect of subclinical PCV2 infection on growth (Duivon et al., 2018). In previous study, a combined vaccine includes capsid-derived virus-like particles of PCV2 and a new recombinant chimera composed of the P97R1, P46 and P42 antigens of *M. hyopneumoniae* could induce humoral and cellular immune responses against both antigens in mice and piglets (Tao et al., 2020). Another study found a novel combination vaccine, including baculovirus expressed PCV2 subunits and inactivated *M. hyopneumoniae* strain J in Emunade adjuvant. They indicated the combined vaccine reduce the severity of lung lesions at slaughter and induce protective reactions in PCV2 viremia and ADWG against both PCV2 and *M. hyopneumoniae* infection (Tassis et al., 2017).

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PART I. Efficacy comparison of commercial porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* monovalent and bivalent vaccines against a dual challenge

ABSTRACT

The objective of this study was to compare the efficacy of commercially available porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* vaccines. A total of 80 pigs was randomly divided into 6 treatment groups; 4 of the groups each received a different vaccine as well as a dual challenge. The remaining 2 groups were used as controls, 1 of which also received a dual challenge. Two of the 4 groups of pigs were administered 2 monovalent vaccines (designated as either monovalent vaccine A or B) of *M. hyopneumoniae* at 7 days old and PCV2 at 21 days old, or *M. hyopneumoniae* and PCV2 at 21 days old. The remaining 2 vaccinated groups of pigs received a bivalent vaccine (designated as either bivalent vaccine A or B) of PCV2 and *M. hyopneumoniae* at 21 days old. All 4 vaccinated groups were challenged with *M. hyopneumoniae* at 42 days old [−14 d post-challenge (dpc)], followed by a PCV2d challenge at 56 days old (0 dpc). All 4 vaccinated/challenged groups displayed a reduction in clinical signs, PCV2d viremia, nasal shedding of *M. hyopneumoniae*, and lung lesions compared with pigs in the unvaccinated and challenged groups. Vaccination and challenge improved growth performance and increased the immunologic responses (*M. hyopneumoniae*- and PCV2-specific antibodies and interferon-γ-secreting cells) when compared to pigs in the unvaccinated/challenged groups. Pigs in groups vaccinated with either a monovalent or bivalent vaccine A treatment and challenge produced a larger amount of *M. hyopneumoniae*- and PCV2d specific interferon-γ-secreting cells within the pigs and simultaneously reduced the nasal shedding of *M. hyopneumoniae* and PCV2d viremia compared with groups vaccinated with either a monovalent or bivalent

vaccine B treatment and challenge. Both the bivalent vaccines and the respective monovalent vaccines were efficacious against a dual challenge of *M. hyopneumoniae* and PCV2d.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is the smallest known virus to autonomously replicate and causes a wide variety of syndromes that are collectively termed porcine circovirus associated disease (PCVAD) (Chae., 2005). At least 5 different genotypes have been identified to date and are designated with lowercase letters (a, b, c, d, and e). This alphanumerical ordering is based on the first identification of the virus (Davies et al., 2016). Among those, PCV2d has become the predominant genotype in North America and Asia, although PCV2a and PCV2b are still prevalent in these regions (Franzo et al., 2018). Although PCV2 is the causative agent of PCVAD, subclinical infection remains the most common form of PCV2 infection worldwide (Seo et al., 2014a).

Mycoplasma hyopneumoniae is the primary causative agent of enzootic pneumonia. It is considered one of the most economically important respiratory pathogens as it results in common and chronic disease in swine herds. *Mycoplasma hyopneumoniae* attaches itself to the ciliated epithelial cells lining the upper respiratory tract. As the bacterium colonizes, the cells are damaged, which predisposes the infected animals to secondary infection (Thacker et al., 2012). Hydrogen peroxide and superoxide radicals are 2 toxic products of mycoplasmal metabolism that may cause further damage to the epithelial cells (Razin et al., 1998).

Porcine circovirus type 2 (PCV2) and *M. hyopneumoniae* are the 2 most prevalent and economically important pathogens in global pig production systems. These agents can have a significant negative impact on several areas of pig performance, such as weight gain and feed efficiency, particularly during the vital grow/finish

phase of pig production. Co-infection with PCV2 and *M. hyopneumoniae* is commonly observed during the development of porcine respiratory disease complex (PRDC), which is responsible for major economic losses in the Asian pork industry.

Vaccination rather than administration of antimicrobial treatment is considered the more effective method to control pathogens associated with PRDC and is the most widely used. Vaccines against PCV2 and *M. hyopneumoniae* are the 2 most commonly administered vaccines in Korea, as in all Asian swine herds. Since the recommended vaccination time against both PCV2 and *M. hyopneumoniae* is similar, the use of a bivalent vaccine is preferred in an effort to reduce the number of injections that need to be administered. Despite this convenience, some swine practitioners and producers still prefer to use monovalent vaccines against the 2 pathogens. The objective of this study was to compare 2 different PCV2-*M. hyopneumoniae* bivalent vaccines with 2 sets of different PCV2 and *M. hyopneumoniae* monovalent vaccines against a dual *M. hyopneumoniae* and PCV2d challenge in swine.

MATERIALS AND METHODS

Animals

A total of 80, 7-day-old conventional piglets was purchased for this study. Piglets were colostrum-fed crossbreds and were selected from a commercial farm that was deemed free from porcine reproductive and respiratory syndrome virus (PRRSV) and *M. hyopneumoniae* based on serological testing of the breeding herd and long-term clinical and slaughter history. Piglets were tested for PCV2 and PRRSV viremia, as well as for nasal shedding of *M. hyopneumoniae*, all by real-time polymerase chain reaction (RT-PCR) upon arrival. Serology testing was also evaluated on the newly arrived piglets for PCV2 (SERELISA PCV2 Ab Mono Blocking; Synbiotics, Lyon, France), PRRSV (HerdCheck PRRS X3 Ab Test; IDEXX Laboratories, Westbrook, Maine, USA), and *M. hyopneumoniae* (*M. hyo* Ab Test; IDEXX Laboratories) antibodies. All piglets tested seronegative for PCV2, PRRSV, and *M. hyopneumoniae*.

Experimental design

The random number generator function (Excel; Microsoft Corporation, Redmond, Washington, USA) was used to randomly assign a total of 80 pigs into 6 groups (Figure 1). The following groups of pigs were then randomly assigned into 9 rooms: VacA-PM/Ch (n = 16, male = 8, female = 8); VacB-PM/Ch (n = 16, male = 8, female = 8); VacA-M+P/Ch (n = 16, male = 8, female = 8); VacB-M+P/Ch (n =16, male = 8, female = 8); and the unvaccinated/challenged group (UnVac/Ch) (n = 8, male = 4, female = 4). Pigs in the unvaccinated/unchallenged group (UnVac/UnCh) (n = 8,

male = 4, female = 4) were randomly assigned into 1 room. Each room contained 2 pens with 4 pigs per pen. Individual pigs were also randomly assigned to pens using the random number generator function (Excel; Microsoft). All rooms and pens were uniform in design and equipment, including a freely accessible feed and water trough.

Pigs in the VacA-PM/Ch group were administered a 2.0-mL dose of Fostera PCV MH (Serial No. 259044A; Zoetis, Parsippany, New Jersey, USA) intramuscularly at 21 d old. Pigs in the VacB-PM/Ch group were administered a 2.0-mL dose of Porcilis PCV M Hyo (Lot No. C530B01; MSD Animal Health, Boxmeer, Netherlands) intramuscularly at 21 d old. Pigs in the VacA-M+P/Ch group were administered 2 monovalents: a 2.0-mL dose of RespiSure-One (Serial No. 263688; Zoetis) intramuscularly on the left side of the neck at 7 d old, followed by a 2.0-mL dose of Fostera PCV MetaStim (Serial No. 306218A; Zoetis) intramuscularly on the right side of the neck at 21 d old. Pigs in the VacB-M+P/Ch group were administered 2 different monovalent vaccines: a 2.0-mL dose of M+Pac (Serial No. 000511244; MSD Animal Health) intramuscularly on the left side of the neck, followed by a 2.0-mL dose of Porcilis PCV (Lot No. A545A01; MSD Animal Health) intramuscularly on the right side of the neck at 21 d old.

Pigs in the unvaccinated/challenged (UnVac/Ch) and unvaccinated/unchallenged (UnVac/UnCh) groups received a 2.0-mL dose of phosphate-buffered saline (PBS, 0.01M, pH 7.4) at 21 d old.

At −14 d post-challenge [(dpc), 42 d old], pigs in the VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, VacB-M+P/Ch, and UnVac/Ch groups were anesthetized with a mixture of 2.2 mg/kg xylazine hydrochloride (Rumpon; Bayer Korea, Seoul, Korea) and 2.2 mg/kg tiletamine hydrochloride and 2.2 mg/kg zolazepam hydrochloride (Zoletil 50; Virbac Korea, Seoul, Korea) by intramuscular injection. Post-

anesthetization, pigs were inoculated intratracheally with 7 mL of *M. hyopneumoniae* (strain SNU98703) culture medium containing 10^7 color-changing units (CCUs)/milliliter, as described in a previous study (Van Reeth K et al ., 2000; Marchioro et al., 2014).

At 0 dpc (56 d old), pigs in the VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, VacB-M+P/Ch, and UnVac/Ch groups were inoculated intranasally with 3 mL of tissue culture supernatant containing 1.2×10^5 TCID50/mL of PCV2d (SNUVR140004, GenBank no. KJ437506) (Seo et al., 2014a).

Blood and nasal swabs were collected at the following timepoints: −49 dpc (7 d old); −35 dpc (21 d old); −14 dpc (42 d old); 0 dpc (56 d old), 7 dpc (63 d old); 14 dpc (70 d old); 35 dpc (91 d old); and 56 dpc (112 d old). At 175 d old (119 dpc), all pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution, as described in a previous study (Beaver et al., 2001). Animal methodology was approved by the Seoul National University Institutional Animal Care and Use Committee.

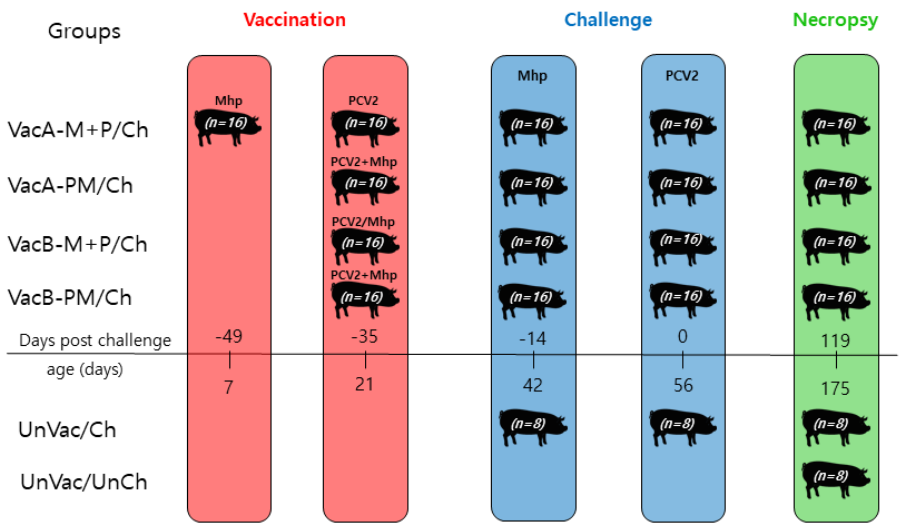

Figure 1. Experimental design. Pigs were administered a vaccine against *M. hyopneumoniae* (Mhp) and/or porcine circovirus type 2 (PCV2) and challenged with *M. hyopneumoniae* and PCV2 on certain days as show. A numbers of pigs were necropsied as shown.

Clinical observation

The pigs were monitored daily for abnormal clinical signs and rated weekly using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur et al., 1995). Observers were blinded to type of vaccine status and vaccination. Pigs that died or were culled as deemed necessary were necropsied. At the end of the study, mortality rate was calculated as the number of pigs that died, divided by the number of pigs initially assigned to that group within the batch.

Growth performance

The live weight of each pig was measured at various timepoints throughout the study as follows: −35 dpc (21 d old); 0 dpc (56 d old); 28 dpc (84 d old); 56 dpc (112 d old); and 119 dpc (175 d old). At the end of the study, the average daily weight gain [(ADWG) grams/pig/day] was calculated over 4 time periods or production stages: i) from 21 to 56 d old; ii) from 56 to 84 d old; iii) from 84 to 112 d old; and iv) from 112 to 175 d old. During the different production stages, ADWG was calculated as the difference between the starting weight and final weight, divided by the number of days spanning the duration of the stage. Data for dead or removed pigs were included in the calculation.

Quantification of *M. hyopneumoniae* in nasal swabs

Real-time PCR was used to quantify the number of genomic deoxyribonucleic acid (DNA) copies for *M. hyopneumoniae* (Dubosson et al., 2004) once DNA was extracted from nasal swabs using a commercial kit (QIAamp DNA Mini Kit; QIAGEN, Valencia, California, USA).

Quantification of PCV2d DNA in blood

Real-time PCR was used to quantify the number of genomic DNA copies for PCV2d (Jeong et al., 2015) once DNA was extracted from serum samples using the same commercial kit as for nasal swabs.

Enzyme-linked immunosorbent assay

Serum samples were tested for both *M. hyopneumoniae* and PCV2 antibodies using 2 commercially available enzyme-linked immunosorbent assay (ELISA) kits (*M. hyo* Ab Test; IDEXX Laboratories and SERELISA PCV2 Ab Mono Blocking; Synbiotics). Serum samples were considered positive for *M. hyopneumoniae* antibody if the sample-to-positive (S/P) ratio was ≥ 0.4 and positive for anti-PCV2 antibodies if the reciprocal ELISA titer was > 350, in accordance with the manufacturer's instructions for each kit.

Enzyme-linked immunospot assay

The numbers of *M. hyopneumoniae* and PCV2d-specific IFN-γ-SCs were evaluated by enzyme-linked immunospot (ELISPOT) assay. PBMCs were stimulated using the challenge *M. hyopneumoniae* and PCV2d strains used in previous studies (Jeong et al., 2015, 2018), with results reported as the numbers of IFN-γ-SCs per million PBMCs.

Pathology

The severity of macroscopic lung lesions was scored by 2 pathologists (Chae and a

graduate student) at Seoul National University (Seoul, Republic of Korea) to estimate the percentage of the lung affected by pneumonia. Scoring was out of 100 total possible points over the entire lung as follows: 10 points each to the right cranial lobe, right middle lobe, left cranial lobe, and left middle lobe; 27.5 points each to the right caudal lobe and left caudal lobe; and 5 points to the accessory lobe (Halbur et al., 1995).

Collected lung and lymphoid tissue sections were examined by 2 blinded veterinary pathologists (Chae and a graduate student). The severity of peribronchiolar and perivascular lymphoid tissue hyperplasia (Thacker et al., 1999) was assessed by scoring mycoplasmal pneumonia lesions (0 to 6), while interstitial pneumonia lesions were scored (0 to 6) based on the severity of interstitial pneumonia, as described in a previous study (Halbur et al., 1995). Mycoplasmal pneumonia lesions were confirmed by RT-PCR from lung lesions, also as described in a previous study (Dubosson et al., 2004).

Statistical analysis

Real-time PCR data were transformed to $\log_{10}$ values prior to statistical analysis evaluation. The Shapiro-Wilk test was then used to test the collected data for a normal distribution. One-way analysis of variance (ANOVA) was also used to examine whether there were statistically significant differences at each timepoint within the 6 groups. A 1-way ANOVA test result with such a statistical significance was further evaluated by conducting a post-hoc test for a pairwise comparison with Tukey's adjustment. If the normality assumption was not met, the Kruskal-Wallis test was conducted. Results from the Kruskal-Wallis test that showed statistical significance were further evaluated with the Mann-Whitney U-test to include

Tukey's adjustment to compare the differences among the groups. Results were reported in P-value, with a value of $P < 0.05$ considered to be significant.

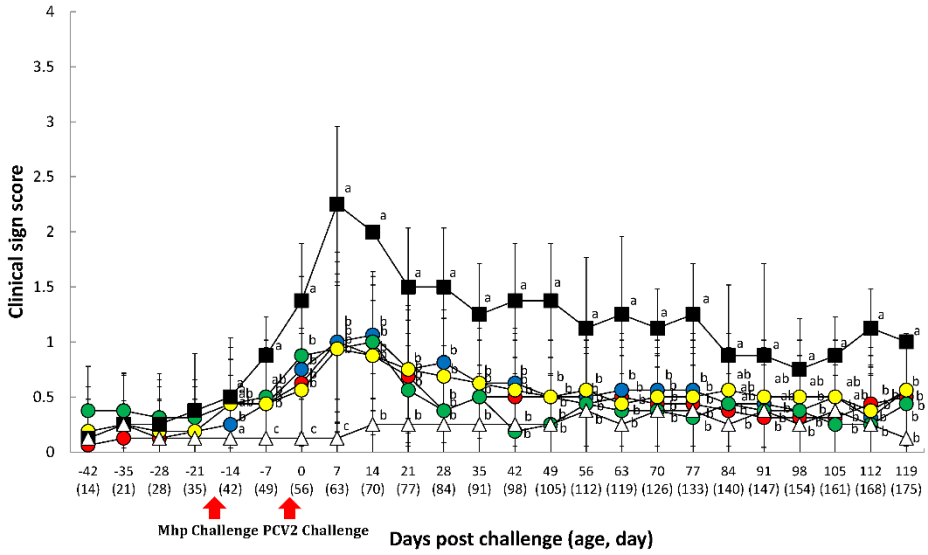

Figure 2. Clinical respiratory sign scores in the different groups: VacA-M+P/Ch (●); VacA-PM/Ch (●); VacB-M+P/Ch (●); VacB-PM/Ch (●); UnVac/Ch (■); and UnVac/UnCh (△). Different letters within a sampling point mean statistically significant differences ($P < 0.05$).

RESULTS

Clinical observations

Pigs in the vaccinated/challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had significantly lower ($P < 0.05$) mean respiratory scores at 0 to 77, 112, and 119 d post-challenge (dpc) than the unvaccinated/challenged group (UnVac/Ch). At 84 dpc, pigs in the VacA-PM/Ch group also had significantly lower ($P < 0.05$) mean respiratory scores than the UnVac/Ch group. At 91 dpc, the mean respiratory scores of pigs in both the VacA-PM/Ch and VacB-M+P/Ch groups were significantly lower ($P < 0.05$) than those of the UnVac/Ch group, while at 98 dpc, pigs in the VacA-PM/Ch, VacB-PM/Ch, and VacA-M+P/Ch groups had significantly lower ($P < 0.05$) mean respiratory scores than the UnVac/Ch group. At 105 dpc, pigs in the VacA-PM/Ch and VacA-M+P/Ch groups had a significantly lower ($P < 0.05$) respiratory score mean than those in the UnVac/Ch group. Pigs in the unvaccinated/unchallenged group (UnVac/UnCh) remained normal throughout the study (Figure 2).

Growth performance

Average body weight (mean ± standard deviation) was calculated at 7 and 21 d old, which resulted in no observed statistical difference among the 6 groups. Average daily weight gain (ADWG) was also measured among the 6 groups. During the 21- to 56-day period, a statistical difference was not found, but this changed during the latter part of the study. The ADWG of pigs in the VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, VacB-M+P/Ch, and UnVac/UnCh groups was significantly higher ($P <$

0.05) than that of the UnVac/Ch group during the 56- to 84-, 84- to 112, and 112- to 175-day periods A significantly higher overall growth rate ($P < 0.05$) was observed in all of these groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, VacB-M+P/Ch, and UnVac/UnCh) when compared to the UnVac/Ch group from 21- to 175-day-old (Table I).

TABLE I. Average daily weight gain (ADWG, mean ± standard deviation) in pigs from 6 groups.

Period between		ADWG (grams/day/pig)[*)]					
days post challenge	age (days)	VacA-M+P/Ch	VacA-PM/Ch	VacB-M+P/Ch	VacB-PM/Ch	UnVac/Ch	UnVac/UnCh
–35 to 0	21 to 56	330.54 ± 23.81	332.85 ± 19.96	326.79 ± 29.54	330.71 ± 28.41	322.86 ± 22.34	326.07 ± 12.82
0 to 28	56 to 84	491.30 ± 38.95[a]	494.42 ± 33.25[a]	474.78 ± 41.13[a]	478.13 ± 29.01[a]	405.36 ± 19.19[b]	500.45 ± 45.71[a]
28 to 56	84 to 112	658.71 ± 37.23[a]	658.26 ± 47.64[a]	690.18 ± 61.47[a]	664.06 ± 48.47[a]	470.09 ± 36.27[b]	698.21 ± 51.37[a]
56 to 119	112 to 177	892.76 ± 43.05[a]	894.35 ± 57.67[a]	881.85 ± 28.77[a]	885.52 ± 74.46[a]	815.87 ± 07.05[b]	909.13 ± 61.61[a]
–35 to 119	21 to 175	649.43 ± 20.02[a]	651.10 ± 25.81[a]	646.84 ± 14.19[a]	645.09 ± 27.87[a]	566.32 ± 09.32[b]	663.96 ± 22.82[a]

[*)]The live weight of each pig was measured at 21 (–35 days post challenge; dpc), 56 (0 dpc), 84 (28 dpc), 112 (56 dpc) and 175 (119 dpc; the time of final necropsy) days of age among 6 groups.

Different superscript letters[a,b] within a sampling point mean statistically significant differences ($P < 0.05$).

Quantification of *M. hyopneumoniae* in nasal swabs

Prior to challenge, pigs in all groups were confirmed negative for *M. hyopneumoniae* (zero genomic copies) by PCR evaluation of nasal swabs. Nasal swabs were evaluated at multiple timepoints post-challenge. Pigs in the vaccinated-challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had a significantly lower ($P < 0.05$) number of genomic copies of *M. hyopneumoniae* in their nasal swabs at 7, 14, 35, and 56 dpc than pigs in the unvaccinated challenged group (UnVac/Ch). At 14 and 35 dpc, pigs in the VacA-PM/Ch and VacA-M+P/Ch groups had a significantly lower ($P < 0.05$) number of genomic copies of *M. hyopneumoniae* in their nasal swabs than those in the VacB-PM/Ch and VacB-M+P/Ch groups (Figure 3A). All pigs in the unvaccinated/ unchallenged group (UnVac/UnCh) remained free from *M. hyopneumoniae* (no genomic copies detected) throughout the nasal swab portion of this study.

Quantification of PCV2d DNA in blood

Prior to challenge, pigs in all 6 groups were screened and found negative for PCV2 DNA. At 7 dpc, pigs in the VacA-PM/Ch and VacA-M+P/Ch groups had a significantly lower ($P < 0.05$) number of genomic copies of PCV2 in their blood than those in the VacB-PM/Ch, VacB-M+P/Ch, and UnVac/Ch groups. At 14, 35, and 56 dpc, pigs in the vaccinated/challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had a significantly lower ($P < 0.05$) number of genomic copies of PCV2 in their blood than pigs in the unvaccinated/challenged group (UnVac/Ch). In the vaccinated/challenged groups, pigs in the VacA-PM/Ch and VacA-M+P/Ch groups had a significantly lower ($P < 0.05$) number of genomic copies of PCV2 in their blood at 14, 35, and 56 dpc than pigs in the VacB-PM/Ch

and VacB-M+P/Ch groups (Figure 3B). No genomic copies of PCV2 were detected in any of the pigs in the unvaccinated/unchallenged group (UnVac/UnCh).

Enzyme-linked immunosorbent assay

Antibody responses against *M. hyopneumoniae* were assessed with ELISA. From −14 to 56 dpc, all vaccinated/challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratio than that of the unvaccinated/challenged group (UnVac/Ch). In the vaccinated/challenged groups, pigs in the VacA-M+P/Ch group had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratio at −14 dpc than pigs in the VacB-PM/Ch and VacB-M+P/Ch groups. From 0 to 14 dpc, pigs in the VacA-M+P/Ch group had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratio than pigs in the VacA-PM/Ch, VacB-PM/Ch, and VacB-M+P/Ch groups. Pigs in the VacA-PM/Ch group had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratio at 35 and 56 dpc than pigs in the VacA-M+P/Ch, VacB-PM/Ch, and VacB-M+P/Ch groups (Figure 4A). No antibodies against *M. hyopneumoniae* were detected in any of the pigs from the unvaccinated/unchallenged group (UnVac/UnCh).

Antibody responses against PCV2 were also assessed with ELISA. From −14 to 56 dpc, pigs in the vaccinated/challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had significantly higher ($P < 0.05$) PCV2 ELISA titers than pigs in the unvaccinated/challenged group (UnVac/Ch) (Figure 4B). No antibodies specific to PCV2 were detected in any of the pigs in the UnVac/UnCh group.

Enzyme-linked immunospot assay

T-cell response was determined as the number of *M. hyopneumoniae* specific interferon-g-secreting cells (IFN-γ-SCs) that was quantified in the PBMCs of individual pigs. From −14 to 56 dpc, pigs in the vaccinated/challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*-specific IFN-γ-SCs in their PBMCs than pigs in the UnVac/Ch group. In the vaccinated/challenged groups, pigs in the VacA-PM/Ch and VacA-M+P/Ch groups had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*-specific IFN-γ-SCs in their PBMCs at −14, 0, and 7 dpc than pigs in the VacB-PM/Ch and VacB-M+P/Ch groups.

At 14 dpc, pigs in the VacA-M+P/Ch group had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*-specific IFN-γ–SCs in their PBMCs than pigs in the VacB-PM/Ch group. Pigs in the VacA-PM/Ch group had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*-specific IFN-γ-SCs in their PBMCs at 35 dpc than pigs in the VacB-M+P/Ch group (Figure 5A). The mean numbers of *M. hyopneumoniae*-specific IFN-γ-SCs in the unvaccinated/unchallenged group (UnVac/UnCh) remained at basal levels (< 20 cells/10^6 PBMC) throughout the study.

T-cell responses were also evaluated by comparing the number of PCV2-specific IFN-γ-SCs among groups. From −14 to 35 dpc, pigs in the vaccinated/challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had a significantly higher ($P < 0.05$) number of PCV2d-specific IFN-γ–SCs in their PBMCs than pigs in the UnVac/Ch group. At 56 dpc, pigs in the VacA-PM/Ch group had a significantly higher ($P < 0.05$) number of PCV2d-specific IFN-γ-SCs in their PBMCs than those in the UnVac/Ch group.

In the vaccinated/challenged groups, pigs in the VacA-PM/Ch and VacA-M+P

groups had a significantly higher ($P < 0.05$) number of PCV2d-specific IFN-γ-SCs in their PBMCs at −14 dpc than pigs in the VacB-M+P/Ch group. At 7, 14, and 35 dpc, pigs in the VacA-PM/Ch and VacA-M+P groups also displayed a significantly higher ($P < 0.05$) number of PCV2d-specific IFN-γ-SCs in their PBMCs than pigs in the VacB-PM/Ch and VacB-M+P/Ch groups (Figure 5B). The mean numbers of PCV2d-specific IFN-γ-SCs in the unvaccinated/unchallenged group (UnVac/UnCh) remained at basal levels (< 20 cells/10^6 PBMC) throughout the study.

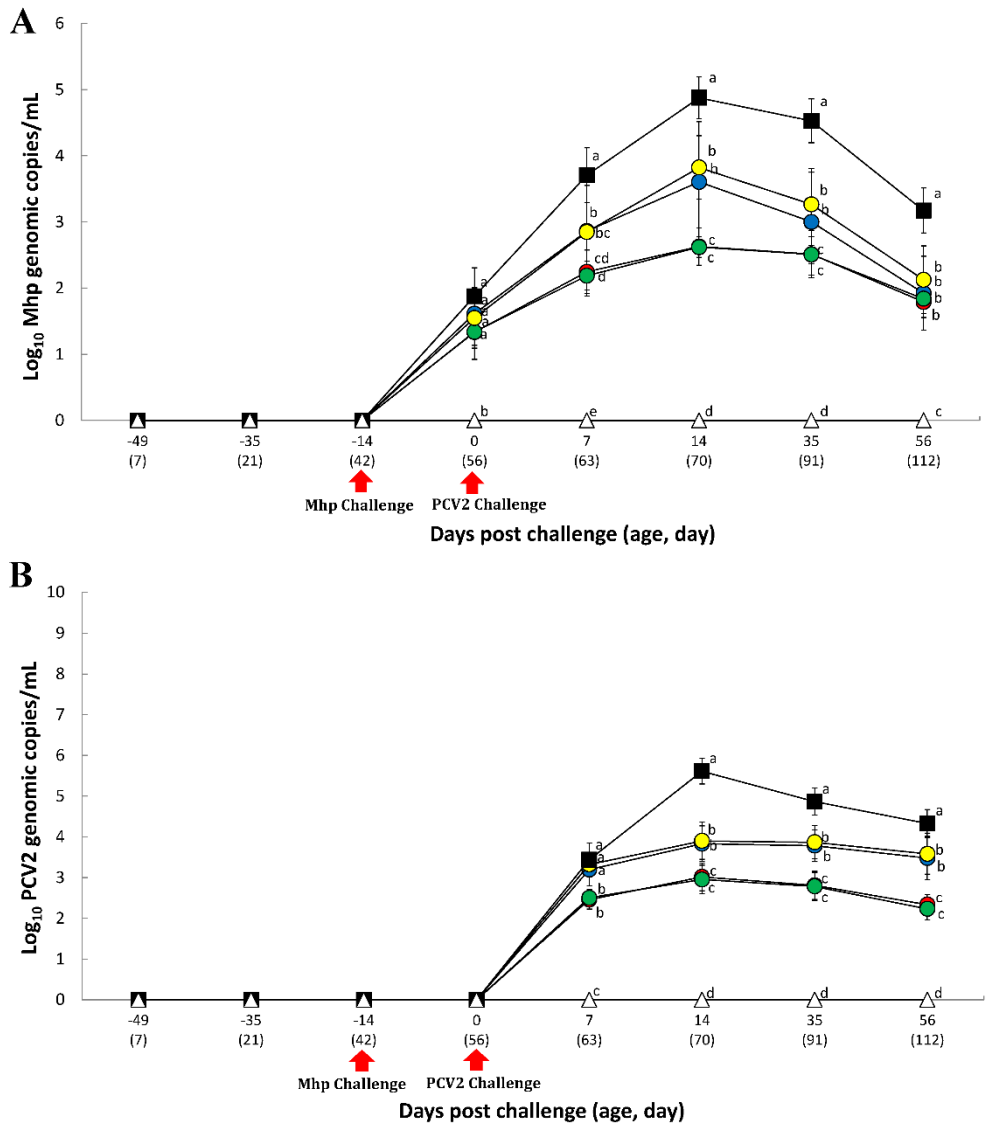

Figure 3. Quantification of *M. hyopneumoniae* (Mhp) DNA and PCV2 DNA. A - Mean values of the genomic copy numbers of *M. hyopneumoniae* DNA in the nasal swabs. B - Mean values of the genomic copy numbers of PCV2 DNA in the serum samples in the different groups: VacA-M+P/Ch (●); VacA-PM/Ch (●); VacB-M+P/Ch (●); VacB-PM/Ch (●); UnVac/Ch (■); and UnVac/UnCh (△). Different letters within a sampling point mean statistically significant differences ($P < 0.05$).

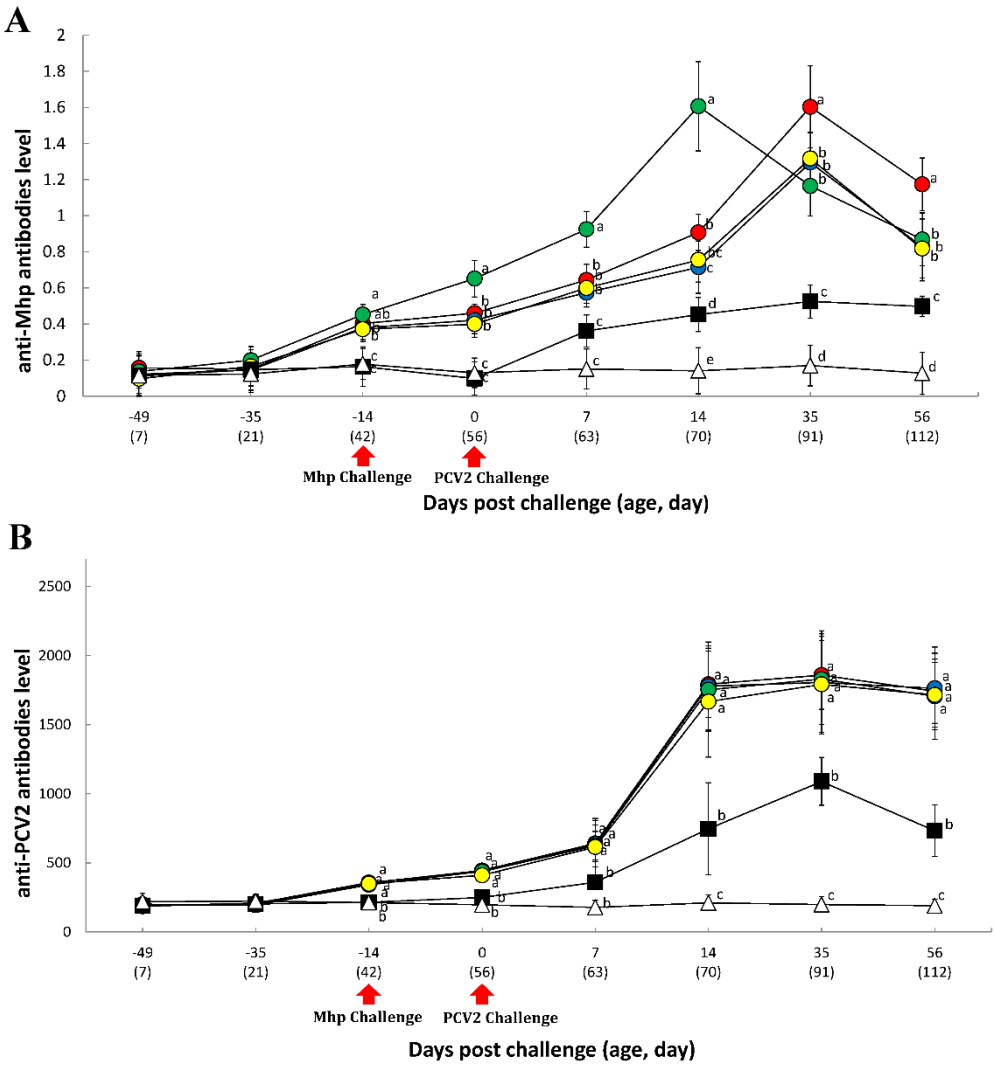

Figure 4. Enzyme-linked immunosorbent assay. A - Mean values of the anti-*M. hyopneumoniae* (Mhp) antibody levels and B - Mean values of the anti-PCV2 antibody levels in the different groups: VacA-M+P/Ch (●); VacA-PM/Ch (●); VacB-M+P/Ch (●); VacB-PM/Ch (●); UnVac/Ch (■); and UnVac/UnCh (△). Different letters within a sampling point mean statistically significant differences ($P < 0.05$).

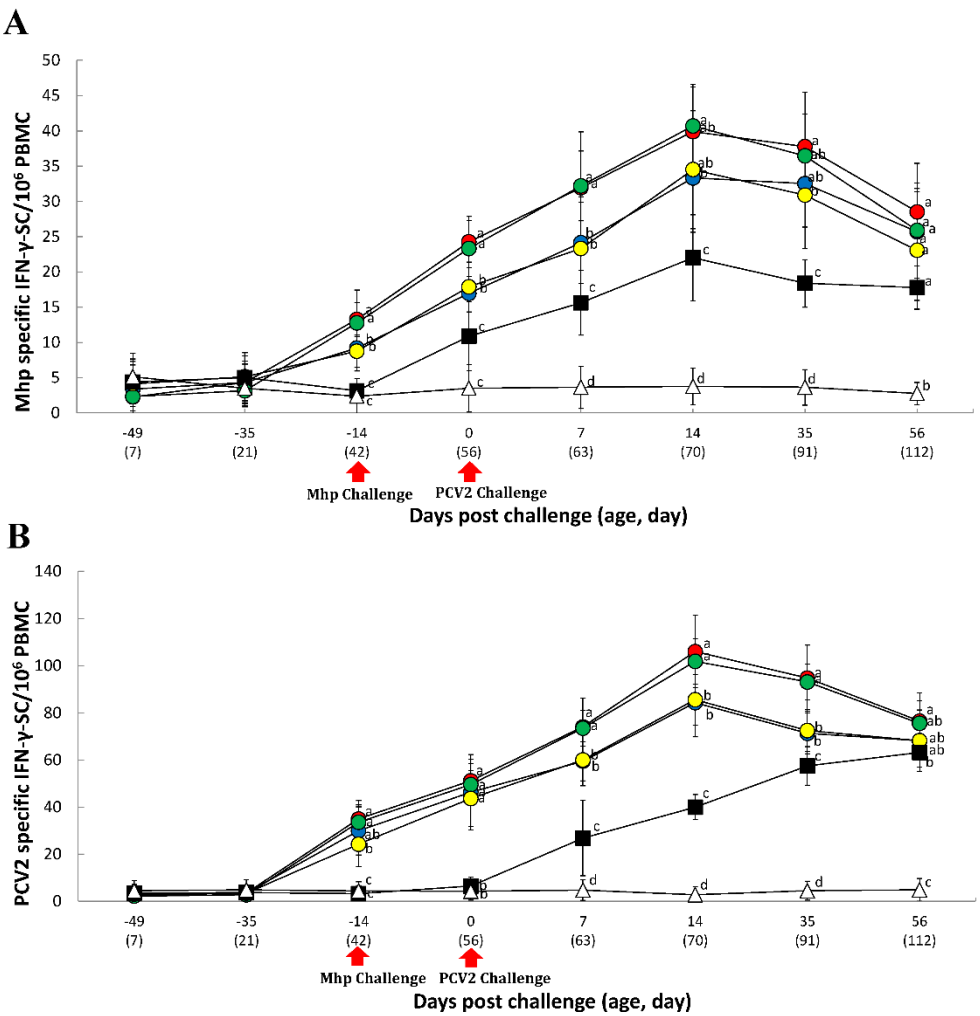

Figure 5. Numbers of interferon-γ-secreting cells (IFN-γ-SC). A - Mean number of *Mycoplasma hyopneumoniae* (Mhp)-specific IFN-γ-SC in peripheral blood mononuclear cells (PBMC). B - Mean number of PCV2-specific IFN-γ-SC in PBMC in the different groups: VacA-M+P/Ch (●); VacA-PM/Ch (●); VacB-M+P/Ch (●); VacB-PM/Ch (●); UnVac/Ch (■); and UnVac/UnCh (△). Different letters within a sampling point mean statistically significant differences ($P < 0.05$).

TABLE II. Pathology (mean ± standard deviation) in pigs from 6 groups at 119 days post challenge (175 days of age).

	Groups					
Pathology	VacA-M+P/Ch	VacA-PM/Ch	VacB-M+P/Ch	VacB-PM/Ch	UnVac/Ch	UnVac/UnCh
Macroscopic lung lesions	25.06 ± 2.02^{b}	25.88 ± 4.16^{b}	27.94 ± 3.94^{b}	26.19 ± 5.50^{b}	57.00 ± 2.67^{a}	4.75 ± 2.92^{c}
Microscopic lung lesions						
mycoplasmal pneumonia	1.44 ± 0.41^{b}	1.40 ± 0.28^{b}	1.43 ± 0.27^{b}	1.41 ± 0.46^{b}	2.98 ± 0.39^{a}	0.15 ± 0.14^{c}
interstitial pneumonia	1.40 ± 0.30^{b}	1.30 ± 0.43^{bc}	0.98 ± 0.44^{c}	1.34 ± 0.31^{bc}	3.18 ± 0.45^{a}	0.23 ± 0.20^{d}
Microscopic lymphoid lesions	0.90 ± 0.24^{b}	0.88 ± 0.22^{b}	0.95 ± 0.45^{b}	0.85 ± 0.42^{b}	2.65 ± 0.52^{a}	0.18 ± 0.17^{c}

Different superscript lettersa,b,c mean statistically significant differences ($P < 0.05$).

Pathology

At 119 dpc, pigs in the vaccinated/challenged groups (VacA-PM/Ch, VacB-PM/Ch, VacA-M+P/Ch, and VacB-M+P/Ch) had significantly lower ($P < 0.05$) macroscopic lung lesions, microscopic lung lesions (mycoplasmal pneumonia and interstitial pneumonia), and lymphoid lesions scores than those of pigs in the unvaccinated/challenged group (UnVac/Ch). There were no macroscopic lung, microscopic lung, or lymphoid lesions in pigs in the unvaccinated/unchallenged group (UnVac/UnCh) (Table II).

DISCUSSION

This comparative trial demonstrates that vaccination against PCV2 and *M. hyopneumoniae* is efficacious in controlling these 2 pathogens, regardless of vaccine type, i.e., monovalent or bivalent. Economic losses related to PCV2 and M. *hyopneumoniae* are mainly associated with pig productivity. PCV2 vaccines are effective in reducing morbidity and mortality, while improving overall growth performance, even in pigs without clinical signs (Fachinger et al., 2008; Horlen et al 2008; Kixmoller et al., 2008; Martelli et al., 2011; Seo et al., 2012). Vaccination against *M. hyopneumoniae* reduces clinical signs and lung lesions while improving performance (Del et al., 2014; Jensen et al., 2002; Maes et al., 1998; 1999; Wilson et al., 2012). Pigs are therefore often vaccinated against PCV2 and *M. hyopneumoniae* to improve growth performance even in the absence of clinical disease.

Evaluation of average daily weight gain (ADWG) is the main indicator of production performance and is therefore a critical parameter for comparing and selecting vaccines. All 4 vaccinated/challenged groups measured significantly higher growth performance than the unvaccinated/challenged group. A numerical difference in growth performance should be noted between the 2 VacA/challenged groups (VacA-PM/Ch and VacA-M+P/Ch) and the 2 VacB/challenged groups (VacB-PM/Ch and VacB-M+P/Ch). From 21 to 175 d old, ADWG was higher overall in the VacA/challenged groups than in the VacB/challenged groups, although the difference was too small to be considered statistically significant. This may be due to either the small number of animals per group or to the overly regulated experimental

management conditions, both of which are different at a laboratory farm and in a commercial farm setting. Although not statistically significant, the observed numerical difference in ADWG may therefore still be clinically meaningful data for pig producers.

Although the exact protective mechanisms of *M. hyopneumoniae* vaccination are not yet fully understood, cell-mediated immunity may alleviate the development of mycoplasmal pneumonia (Thacker et al., 2000). In this study, vaccination-generated interferon-γ–secreting cells (IFN-γ-SCs) within blood, which provides evidence that IFN-γ-SCs may play a role in protective cell-mediated immunity against M. hyopneumoniae (Thacker et al., 2000). Pigs in the VacA/challenged groups (VacA-PM/Ch and VacA-M+P/Ch) generated significantly higher numbers of IFN-γ-SCs in their blood, while exhibiting less nasal shedding and mycoplasma pneumonia than those in the VacB/challenged groups (VacB-PM/Ch and VacB-M+P/Ch). It has been suggested that transmission through nasal secretions is a potential mode of horizontal spreading (Clark et al., 1993; Fano et al., 2005). As horizontal transmission is considered a main risk factor in chronically infected populations (Clark et al., 1993; Fano et al., 2005), minimizing nasal shedding of *M. hyopneumoniae* should be emphasized.

There are several potential reasons that the *M. hyopneumoniae* vaccines elicited different responses throughout the study. Each commercially available vaccine has unique antigen and adjuvant formulations (Maes et al., 2018). The vaccine strain used in the VacA/challenged groups (VacA-PM/Ch and VacA-M+P/Ch) also differed from the vaccine strains used in the VacB/challenged groups (VacB-PM/Ch and VacB-M+P/Ch) (Tao et al., 2019). Adjuvant formulation in particular is known to affect the immunogenicity and protective effect of inactivated whole-cell *M.*

hyopneumoniae bacterins (Galliher-Beckley et al., 2015). The efficacy of an *M. hyopneumoniae* vaccine also differs depending on the challenge strain used to inoculate the animals (Villarreal et al., 2011). Each of these factors may have contributed to the outcome of this study. Further studies are needed to elucidate the difference in protective immune mechanisms and nasal shedding between *M. hyopneumoniae* vaccines.

For the *M. hyopneumoniae* vaccination, pigs in the monovalent groups (VacA-M+P/Ch and VacB-M+P/Ch) were vaccinated 2 calendar weeks before those in the bivalent groups (VacA-PM/Ch and VacB-PM/Ch). There was no significant difference, however, between vaccinating with either a monovalent vaccine at 1 wk of age or with a bivalent vaccine at 3 wk of age. It has been shown that vaccinating pigs at 7 d old with the same *M. hyopneumoniae* vaccine as used in this study was effective in reducing lung lesions, even in the presence of maternally derived antibodies (MDA) at a titer considerably higher than what is typically seen in the field (Thacker et al., 2000). These antibodies therefore do not interfere with the onset of immunity in regard to the *M. hyopneumoniae* fraction of bivalent vaccines or with *M. hyopneumoniae* monovalent vaccines.

Vaccinating pigs for PCV2 has been proven effective for controlling PCVAD in pigs and inducing a strong protective immunity. In particular, as part of cell-mediated immune responses, IFN-γ–SCs production may be a key component in developing protective immunity against PCV2 infection (Fort et al., 2009). Failure to produce sufficient IFN-γ-SCs may result in lower titers of neutralizing antibodies and greater PCV2 viremia (Meerts et al., 2005), which has been correlated to the severity of PCVAD (Fort et al., 2009; Martelli et al., 2011; Seo et al., 2012). Increasing IFN-γ-SCs production therefore results in heightened protective immunity against PCV2

infection.

The present study demonstrates that the inactivated chimeric PCV1-2a vaccine in the VacA/challenged groups (VacA-PM/Ch and VacA-M+P/Ch) induced significantly higher numbers of PCV2d-specific IFN-γ-SCs than the inactivated subunit PCV2a vaccine based on the PCV2a capsid protein in the VacB/challenged groups (VacB-PM/Ch and VacB-M+P/Ch). The generation of higher amounts of IFN-γ-SCs by inactivated chimeric PCV1-2a vaccine results in less PCV2d viremia than that generated by the inactivated subunit PCV2a vaccine. Similarly, previous studies have resulted in inactivated chimeric PCV1-2a vaccine inducing higher numbers of PCV2b-specific IFN-γ-SCs and lower levels of PCV2b viremia (Seo et al., 2014b). The different antigen and adjuvant formulations of the 2 PCV2 vaccines may account for these differences.

Most PCV2a-based monovalent vaccines now available provide cross-protection against PCV2d infection (Opriessnig et al., 2014; Park et al., 2019). The 2 PCV2a-based monovalent and bivalent vaccines used in this study also provide cross-protection against PCV2d. This was a critical parameter as PCV2d is the predominant genotype in Asia, including Korea. According to the Korea Animal Health Products Association (http://www.kahpa.or.kr), almost all piglets farrowed in 2018 were administered some form of a PCV2 vaccine.

Although antigen interference is always a concern within a bivalent vaccine, the present study showed similar generation of protective immunity, such as neutralization antibodies and IFN-γ-SCs, and reduction of PCV2d viremia regardless of whether the vaccine was a bivalent or a PCV2 monovalent. In addition, no significant difference was observed between bivalent and mycoplasmal monovalent in the generation of IFN-γ-SCs and reduction of nasal shedding of *M.*

hyopneumoniae, which suggests that there was no antigen interference.

Porcine circovirus associated disease (PCVAD) and enzootic pneumonia are considered the 2 most prominent and costly diseases in pigs at the present time. Certain factors should be considered when interpreting the results of this study. The results obtained from the experimental conditions described in this study are not indicative of field conditions. The immunological background (i.e., maternally derived antibodies), timing of infection, and other factors, such as management, production system, and facility, including ventilation, can greatly influence the efficacy of a vaccination program. Swine practitioners and producers therefore need to consider variables when applying the results of this study to commercial pig farms.

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PART II. Experimental efficacy of a trivalent vaccine containing porcine circovirus types 2a/b (PCV2a/b) and *Mycoplasma hyopneumoniae* against PCV2d and *M. hyopneumoniae* challenges

ABSTRACT

The purpose of this experimental study was to evaluate the efficacy of a new trivalent vaccine containing porcine circovirus types 2a/b (PCV2a/b) and *Mycoplasma hyopneumoniae*. Pigs were administered the vaccine intramuscularly as either at 3 and 24 days of age with 1.0 mL or at 21 days of age with 2.0 mL according to the manufacturer's recommendations. The pigs were challenged at 42 days of age with either PCV2d (intranasal route) or *M. hyopneumoniae* (intratracheal route), or both. No statistical differences were observed between the one-dose and two-dose experiments based on clinical (growth performance), immunological (protective immunity), microbiological (viremia and laryngeal swab), and pathological (pulmonary and lymphoid lesion) outcomes. Pigs in vaccinated/challenged and unvaccinated/unchallenged groups showed significant difference in growth performance compared to pigs in the unvaccinated/challenged group in both dosage experiments. Vaccinated pigs elicited a significant amount of protective immunity for PCV2d-specific neutralizing antibodies and IFN-γ-SC as well *as M. hyopneumoniae*-specific IFN-γ-SC significantly post-challenge compared to unvaccinated/challenged pigs. Vaccination and challenge reduced the viral load amount of PCV2d in the blood and reduced the *M. hyopneumoniae* load in laryngeal swab, while simultaneously reducing both pulmonary and lymphoid lesion severity when compared to unvaccinated/challenged pigs. Trivalent vaccination provided good protection against a single PCV2d challenge, single *M. hyopneumoniae* challenge, and a PCV2d/*M. hyopneumoniae* dual challenge.

INTRODUCTION

Porcine circovirus type 2 (PCV2), a single-stranded DNA virus in the family *Circoviridae*, contributes to a group of syndromes collectively termed porcine circovirus-associated disease (PCVAD) (Chae, 2004; 2005). PCVAD was first described clinically in the early 1990s as postweaning multisystemic wasting syndrome (PMWS), but knowledge of specific antibody detection dates to the 1960s and the mid-1980s in Europe and North America, respectively (Gillespie et al., 2009). Today, at least eight PCV2 genotypes have been described in swine based on the open reading frame 2 (ORF2) capsid protein sequence and designated consecutively based on the time of first identification with lower case letters, PCV2a-PCV2h (Franzo and Segalés, 2018). Among these, PCV2d has become the predominant strain currently circulating in global pig populations (Xiao et al., 2015; Franzo et al., 2016; Kwon et al, 2017).

Mycoplasma hyopneumoniae is widespread in pig populations and is the primary etiological pathogen of enzootic pneumonia that mainly affects growing and finishing pigs. This disease remains one of the most economically important respiratory diseases and causes a reduction of growth performance and feed conversion (Maes et al., 1996). *M. hyopneumoniae* infection increases susceptibility of the pig to secondary bacterial infections, resulting in further economic losses to producers (Maes et al., 1996).

Due to their long-term world-wide prevalence, PCV2 and *M. hyopneumoniae* remain a constant challenge. Coinfection of pigs with PCV2 and *M. hyopneumoniae* has contributed to cause porcine respiratory disease complex, the most devastating

and economically important disease in the Asian pork industry. Combined vaccines containing PCV2 and *M. hyopneumoniae* are gaining in popularity and becoming part of routine management practices in Asian farms. A new trivalent PCV2a/b and *M. hyopneumoniae* vaccine (Fostera® Gold PCV MH in USA and Asia/CircoMax® Myco in Europe, Zoetis, Parsippany, NJ, USA) has recently been introduced. This is of clinical interest to pig producers as PCV2b is genetically similar to PCV2d; currently the most prevalent field genotype formerly referred to as "mutant PCV2b". Most available vaccines are PCV2a antigen, and while cross-protection against PCV2d has been demonstrated (Opriessnig et al., 2014a, 2014b, 2017; Park et al., 2019), PCVAD outbreaks have been still reported in PCV2a-vaccinated herds (Opriessnig et al., 2013; Ramos et al., 2015; Seo et al., 2014). Another cross-protection study demonstrated that PCV2b-based vaccines are more effective than PCV2a-based vaccines at protecting against the PCV2d genotype (Huan et al., 2018). These results suggest that PCV2b-based vaccines may offer superior cross-protection over PCV2a-based vaccines against currently predominant PCV2d infection. The objective of this study was to evaluate the efficacy of a trivalent PCV2a/b and *M. hyopneumoniae* vaccine against PCV2d and *M. hyopneumoniae* challenges under experimental conditions.

MATERIALS AND METHODS

Animals

Colostrum-fed, conventional piglets were sources from sows that had never been vaccinated for PCV2. Seventy of these clinically healthy piglets were purchased from this farm at 2 days of age. The commercial sow farm was also free of porcine reproductive and respiratory syndrome virus (PRRSV) and *M. hyopneumoniae* as determined by serology, while providing a comprehensive long-term clinical and slaughter of their animals. Upon arrival, piglets were screened and tested seronegative for PCV2 (SERELISA PCV2 Ab Mono Blocking, Synbiotics, Lyon, France) and PRRSV (HerdCheck PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA). They were also screened using real-time polymerase chain reaction (PCR), and tested negative for PCV2 and PRRSV viremia. *M. hyopneumoniae* screening was also conducted on the piglets, which tested as seronegative by ELISA (*M. hyopneumoniae* (*M. hyo* Ab test, IDEXX Laboratories Inc.) and negative by real-time PCR for *M. hyopneumoniae* in pharynx.

Experimental design

Experimental design was based on the global study and conducted in accordance with the registration guidelines of the Republic of Korea's Animal, Plant & Fisheries Quarantine & Inspection Agency (QIA, http://qia.go.kr). The random number generator function (Excel, Microsoft Corporation, Redmond, Washington, USA) was used to assign 35 total pigs into 7 groups (5 piglets per groups, 2 = male and 3 = female) for each of two experiments (one-dose and two-dose study). The one-dose

experiment housed 5 pigs per room in groups as follows: Vac1/ChPM, UnVac1/ChPM, Vac1/ChP, UnVac/CHP, Vac1/ChM, UnVac1/ChM, and UnVac1/UnCh. The two-dose experiment also housed 5 pigs per room in groups as follows: Vac2/ChPM, UnVac2/ChPM, Vac2/ChP, UnVac2/ChP, Vac2/ChM, UnVac2/ChM, and UnVac2/UnCh (Table 1). All rooms and pens were uniform in design and allowed piglets free access to feed and water troughs.

For one-dose experiment, pigs in the Vac1/ChPM, Vac1/ChP, and Vac1/ChM groups were administered a 2.0 mL intramuscular injection of trivalent vaccine (Fostera® Gold PCV MH, Serial No: 413369A, Expiration date: 03-Feb-2022, Zoetis) on the right side of the neck at 21 days of age. The remaining one-dose unvaccinated groups (UnVac1/ChPM, UnVac1/ChP, UnVac1/ChM, and UnVac1/UnCh) received a 2.0 mL injection of phosphate buffered saline (PBS, 0.01M, pH 7.4) in the same anatomical location as the vaccinated groups at 21 days of age.

For two-dose experiment, pigs in the Vac2/ChPM, Vac2/ChP, and Vac2/ChM groups were vaccinated intramuscularly on the right side of the neck at 3 and 24 days of age with a 1.0 mL dose of the same trivalent vaccine. The remaining two-dose unvaccinated groups (UnVac2/ChPM, UnVac2/ChP, UnVac2/ChM), and UnVac2/UnCh group each received a 1.0 mL injection of phosphate buffered saline (PBS, 0.01M, pH 7.4) also in the same anatomical location as the vaccinated groups at 3 and 24 days of age.

At 0 days post challenge (dpc, 42 days old), pigs in the Vac1/ChPM, Vac2/ChPM, UnVac1/ChPM, and UnVac2/ChPM groups were inoculated with PCV2d (SNUVR140004, GenBank no. KJ437 506, 5th passage in PCV-free PK-15 cell lines). Five hours later an *M. hyopneumoniae* (strain SNU98703) challenge was administered. The five-hour interval between the two challenges was implemented

in order to avoid mixing the two pathogens which could have resulted in a decrease in infectivity. During the PCV2d challenge, a 3 mL inoculation containing 1.2 x 10^5 (50% tissue culture infective dose/mL) was administered intranasally. Five hours post-PCV2 inoculation, pigs were intramuscularly anesthetized with a mixture of 2.2 mg/kg xylazine hydrochloride (Rompun, Bayer), 2.2 mg/kg tiletamine hydrochloride, and 2.2 mg/kg zolazepam hydrochloride (Zoletil 50, Virbac). Pigs were then inoculated intratracheally with 7 mL of *M. hyopneumoniae* (strain SNU98703) culture medium containing 10^7 color changing units/mL as previously described (Marchioro et al., 2014; Van Reeth et al., 2000). Pigs in the Vac1/ChP, Vac2/ChP, UnVac1/ChP, and UnVac2/ChP groups were only administered the same PCV2d intranasal challenge, while pigs in the Vac1/ChM, Vac2/ChM, UnVac1/ChM, and UnVac2/ChM groups were inoculated intratracheally with the same *M. hyopneumoniae* challenge as described above for the dually-challenged groups.

At 21 dpc (63 days old), all pigs were sedated by an intravenous injection of sodium pentobarbital prior to euthanization by electrocution as previously described (Beaver et al., 2001). Tissues were collected from each pig at necropsy, then fixed in a 10% neutral buffered formalin solution, before they were embedded in paraffin. All study methods were approved previously by the Seoul National University Institutional Animal Care and Use, and Ethics Committee (SNU-200323-5).

Table 1

Experiment design with vaccination and challenge of porcine circovirus type 2d (PCV2d) and *Mycoplasma hyopneumoniae* (Mhp).

Group	Vaccination (days	Challenge (days of age)	
		PCV2	Mhp
One-dose			
Vac1/ChPM	21 (2.0 mL)	42	42
Vac1/ChP	21 (2.0 mL)	42	–
Vac1/ChM	21 (2.0 mL)	–	42
UnVac1/ChPM	-	42	42
UnVac1/ChP	-	42	–
UnVac1/ChM	-	–	42
UnVac1/UnCh	-	–	–
Two-dose			
Vac2/ChPM	3, 24 (1.0 mL)	42	42
Vac2/ChP	3, 24 (1.0 mL)	42	–
Vac2/ChM	3, 24 (1.0 mL)	–	42
UnVac2/ChPM	-	42	42
UnVac2/ChP	-	42	–
UnVac2/ChM	-	–	42
UnVac2/UnCh	-	–	–

Sampling collection

Blood and laryngeal swabs were collected from all pigs at –39, –18, 0, 7, 14, and 21 dpc. Blood were also collected from pigs in two-dose experiment group at –21 dpc (time of vaccination). Laryngeal swabs were conducted as previous described (Pieters et al., 2017).

Clinical observation

Pigs were monitored daily and scored weekly for clinical signs. Briefly, scoring was defined as follows: 0 (normal), 1 (rough haircoat), 2 (rough haircoat and dyspnea), 4 (severe dyspnea and abdominal breathing), and 6 (death). Scoring observers were blinded to vaccination status.

Average daily weight gain

The live weight of each pig was measured at various time points throughout the study as follows: –39 dpc (3 days of age), –18 dpc (24 days of age), 0 dpc (42 days of age), and 21 dpc (63 days of age). Average daily weight gain (ADWG = grams/pig/day) was calculated over two time points upon conclusion of the study: (i) between 3 and 24 days of age, (ii) between 24 and 42 days of age, (iii) between 42 and 63 days of age, and (iv) between 3 and 63 days of age, each of which represented a stage of production. ADWG during the two production stages was calculated as the difference between the starting and final weight divided by the number of days spanning the duration of the stage.

Quantification of PCV2 and *M. Hyopneumoniae* DNA

A commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA) was use to extract DNA from serum samples for PCV2 and laryngeal swabs for *M. hyopneumoniae*. The number of genomic DNA copies for both PCV2d (Jeong et al., 2015) and *M. hyopneumoniae* (Dubosson et al., 2004) was then quantified by real-time PCR.

Serology

The presence of PCV2 and *M. hyopneumoniae* antibodies were evaluated in serum samples by use of commercially available enzyme-linked immunosorbent assay (ELISA) kits (SERELISA PCV2 Ab Mono Blocking, Synbiotics, Lyon, France, and *M. hyo* Ab test, IDEXX Laboratories Inc. Westbrook, Maine, USA). Testing was conducted in accordance with each manufacturer's kit instructions, where samples were considered as positive for anti-PCV2 antibodies if the reciprocal ELISA titer was > 350 and as positive for *M. hyopneumoniae* antibody if the sample-to-positive (S/P) ratio was ≥ 0.4 Serum samples were also tested for serum virus neutralization against PCV2d (Pogranichnyy et al., 2000).

Enzyme-linked immunospot

An enzyme-linked immunospot (ELISpot) assay was conducted to measure the numbers of PCV2d- and *M. hyopneumoniae*-specific IFN-γ-SCs. PBMCs were stimulated using the aforementioned challenge strains for PCV2d and *M. hyopneumoniae* (Jeong et al., 2015, 2018) with results reported as the number of IFN-γ-SCs per million PBMC.

Pathology

Two pathologists at the Seoul National University scored the severity of macroscopic lung lesions in order to estimate the percentage of the lung affected by pneumonia (Halbur et al., 1995). Two blinded veterinary pathologists then examined the collected lung and lymphoid tissue sections and scored the severity of peribronchiolar and perivascular lymphoid tissue hyperplasia by mycoplasmal pneumonia lesions (0 to 6) (Opriessnig et al., 2004). Real-time PCR confirmed mycoplasmal pneumonia lung lesions as previously described (Dubosson et al., 2004). Lymphoid lesion severity was scored (0 to 5) based on lymphoid depletion and granulomatous inflammation (Kim and Chae, 2004).

Immunohistochemistry

Immunohistochemistry for PCV2 was performed as previously described (Park et al., 2013). Three sections were cut from each of three blocks of tissue from a lymph node of each pig and prepared on slides for the morphometric analyses of immunohistochemistry. Quantitative data was analyzed from the prepared immunohistochemistry slides using the NIH Image J 1.45s Program (http://imagej.nih.gov/ij/download.html). PCV2 analysis was conducted by the random selection of 10 fields, where number of the positive cells per unit area (0.25 mm^2) was determined as previously described (Kim et al., 2003). The mean values were also calculated.

Statistical analysis

Statistical analyses were performed IBM SPSS Statistics for Windows version 23.0 (IBM Corp., Armonk, NY, USA). All real-time PCR data and neutralizing antibody titers were transformed to $\log_{10}$ and $\log_2$, respectively, values prior to statistical analysis. The Shapiro-Wilk test evaluated data for normal distribution. One-way analysis of variance (ANOVA) was used to examine differences in variables with normal distribution (e.g., ADWG, growth performance, PCV2 DNA, *M. hyopneumoniae* DNA, PCV2 antibody titer, *M. hyopneumoniae* antibody titer, number of IFN-γ-SC, macroscopic lung lesion scores, and PCV2 antigen in lymph node) and Kruskal-Wallis test was used for variables without a normal distribution (e.g., clinical signs, neutralizing antibody titers against PCV2d, and microscopic lung and lymphoid lesion scores). If a one-way ANOVA test resulted in a statistical significance, data was further evaluated by conducting a post-hoc test for a pairwise comparison with Bonferroni's adjustment. Kruskal Wallis test results which showed a statistical significance were further evaluated with a Dunn's nonparametric comparison for post hoc test (Dunn, 1964). Results were reported in P-values where a value of $P < 0.05$ was considered to be significant.

RESULTS

Decreased respiratory clinical outcomes

Pigs were not observed for clinical signs at the time of PCV2d and *M. hyopneumoniae* challenge. Post PCV2d and *M. hyopneumoniae* challenge, pigs in the UnVac1/ChPM and UnVac2/ChPM groups displayed mild to moderate respiratory disease characterized by sneezing, coughing and clear nasal discharged with a marked decreased in appetite. Pigs in the UnVac1/ChM and UnVac2/ChM groups displayed mild respiratory disease characterized by sneezing and coughing, while pigs in the UnVac1/ChP and UnVac2/ChP groups were mildly lethargic.

Pigs in the one-dose groups were compared for clinical scores with the following results: the UnVac1/UnCh group had significantly lower ($P < 0.05$) clinical scores between 7 and 21 dpc compared with pigs in the UnVac1/ChPM and UnVac1/ChM groups. Pigs in the UnVac1/UnCh group also had significantly lower ($P < 0.05$) clinical scores between 14 and 21 dpc compared with pigs in the UnVac1/ChP group. Pigs in the Vac1/ChPM, Vac1/ChP, and Vac1/ChM groups had significantly lower ($P < 0.05$) clinical scores at 21 dpc compared with pigs in the UnVac1/ChPM group (Supplementary Fig. 1A).

Pigs in the two-dose groups were compared for clinical scores with the following results: the UnVac2/UnCh group had significantly lower ($P < 0.05$) clinical scores between 7 and 21 dpc compared with pigs in the UnVac2/ChPM and UnVac2/ChM groups. Pigs in the UnVac2/UnCh group also had significantly lower ($P < 0.05$) clinical scores at 14 and 21 dpc compared with pigs in the UnVac2/ChP group. Pigs in the Vac2/ChPM, Vac2/ChP, and Vac2/ChM groups had significantly lower ($P <$

0.05) clinical scores at 14 dpc compared with pigs in the UnVac2/ChPM and UnVac2/ChM groups. Pigs in the Vac2/ChP and Vac2/ChM groups had significantly lower ($P < 0.05$) clinical scores at 14 dpc compared with pigs in the UnVac2/ChPM and UnVac2/ChM groups (Supplementary Fig. 1B).

Improved growth performance

Within the one-dose experiment, pigs in the Vac1/ChPM, Vac1/ChP, Vac1/ChM, UnVac1/ChP, UnVac1/ChM, and UnVac1/UnCh groups had significantly higher ($P < 0.05$) ADWG between 42 and 63 days old, and between 3 and 63 days old than the pigs in the UnVac1/ChPM group. Within the two-dose experiment, pigs in the Vac2/ChP, Vac2/ChM, UnVac2/ChP, UnVac2/ChM, and UnVac2/UnCh groups had significantly higher ($P < 0.05$) ADWG between 42 and 63 days old, and between 3 and 53 days old than the pigs in the UnVac2/ChPM group (Table 2).

Table 2. Mean (± standard deviation) of average daily weight gain in different groups.

Group	Average daily weight gain (days, unit = gram/pig/day)			
	3-24	24-42	42-63	3-63
One-dose				
Vac1/ChPM	140.00 ± 28.29	272.22 ± 21.87	330.48 ± 14.13 [a]	246.33 ± 3.80 [a]
Vac1/ChP	144.76 ± 9.87	263.33 ± 29.55	344.76 ± 24.42 [a]	250.33 ± 8.45 [a]
Vac1/ChM	142.86 ± 17.17	261.11 ± 32.16	347.62 ± 13.04 [a]	250.00 ± 4.56 [a]
UnVac1/ChPM	139.05 ± 15.58	263.33 ± 20.64	300.00 ± 6.73 [b]	232.67 ± 4.01 [b]
UnVac1/ChP	141.90 ± 20.03	266.67 ± 17.12	337.14 ± 13.21 [a]	247.67 ± 6.08 [a]
UnVac1/ChM	137.14 ± 15.58	268.89 ± 7.45	333.33 ± 16.84 [a]	245.33 ± 1.83 [a]
UnVac1/UnCh	146.67 ± 16.97	273.33 ± 14.91	340.95 ± 18.63 [a]	252.67 ± 5.35 [a]
Two-dose				
Vac2/ChPM	145.71 ± 13.72	271.11 ± 15.91	323.81 ± 13.47 [ab]	245.67 ± 3.03 [a]
Vac2/ChP	144.76 ± 14.91	261.11 ± 39.09	345.71 ± 13.30 [a]	250.00 ± 6.67 [a]
Vac2/ChM	140.95 ± 10.43	266.67 ± 23.90	340.95 ± 12.42 [a]	248.67 ± 4.31 [a]
UnVac2/ChPM	140.00 ± 18.01	277.11 ± 32.01	297.14 ± 7.22 [b]	234.33 ± 7.69 [b]
UnVac2/ChP	141.90 ± 17.63	268.89 ± 44.72	335.24 ± 20.92 [a]	247.67 ± 3.46 [a]
UnVac2/ChM	140.95 ± 8.65	263.33 ± 43.14	336.19 ± 18.32 [a]	246.00 ± 5.73 [a]
UnVac2/UnCh	141.90 ± 17.30	273.33 ± 40.33	340.95 ± 16.36 [a]	251.00 ± 6.41 [a]

Reduced genomic copies of PCV2d in blood

Genomic copies of PCV2d were not detected in any pigs at the time of PCV2d challenge. No genomic copies of PCV2d were detected in negative control groups (UnVac1/UnCh and UnVac2/UnCh) throughout the duration of the experiment. The one-dose experiment resulted in a significantly lower ($P < 0.05$) amount of PCV2d genomic copies in the blood of vaccinated-challenged groups (Vac1/ChPM and Vac1/ChP) at 7, 14, and 21 dpc when compared to unvaccinated-challenged groups (UnVac1/ChPM and UnVac1/ChP) (Fig. 1A). The two-dose experiment resulted in a significantly lower ($P < 0.05$) amount of PCV2d genomic copies in the blood of vaccinated-challenged groups (Vac2/ChPM and Vac2/ChP) when compared to unvaccinated-challenged groups (UnVac2/ChPM and UnVac2/ChP) at 7, 14, and 21 dpc (Fig. 1C).

Reduced genomic copies of *M. Hyopneumoniae* in laryngeal swabs

Genomic copies of *M. hyopneumoniae* were not detected in any pigs at the time of *M. hyopneumoniae* challenge. No genomic copies of *M. hyopneumoniae* were detected in the negative control groups (UnVac1/UnCh and UnVac2/UnCh) throughout the duration of the experiment. The one-dose experiment had significantly lower ($P < 0.05$) amounts of *M. hyopneumoniae* genomic copies in laryngeal swabs of vaccinated-challenged groups (Vac1/ChPM and Vac1/ChM) compared to unvaccinated-challenged groups (UnVac1/ChPM and UnVac1/ChM) at 7, 14 and 21 dpc (Fig. 1B). The two-dose experiment resulted in significantly lower ($P < 0.05$) amounts of *M. hyopneumoniae* genomic copies in laryngeal swabs of vaccinated-challenged groups (Vac2/ChPM and Vac2/ChM) when compared to unvaccinated-challenged groups (UnVac2/ChPM and UnVac2/ChM) at 7, 14 and 21 dpc (Fig. 1D).

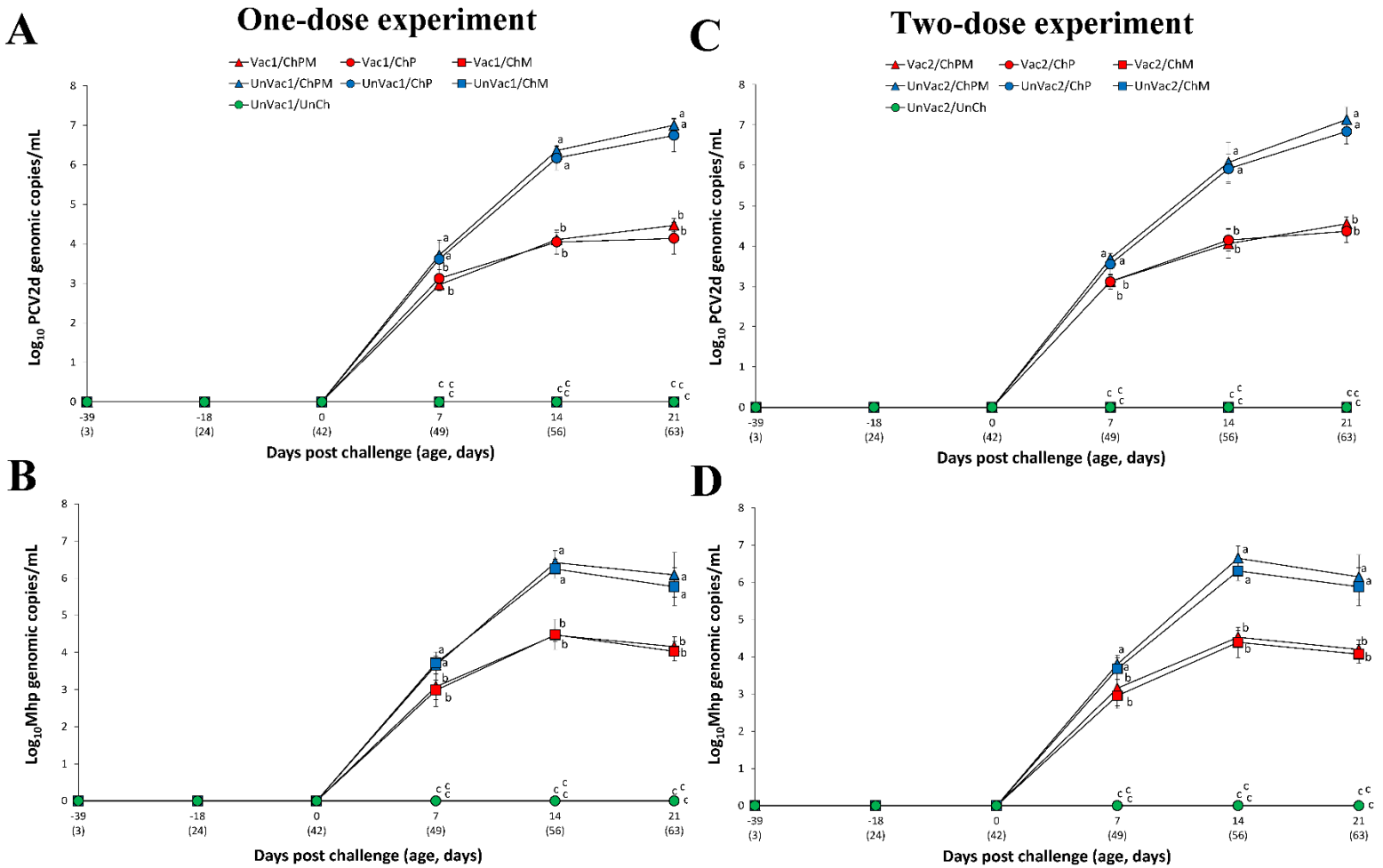

A
One-dose experiment
Vac1/ChPM
Vac1/ChP
Vac1/ChM
UnVac1/ChPM
UnVac1/ChP
UnVac1/ChM
UnVac1/UnCh
Log10 PCV2d genomic copies/mL
Days post challenge (age, days)
B
Log10Mhp genomic copies/mL
Days post challenge (age, days)
C
Two-dose experiment
Vac2/ChPM
Vac2/ChP
Vac2/ChM
UnVac2/ChPM
UnVac2/ChP
UnVac2/ChM
UnVac2/UnCh
Log10 PCV2d genomic copies/mL
Days post challenge (age, days)
D
Log10Mhp genomic copies/mL
Days post challenge (age, days)
-39 (3)
-18 (24)
0 (42)
7 (49)
14 (56)
21 (63)

Figure 1. Mean values of the genomic copy numbers of PCV2d DNA in serum and *M. hyopneumoniae* in larynx from one-dose experiment (A and B) in Vac1/ChPM (▲), Vac1/ChP (●), Vac1/ChM (■), UnVac1/ChPM (▲), UnVac1/ChP (●), UnVac1/ChM (■), and UnVac1/UnCh (●) groups and from two-dose experiment (C and D) in Vac2/ChPM (▲), Vac2/ChP (●), Vac2/ChM (■), UnVac2/ChPM (▲), UnVac2/ChP (●), UnVac2/ChM (■), and UnVac2/UnCh (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b and c) indicate significant ($P < 0.05$) different among 7 groups in each of experiments.

Induced immunological responses against PCV2d by a trivalent vaccine

PCV2 ELISA titers, PCV2d-specific NA titers, and PCV2d-specific IFN-γ-SC were not detected among the groups at the time of vaccination (one-dose = 24 days of age and two-dose = 3 days of age). PCV2 ELISA titers, PCV2d-specific NA titers, and PCV2d-specific IFN-γ-SC were not detected in negative control groups (UnVac1/UnCh and UnVac2/UnCh) throughout the experiment.

Within the one-dose experiment, PCV2 ELISA (Fig. 2A) and PCV2d-specific NA titers (Fig. 2B) in the vaccinated-challenged groups (Vac1/ChPM and Vac1/ChP) were significantly higher ($P < 0.05$) than those of their unvaccinated-challenged (UnVac1/ChPM and UnVac1/ChP) counterparts at 0 dpc. These statistical differences were evident until the end of the study. The levels of PCV2d-specific IFN-γ-SC in vaccinated-challenged (Vac1/ChPM and Vac1/ChP) groups were significantly higher ($P < 0.05$) than in those of their unvaccinated-challenged (UnVac1/ChPM and UnVac1/ChP) counterparts at 0, 14, and 21 dpc (Fig. 2C).

Within the two-dose experiment, PCV2 ELISA (Fig. 2D) and PCV2d-specific NA titers (Fig. 2F) in the vaccinated-challenged groups (Vac2/ChPM and Vac2/ChP) were significantly higher ($P < 0.05$) than in those of their unvaccinated-challenged (UnVac2/ChPM and UnVac2/ChP) counterparts at −18, 0, 14, and 21 dpc. The levels of PCV2d-specific IFN-γ-SC in vaccinated-challenged groups (Vac2/ChPM and Vac2/ChP) were significantly higher ($P < 0.05$) than in those of their unvaccinated-challenged (UnVac2/ChPM and UnVac2/ChP) counterparts at −18, 0, 14, and 21 dpc (Fig. 2G).

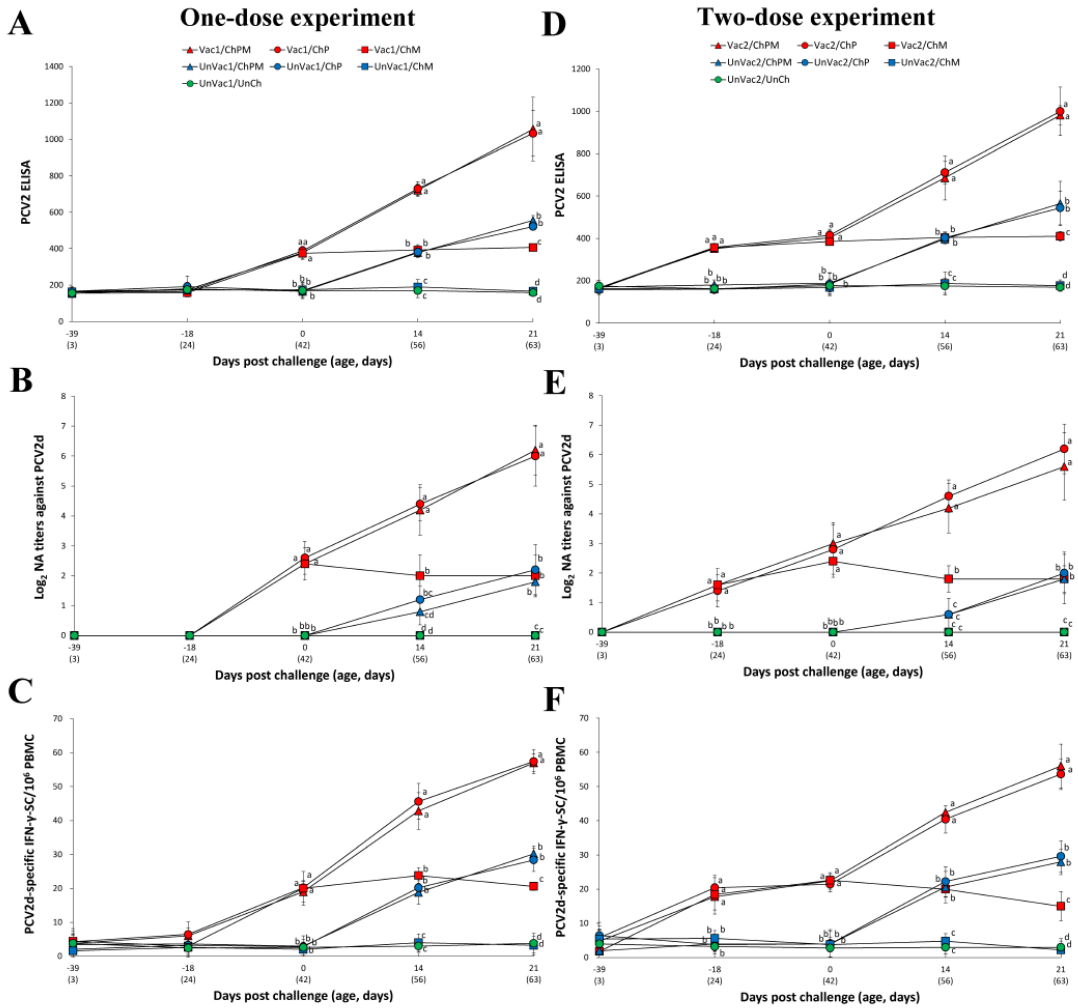


Figure 2. Immune responses of PCV2-specific enzyme-linked immunosorbent aassay (ELISA) antibody levels in serum, neutralizing antibody (NA) titers against PCV2d in serum, and frequency of PCV2d-specific interferon-γ secreting cells (IFN-γ-SC)/10^6 in peripheral blood mononuclear cells (PBMC) from one-dose experiment (A, B, and C) in Vac1/ChPM (▲), Vac1/ChP (●), Vac1/ChM (■), UnVac1/ChPM (▲), UnVac1/ChP (●), UnVac1/ChM (■), and UnVac1/UnCh (●) groups and from two-dose experiment (D, E, and F) in Vac2/ChPM (▲), Vac2/ChP (●), Vac2/ChM (■), UnVac2/ChPM (▲), UnVac2/ChP (●), UnVac2/ChM (■), and UnVac2/UnCh (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b and c) indicate significant ($P < 0.05$) different among 7 groups in each of experiments.

Induced immunological responses against *M. Hyopneumoniae* by a trivalent vaccine

M. hyopneumoniae ELISA S/P ratio and *M. hyopneumoniae*-specific IFN-γ-SC were not detected among the groups at the time of vaccination (one-dose = 24 days of age and two-dose = 3 days of age). *M. hyopneumoniae* ELISA S/P ratios and *M. hyopneumoniae*-specific IFN-γ-SC levels were not detected in negative control groups (UnVac1/UnCh and UnVac2/UnCh) throughout the experiment.

The one-dose experiment resulted in significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratios (Fig. 3A) and *M. hyopneumoniae*-specific IFN-γ-SC levels (Fig. 3B) in vaccinated-challenged groups (Vac1/ChPM and Vac1/ChM) than in those of their unvaccinated-challenged (UnVac1/ChPM and UnVac1/ChM) counterparts at 0, 14, and 21 dpc.

The two-dose experiment resulted in significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratios (Fig. 3C) and *M. hyopneumoniae*-specific IFN-γ-SC levels (Fig. 3D) in vaccinated-challenged (Vac2/ChPM and Vac2/ChM) groups than in those of their unvaccinated-challenged (UnVac2/ChPM and UnVac2/ChM) counterparts at 0, 14, and 21 dpc.

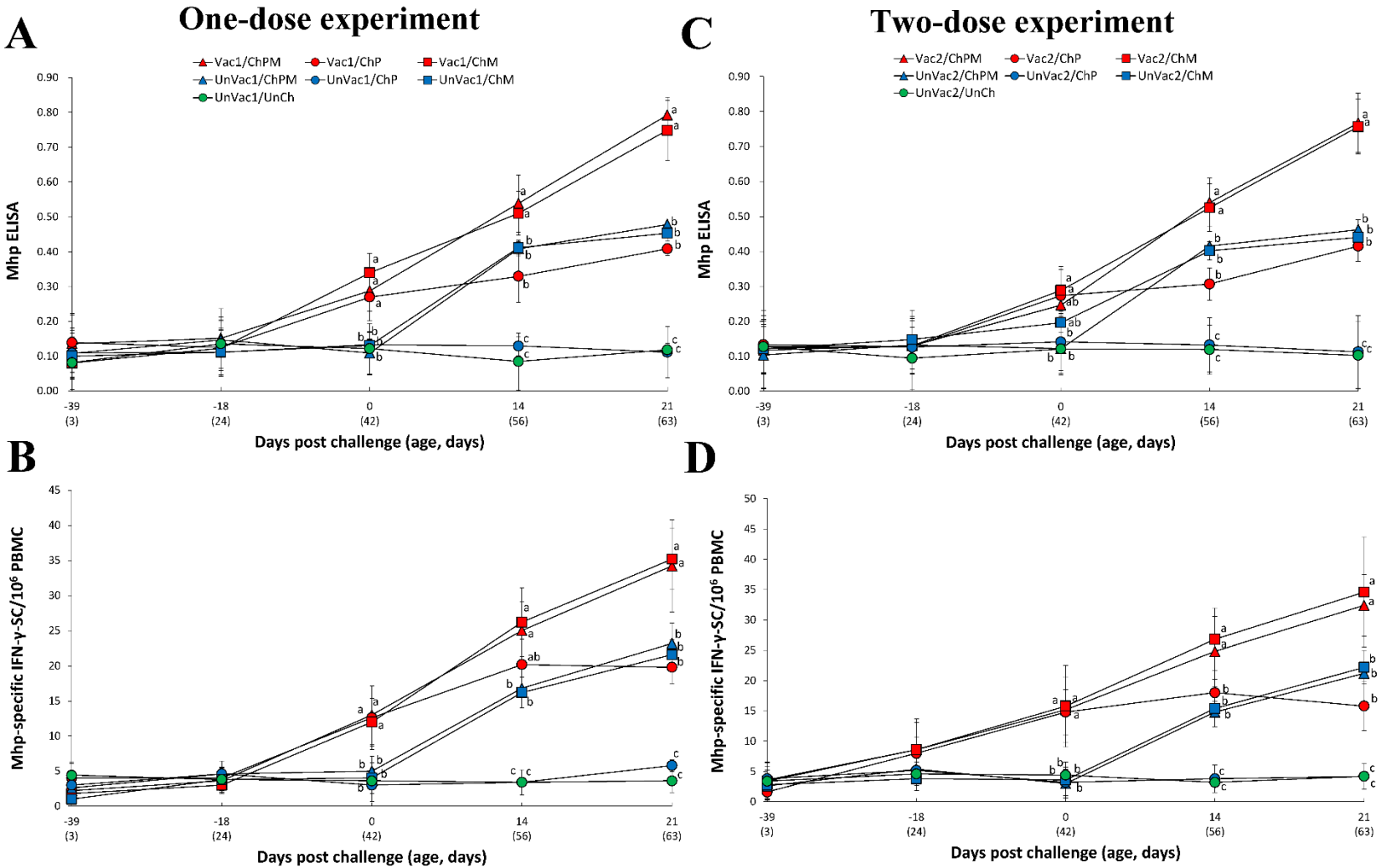

A
One-dose experiment
Vac1/ChPM
Vac1/ChP
Vac1/ChM
UnVac1/ChPM
UnVac1/ChP
UnVac1/ChM
UnVac1/UnCh
Mhp ELISA
Days post challenge (age, days)
B
Mhp-specific IFN-γ-SC/10⁶ PBMC
Days post challenge (age, days)
C
Two-dose experiment
Vac2/ChPM
Vac2/ChP
Vac2/ChM
UnVac2/ChPM
UnVac2/ChP
UnVac2/ChM
UnVac2/UnCh
Mhp ELISA
Days post challenge (age, days)
D
Mhp-specific IFN-γ-SC/10⁶ PBMC
Days post challenge (age, days)

Fig. 3. Immune responses of *M. hyopneumoniae*-specific enzyme-linked immunosorbent assay (ELISA) antibody levels in serum and frequency of *M. hyopneumoniae*-specific interferon-γ-secreting cells (IFN-γ-SC)/10^6 in peripheral blood mononuclear cells (PBMC) from one-dose experiment (A and B) in Vac1/ChPM (▲), Vac1/ChP (●), Vac1/ChM (■), UnVac1/ChPM (▲), UnVac1/ChP (●), UnVac1/ChM (■), and UnVac1/UnCh (●) groups and from two-dose experiment (D, E, and F) in Vac2/ChPM (▲), Vac2/ChP (●), Vac2/ChM (■), UnVac2/ChPM (▲), UnVac2/ChP (●), UnVac2/ChM (■), and UnVac2/UnCh (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) differences among 7 groups in each of experiments.

Decreased the severity of lung and lymphoid lesions

Within the one-dose experiment, vaccinated-challenged pigs in the Vac1/ChPM and Vac1/ChP groups had significantly lower ($P < 0.05$) lymphoid lesion severity and lower numbers of lymphoid PCV2-positive antigen compared to unvaccinated groups (UnVac1/ChPM and UnVac1/ChP). Vaccinated-challenged pigs in the Vac1/ChPM and Vac1/ChM groups had significantly lower ($P < 0.05$) macroscopic and microscopic pulmonary lesions compared to unvaccinated groups (UnVac1/ChPM and UnVac1/ChM) at 21 dpc (Table 3).

Within the two-dose experiment, vaccinated-challenged pigs in the Vac2/ChPM and Vac2/ChP groups had significantly lower ($P < 0.05$) amounts of lymphoid lesions and lower numbers of lymphoid PCV2-positive antigen compared to unvaccinated groups (UnVac2/ChPM and UnVac2/ChP) at 21 dpc. Vaccinated-challenged pigs in the Vac2/ChPM and Vac2/ChM groups had significantly less ($P < 0.05$) macroscopic and microscopic pulmonary lesions compared to unvaccinated groups (UnVac2/ChPM and UnVac2/ChM) at 21 dpc (Table 3).

A minimal amount of macroscopic pulmonary lesions were observed in the negative control groups (UnVac1/UnCh and UnVac2/UnCh) throughout the experiment. Microscopic pulmonary, and lymphoid lesions were not observed in any negative control groups (UnVac1/UnCh and UnVac2/UnCh) throughout the experiment.

Table 3 Tathological outcomes in different groups at 21 days post challenge*.

	Group	Gross lung lesions (%)	Histopathology		PCV2 antigen in lymph node (+cell / 0.25 mm^2)
			Mycoplasmal lung lesions (score 0-6)	Lymph node (score 0-5)	
One-dose	Vac1/ChPM	17.2 ± 2.20 [ab]	1.00 ± 0.30 [a]	0.80 ± 0.60 [a]	9.40 ± 1.14 [a]
	Vac1/ChP	9.6 ± 3.01 [c]	0 ± 0 [b]	0.60 ± 0.80 [a]	11.20 ± 1.69 [a]
	Vac1/ChM	11.9 ± 1.64 [bc]	0.80 ± 0.70 [a]	0 ± 0 [b]	0 ± 0 [b]
	UnVac1/ChPM	27.6 ± 2.77 [a]	3.40 ± 1.00 [c]	2.20 ± 0.90 [c]	32.93 ± 5.88 [c]
	UnVac1/ChP	13.6 ± 5.74 [bc]	0 ± 0 [b]	2.00 ± 0.40 [c]	30.07 ± 3.11 [c]
	UnVac1/ChM	25.4 ± 5.80 [a]	2.80 ± 0.90 [c]	0 ± 0 [b]	0 ± 0 [b]
	UnVac1/UnCh	0.2 ± 0.45 [d]	0 ± 0 [b]	0 ± 0 [b]	0 ± 0 [b]
Two-dose	Vac2/ChPM	14.3 ± 1.68 [a]	1.00 ± 0.80 [a]	0.80 ± 0.70 [a]	11.40 ± 2.34 [a]
	Vac2/ChP	8.5 ± 4.14 [a]	0 ± 0 [b]	1.00 ± 0.40 [a]	10.93 ± 3.12 [a]
	Vac2/ChM	11.3 ± 1.92 [a]	0.80 ± 0.70 [a]	0 ± 0 [b]	0 ± 0 [b]
	UnVac2/ChPM	25.6 ± 1.47 [b]	3.20 ± 1.30 [c]	2.20 ± 0.80 [c]	31.93 ± 4.96 [c]
	UnVac2/ChP	13.4 ± 4.20 [a]	0 ± 0 [b]	2.20 ± 0.20 [c]	30.87 ± 2.99 [c]
	UnVac2/ChM	25.2 ± 5.26 [b]	3.00 ± 0.60 [c]	0 ± 0 [b]	0 ± 0 [b]
	UnVac2/UnCh	0.4 ± 0.89 [c]	0 ± 0 [b]	0 ± 0 [b]	0 ± 0 [b]

[a,b,c] Significant ($P < 0.05$) difference between groups.

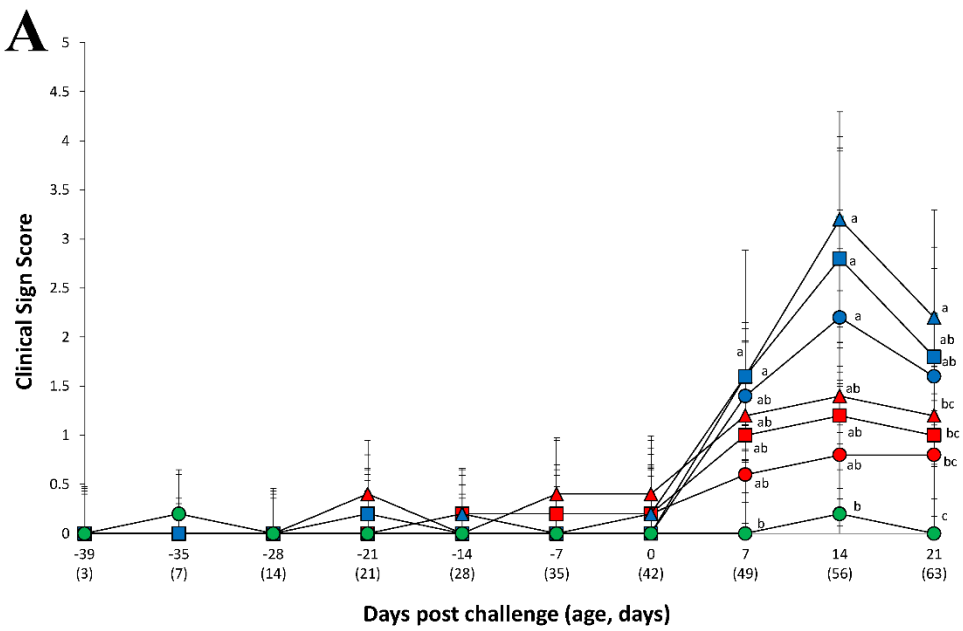
*Gross lung lesion and PCV2 antigen in lymph node: mean ± standard deviation, and histopathology : median ± interquartile range (IQR).

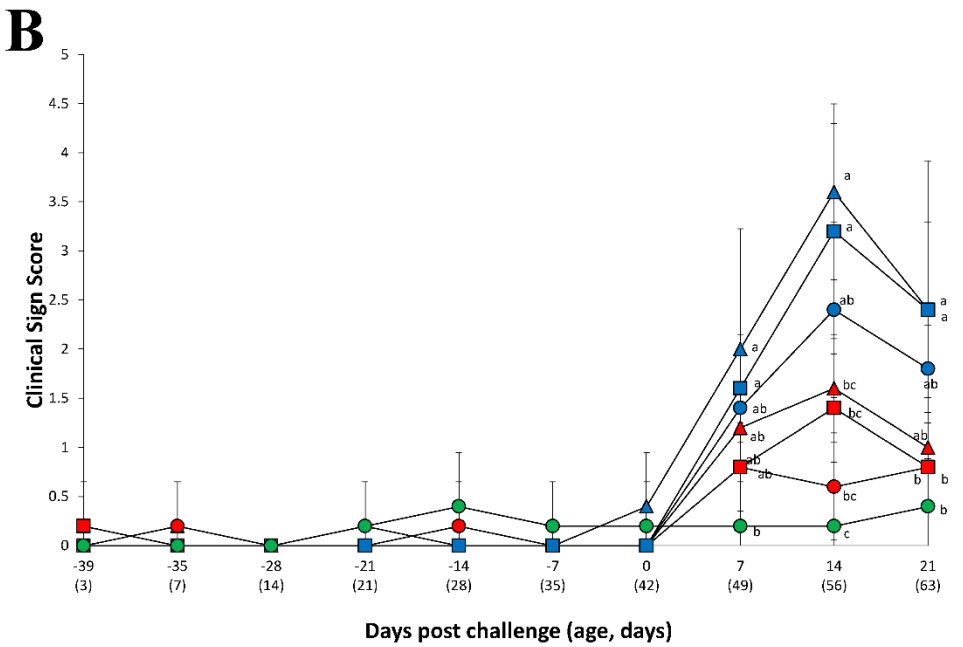
No differences of effect between one-dose and two-dose experiment

No statistical differences in clinical scores (Fig. S1A and S1B), ADWG (Table 2), levels of PCV2d viremia (Supplementary Fig. 2A), levels of laryngeal *M. hyopneumoniae* (Supplementary Fig. 2B), macroscopic pulmonary lesions (Table 3), microscopic pulmonary and lymphoid lesions (Table 3), and numbers of PCV2-antigen (Table 3) were observed between the one-dose and two-dose experiments.

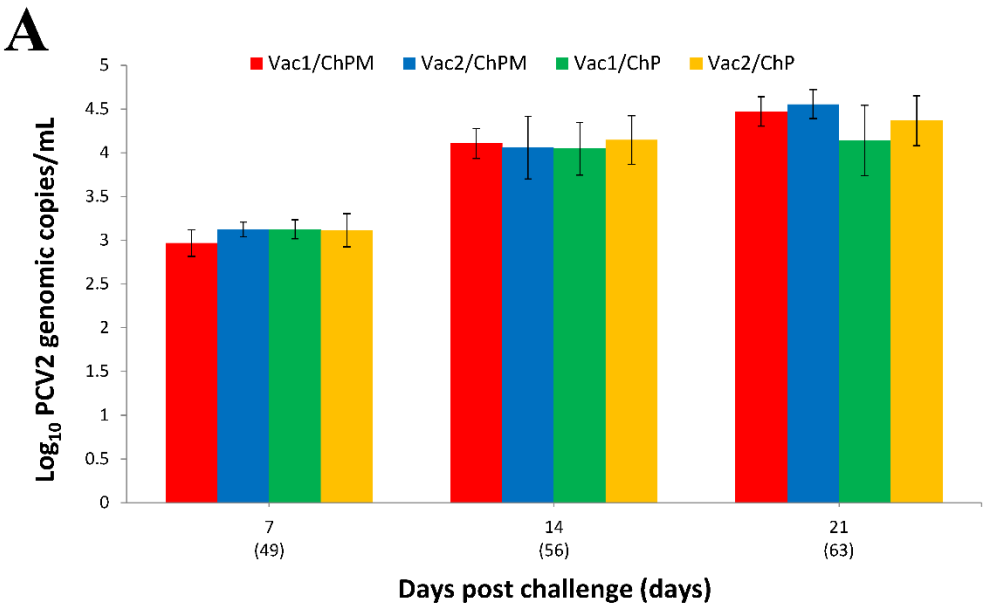
The inter-group comparison resulted in significantly higher ($P < 0.05$) PCV2 ELISA (Supplementary Fig. 3A), PCV2d-specific NA titers (Supplementary Fig. 3B), and PCV2d-specific IFN-γ-SC (Supplementary Fig. 3A) between the different dosages.

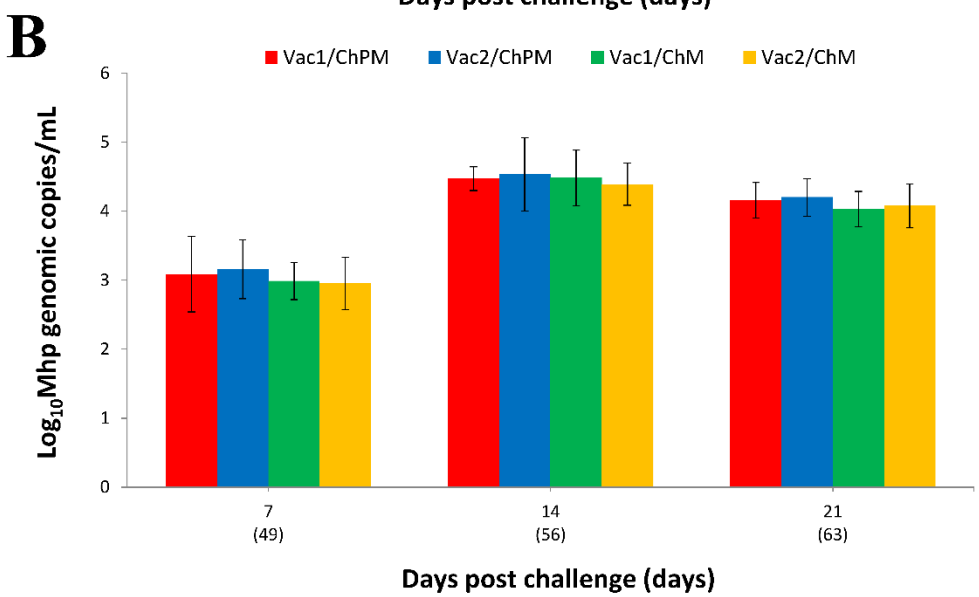
The inter-group comparison between the one-dose and two-dose experiments resulted in significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA (Supplementary Fig. 4A) and levels of *M. hyopneumoniae*-specific IFN-γ-SC (Supplementary Fig. 4B).


A
Clinical Sign Score
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-39 (3)
-35 (7)
-28 (14)
-21 (21)
-14 (28)
-7 (35)
0 (42)
7 (49)
14 (56)
21 (63)
Days post challenge (age, days)

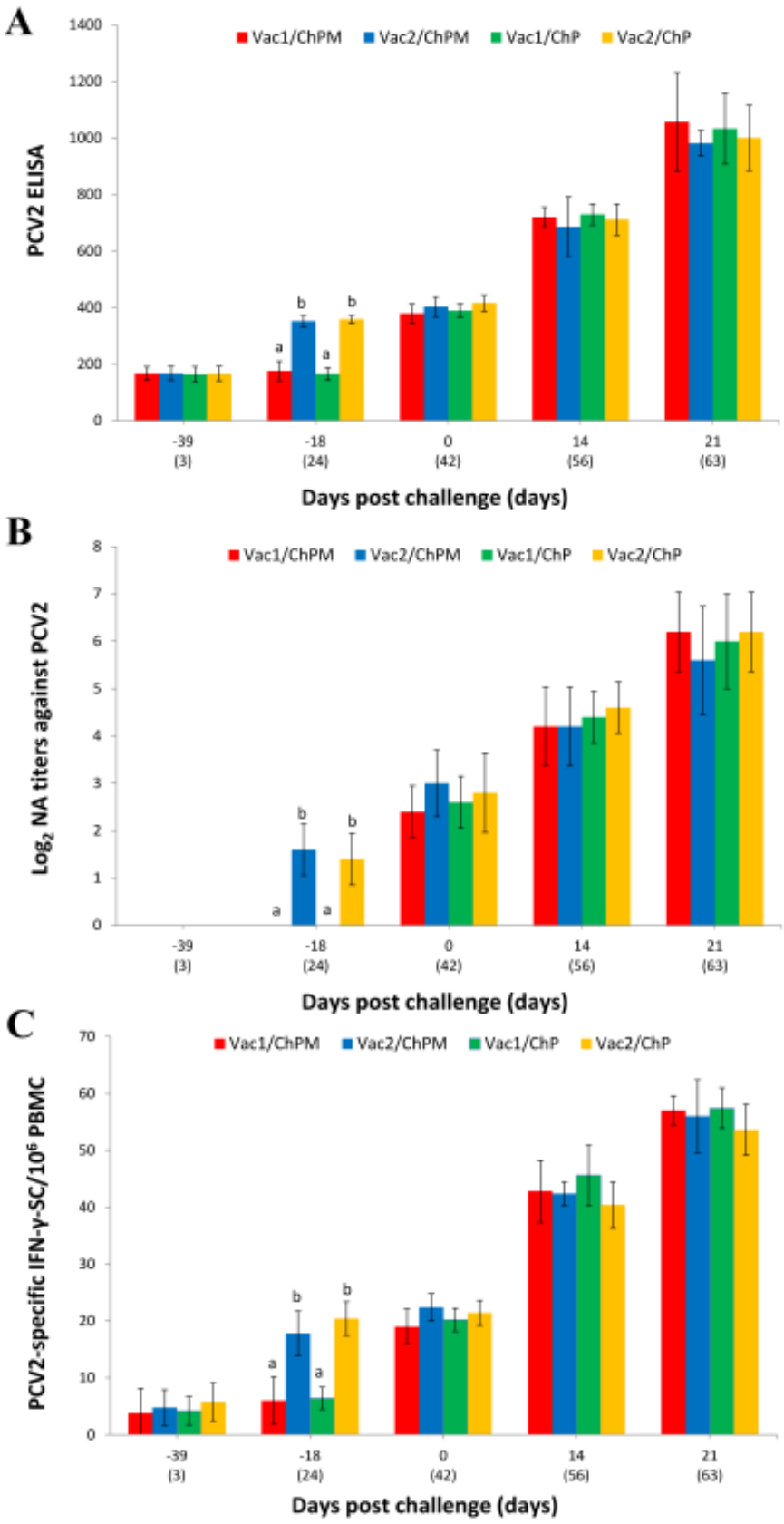

B
Clinical Sign Score
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-39 (3)
-35 (7)
-28 (14)
-21 (21)
-14 (28)
-7 (35)
0 (42)
7 (49)
14 (56)
21 (63)
Days post challenge (age, days)

Supplementary Fig. 1. Clinical sign scores from one-dose experiment (A) in Vac1/ChPM (▲), Vac1/ChP (●), Vac1/ChM (■), UnVac1/ChPM (▲), UnVac1/ChP (●), UnVac1/ChM (■), and UnVac1/UnCh (●) groups and from two-dose experiment (B) in Vac2/ChPM (▲), Vac2/ChP (●), Vac2/ChM (■), UnVac2/ChPM (▲), UnVac2/ChP (●), UnVac2/ChM (■), and UnVac2/UnCh (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) differences among 7 groups in each of experiments.

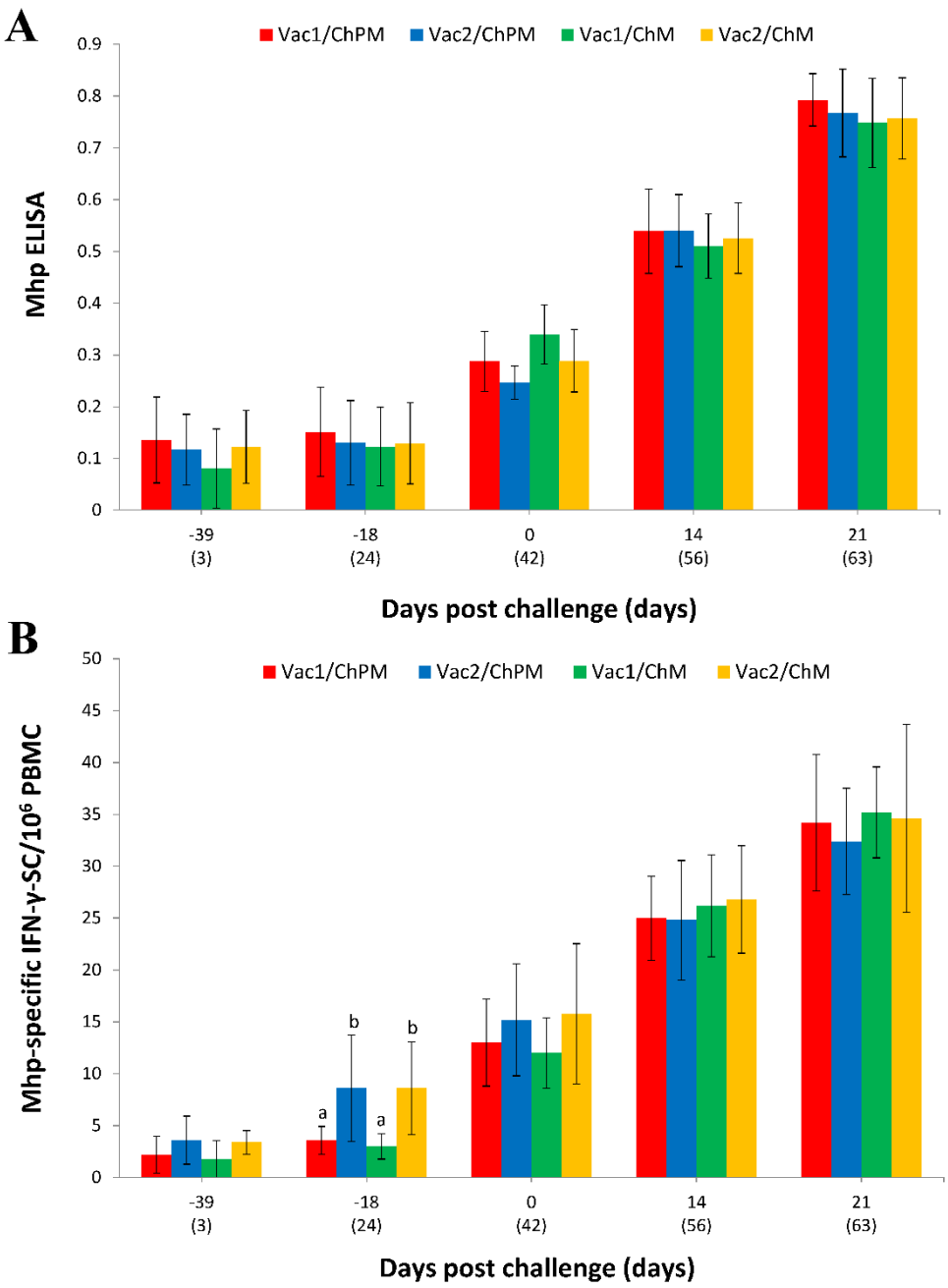

A
Vac1/ChPM
Vac2/ChPM
Vac1/ChP
Vac2/ChP
Log10 PCV2 genomic copies/mL
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
7
(49)
14
(56)
21
(63)
Days post challenge (days)


B
Vac1/ChPM
Vac2/ChPM
Vac1/ChM
Vac2/ChM
Log10Mhp genomic copies/mL
6
5
4
3
2
1
0
7
(49)
14
(56)
21
(63)
Days post challenge (days)

Supplementary Fig. 2. Comparison of mean values of the genomic copy numbers of PCV2d DNA in serum between one-dose and two-dose experiment (A) in Vac1/ChPM (■), Vac2/ChPM (■), Vac1/ChP (■), and Vac1/ChP (■) groups and mean values of the genomic copy numbers of *M. hyopneumoniae* in larynx between one-dose and two-dose experiment (B) in Vac1/ChPM (■), Vac2/ChPM (■), Vac1/ChM (■), and Vac1/ChM (■) groups. Variation is expressed as the standard deviation.


A
Vac1/ChPM
Vac2/ChPM
Vac1/ChP
Vac2/ChP
PCV2 ELISA
1400
1200
1000
800
600
400
200
0
a
b
a
b
-39
(3)
-18
(24)
0
(42)
14
(56)
21
(63)
Days post challenge (days)
B
Vac1/ChPM
Vac2/ChPM
Vac1/ChP
Vac2/ChP
Log2 NA titers against PCV2
8
7
6
5
4
3
2
1
0
a
b
a
b
-39
(3)
-18
(24)
0
(42)
14
(56)
21
(63)
Days post challenge (days)
C
Vac1/ChPM
Vac2/ChPM
Vac1/ChP
Vac2/ChP
PCV2-specific IFN-γ-SC/10^6 PBMC
70
60
50
40
30
20
10
0
a
b
a
b
-39
(3)
-18
(24)
0
(42)
14
(56)
21
(63)
Days post challenge (days)

Supplementary Fig. 3. Comparison of immune responses of PCV2-specific enzyme-linked immunosorbent assay (ELISA) antibody levels in serum (A), neutralizing antibody (NA) titers against PCV2d in serum (B), and frequency of PCV2d-specific interferon-γ-secreting cells (IFN-γ-SC)/10^6 in peripheral blood mononuclear cells (PBMC) (C) between one-dose and two-dose experiment in Vac1/ChPM (■), Vac2/ChPM (■), Vac1/ChP (■), and Vac1/ChP (■) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) differences between one-dose and two-dose experiment.


A
Vac1/ChPM
Vac2/ChPM
Vac1/ChM
Vac2/ChM
Mhp ELISA
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-39
(3)
-18
(24)
0
(42)
14
(56)
21
(63)
Days post challenge (days)
B
Vac1/ChPM
Vac2/ChPM
Vac1/ChM
Vac2/ChM
Mhp-specific IFN-γ-SC/10^6 PBMC
50
45
40
35
30
25
20
15
10
5
0
a
b
a
b
-39
(3)
-18
(24)
0
(42)
14
(56)
21
(63)
Days post challenge (days)

Supplementary Fig. 4. Comparison of immune responses of *M. hyopneumoniae*-specific enzyme-linked immunosorbent assay (ELISA) antibody levels in serum (A) and frequency of *M. hyopneumoniae*-specific interferon-γ secreting cells (IFN-γ-SC)/10^6 in peripheral blood mononuclear cells (PBMC) (B) between one-dose and two-dose experiment in in Vac1/ChPM (■), Vac2/ChPM (■), Vac1/ChM (■), and Vac1/ChM (■) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant (P < 0.05) differences between one-dose and two-dose experiment.

DISCUSSION

A new trivalent PCV2a/b and *M. hyopneumoniae* vaccine is available for the first time. By contrast, other commercially available PCV2 vaccines contain either PCV2a or PCV2b, (but not both). The trivalent vaccine was efficacious in protecting pigs against PCV2d and *M. hyopneumoniae* infection. No statistical differences were observed in the present study between the one-dose and two-dose experiments based on clinical (clinical signs and ADWG), immunological (protective immunity), microbiological (viremia and laryngeal swab), and pathological (pulmonary and lymphoid lesion) analysis. Pig producers are therefore able to select the preference of administration (one or two dose) for their pig farm conditions.

A common clinical feature of PCV2 and *M. hyopneumoniae* co-infection is retardation of growth. Growth performance was therefore considered the most critical parameter when evaluating the efficacy of this trivalent vaccine. Statistical differences in growth performance were observed between the vaccinated/dually-challenged groups (Vac1/ChPM and Vac2/ChPM) and unvaccinated/dually-challenged groups (UnVac1/ChPM and UnVac2/ChPM) in both dosage experiments. By contrast, the single challenge did not improve the growth performance between the vaccinated/singularly-challenged groups (Vac1/ChP and Vac2/ChP) and unvaccinated/singularly-challenged groups (UnVac1/ChP and UnVac2/ChP) in either dosage experiment. Even though PCV2 is the primary causative agent of PCVAD, a single infection with PCV2 cannot produce the full manifestation of clinical PCVAD (Opriessnig et al., 2004). This explains why growth performance was not statistically different between the vaccinated/challenged and

unvaccinated/challenged groups that were only challenged with PCV2. A significant difference in ADWG did not exist between vaccinated/challenged (Vac1/ChM and Vac2/ChM) and unvaccinated/challenged (UnVac1/ChM and UnVac2/ChM) groups in either dosage experiments. This is attributed to the short duration of observation post *M. hyopneumoniae* challenge. These results agree with previous studies where the *M. hyopneumoniae* vaccine did not produce significant improvement in growth performance under experimental conditions (Michiels et al., 2017).

The efficacy of the trivalent vaccine depends on the induction of protective immunity. Neutralizing antibody and IFN-γ-SC are well known to decrease the amount of PCV2 loads in the blood while simultaneously reducing the severity of lymphoid lesions and the amount of PCV2 antigen within the lymphoid lesions (Fort et al., 2008, 2012; Martelli et al., 2011; Seo et al., 2012). The trivalent vaccine evaluated in this study elicited protective immunity, which in-turn reduced blood viral loads and reduced the severity of lymphoid lesions. These are the mechanisms that ultimately protect pigs from PCV2d infection. In the case of *M. hyopneumoniae* infection, protective immunity is not well known, but it has been reported that cell-mediated immunity is related to disease protection (Djordjevic et al., 1997; Thacker et al., 2000). The evaluated trivalent vaccine elicited the protective cell-mediated immunity. This cell-mediated reduced the amount of *M. hyopneumoniae* load in larynx and reduced the severity of mycoplasmal pneumonia lesions, providing that vaccinated pigs were protected from *M. hyopneumoniae* infection.

Since the discovery of PCV2 in the late 1990s, PCV2 has been among the highest-mutating DNA viruses in animals (Karuppannan and Opriessnig, 2017) and continues to evolve. Two major genotype shifts have been observed over the past three decades. First, a shift from PCV2a to PCV2b in 2004/2005 occurred (Carman

et al., 2006; Cortey et al., 2011; Timmusk et al., 2008; Wiederkehr et al., 2009). Since 2012, PCV2b has been gradually replaced by the PCV2d genotype in global pig populations in what is considered the second shift (Xiao et al., 2015). In the present study, the vaccine's efficacy against PCV2d, the most current prevalent PCV2 genotype, is demonstrated under experimental challenge conditions. This trivalent PCV2a/b and *M. hyopneumoniae* vaccine provides the broad range of protection in swine against evolving PCV2 viruses (as evaluated against a heterologous PCV2d challenge) as well as good protection against *M. hyopneumoniae*. The results of this study demonstrate that a trivalent PCV2a/b and *M. hyopneumoniae* vaccine may be a good candidate to replace the bivalent PCV2a and *M. hyopneumoniae* vaccine in swine herds to protect against PCV2d and *M. hyopneumoniae* infection.

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GENERAL CONCLUSION

Porcine circovirus type 2 (PCV2) and *Mycomplasma hyopneumoniae* are the most prevalent and economically important pathogens in worldwide pig production systems. Coinfection of pigs with PCV2 and *M. hyopneumoniae* has contributed to cause porcine respiratory disease complex, and this in turn results in an increase in animal treatment costs as well as economic losses. Vaccines against PCV2 and *M. hyopneumoniae* are the 2 most commonly administered vaccines in Korea, as in all global swine herds. The use of combined vaccine is preferred as the recommended vaccination time against both PCV2 and *M. hyopnuemoniae* is similar.

The objective of this experimental study was to evaluate the efficacy of combined vaccines. Part Ⅰ study was to compare the efficacy of 2 different PCV2 – *M. hyopneumoniae* bivalent vaccines with 2 sets of different PCV2 and *M. hyopneumoniae* monovalent vaccines against a dual *M. hyopneumoniae* and PCV2d challenge in swine. The results indicate that vaccination against PCV2 and *M. hyopneumoniae* is efficacious in controlling these 2 pathogens, regardless of vaccine type, monovalent or bivalent. All vaccinated groups measured higher growth performance than unvaccinated groups. Both monovalent vaccine and bivalent vaccine groups show similar generation of protective immunity results. There were some differences within bivalent vaccines vaccinated groups, although too small to be considered statistically significant. Pigs in VacA/challenged groups generated significantly higher numbers of IFN-γ-SCs in their blood, while exhibiting less nasal shedding and mycoplasma pneumonia than those in the VacB/challenged groups. There are several potential reasons for these differences including each bivalent

vaccine's unique antigen and adjuvant. These results demonstrate that the inactivated chimeric PCV1-2a vaccine induced significantly higher numbers of PCV2d-specific IFN-γ-SCs capsid protein than inactivated subunit PCV2a vaccine. The higher amount of IFN-γ-SCs results less PCV2d viremia.

Part II study was to compare the efficacy of a new trivalent PCV2a/b and *M. hyopneumoniae* vaccine for the first time. Trivalent vaccine was efficacious in protecting pig against PCV2d and *M. hyopneumoniae* infection. The trivalent vaccine elicited protective immunity, which reduced amount of virus loads in blood and the severity of lymphoid lesions. In addition, the evaluated trivalent vaccine elicited the protective cell-mediated immunity and this reduced the amount of *M. hyopneumoniae* load in larynx. The trivalent vaccine provided efficacious immunity against both PCV2d and *M. hyopneumoniae*.

In growth performance, there were statistical significant differences between vaccinated/dually challenged groups and unvaccinated/dually challenged groups. In contrast, the single challenge did not improve the growth performance between vaccinated/singularly-challenged groups and unvaccinated/singularly-challenged groups in either dosage experiment. This indicated that the single infection with PCV2 cannot produce the full manifestation of clinical PCVAD. In singularly challenge with *M. hyopneumoniae* groups also showed no differences in growth performance between vaccinated groups and unvaccinated groups. This might be due to short duration of observation post *M. hyopneumoniae* challenge.

In several studies, PCV2a-based monovalent vaccines provide crosss-protection against PCV2d infection. This study also indicated bivalent and trivalent vaccine also show similar generation of protective immunity, such as neutralization antibodies and IFN-γ-SCs, and reduction of PCV2 viremia. No significant difference

was observed between conbined vaccine and mycoplasmal monovalent in the generation of IFN-γ-SCs and reduction of nasal shedding of *M. hyopneumoniae*, which suggests that there was no antigen interference. Combined vaccine reduces the number of infected pigs and is more convenient and more efficient than working with multiple products. Swine practitioners and producers could consider variable vaccination program by using these experimental study results.

국문 논문 초록

돼지 써코바이러스 2형, 마이코플라즈마 백신의 임상학적, 미생물학적, 면역학적, 병리학적 분석을 통한 평가

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본 실험의 목적은 실험적으로 유발시킨 PCV2-*M. hyopneumoniae* 복합감염 모델에서 돼지 써코바이러스 2형과 마이코플라즈마 복합 백신의 효능을 임상학적, 미생물학적, 면역학적, 병리학적 기법을 통하여 분석하고, 백신 종류와 접종 방법에 따른 효능 차이를 평가하는 것이다. 백신의 효과는 PCV2의 바이러스혈증, *M. hyopneumoniae*의 비강 배출과 후두부 스왑 결과를 지표로 미생물학적으로 평가하였고, 중화항체와 인터

페론 감마 분비세포 측정 실험으로 얻은 면역학적 지표를 통해 체액성 면역 및 세포매개성 면역 효능을 평가하였다. 폐와 림프조직의 육안병변, 조직병변 및 병변 내 PCV2 항원을 지표로 병리학적 평가를 하였다.

첫 번째 실험에서는 PCV2와 마이코플라즈마 2가 백신과 단일 백신의 효능 차이를 평가하였다. 실험 결과, 백신을 접종한 모든 그룹에서 접종하지 않은 그룹보다 높은 증체율과 유의적인 차이의 면역반응을 유도하였다. 하지만, 21일령 돼지에 PCV2와 마이코플라즈마 2가 백신을 접종한 그룹과 7일령, 21일령에 마이코플라즈마 백신과 PCV2 백신을 각각 단일 접종한 그룹 사이에 미생물학적, 면역학적, 병리학적 지표에서 유의미한 차이를 보이지 않았다. 또한 이 실험에서 돼지 써코바이러스 1형의 ORF1 과 돼지 써코바이러스 2형의 ORF2 부위를 재조합하여 생산된 키메라 백신이 돼지 써코바이러스 2형 재조합 백신보다 높은 인터페론 감마 분비세포를 유도하여 더 많은 PCV2 바이러스 혈증과 *M. hyopneumoniae* 비강 배출 감소를 보이는 것으로 확인되었다.

두 번째 실험에서는 PCV2a/2b, 마이코플라즈마 3가 백신에 대한 효능을 평가하였다. 3가 백신을 3일령과 24일령에 2회 접종한 군과 21일령에 1회 접종한 군으로 나누었고, 각각 PCV2와 마이코플라즈마 복합 공격 접종 군과 두 개 병원체 단일 공격 접종 군으로 나누었다. 실험 결과, 백신을 접종한 그룹이 접종하지 않은 그룹에 비해 세포매개성 면역 과 체액성 면역을 유의적으로 높게 유도하여, 3가 백신이 세가지 병원체에

대한 각각의 능동면역을 유발할 수 있음을 증명하였다. 또한, 백신을 접종하지 않은 그룹 내에서 PCV2와 마이코플라즈마 단일 공격 접종 군은 백신을 접종한 군과 비슷한 증체율을 보여, PCV2가 PCVAD의 일차적인 요소이지만 단독 감염만으로는 확연한 임상증상을 보이지 않음을 입증하였다.

최근 경제적 측면을 고려하여, 백신 접종 횟수를 줄이기 위해 다른 종류의 항원을 함유한 복합 백신 개발이 활발하다. 하지만 복합 백신은 이종 항원의 간섭현상으로 인해 단일 백신에 비해 각각의 병원체에 대한 충분한 면역 유도를 하지 못할 수 있다는 우려가 존재한다. 이번 실험 실내 공격 접종 실험을 통하여 백신의 균주와 면역증강제 종류에 따른 차이가 있을 수 있지만 PCV2와 마이코플라즈마 복합 백신이 단일 백신과 비교하여 충분한 면역반응을 유도할 수 있으며, 이를 통해 더욱 효과적인 백신 프로그램을 성립할 수 있다는 결론을 도출하였다.

주요어 : 돼지 써코바이러스 2형; 마이코플라즈마; 돼지 호흡기 복합 질병; 혼합감염; 복합백신; 백신효능

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