수의학박사 학위논문

Pathological and Immunological Evaluation of Bivalent Vaccine Containing Porcine Circovirus Type 2b and *Mycoplasma hyopneumoniae*

돼지써코바이러스 2b형과 마이코플라즈마 복합백신의 병리학적 및 면역학적 효능 평가 연구

2023 년 2월

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학전공

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獸 醫 學 博 士 學 位 論 文

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이 논문을 수의학 박사 학위논문으로 제출함
2022 년 11 월

서울대학교 대학원
수의학과 수의병인생물학 및 예방수의학 전공
안 용 준

안용준의 수의학박사 학위논문을 인준함
2022 년 12 월

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A dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2022

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Abstract

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In countries with a developed pig industry such as Korea, porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* are very important pathogens that cause complex infections like porcine respiratory disease complex (PRDC) and impact economic damage to the pig industry. Therefore, by vaccinating against both pathogens, they have been trying to control disease and reduce damage to farms. Vaccines against both pathogens have been proven effective and are supplied by several companies. In order to reduce the number of

injections, combination vaccines using two pathogens as antigens or vaccines that can be used by mixing each vaccine are being supplied. Most PCV2 vaccines were based on PCV2a. However, the prevalence of PCV2d, which was called 'mutant PCV2b', is increasing. Recently, a new single dose bivalent vaccine containing PCV2b and *M. hyopneumoniae* has been developed and introduced into Korea. This vaccine contains two types of pathogens, and is unlike existing other vaccines that are injected 2ml dose at 3 weeks of age, it can be vaccinated 1ml dose from 10 days of age, so it was necessary to evaluate the effectiveness and efficacy of the vaccine. Therefore, in this study, we tried to verify the effectiveness and efficacy of this new bivalent vaccine through experimental dual challenge with PCV2 and *M. hyopneuomoniae* and field evaluation

In chapter I, to evaluate the efficacy of the vaccine, 24 pigs were purchased and divided into 3 groups as Vaccinated/Challenged (Vac/Ch), Unvaccinated/Challenged (UnVac/Ch) and Unvaccinated/ Unchallenged (UnVac/UnCh), vaccination were conducted at 10 days of age (-25 days post-challenge (dpc)). Vac/Ch and UnVac/Ch were challenged by inoculation with PCV2b and *M. hyopneumoniae* at 5 hours intervals. Pigs were observed from 10 days of age (-25 dpc) to 56 days of age (21 dpc), and euthanized at 21 dpc then lung lesions and lymphoid lesions were evaluated. As a result, the Vac/Ch group showed significantly lower respiratory symptoms during the study compared to the UnVac/Ch group, and the Vac/Ch group and the

UnVac/UnCh group showed significantly higher average daily weight gain (ADWG) compared to the UnVac/Ch group for weight gain. In evaluation of blood samples and nasal swabs collected at -15, -14, 0, 7, 14 and 21 dpc, the Vac/Ch group showed significantly less PCV2 genomic copies and *M. hyopneumoniae* genomic copies than UnVac/Ch group. The Vac/Ch group showed significantly higher humoral and cell-mediated immune responses and lower score of lung and lymphoid lesion than the UnVac/Ch group.

In chapter II, for field evaluation, 10-day-old pigs were received 1ml single dose of bivalent vaccine in 3 farms which had a history of subclinical PCV2 and clinical *M. hyopneumoniae*, and compared with a control group received phosphate buffered-saline at the same time. In order to evaluate the effectiveness of the vaccine, clinical signs were observed and recorded weekly until 175 days of age, and the live weight of each pig was measured at the test start date (10 days of age), 70, 112 and 175 days of age, and ADWG was analyzed for each time section. To evaluate the efficacy of vaccine, levels of PCV2d viremia and nasal shedding of *M. hyopneumoniae* were measured through samples of blood and nasal swabs at 0, 18, 39, 81 and 102 dpv, and indicators of humoral and cell-mediated immunity were measured. As a result, the clinical score was significantly lower in the vaccinated group compared to the unvaccinated group, and the ADWG and the growth performance of vaccinated groups were higher than those of unvaccinated group. The vaccinated group

showed significantly lower levels of PCV2d viremia and nasal shedding of *M. hyopneumoniae,* and the immune response had significantly higher markers of humoral and cell-mediated immunity compared to the unvaccinated group.

Through these two studies, it was confirmed that the new single dose bivalent vaccine containing PCV2b and *M. hyopneumoniae* was effective against subclinical PCV2 and clinical *M. hyopneumoniae* even when vaccinated at 10 days of age, and that the immune response to each pathogen was also well established. It can be concluded that it can be used for the purpose of prevention in the field in Korea, where PCV2d is most prevalent genotype.

Keywords: Porcine circovirus type 2b (PCV2b); *Mycoplasma hyopneumoniae*; Bivalent vaccine; Dual challenge; Porcine respiratory disease complex (PRDC)

Student number: 2020-20725

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LIST OF ABBREVIATIONS

ADG/ADWG	Average daily gain / Average daily weight gain
dpc	days post-challenge
dpi	days post-inoculation
dpv	days post-vaccination
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
IFN-ɣ-SC	Interferone-ɣ-secreting cells
IHS	Immunohistochemistry
IL	Interleukin
ISH	In-situ hybridization
LAMP	Lipid associated membrane proteins
MDA	Maternally derived antibody
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PCV2	Porcine circovirus type 2
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PMWS	Postweaning multisystemic wasting syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

GENERAL INTRODUCTION

Porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* are important pathogens causing economic impact to the global pig industry. They can cause disease on their own, but usually infect with other pathogens, making the disease more serious. PCV2 is the primary causative agent of porcine circovirus-associated diseases (PCVAD). Representative PCVADs include Postweaning multisystemic wasting syndrome (PMWS), Porcine dermatitis and nephropathy syndrome (PDNS), Porcine respiratory disease complex (PRDC) and reproductive failure. PCV2 is genetically very diverse, and some studies have classified it into 8 groups. Coinfection of pigs with PCV2 and *M. hyopneumoniae* is the most frequent combination in field PRDC cases.

Vaccines have been using to control these pathogens in countries with developed pig industries. The use of vaccines against both pathogens improves growth performance and alleviates clinical symptoms of disease associated with both pathogens, or suppresses the spread of diseases. Currently, in order to reduce the number of injections, the use of a bivalent vaccine or mixable vaccines is increasing in pig farms.

Recently, the single-dose bivalent vaccine containing PCV2b and *M. hyopneumoniae* has been developed. Most of the commercial

vaccines targeting PCV2 are based on PCV2a, and the administration is from 3 weeks of age. However, the dominant virus worldwide has been changing from PCV2a to PCV2b and PCV2d, also, there are studies that Mycoplasma start infection a little earlier than porcine circovirus. This new vaccine contains a different antigen (PCV2b) and is recommended administration earlier than the existing vaccines, thus it was thought there is necessary to evaluate the efficacy of the vaccine.

It is expected that vaccination could reduce clinical symptoms caused by PCV2 and *M. hyopneumoniae* and improve growth performance. In addition, it is expected that the immune response to the two pathogens will be achieved, so that viremia of PCV2 and nasal shedding of *M. hyopneumoniae* will be reduced, and lung and lymphoid lesions will be less.

The objective of this dissertation was to evaluate the new single-dose bivalent vaccine containing PCV2b and *M. hyopneumoniae* based on clinical, microbiological, immunological and pathological analysis under field conditions and experimental dual challenge.

LITERATURE REVIEW

1. Porcine circovirus type 2

1.1. Background

Since postweaning multisystemic wasting syndrome (PMWS) was reported as a new syndrome [1, 2], porcine circovirus type 2 (PCV2) has been considered as the most important pathogen in pig industry.

Research of PCV began actively in 1990's, since then, additional PCVs have been identified. There are four types: Porcine circovirus type 1 (PCV1) [3, 4], Porcine circovirus type 2 (PCV2) [5], Porcine circovirus type 3 (PCV3) [6] and Porcine circovirus type 4 (PCV4) [7]. PCV3 and PCV4 were identified recently, so there are lack of research results.

Although PCV1 has been found in both wild and farmed pigs, it is generally considered non-pathogenic for swine. Because no disease associated with PCV1 was observed in naturally or experimentally infected pigs [3, 4]. In contrast pathogenicity of PCV2 was established by virus isolation from PMWS affected pigs, experimental infection, histopathology, in addition to immunohistochemistry (IHC) and in situ hybridization (ISH) to detect antigen or genome [8 - 11].

In case of PCV3, it was discovered by metagenomic sequencing on tissue samples from pigs with PMWS, PDNS, reproductive failure,

myocarditis and multi-systemic inflammation [6, 12], but despite there were a lot of attention and research, PCV3 isolation in cell culture has only been successful by Korean researcher [13]. PCV4 was also identified by sequencing in pigs with various health conditions from two farms in Hunan province, China [7].

This review concentrates PCV2 and PCV2-associated syndromes and diseases.

1.2. Etiology

Porcine circovirus is a small, non-enveloped virus with a single-strand circular DNA genome in the genus Circovirus, family Circoviridae [14]. The genome of PCV2 contains 1767 - 1768 nucleotides and the nucleocapsid of PCV2 contains 60 capsid (Cap) protein elements exhibit icosahedral symmetry [15]. PCV2 genes are arranged in 11 putative open reading frames (ORFs) [16], but protein expression has been described for only three. ORF1 (Rep gene) is located on the positive strand and in clockwise orientation and encodes two viral replication-associated proteins, Rep and Rep′, by differential splicing and these are essential for viral replication [17 - 19]. ORF2 (Cap gene) is on the complementary strand and oriented counterclockwise and encodes the capsid protein, which has the ability to bind to host cell receptor [20 - 22]. The capsid protein is the only structural protein and the primary immunogenic protein [23,

24], thus it has been the target for development of vaccine and sero-diagnostic assays for tracking PCV2-specific immune responses [25 - 27]. ORF3 encodes for a nonstructural protein. An ORF3-deficient PCV2 mutant was shown to be less virulent in pigs compared with wild-type PCV2 [28].

The research on the genetic diversity of PCV2 has been conducted actively. Initially, several research groups identified in their own terms many PCV2 genotypes that lacked uniformity. For this reason, the EU Consortium on Porcine Circovirus Diseases has proposed a standardized nomenclature for the PCV2 genotypes: PCV2a, PCV2b and PCV2c [29, 30]. PCV2a and PCV2b have both been associated with clinical Porcine circovirus associated disease (PCVAD) of varying degrees of severity [31 - 37]. After introduction of PCV2 vaccine using PCV2a-based antigen, the prevalence of PCV2 showed a tendency to shift from PCV2a to PCV2b between roughly 2003 and 2006 [37], then PCV2b shifted to PCV2d between 2010 and 2015 [38, 39]. Some researcher diverse the genotype of PCV2 to eight (a through h) by analyzing ORF2 sequences [38], otherwise another researcher proposed five genotypes (a through e), they defined PCV2f and PCV2h as intermediate (IM) groups IM3 and IM1, respectively, and PCV2d and PCV2g as same cluster [40]. Despite the diverse genotypes of PCV2, immunity induced by one genotype has a protective effect against the other [36].

1.3. Epidemiology

PCV2 has been found in both domestic and wild pigs [41], and pig-to-pig transmission was experimentally confirmed [42 - 44]. In case of systemic infections, it can be expected that PCV2 can be shed in many body secretions. In naturally and experimentally infected pigs, PCV2 virus shed in oral and nasal secretions, feces and urine [45, 46]. In addition, it has been found in tonsillar, bronchial, and ocular secretion, colostrum, milk and semen [46 - 48]. The virus shedding period in pig naturally infected with PCV2 at around 13 day old was shown to be at least 209 days [43] or 69 days after experimental PCV2 infection [45]. Through the colostrum of infected sows, naïve piglet can be infected [49]. Naïve sow can be infected by insemination with PCV2 contaminated semen, but it is considered dose dependent. High level dose could induce reproductive failure and infect their fetuses, but low-level dose failed to infect sow [50, 51]. Transplacental transmission by intranasal exposing [52] and intrauterine transmission [53] are confirmed. Horizontal transmission of PCV2 can occur through infected pigs direct contact with naïve pigs when they are mixed [54]. Longitudinal studies quantifying PCV2 in serum, nasal excretions, and feces found that most pigs became infected at 4 - 11 weeks of age, depending on the farm [55, 56].

Because there are many shedding route of PCV2, traces of PCV2 can be found in various places or objects. Therefore, the environmental load of PCV2 can be considered as an important

indicator of herd health. A study shows that high levels of PCV2 DNA have been detected in dust particles in the air of production facilities, up to 10^7 genome copies/m^3 air [57]. The possibility of airborne transmission cannot be excluded in high-density pig farm areas. Another study found PCV2 DNA in houseflies that were captured in pig farms, which was matched to those in fecal samples from pigs in each farm. It seems possible that flies can serve as mechanical vectors in pig-to-pig or farm-to-farm transmission [58].

1.4. Pathogenesis

The mechanism of PCV2 pathogenesis is understood poorly and there is little information regarding the target cells for initial viral replication, the early events of viral infection. PCV2 does not code for its own DNA polymerase, so they may need cells in the S phase for the virus to complete its infectious cycle [59], if so, cells with a high mitotic rate should be the most efficient for viral replication. Macrophages and lymphocytes were found that they may support replication [60]. PCV2-infected leukocyte from peripheral blood mononuclear cells (PBMCs) showed that circulating T lymphocytes (CD4+ and CD8+) and B lymphocytes support PCV2 replication, but not PBMC-derived monocytes [60]. Monocytes seem to serve as a mechanism to disseminate PCV2 rather than replication of PCV2 within the host [61].

PCV2 viremia is first detected around 7 days post-inoculation (dpi) and peaks at 14 - 21 dpi [41, 62]. PCV2 is mainly detected in lymphoid tissue [63], but it may also be detected in epithelial cells from the kidney and respiratory tracts, endothelial cells, lymphocytes, enterocytes, hepatocytes, smooth muscle cells, and pancreatic acinar and ductal cells [63, 64]

1.5. Postweaning multisystemic wasting syndrome (PMWS)

Postweaning multisystemic wasting syndrome (PMWS) is the most important porcine circovirus associated disease (PCVAD). Since its first description is healthy herds in Canada in 1991 [1, 2], PMWS has been reported from all major pig-producing countries. Infection with PCV2 is necessary for PMWS to develop, but most research has shown that PCV2 needs one or more co-factors for PMWS to develop into severe and even fatal disease [65]. Several pathogens such as porcine parvovirus [66], PRRSV [67] and *Mycoplasma hyopneumoniae* [68] have been shown to enhance the severity of PCV2 infections.

PMWS most commonly affects pigs at 2 - 4 months of age [56]. Clinically affected pigs have a higher concentration of virus in the serum, shed virus and demonstrate a weaker antibody response compared with subclinically infected pigs [56]. During PMWS, most affected herds experienced heavy losses mainly due to a significant

increase in post-weaning mortality. A study demonstrates mortality ranges from 4 % to 20 % [69]. PMWS is characterized clinically by wasting, pallor of the skin, respiratory distress, diarrhea and icterus [1, 2] In the early clinical phase, PMWS lesions are primarily found in lymphoid tissues, and enlarged lymph nodes are the most prominent feature [1, 63]. Normal sized, or even atrophied, lymph nodes are usually seen in more advanced phases of PMWS [70] and thymus is frequently atrophied in affected pigs. Enlarged and non-collapsed lungs and pulmonary consolidation may be found, these findings correspond microscopically to interstitial pneumonia [1, 70]

A diagnosis of PMWS in a herd level should be based on two conditions: (i) a significant increase in mortality associated with clinical signs of PMWS, and (ii) an individual diagnosis in at least one of the three to five necropsied pigs [71].

Another diagnostic criteria are as follow: (i) growth retardation and wasting, frequently with dyspnea and enlargement of inguinal lymph nodes and occasionally with jaundice; (ii) moderate-to-severe characteristic histopathological lesions in lymphoid tissues; (iii) moderate-to-high amounts of PCV2 within the lesions in lymphoid and other tissues of affected pigs.

1.6. Porcine dermatitis and nephropathy syndrome (PDNS)

Porcine dermatitis and nephropathy syndrome (PDNS) affects

nursery to adult pigs [72] and PDNS-affected pigs have significant gross lesions in skin and kidneys [73, 74]. Skin lesions are several round to irregularly shaped red-purple macules and papules that coalesce over the hind limb and perineal area. The kidneys are bilaterally enlarged and have pale cortices with multiple red circular haemorrhagic cortical foci.

The microscopic lesions of PDNS are characterized by generalized severe necrotizing vasculitis and fibrinonecrotic glomerulonephritis [73, 75]. The vasculitis associated with PDNS is thought to involve an immune-mediated mechanism [74, 76]. Some studies showed PCV2 was not detected in endothelial cells in PDNS-affected pigs [73, 75, 76], it suggest that PCV2 causes vascular lesions indirectly such as immune-mediated mechanism.

PMWS and PDNS are PCV2-associated disease, but there is no direct relationship. The pigs with one of them never progress into the others. As a result of one study, a large amount of PCV2 nucleic acid was detected in lymph nodes in the case of PMWS and in the kidneys in the case of PDNS [75]. It seems that the disease depends on tissue tropism of PCV2.

1.7. Porcine respiratory disease complex (PRDC)

Porcine respiratory disease complex (PRDC) is a serious health problem in growing and finishing pigs. PRDC caused by a

combination of infectious viral or bacterial pathogens, environmental stressors and various management practices. PRDC is characterized by respiratory symptoms, anorexia, poor growth and decreased feed efficiency [77, 78]. PCV2 plays a very important role in the development of PRDC, and there are many viral and bacterial pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), *M. hyopneumoniae, Actinobacillus pleuropneumoniae,* and *Pasteurella multocida* [77, 78]. Especially PCV2, PRRSV and *M. hyopneumoniae* are considered to be the most clinically important pathogens for PRDC. The infection of PCV2 has been identified in most PRDC cases, and coinfection with one or more other pathogens produces more severe PRDC symptoms.

In case of coinfection with PCV2 and *M. hyopneumoniae, M. hyopneumoniae* potentiated the severity of PCV2-associated lung and lymphoid lesions in pigs inoculated with *M. hyopneumoniae* 2 weeks prior to PCV2 [68].

In a study of the interaction between PCV2 and *M. hyopneumoniae*, *M. hyopneumoniae* enhanced the level of PCV2 viremia but PCV2 did not enhance Mycoplasmal nasal shedding in dually infected pigs compared to singular infected pigs under experimental conditions [79]. In field condition, *M. hyopneumoniae* infection in pigs occurs slightly prior to PCV2 infection [80, 81].

1.8. Reproductive failure

PCV2-associated reproductive failure is characterized by late-term abortion and delivery of stillborn or immature piglets.

It was confirmed PCV2 can induce reproductive failure by the experimental studies such as direct intrafetal inoculation [82], artificial insemination with PCV2-contaminated semen [83] and intranasal inoculation to pregnant sows [52]. Direct intrafetal inoculation at 57, 75, and 92 days of gestation showed that PCV2 replicated in all inoculated fetuses, with significantly higher replication in fetuses inoculated at 57 days of gestation [82]. Another study of direct intrafetal inoculation showed different results depending on the time of inoculation. The study reported mummification in fetuses inoculated at 57 days of gestation and transmission of PCV2 to noninoculated fetuses. At farrowing, there were stillborn in fetuses inoculated at 75 days of gestation and there was no lesion in fetuses inoculated at 92 days of gestation [84]. Sows inseminated with PCV2a-contaminated semen failed to be pregnant whileas Sows inseminated with PCV2b-contaminated semen became pregnant and maintained pregnancy to farrow but there were mummified fetuses with myocardial lesion [83]. Intranasal inoculation to pregnant sows at around 3 weeks before the farrowing date induced abortion or premature farrowing [52].

1.9. Vaccine and Immune response

Both neutralizing antibodies and cell-mediated immune responses are important to protect against PCV2 infection or improve growth performance. PCV2-infected pigs develop PCV2-specific neutralizing antibodies at 10 - 28 dpi [85, 86] and interferon gamma (IFN-ɣ) gene expression in PBMCs were detected [85]. A poor neutralizing antibody response is associated with increased viral load in serum, severe lymphoid lesions [85, 86].

Most commercial PCV2 vaccines are based on inactivated PCV2 virus or are subunit vaccines containing capsid protein as antigen. These also induce to develop neutralizing antibodies and cell-mediated immune responses. The obvious effects of the PCV2 vaccine on pig farms are the improvement of the average daily gain (ADG) and the reduction of mortality. Most commercial PCV2 vaccines are for piglets, it must be possible to minimize or overcome interference from maternal derived antibodies.

PCV2 vaccines supplied by multinational companies registered in Korea are shown in the Table 1 [87]. All vaccines are recommended to inject piglets from at least 3 weeks of age, and most of the administrative route is intramuscular method, but recently, intradermal vaccine was also introduced.

Table 1 PCV2 vaccines registered in Korea.

Vaccine	Manufacturer	Antigen	Usage	Administration
Circovac	Merial Ceva-Phylaxia	Inactivated PCV2	Sows Piglets > 3 weeks old	IM 2 ml IM 0.5 ml
Circumbent PCV	Intervet (MSD)	PCV2a subunit (ORF2)	Piglets > 3 weeks old	IM 2 ml 3 week-interval
Fostera™ PCV	Zoetis	Inactivated chimeric PCV1-2a	Piglets > 3 weeks old	IM 2 ml once or IM 1 ml twice 3 week- interval
Fostera™ PCV Metastim®	Zoetis	Inactivated cPCV1-2	Piglets > 3 weeks old	IM 2 ml once
CircoMax (Fostera™ Gold PCV)[1]	Zoetis	Inactivated chimeric PCV1-2a & Inactivated chimeric PCV1-2b	Piglets > 3 weeks old	IM 2 ml once or IM 1 ml twice 3 week-interval
Ingelvac CircoFLEX®	Boehringer Ingelheim	PCV2a subunit (ORF2)	Piglets > 3 weeks old	IM 1 ml once
Porcigen® PCV2 (Suigen PCV2)[1]	Virbac	Inactivated PCV2d (H strain)	Piglets > 3 weeks old	IM 1 ml once
Porcilis® PCV	Intervet (MSD)	PCV2 subunit (ORF2)	Piglets > 3 weeks old	IM 2 ml once
Porcilis® PCV ID	Intervet (MSD)	PCV2 subunit (ORF2)	Piglets > 3 weeks old	ID 0.2 ml

[1] Product name in the country of manufacture

2. *Mycoplasma hyopneumoniae*

2.1. Background

Mycoplasma hyopneumoniae is the primary pathogen of enzootic pneumonia and plays a role in the porcine respiratory diseases complex (PRDC), economically one of the most important disease in pig industry. The prevalence of *M. hyopneumoniae* is very high in almost all swine producing areas.

M. hyopneumoniae has small sized genome, lacks a cell wall and is a pleomorphic. *M. hyopneumoniae* is very difficult to isolate because of its slow growth and overgrowth of other swine Mycoplasmas. *M. hyopneumoniae* is primarily found on the mucosal surface of the trachea, bronchi, and bronchioles, and adherence of *M. hyopneumoniae* to the ciliated epithelium is precondition for initiation of the infection [88].

M. hyopneumoniae affects the mucosal clearance system by disturbing the cilia, it modulated the immune system of the respiratory tract [89]. Therefore *M. hyopneumoniae* predisposes pigs to concurrent infections with other pathogens including bacteria, parasites and viruses.

2.2. Etiology

Adhesion on *M. hyopneumoniae* to the ciliated epithelial cells is followed by the induction of ciliostasis, loss of cilia, and eventually epithelial cell death [90]. *M. hyopneumoniae* proteins have been associated with cell adhesion, including several related to the P97/P102 paralog families and other surface proteins that moonlight as adhesins [91, 92]. P97 is one of the most important adhesins of *M. hyopneumoniae* and contains two repeat regions (R1 and R2), located in the C-terminal portion. Both R1 and R2 are involved in the attachment of *M. hyopneumoniae* to the extracellular matrix of the respiratory tract [93]. Some swine extracellular matrix molecules, such as fibronectin and plasminogen, also provide binding sites for surface adhesins of *M. hyopneumoniae* [94] and extracelluar actin is also used as a surface receptor by different proteoforms of *M. hyopneumoniae* P97 adhesin and other proteins [95].

It was confirmed that *M. hyopneumoniae* can produce H_2O_2 in the presence of glycerol in vitro, but this is strain dependent and further investigation is required [96].

Lipid associated membrane proteins (LAMP) have also been implicated in the pathogenicity of mycoplasmas. They interact with the hos immune system mainly through Toll-like receptors (TLRs), such as TLR2 [97]. In *M. hyopneumoniae*, whole membrane lipoprotein fractions induced apoptosis in various cell types, including porcine peripheral blood mononuclear cells (PBMCs) [98].

2.3. Epidemiology

The main route of *M. hyopneumoniae* transmission is close contact between infected and susceptible pigs through nose-to-nose. Because in-utero transmission has not been reported, piglets are considered free from *M. hyopneumoniae*, and first exposure events occur during the lactation period by contacting with dams shedding *M. hyopneumoniae* [99]. Infection with *M. hyopneumoniae* in most herds by transmission via nose-to-nose contact from sow to pigs. So the length of the lactation period has been considered as one risk factor for infection of piglet with *M. hyopneumoniae.*

Sows and piglets in the breeding herds are considered the reservoir of *M. hyopneumoniae* infections for the entire production system. Transmission of *M. hyopneumoniae* occur between existing sow and incoming gilt, which is way to maintain the pathogen within the farm and the bacterial shedding to newborn piglets [99, 100]. In this way, addition of gilts and birth of piglets constantly provide susceptible populations needed to maintain pathogen transmission. *M. hyopneumoniae* infects individuals for long periods and there is report to be isolated from the respiratory tract for 214 days [101]. A critical aspect of the epidemiology of *M. hyopneumoniae* is based on the long pathogen persistence.

In a majority of herds, significant transmission among pen-mates begins at weaning, although *M. hyopneumoniae* can be isolated as early as 1 week of age [102]. However, overt signs of mycoplasmal

pneumonia are typically not observed in pigs under 6 weeks of age, even though all ages of pigs are susceptible [103].

Airborne transmission of *M. hyopneumoniae* has been documented. An experimental report demonstrated *M. hyopneumoniae* can travel for at least 300 m [104], and a report showed aerosol transport of *M. hyopneumoniae* for up to 9.2 km [105].

2.4. Pathogenesis and immune responses

The disease initiates by binding to the cilia of epithelial cells and colonizing of *M. hyopneumoniae* in the airways of the pig [106]. The P97 protein is involved in adherence of *M. hyopneumoniae* to cilia, as monoclonal antibodies to this protein block adherence of the organism in vitro [107]. While P97 has been recognized as important in adherence to the cilia, vaccination against this protein alone was not protective against clinical disease or colonization in vivo [108]. Colonizing of *M. hyopneumoniae* also modulate the innate and adaptive respiratory immune response [78]. This altered immune response prevents systemic spread of *M. hyopneumoniae*, but cannot rapidly clear the infection that results in chronic colonization of the airways and a prolonged pulmonary inflammatory response.

Toll-like receptor 2 (TLR2) and TLR6 are important in the recognition of *M. hyopneumoniae* by porcine alveolar macrophages [109]. The activated signal induces macrophages to produce

pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and IL-8 [111, 112]. TLR2 and TLR6 receptors were blocked, it led to less TNF-α production by macrophages. It indicates that alveolar macrophages are involved in inflammatory and innate immune responses during *M. hyopneumoniae* infection.

After experimental infection, *M. hyopneumoniae*-specific serum IgG antibodies are detected 3 - 4 weeks post-infection, peak after 11 - 12 weeks and the decrease very gradually [112]. *M. hyopneumoniae* -specific IgM can be detected as early as 9 days post-infection, peak after 14 days and rapidly decreases afterwards [113].

T cell-mediated immune responses are generally considered important for protection against *M. hyopneumoniae* [89], T cells are key in the regulation of immune responses and have a critical impact on the development of Mycoplasma induced pneumonia [114].

A study showed that the level of IFN-ɣ-secreting blood lymphocytes in vaccinated pigs is higher than non-vaccinated ones before and after experimental infection [110]. Another study for vaccination of *M. hyopneumoniae* found a lower $CD4^+/CD8^+$ ratio, and thus a higher relative number of $CD8^+$ cells in pigs vaccinated comparing to in pigs controlled, which suggest $CD8^+$ T cells have a protective role in *M. hyopneumoniae* infections [115].

2.5. Clinical signs and lesions

M. hyopneumoniae causes disease by itself, but when infected with other pathogens, it induces more complex PRDCs and acts as a promoter for other pathogens. A typical characteristic of mycoplasmal pneumonia is a dry, nonproductive cough which may last from weeks to months. Coughing is the direct result of the lung lesions observed in affected animals, which consist of purple to grey consolidated areas affecting the apical and middle lobes and cranial part of diaphragmatic lobes [116]. In mono infection, the lesions affect a smaller portion of the lungs and the parenchyma is relatively uniform in color on cut surface, and the catarrhal exudate is expressed from the airways. But in case of secondary pathogenic bacterial infection, the lesions affect a larger portion of the lungs and affected lungs are firmer and heavier, and grey-to-white mucopurulent exudate in the airways. In recovered lesions, whitish firm and thickened interlobular connective tissue formation can be observed [117]. At microscopic level, *M. hyopneumoniae* causes well-differentiated broncho-interstitial pneumonia. In the initial stages of infection, perivascular and peri-bronchial lymphoid hyperplasia, alveolar type II hyperplasia and edema fluid are observed in the alveolar space with neutrophils, macrophages and plasma cells [88]. In more chronic lesions, lymphocytic cuffs are more prominent and contain lymphoid nodules. Increased numbers of goblet cells and hyperplasia of submucosal glands may be observed in bronchi. In recovering lesions, alveoli are collapsed and/or

emphysematous, and lymphoid nodules as well as fibrosis are common in peribronchial regions [117].

2.6. Diagnosis

Although typical clinical signs and lung lesions can lead to diagnosis, a definitive diagnosis requires laboratory tests. Detection of *M. hyopneumoniae* in lung tissue can be accomplished with several techniques such as immunohistochemistry (IHC) [68], in situ hybridization (ISH) [118] and polymerase chain reaction (PCR) [119]. IHC and ISH allow for detection of *M. hyopneumoniae* on respiratory epithelium lining airways. The application of PCR has significantly increased the detection of *M. hyopneumoniae* in various sample types, and recently real-time PCR constitutes one of the most common methods for the detection of *M. hyopneumoniae*. Lung tissue, bronchial swabs, or bronchial washings are the most useful samples, while detection of *M. hyopneumoniae* from nasal swabs is more variable.

Serology is the most common tool used to determine herd status as positive or negative. Antibodies against *M. hyopneumoniae* are developed several weeks after initial infection [117] or vaccination and can be detected with ELISA test. Although based on different antigens and ELISA platforms, commercial ELISA kits are available for *M. hyopneumoniae* antibody detection, they exhibit similar

accuracy [113, 120]. They are equally unable to differentiate vaccine-induced antibodies from those of infection. There is no correlation between vaccine-induced antibody levels and protection from colonization and disease has been observed [121, 122]

2.7. Vaccines

Vaccination is a very effective and widely used tool to control the diseases associated with *M. hyopneumoniae*. Most commercial vaccines consist of inactivated whole-cell of *M. hyopneumoniae*. Vaccination can reduce clinical signs and lung lesions and improve the growth performance. Vaccines induce production of antibodies and cell-mediated immune responses [110, 115]. Although the serum *M. hyopneumoniae*-specific antibodies raised after vaccination, it is not suited to evaluate protective immunity [121].

Different vaccination strategies should adopted, depending on the type of herd, the production system, management practices and the infection pattern. Usually, piglets are exposed first from carrier sow during the lactation period and horizontal transmission among pen-mates occurs after weaning.

Table 2 shows the vaccines registered in Korea and their summary [87]. These are all inactivated whole-cell bacterin vaccines. It can be seen that the recommended administrative method and age of vaccination vary.

Table 2 *Mycoplasma hyopneumoniae* vaccines registered in Korea.

Vaccine	Manufacturer	Antigen	Usage	Administration
Hyogen	Ceva-Phylaxia	*M. hyopneumoniae* (Strain 2940)	Piglets > 3 weeks old Piglets > 1 week old	IM 2 ml once or IM 1 ml twice 2-3 week-interval
Ingelvac MycoFLEX®	Boehringer Ingelheim	*M. hyopneumoniae*	Piglets at 3-4 weeks old	IM 1 ml once
M+ PAC®	Schering-Plough (MSD)	*M. hyopneumoniae*	Piglets at 7-10 days old Piglets at 6 weeks old	IM or SC 1 ml twice 2 week-interval IM 2 ml once
MycoGard™-1 TIME	Pharmgate BIOLOGICS INC	*M. hyopneumoniae* (ATCC strain #25095)	Piglets > 2 weeks old	IM 1ml once
MYPRAVAC	HIPRA	*M. hyopneumoniae* (Strain J)	Piglets at 7-10 days old	IM 2 ml twice 3 week-interval
Porcilis® M hyo ID Once	Intervet (MSD)	*M. hyopneumoniae* (Strain 11)	Piglets at 2 weeks old	ID 0.2 ml once
Respisure ONE	Zoetis	*M. hyopneumoniae* (Strain P5722-3)	Piglets > 1week old	IM 2 ml once

3. Coinfection of PCV2 and *M. hyopneumoniae*

As mentioned earlier, PCV2 and *M. hyopneumoniae* are very important pathogens in the swine industry. These two pathogens can cause clinical lesions by themselves, but under general field conditions, they infect together with other pathogens and promote them, resulting in serious situation. In addition, these two pathogens are the causative agents of PRDC, and there have been many cases of simultaneous infection of the two pathogens.

A study of experimental dual infection demonstrates that *M. hyopneumoniae* potentiated the severity of PCV2-associated lung and lymphoid lesions, and increased the incidence of PCVAD in pigs that were innoculated with *M. hyopneumoniae* in advance and then inoculated with PCV2 weeks later [68]. Another study as same as a sequential dual infection method shows that *M. hyopneumoniae* enhanced the level of PCV2 viremia compared to PCV2 singular infection, whereas PCV2 didn't enhance the level of *M. hyopneumoniae* nasal shedding compare to *M. hyopneumoniae* singular infection [79].

Vaccines must be used appropriately for simultaneous protection against these two pathogens. PCV2 is infected usually after weaning or at the beginning of grower, while infection period of *M. hyopneumoniae* varies from lactation to grower, so it is important to accurately diagnose the farm situation first.

3.1. Bivalent vaccines

In the case of vaccines, a strategy should be established according to the farm situation and the most effective vaccine program should be operated. As labor costs accounted for a significant portion of the farm's production cost, reducing the labor force became a concern of the farm. These two pathogens must be controlled as they have a significant impact on farm productivity. For this reason, recently, there is a demand for effective vaccines against both pathogens while reducing the labor force in farms. Among commercial vaccines, there are bivalent vaccines composed of two antigens and vaccines that can be used by mixing the two vaccines as needed.

The previous bivalent vaccine used PCV2a as PCV2 antigen because vaccines were developed when PCV2a was predominant virus. PCV2d virus is increasing in the field, which is genetically closely related to PCV2b, and has been formerly called 'mutant PCV2b'. The bivalent vaccine used in these studies contains PCV2b as PCV2 antigen for the first time. Theoretically, it is expected that the PCV2b-based bivalent vaccine will be more useful than the PCV2a-based bivalent vaccine in preventing PCV2d and *M. hyopeumoniae* in pig farms.

Table 3 shows the vaccines registered in Korea [87]. There are bivalent vaccines and mixable vaccines. CircoMycoGard® is a PCV2b-based bivalent vaccine using in these studies and CircoMax Myo containing inactivated chimeric virus is the latest registered

combination vaccine. While other vaccines recommend a vaccination age of at least 3 weeks and one dose of 2 ml as same as PCV2 monovalent vaccine, CircoMycoGard® recommends administration to piglet from 10 days of age with 1 ml dose. In coinfection of PCV2 and *M. hyopneumoniae*, veterinarians recommended using each monovalent vaccine when infection of *M. hyopneumonae* was confirmed at earlier age, but if the bivalent vaccine can be administered from 10 days of age, it can be applied without change.

Table 3 Bivalent vaccines of PCV2 and *M. hyopneumoniae* registered in Korea.

Vaccine	Manufacturer	Antigen	Usage	Administration
CircoMycoGard®[1]	Pharmgate BIOLOGICS INC	PCV2b subunit (ORF2) *M. hyopnuemoniae*	Piglets > 10 days old	IM 1 ml once
CircoMax Myco (Fostera Gold PCV MH)	Zoetis	Inactivated chimeric PCV1-2a & PCV1-2b *M. hyopneumoniae* (strain P5722-3)	Piglets > 3 weeks old	IM 2 ml once or IM 1 ml twice 3 week-interval
Fostera PCV MH	Zoetis	Inactivated chimeric PCV1-2a *M. hyopneumoniae* (strain P5722-3)	Piglets >3 weeks old	IM 2 ml once
Porcilis® PCV M Hyo	Intervet (MSD)	PCV2 subunit (ORF2) Inactivated *M. hyopneumoniae* (strain J)	Piglets > 3 weeks old	IM 2 ml once
Ingelvac CircoFLEX®* Ingelvac MycoFLEX®*	Boehringer Ingelheim	PCV2a subunit (ORF2) *M. hyopneumoniae*	Piglets > 3 weeks old	IM 2 ml once

1 New bivalent vaccine, * mixable 1:1

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CHAPTER I

Efficacy evaluation of a bivalent vaccine containing porcine circovirus type 2b and *Mycoplasma hyopneumoniae* against an experimental dual challenge

Abstract

The purpose of this study was to evaluate the efficacy of a new, single-dose bivalent vaccine containing porcine circovirus type 2b (PCV2b) and *Mycoplasma hyopneumoniae* against a dual PCV2b and *M. hyopneumoniae* challenge. At -25 days post challenge (dpc, 10 days of age), one pig group (designated as the vaccinated/challenged group) received a single, 1.0 ml dose of bivalent vaccine. Pigs in both the vaccinated/challenged and unvaccinated/challenged groups were then inoculated intranasally with PCV2b and *M. hyopneumoniae* at 0 dpc (35 days of age). Pigs in vaccinated/challenged group induced significantly higher levels of neutralizing antibodies against PCV2b and cell-mediated immunity against PCV2b and *M. hyopneumonia* when compared with pigs in unvaccinated/challenged group. The vaccination of pigs with a bivalent vaccine also reduced PCV2b viremia, reduced mycoplasmal nasal shedding, and decreased the severity of both lung and lymphoid lesions for PCV2b and *M. hyopneumoniae* infection, respectively. The results of this study demonstrated that the evaluated bivalent vaccine was effective in protecting pigs against PCV2b and *M. hyopneumoniae* infection.

Keywords: *Mycoplasma hyopneumoniae*, porcine circovirus type 2, porcine respiratory disease complex

Introduction

Pneumonia caused by multiple infectious agents has been described with the term “Porcine Respiratory Disease Complex (PRDC)”. PRDC infection is categorized by reduced pig performance, increased medication costs to the producer, and an increased mortality rate during the finishing process (15 to 20 weeks of age) [1]. The etiology of PRDC is extremely diverse and occurs in both all-in-all-out as well as in continuous production systems. The main three causative agents of PRDC are porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), and *Mycoplasma hyopneumoniae*, which are known to be responsible for serious economic damages within the global pig industry [1]. Among the three, coinfection of pigs with PCV2 and *M. hyopneumoniae* is the most frequent combination in field PRDC cases and is the most rapidly increasing within the Asian pork industry [2]. Vaccination is routinely implemented to control PRDC in relation to PCV2 and *M. hyopneumoniae* infection [3]. This increase in vaccination numbers has led to a demand for single-dose bivalent vaccines containing PCV2 and *M. hyopneumoniae*. This experimental challenge study was designed to help meet this demand by evaluating the efficacy of a new bivalent PCV2b and *M. hyopneumoniae* vaccine (Circo/Myco Gard, Pharmgate Animal Health, Wilmington, NC, USA) containing killed Baculovirus vector and *M. hyopneumoniae* bacterin vaccine with a trivalent-adjuvanted formulation against an experimental challenge of PCV2b and *M. hyopneumoniae.*

Material and methods

Animals

A total of 24 colostrum-fed, cross-bred, conventional piglets were purchased at 7 days of age from a PRRSV- and *M. hyopneumoniae*-free commercial farm. The negative status of the farm was based on serological testing of the breeding herd, and long term clinical and slaughter history. Sows residing on the commercial farm were n aïve to vaccination against PCV2 and *M. hyopneumoniae*, while all piglets were vaccinated against both pathogens at 21 days of age. The pigs were weaned early at 7 days of age before vaccination of PCV2 and *M. hyopneumoniae* and selected for this study based on their seronegative results for PRRSV (IDEXX PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, Maine, USA), *M. hyopneumoniae* (*M. hyo.* Ab test, IDEXX Laboratories Inc.), and PCV2 (PCV2 Ab Mono Blocking, Synbiotics, Lyon, France). In addition, negative results for viral and mycoplasmal infections were also obtained for PCV2 and PRRSV from sera samples and for *M. hyopneumoniae* from nasal swabs as tested by real-time polymerase chain reaction (PCR) [4 - 6].

Experimental Design

A total of 24 pigs were randomly allocated into 3 groups that contained 8 piglets per groups (4 = male and 4 = female). Three rooms, uniform in design that allowed free access to feed and water troughs each contained 2 pens in facility of Seoul National University.

Four pigs were randomly assigned to the pens from each of the three groups. All randomizations were performed using the random number generator function (Excel, Microsoft Corporation, Redmond, WA, USA).

At -25 days post challenge (dpc, 10 days of age), pigs in the Vaccinated/Challenged (Vac/Ch) group were vaccinated intramuscularly on the right side of the neck with 1.0 mL of a bivalent vaccine containing PCV2b and *M. hyopneumoniae* (Circo/MycoGard, Serial No: CMG-18007, Expiration date: 02.28.2020). Pigs in the Unvaccinated/Challenged (UnVac/Ch) and Unvaccinated/Unchallenged (UnVac/UnCh) groups received a 1.0 mL injection of phosphate buffered saline (PBS, 0.01M, pH 7.4) in the same anatomical location as the Vac/Ch group.

At 0 dpc (35 days old), pigs in the Vac/Ch and UnVac/Ch groups were challenged by inoculation with PCV2b (strain SNUVR000463, GenBank no. KF871068). Five hours later, an *M. hyopneumoniae* (strain SNU98703) challenge was administered. Coinfection with PCV2b (strain SNUVR000463) and *M. hyopneumoniae* (strain SNU98703) induced severe pneumonia in lungs and lymphoid depletion in the lymph node in infected pigs [7]. The wait interval was performed to avoid the mixture of two pathogens which could have resulted in an infectivity decrease. During the PCV2b challenge, a 3 mL inoculation containing 1.2 X 10^5 (50 % tissue culture infective dose ($TCID_{50}$)/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). Then, pigs were inoculated intratracheally with 7 mL of *M. hyopneumoniae* (strain SNU98703) culture medium containing 10^7 color changing units (CCU)/mL. All study methods were approved previously by the Seoul National University Institutional Animal Care and Use, and Ethics Committee (SNU-181018-8-2).

At 21 dpc (56 day old), all pigs were sedated with an intravenous injection of sodium pentobarbital prior to euthanasia by electrocution as previously described [8]. Tissues were collected from each pig at necropsy. Tissue preparation included fixation in a 10% neutral buffered formalin solution for 24 hours before they were routinely processed and embedded in paraffin.

Clinical Observations

Pigs were monitored daily and scored weekly for clinical signs as previously described [9]. Scores ranged from 0 to 6: 0 = normal; 1 = rough haircoat; 2 = rough haircoat and dyspnea; 3 = mild dyspnea and abdominal breathing; 4 = moderate dyspnea and abdominal breathing; 5 = severe dyspnea and abdominal breathing; 6 = death.

Growth Performance

Pigs were weighed at 10 (-25 dpc) and 56 (21 dpc) days of age. Average daily gain (ADG) was calculated as the difference between

the starting and final weight divided by the number of days spanning the duration of the stage, and included data for pigs that died or were removed from the study.

Quantification of *M. hyopneumoniae* DNA in the Nasal Swab

Blood and nasal swabs were collected from all pigs at -25, -14, 0, 7, 14, and 21 dpc. A commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA) was use to extract DNA from serum samples and nasal swabs. The number of genomic DNA copies for *M. hyopneumoniae* was quantified by real-time PCR [4]. To construct a standard curve, real-time PCR was performed in quadruplicate in 10-fold serial dilution of chromosomal DNA from *M. hyopneumoniae*s strain SNU98703, with concentrations ranging from 10 ng/mL to 1 fg/mL. One fetogram of chromosomal DNA from *M. hyopneumoniae* is considered to be approximately one genome equivalent [10]. A negative control was included in each run using double distilled water as the template.

Quantification of PCV2b in the Blood

The number of genomic DNA copies for PCV2b was quantified by real-time PCR [3]. To construct a standard curve, real-time PCR was performed in quadruplicate in two different assays: (i) 10-fold serial dilutions of the PCV2b plasmid were used as the standard, with concentrations ranging from 10^{10} to 10^{2} copies/mL, and (ii) 10-fold serial dilutions of PCV2b cultured in PCV1-free PK-15 cells were

used at concentrations ranging from $10^{4.5}$ $TCID_{50}$/mL to $10^{-3.5}$ $TCID_{50}$/mL. The PCV2b plasmid was prepared as described previously [3]. Culture supernatants of PCV1-free PK-15 cells were used as negative control.

Serology

The presence of *M. hyopneumoniae* and PCV2 antibodies were evaluated in serum samples by use of commercially available enzyme-linked immunosorbent assay (ELISA) kits (M. hyo Ab test, IDEXX Laboratories Inc and SERELISA PCV2 Ab Mono Blocking, Synbiotics). Testing was conducted in accordance with each manufacturer's kit instructions, where samples were considered as positive for *M. hyopneumoniae* antibody if the sample-to-positive (S:P) ratio was $\geq$ 0.4 and as positive for PCV2 antibodies if the reciprocal ELISA titer was > 350. Serum samples were also tested for serum virus neutralization against PCV2b [11].

Enzyme-Linked Immunospot Assay

Inactivated *M. hyopneumoniae* and PCV2b antigens using challenge strains for *M. hyopneumoniae* and PCV2b were prepared for enzyme-linked immunospot (ELISpot) assay as previously described [12, 13]. An ELISpot assay was conducted to measure the numbers of *M. hyopneumoniae*- and PCV2b-specific interferon-γ secreting cells (IFN-γ-SC). Peripheral blood mononuclear cells (PBMC) were stimulated with inactivated *M. hyopneumoniae* and PCV2b antigens

and results reported as the number of IFN-ɣ-SC per million PBMC [12, 14].

Phytohemagglutinin (Roche Diagnostics GmbH, Mannheim, Germany) and PBS used as a positive and negative control, respectively.

Pathology

Lung lesion scoring was performed for *M. hyopneumoniae* infection and lymphoid lesion for PCV2b infection by two veterinary pathologists. Severity of lung lesion was scored (0 to 6) based on peribronchiolar and perivascular lymphoid tissue hyperplasia [15]. Severity of lymphoid lesion was scored (0 to 5) based on lymphoid depletion and granulomatous inflammation [16].

Statistical Analysis

All real-time PCR data was transformed to log10 values prior to statistical analysis. The Shapiro-Wilk test evaluated data for normal distribution. One-way analysis of variance (ANOVA) was used to examine whether there were statistically significant differences at each time point within the three groups. 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 Tukey's adjustment. The Kruskal-Wallis test was performed if the normality assumption was not met. Kruskal Wallis test results which showed a statistical significance were further evaluated with the Mann-Whitney test to include Tukey's adjustment to compare the differences among

the groups. The Pearson's correlation coefficient was used to assess the correlation of PCV2b viremia with neutralizing antibody titers against PCV2b, PCV2b viremia with PCV2b-specific IFN-ɣ-SC, and nasal shedding of *M. hyopneumoniae* and *M. hyopneumoniae*-specific IFN-ɣ-SC. Results were reported in P-value where a value of $P < 0.05$ was considered to be significant.

Results

Clinical Observations

The mean scores for respiratory disease were significantly lower ($P < 0.05$) in pigs from the Vac/Ch group (mean ± standard deviation, 0.38 ± 0.52 for 7 dpc, 0.75 ± 0.71 for 14 dpc, and 0.63 ± 0.52 for 21 dpc) when compared with the UnVac/Ch group (mean ± standard deviation, 1.00 ± 0.53 for 7 dpc, 2.13 ± 0.83 for 14 dpc, and 2.13 ± 0.83 for 21 dpc) at 7, 14, and 21 dpc. Pigs from the Group 3 remained normal throughout the experiment.

Growth Performance

The body weight of the pigs did not differ significantly among 3 groups at study day 0 (the time of vaccination, 10 days of age). Pigs from the Vac/Ch (mean ± standard deviation, 301.1 ± 11.3) and UnVac/UnCh (mean ± standard deviation, 309.2 ± 4.8) groups had significantly higher ($P < 0.05$) ADG (unit = gram/pig/day) between 10 and 56 days of age when compared with those from UnVac/Ch (mean ± standard deviation, 277.5 ± 16.4) group.

Quantification of *M. hyopneumoniae* and PCV2b DNA

Nasal swabs evaluation between 7 to 21 dpc reported significantly less ($P < 0.05$) *M. hyopneumoniae* genomic copies in pigs from the Vac/Ch group when compared with the UnVac/Ch group (Fig. 1A). Blood sample evaluation from the same timeframe (7 to 21 dpc) also reported significantly less ($P < 0.05$) PCV2d genomic copies in the

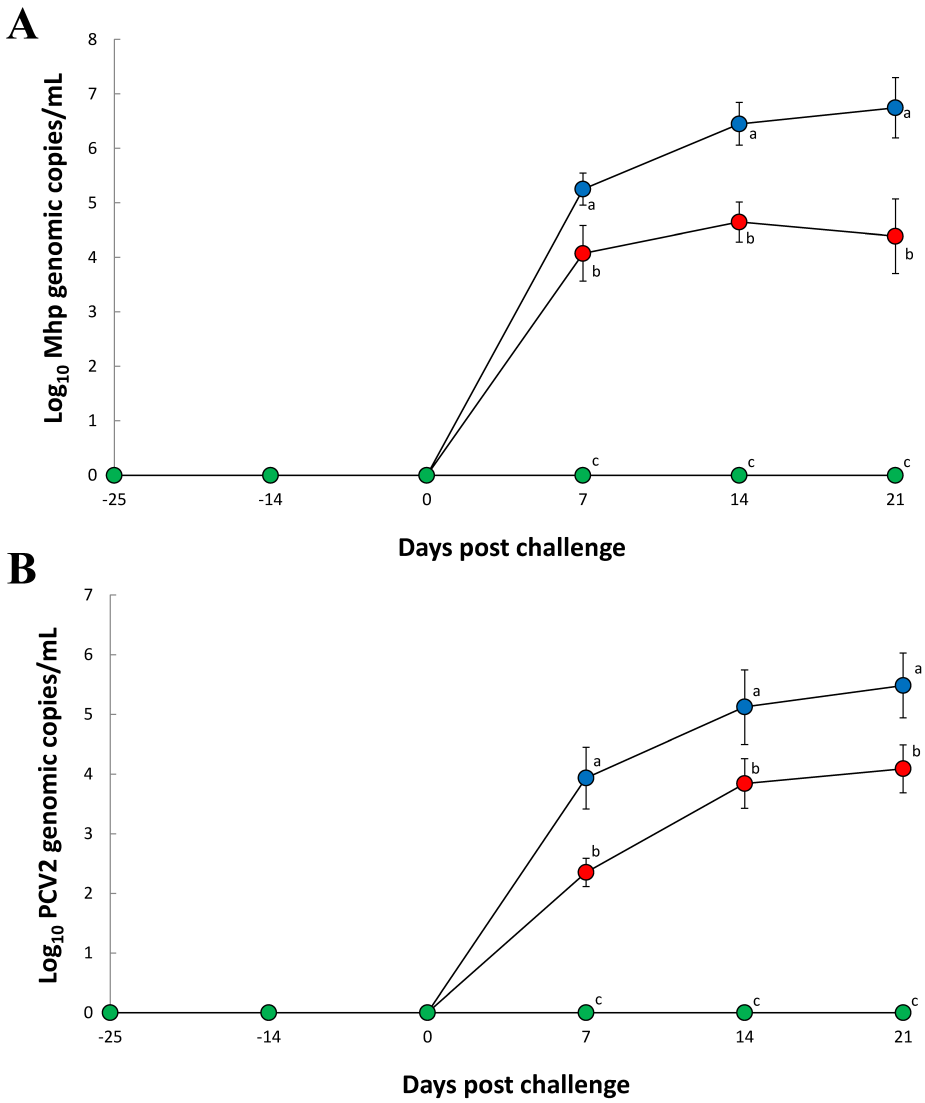

FIGURE 1 Mean values of the genomic copy number of *Mycoplasma hyopneumoniae* DNA in nasal swabs (A) and porcine circovirus type 2b in blood (B) from Vac/Ch (●), UnVac/Ch (●), and UnVac/UnCh (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 3 groups.

Vac/Ch group when compared with the UnVac/Ch group (Fig. 1B). No *M. hyopneumoniae* or PCV2 were detected in the pigs from the UnVac/UnCh group.

Serology

Pigs in the Vac/Ch group had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratio in their -14 to 21 dpc serum samples when compared with the UnVac/Ch group (Fig. 2A). Pigs in the Vac/Ch group had a significantly higher ($P < 0.05$) PCV2 ELISA titer from -14, to 21 dpc in their serum samples when compared with the UnVac/Ch group (Fig. 2B). Pigs in the Vac/Ch group had significantly higher ($P < 0.05$) neutralizing antibody titers against PCV2b from -14 to 21 dpc when compared with the UnVac/Ch group (Fig. 2C). There was a correlation between number of genomic copies of PCV2b in the blood and neutralizing antibody titers against PCV2b ($r = -0.810$, $P = 0.015$). *M. hyopneumoniae* and PCV2 antibodies were not detected in pigs from the UnVac/UnCh group.

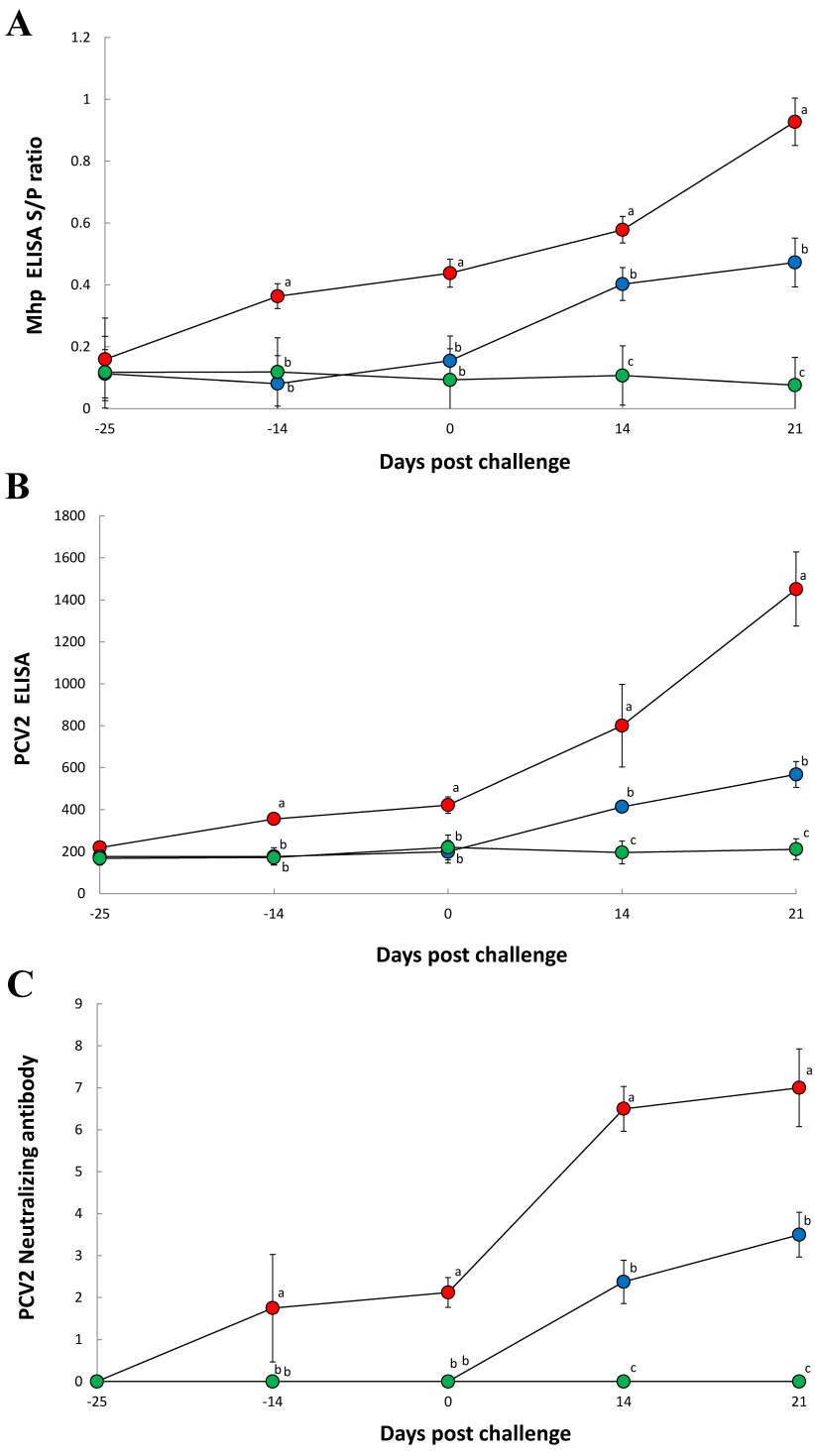
A
Mhp ELISA S/P ratio
1.2
1
0.8
0.6
0.4
0.2
0
-25
-14
0
14
21
Days post challenge
B
PCV2 ELISA
1800
1600
1400
1200
1000
800
600
400
200
0
-25
-14
0
14
21
Days post challenge
C
PCV2 Neutralizing antibody
9
8
7
6
5
4
3
2
1
0
-25
-14
0
14
21
Days post challenge

FIGURE 2 ELISA antibody levels of *Mycoplasma hyopneumoniae*(A), ELISA antibody levels of porcine circovirus type 2 (B), and neutralizing antibody titers against porcine circovirus type 2b (C) in serum from Vac/Ch (●), UnVac/Ch (●), and UnVac/UnCh (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 3 groups.

Enzyme-Linked Immunospot Assay

Pigs in the Vac/Ch group had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*- (Fig. 3A) and PCV2b- (Fig. 3B) specific IFN-ɣ-SC in their PBMC from -14, 0, 14, and 21 dpc when compared with the UnVac/Ch group. There was a correlation between the number of genomic copies of *M. hyopneumoniae* in the nasal swabs and numbers of *M. hyopneumoniae*-specific IFN-ɣ-SC ($r = -0.758$, $P = 0.029$). *M. hyopneumoniae* and PCV2b-specific IFN-ɣ-SC were not detected in pigs from the UnVac/UnCh group.

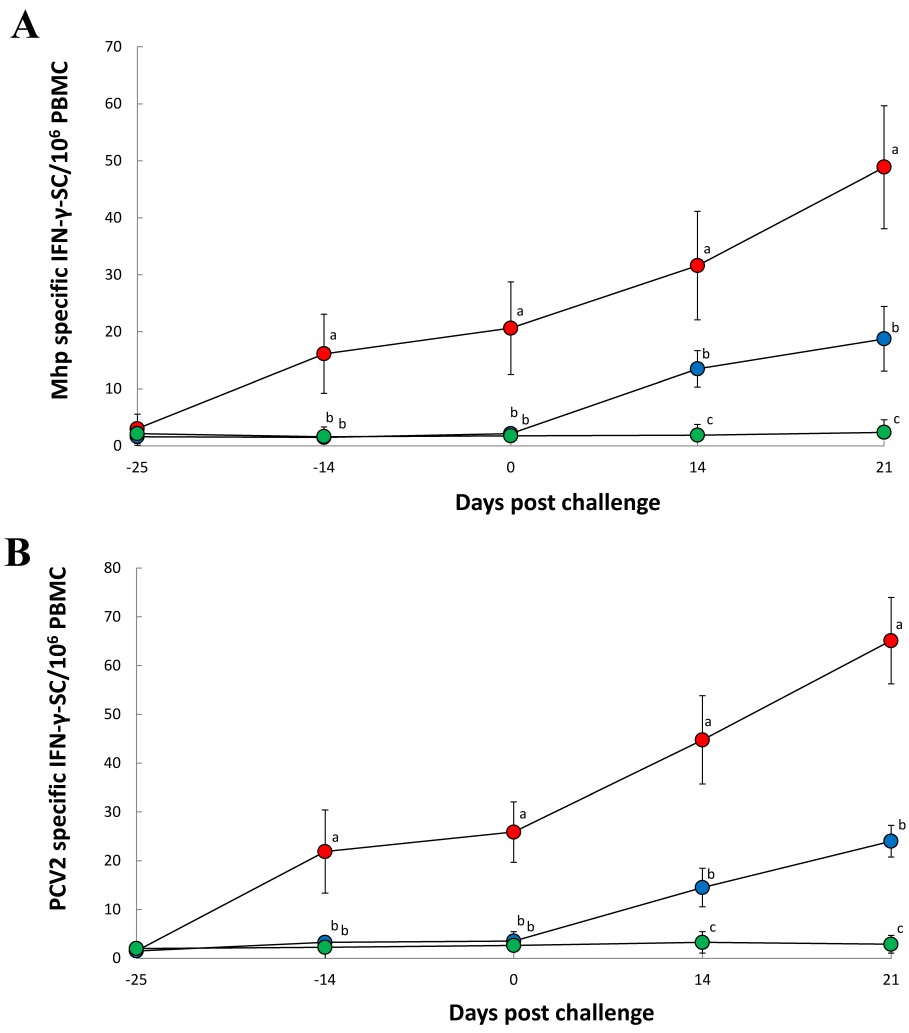

FIGURE 3 Frequency of *Mycoplasma hyopneumoniae*-specific interferon-ɣ secreting cells (IFN-ɣ-SC) (A) andporcine circovirus type 2b-specific IFN-ɣ -SC (B) in peripheral blood mononuclear cells (PBMC) from Vac/Ch (●), UnVac/Ch (●), and UnVac/UnCh (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 3 groups.

Pathology

Pigs in the Vac/Ch group had significantly lower ($P < 0.05$) macroscopic (mean ± standard deviation, 17.4 ± 5.3) and microscopic (mean ± standard deviation, 0.9 ± 0.34) lung lesion scores for *M. hyopneumoniae* infection at 21 dpc when compared with the UnVac/Ch group with macroscopic (mean ± standard deviation, 45.8 ± 3.2) and microscopic (mean ± standard deviation, 3.5 ± 0.4) lung lesion scores (Table 1). Pigs in the Vac/Ch group also had significantly lower ($P < 0.05$) microscopic lymphoid lesions scores (mean ± standard deviation, 0.7 ± 0.2) for PCV2b infection at 21 dpc when compared with the UnVac/Ch group with microscopic lymphoid lesions scores (mean ± standard deviation, 3.5 ± 0.5). Macroscopic and microscopic lung lesions, and microscopic lymphoid lesions were not observed in pigs from the UnVac/UnCh group (Table 1).

Table 1 Lung lestion score for *M. hyopneumoniae* and lymphoid lesion score for PCV2

	Lung lesion score for *M. hyopneumoniae*		Lymphoid lesion score for PCV2
	Macroscopic	Microscopic	Microscopic
Vac/Ch	17.4 ± 5.3*	0.9 ± 0.34*	0.7 ± 0.2*
UnVac/Ch	45.8 ± 3.2	3.5 ± 0.4	3.5 ± 0.5

*Significant difference ($P < 0.05$) between Vac/Ch group and UnVac/Ch group.

Discussion

The study results demonstrated that the evaluated bivalent vaccine containing PCV2b and *M. hyopneumoniae* against a dual challenge of PCV2b and *M. hyopneumoniae* is efficacious in protecting pigs. The evaluation of growth performance was identified as the critical factor in determining the efficacy of this bivalent vaccine as PCV2 and *M. hyopneumoniae* coinfection is mainly characterized by poor growth performance. Vaccination of pigs with the evaluated bivalent vaccine resulted in improved growth performance when compared with unvaccinated pigs.

The bivalent vaccine tested in this study was administered to piglets at 10 days of age. There was potential, therefore, for interference with maternally derived antibodies. In this study, interference by maternally derived antibodies was not evaluated as pigs tested as seronegative against PCV2 prior to study initiation. It can be deduced, however, maternally derived antibodies did not significantly interfere with the induction of both humoral and cell-mediated immunity in piglets post PCV2 vaccination [17].

M. hyopneumoniae is known to have a potentiating effect on the level of PCV2 viremia in pigs coinfected with *M. hyopneumoniae* and PCV2 [15]. The reduction of PCV2b viremia was therefore an effect of bivalent vaccination but not contribute to an immunosuppressive effect of *M. hyopneumoniae* infection in vaccinated pigs challenged with PCV2b and *M. hyopneumoniae*.

Neutralizing antibodies and IFN-γ-SC are responsible for the

reduction of PCV2 viremia [18 – 20], where these levels are considered measurements of protective immunity [19, 20]. The amount of neutralizing antibodies present correlated with the PCV2b viremia reduction in the present study, while frequency of IFN-ɣ-SC did not. On other hands, it should be noted that the protective immunity mechanism against *M. hyopneumoniae* is not fully understood, and therefore caution should always be exercised with evaluating it against study conclusions. Cell-mediated immunity has proven to play an important role in controlling *M. hyopneumoniae* infection [21]. In the present study, cell-mediated immunity as measured by IFN-ɣ-SC correlated with a significant reduction in the amount of *M. hyopneumoniae* loads through nasal shedding. This study demonstrated that pig vaccination and challenge induced high levels of protective immunity, reduced the amount of PCV2b viremia and severity of lymphoid lesions, and reduced the amount of *M. hyopneumoniae* nasal shedding and severity of lung lesions.

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CHAPTER II

Field evalustion of a single-dose bivalent vaccine of porcine circovirus type 2b and *Mycoplasma hyopneumoniae*

Abstract

The field efficacy of a bivalent vaccine containing porcine circovirus type 2b (PCV2b) and *Mycoplasma hyopneumoniae* was evaluated on three pig farms.

Three pig farms were used, two of which had a history of subclinical PCV2 and clinical *M. hyopneumoniae* infections between 84 and 126 days of age while concurrent porcine circovirus-associated disease and clinical *M. hyopneumoniae* infection between 70 and 105 days of age. Each farm vaccinated pigs with a single dose of a bivalent vaccine at 10 days of age while unvaccinated pigs were administered a single dose of phosphate buffered-saline at the same age.

Vaccination improved growth performance and reduced clinical scores significantly ($p < 0.05$) when compared with unvaccinated animals. The amount of PCV2d loads in blood and *M. hyopneumoniae* loads in nasal swabs of vaccinated animals were also significantly lower ($p < 0.05$) when compared with unvaccinated animals. Immunologically, vaccinated groups elicited a significantly higher ($p < 0.05$) level of protective immunity against PCV2d such as neutralizing antibodies and interferon-ɣ secreting cells (IFN-ɣ-SC), as well as protective immunity against *M. hyopneumoniae* such as IFN-ɣ-SC when compared with unvaccinated animals. Pathologically, vaccination significantly lowered ($p < 0.05$) the scores of *M. hyopneumoniae*-induced pneumonia and PCV2-associated lymphoid lesions when compared with unvaccinated animals.

The evaluated bivalent vaccine provided good protection against PCV2d and *M. hyopneumoniae* infection under field conditions.

KEYWORDS

Enzootic pneumonia, *Mycoplasma hyopneumoniae*, Porcine circovirus type 2, Porcine circovirus-associated disease, Subclinical porcine circovirus type 2 infection

Introduction

Porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* are two worldwide economically important dominating pathogens. PCV2 is a very small DNA virus which can be divided into at least eight different genotypes ('a' to 'h') based on its sequence identity in open reading frame 2 (ORF2) [1 - 4]. Currently, PCV2d is the pre-dominant genotype in Asia and North America [2]. PCV2 causes different clinical diseases and syndromes which are collectively referred to as porcine circovirus-associated disease (PCVAD). In Korea, PCV2 infection is so widespread that essentially all pig herds are infected with the pathogen. The decrease in PCVAD outbreaks since 2008 is attributed to the successful introduction of efficacious PCV2 vaccines to the market. Near or fully-asymptomatic subclinical PCV2 infection remains the most common disease, leading to poor growth that increases days to market as described by other countries [5 - 8].

Mycoplasmas including *M. hyopneumoniae*, are the smallest self-replicating bacteria and are distinguished by the lack of a cell wall that is present in most bacteria [9]. *M. hyopneumoniae* is the primary causative agent for enzootic pneumonia; one of the most widespread and costly diseases in the swine respiratory disease worldwide today. It is characterized by significant economic losses due to slower growth and poor feed conversion.

Coinfection with PCV2 and *M. hyopneumoniae* is one of the most devastating and damaging combinations of pig diseases. Vaccination against PCV2 and *M. hyopneumoniae* is one of the most cost-effective strategies and is widely used in Asian pork production. A new bivalent vaccine containing PCV2b and *M. hyopneumoniae* (Circo/MycoGard, Pharmgate Animal Health, Wilmington, NC, USA) was first introduced into the Asian market. In particular, this bivalent vaccine is clinically interesting because it contains the PCV2b genotype. Genetically, PCV2b and PCV2d (initially called a mutant of PCV2b) are more closely related than PCV2a and PCV2d [4]. The objective of this study was to evaluate the new single-dose bivalent vaccine containing PCV2b and *M. hyopneumoniae* based on clinical, microbiological, immunological, and pathological analysis under field conditions in accordance with the registration guidelines of the Republic of Korea's Animal, Plant & Fisheries Quarantine & Inspection Agency (QIA, http://www.qia.go.kr).

Materials and methods

Farm history

The clinical field trial was conducted on three farms (A, B, and C) between May-October of 2019. Farms A, B, and C were each a 380-sow, 160-sow, and 430-sow, (respectively) farrow-to-finish swine farm with an all-in-all-out production system. All three farms are located in Chungcheongnam-do. Sows from these selected farms had not received vaccines against PCV2 or *M. hyopneumoniae*, while all piglets from this production system were immunized against both pathogens. Farms A and C were selected based on history of concurrent subclinical PCV2 infection and enzootic pneumonia. Herds approximately 12 - 18 weeks of age displayed subclinical PCV2 infection and enzootic pneumonia characterized by respiratory signs and growth retardation which were mainly associated with a marked increase in the mortality rate (approximately 7 % and 5 % of Farms A and C, respectively) from 21 to 140 days of age. Lungs were examined at the slaughterhouse where it was confirmed that 12 out of 20 Farm A pigs and 15 out of 20 Farm C pigs had mycoplasmal pneumonia lesions based on scoring from a previous method [10]. Submitted cases from two farms met the definition of subclinical PCV2 infection [8] including decreased average daily gain without overt clinical signs, no or minimal histopathological lesions in inguinal lymph nodes, and the presence of low amounts of PCV2 in inguinal lymph nodes by immunohistochemistry in 4 out of 5 suspected pigs

from Farm A, and 5 out of 5 suspected animals on Farm C. Histopathologicial mycoplasmal lung lesions were observed in three of the five pigs submitted from Farm A, and four of the five pigs submitted from Farm C. Farm B was selected based on a history of concurrent clinical PCVAD and enzootic pneumonia. Farm C swine displayed clinical signs of PCVAD and *M. hyopneumoniae* infection characterized by wasting, respiratory signs, and growth retardation which were mainly associated with a marked increase in the mortality rate (approximately 13 %) at approximately 13 - 18 weeks of age. A lung examination was performed at the slaughterhouse, which confirmed that 8/10 pigs had mycoplasmal pneumonia lesions. Submitted cases from Farm B met the definition of PCVAD [11] including wasting and growth retardation, lymphoid granulomatous inflammation with grape-like intracytoplasmic inclusion bodies, and the presence of PCV2 antigen in lymphoid lesions by immuno-histochemistry in 4 out of 5 suspected animals.

Clinical field study design

The experimental design for the field study strictly adhered to the guidelines set by QIA. QIA guidelines required that 20 piglets (male = 10 and female = 10) were selected and assigned to each group of vaccinated and unvaccinated animals. In an effort to minimize sow variation, four to six 7-day-old piglets were randomly selected from each sow and assigned evenly to either the vaccinated or unvaccinated group using the random number generation function in

Excel (Microsoft Corporation, Redmond, WA, USA). The pigs in the vaccinated (VacA, VacB, and VacC) groups were injected intramuscularly in the right side of the neck with 1.0 mL of the bivalent vaccine containing PCV2b and *M. hyopneumoniae* (Circo/MycoGard, Serial No: CMG-18007, Expiration date: 02.28.2020, Pharmgate Animal Health) at 10 days of age. An equal volume of phosphate buffered saline (PBS, 0.01M, pH 7.4) was injected in the same anatomical location in pigs of the unvaccinated (UnVacA, UnVacB, and UnVacC) groups. Both vaccinated and unvaccinated pigs were comingled before they were randomly distributed into 4 different pens kept within 1 room. Each pen contained 10 pigs with a similar proportion of each treatment per pen. Pens were identical in design and equipment which included free access to a feed and water trough. Standard farm procedures were followed regarding the feeding and watering of study animals. Blood and nasal swabs were collected at 0 (10 days of age), 18 (28 days of age), 39 (49 days of age), 81 (91 days of age), and 102 (112 days of age) days post vaccination (dpv). All methods used in this study were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-180625-3).

Clinical observations

Pigs were monitored and scored weekly for clinical signs as previously described [12]. Briefly, scoring was defined as follows 0 (normal), 1 (rough haircoat), 2 (rough haircoat and dyspnea), 4

(severe dyspnea and abdominal breathing), and 6 (death). Observers were blinded to vaccination status.

Growth performance

The live weight of each pig was measured at 10 (0 dpv), 70 (60 dpv), 112 (102 dpv), and 175 (165 dpv) days of age. The average daily weight gain (ADWG; gram/pig/day) was analyzed over three time periods: (1) between 10 and 70 days of age, (2) between 70 and 112 days of age, and (3) between 112 and 175 days of age. ADWG during the different production stages was calculated as the difference between the starting and final weight divided by the duration of the stage. Data for dead or removed pigs were included in the calculation.

Quantification of PCV2d DNA in blood

DNA was extracted from serum samples by use of a commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA). Real-time PCR was used to quantify the number of genomic DNA copies for PCV2d [13].

Quantification of *M. hyopneumoniae* in nasal swabs

DNA was extracted from nasal swabs by use of a commercial kit (QIAamp DNA Mini Kit, QIAGEN). Real-time PCR was used to quantify the number of genomic DNA copies for *M. hyopneumoniae* were quantified by real-time PCR [14].

Serology

Enzyme-linked immunosorbent assay (ELISA) was used to test for both PCV2 and *M. hyopneumoniae* antibodies with commercial ELISA kits (SERELISA PCV2 Ab Mono Blocking, Synbiotics, Lyon, France, and *M. hyo* Ab test, IDEXX Laboratories Inc. Westbrook, ME, USA). Serum 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 in accordance with the manufacturer's instructions for each kit. Serum samples were also tested for neutralizing antibodies (NA) against PCV2d [15].

Enzyme-linked immunospot assay

Enzyme-linked immunospot assay was used to measure the numbers of *M. hyopneumoniae* and PCV2d-specific interferon-ɣ secreting cells (IFN-ɣ-SC). Peripheral blood mononuclear cells (PBMC) were stimulated using the aforementioned challenge *M. hyopneumoniae* and PCV2d strains [16] with results reported as the numbers of IFN-ɣ-SC per million PBMC.

Pathology

The severity of macroscopic lung lesions was scored by two pathologists (Chae and one graduate student) at the Seoul National University (Seoul, Republic of Korea) to estimate the percentage of the lung affected by pneumonia. Scoring was performed 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) [17].

Collected lung and lymphoid tissue sections were examined by two blinded veterinary pathologists (Chae and one graduate student). The severity of peribronchiolar and perivascular lymphoid tissue hyperplasia was assessed by scoring mycoplasmal pneumonia lesions (0 to 6) [18]. Mycoplasmal pneumonia lesions were confirmed by real-time PCR from lung lesions [14]. The severity of lymphoid lesions were scored (0 to 5) based on the severity of lymphoid depletion and granulomatous inflammation [19].

Immunohistochemistry

Immunohistochemistry for PCV2 was performed as previously described [20]. For the morphometric analyses of immuno-histochemistry, 3 sections were cut from each of three blocks of tissue from lymph node of each pig. The slides were analyzed using the NIH Image J 1.45s Program (http://imagej.nih.gov/ij/download.html) to obtain the quantitative data. For the analysis of PCV2, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm^2) was determined as previously described [21]. The mean values were also calculated.

Statistical analysis

Prior to statistical analysis, real-time PCR and neutralizing antibody data were transformed to log10 and log2 values, respectively. Data was tested for the normal distribution using the Shapiro-Wilk test and either the Student's t-test or Mann-Whitney test were used to examine whether significant statistical differences existed between the two groups at each time point. The student's t-test was conducted to compare the difference between the two groups, only if the normality assumption was met, while the Mann-Whitney test was performed to compare the differences between the two groups when the normality assumption was not met. A value of $P < 0.05$ was considered to be significant.

Results

Clinical evaluation

The vaccinated group on Farm A had the significantly lower ($p < 0.05$) clinical scores between 25 and 74 dpv when compared with the unvaccinated group. Clinical scores were significantly lower ($p < 0.05$) in the Farm B vaccinated group between 32 and 74 dpv, and at 95 and 102 dpv when compared with the unvaccinated group. Farm C clinical scores were significantly lower ($p < 0.05$) between 32 and 95 dpv in the vaccinated group when compared with the unvaccinated group (Fig. 1).

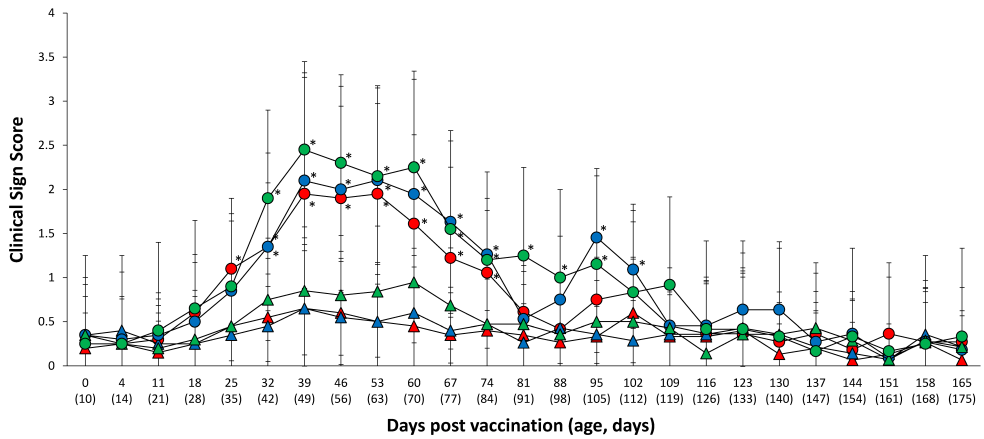

FIGURE 1 Mean respiratory score from VacA (▲) and UnVacA (●), VacB (▲) and UnVacB (●), and VacC (▲) and UnVacC (●) groups. Variation is expressed as the standard deviation. *Significant difference ($p < 0.05$) between vaccinated and unvaccinated group within the same farm.

Growth performance

A significant difference ($p < 0.05$) in the ADWG was observed on Farm A between vaccinated and unvaccinated group between 10 and 70, between 70 and 112, and between 112 and 175 days of age. The ADWG in the Farm B vaccinated group during the 10 - 70 and 112 - 175 days period was significantly higher ($p < 0.05$) than that of the unvaccinated group. The overall growth performance of all three farms (10 to 175 days of age) of vaccinated groups was significantly higher ($p < 0.05$) than that of unvaccinated group (Table 1).

Mortality

Farm A mortality included one unvaccinated pig that died of suppurative leptomeningitis as determined by the isolation of *Streptococcus suis* from the meninges at 63 days of age, and one unvaccinated pig that died of Glasser's disease as determined by the isolation of *Glaesserella parasuis* from the pericardium at 70 days of age. Two additional unvaccinated pigs died of enzootic pneumonia as determined by a combination of *M. hyopneumoniae* that was detected with PCR and *Pasteurella multocida* that was isolated from the lungs at 92 and 117 days of age. Causes of death on Farm B included one vaccinated pig that died of hemorrhagic enteritis by unknown etiology at 84 days of age, and one unvaccinated pig that died of colibacillosis that was determined by the isolation of *Escherichia coli* from the small intestine at 53 days of age. Two additional unvaccinated pigs died of suppurative bronchopneumonia and *M. hyopneumoniae* that

Table 1

Average daily weight gain (ADWG), mortality rate, and pathology between vaccinated (Vac) and unvaccinated (UnVac) animals on 3 Farms.

	Age (day)	Farm A		Farm B		Farm C	
		VacA	UnVacA	VacB	UnVacB	VacC	UnVacC
ADWG (gram/pig/day)							
	10-70	369 ± 20*	344 ± 22	375 ± 20*	350 ± 26	360 ± 27	343 ± 29
	70-112	751 ± 37*	720 ± 25	738 ± 36	733 ± 30	770 ± 43	761 ± 59
	112-175	774 ± 28*	748 ± 37	771 ± 28*	742 ± 36	741 ± 36	730 ± 31
	10-175	620 ± 10*	594 ± 14	620 ± 15*	600 ± 15	610 ± 27*	519 ± 24
Initial body weight (kg)	10	3.3 ± 0.3	3.4 ± 0.2	3.3 ± 0.4	3.4 ± 0.3	3.3 ± 0.4	3.3 ± 0.4
Market body weight (kg)	175	105.6 ± 1.6*	101.4 ± 2.4	105.5 ± 2.4*	102.1 ± 2.5	104.1 ± 1.1*	101.5 ± 2.4
Mortality rate		0/20	4/20	1/20	4/20	1/20	3/20
Lung lesion score							
Macroscopic	175	12 ± 4.06*	42 ± 10.95	15 ± 2.71*	48 ± 7.76	16 ± 5.24*	53± 14.60
Microscopic	175	0.8 ± 0.28*	2.5 ± 0.83	0.8 ± 0.33*	3.0 ± 0.50	1.3 ± 0.33*	2.7 ± 0.54
Lymphoid lesion score	175	0.4 ± 0.41*	1.6 ± 0.43	1.0 ± 0.48*	2.3 ± 0.44	0.9 ± 0.54*	1.7 ± 0.41
PCV2-antigen positive cells	175	3 ± 1.08*	7.6 ± 6.14	4.1 ± 1.34*	7.6 ± 3.1	4.6 ± 2.13*	16 ± 4.40

*Significant difference ($P < 0.05$) between vaccinated and unvaccinated groups within the same farm.

was detected by PCR and *Trueperella pyogenes* that was isolated from the lungs at 89 and 90 days of age. A fourth unvaccinated pig died of pneumonia, as determined by *M. hyopneumoniae* detection with PCR. PCV2 was also detected by immunohistochemistry methodology in this fourth unvaccinated pig at 104 days of age. Farm C mortality included one vaccinated pig that died of exudative epidermitis that was determined by the isolation of *Staphylococcus hyicus* from skin at 62 days of age, and one unvaccinated pig that died of pneumonic pasterellosis that was determined by the isolation of *P. multocida* from the lung at 93 days of age. A second unvaccinated pig died of pneumonia complications. *M. hyopneumoniae* was detected by PCR along with PCV2 as detected with immuno-histochemistry methodology at 101 days of age in the second unvaccinated pig. A third and final unvaccinated pig died of suppurative bronchopneumonia, where *M. hyopneumoniae* was determined as the causative agent through PCR. *T. pyogenes* was also isolated from the lung of this third unvaccinated pig at 111 days of age.

Quantification of PCV2d DNA in blood

PCV2 DNA was not detected in the blood of either vaccinated or unvaccinated animals at 0 and 18 dpv at any of the three farms. PCV2d was detected in blood at 39 dpv (49 days of age) at all three farms. Vaccinated pigs from Farms A and C had a significantly lower ($p < 0.05$) number of genomic copies of PCV2d in their blood

at 39 dpv compared to that of unvaccinated animals. Vaccinated pigs from all three farms had a significantly lower ($p < 0.05$) number of genomic copies of PCV2d in their blood between 81 and 102 dpv compared to that of unvaccinated animals (Fig. 2a).

Quantification of *M. hyopneumoniae* DNA in nasal swabs

M. hyopneumoniae DNA was not detected in nasal swabs from either vaccinated or unvaccinated animals at 0 and 18 dpv from any of the three farms. Vaccinated animals had a significantly lower ($p < 0.05$) number of *M. hyopneumoniae* genomic copies in their nasal swabs when compared with unvaccinated animals at 39 and 81 dpv (Farm A), and 81 dpv (Farms B and C) (Fig. 2b).

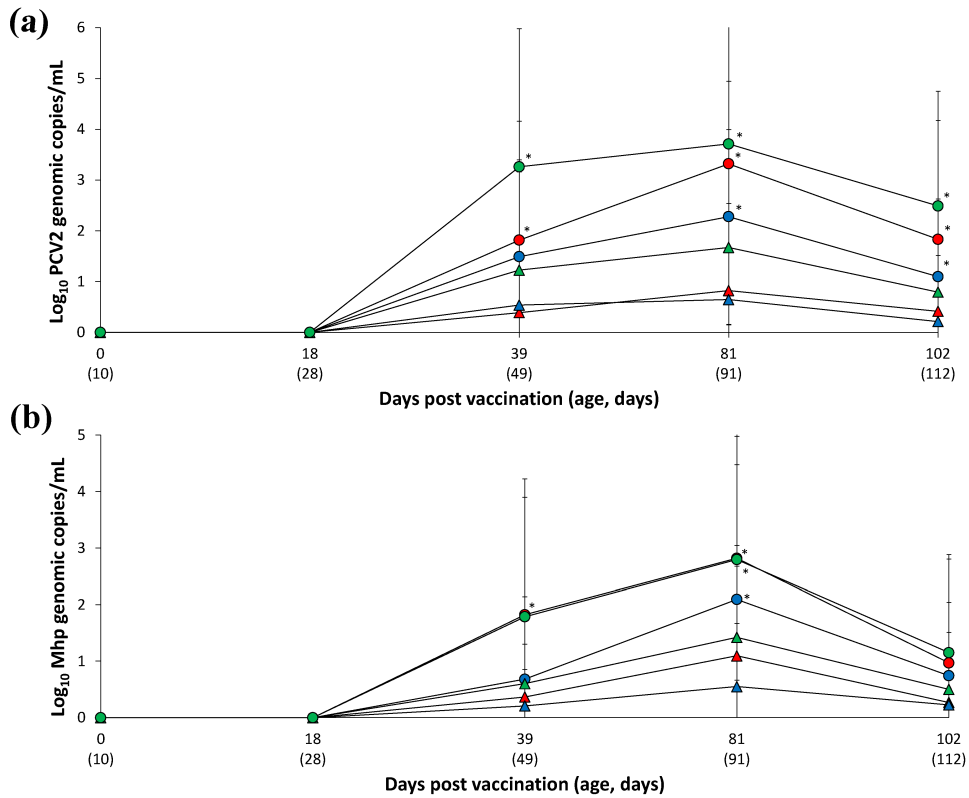

FIGURE 2 Mean values of the genomic copy number of PCV2d DNA in serum of pigs (a) and of *M hyopneumoniae* DNAin nasal swab of pigs (b) from VacA (▲) and UnVacA (●), VacB (▲) and UnVacB (●), and VacC (▲) and UnVacC (●) groups. Variation is expressed as the standard deviation. ∗Significant difference ($p < 0.05$) between vaccinatedand unvaccinated group within the same farm.

Immunological responses against PCV2

At the time of vaccination (10 days of age; 0 dpv), significant differences between vaccinates and non-vaccinates were not detected at any of the three farms in regard to anti-PCV2 antibodies. The PCV2 ELISA titers (Fig. 3a) and PCV2-specific NA (Fig. 3b) were significantly higher ($p < 0.05$) in the vaccinated group when compared with the unvaccinated group at 18 - 102 dpv in three farms. The mean frequencies of PCV2-specific IFN-ɣ-SC remained at basal levels (< 20 cells/10^6 PBMC) in both groups until 0 dpv. Thereafter, the mean number of PCV2-specific IFN-ɣ-SC was significantly higher ($p < 0.05$) in the vaccinated group when compared with the unvaccinated group from 18 to 81 dpv (Farms A and C) and from 18 to 102 dpv at Farm B (Fig. 3c).

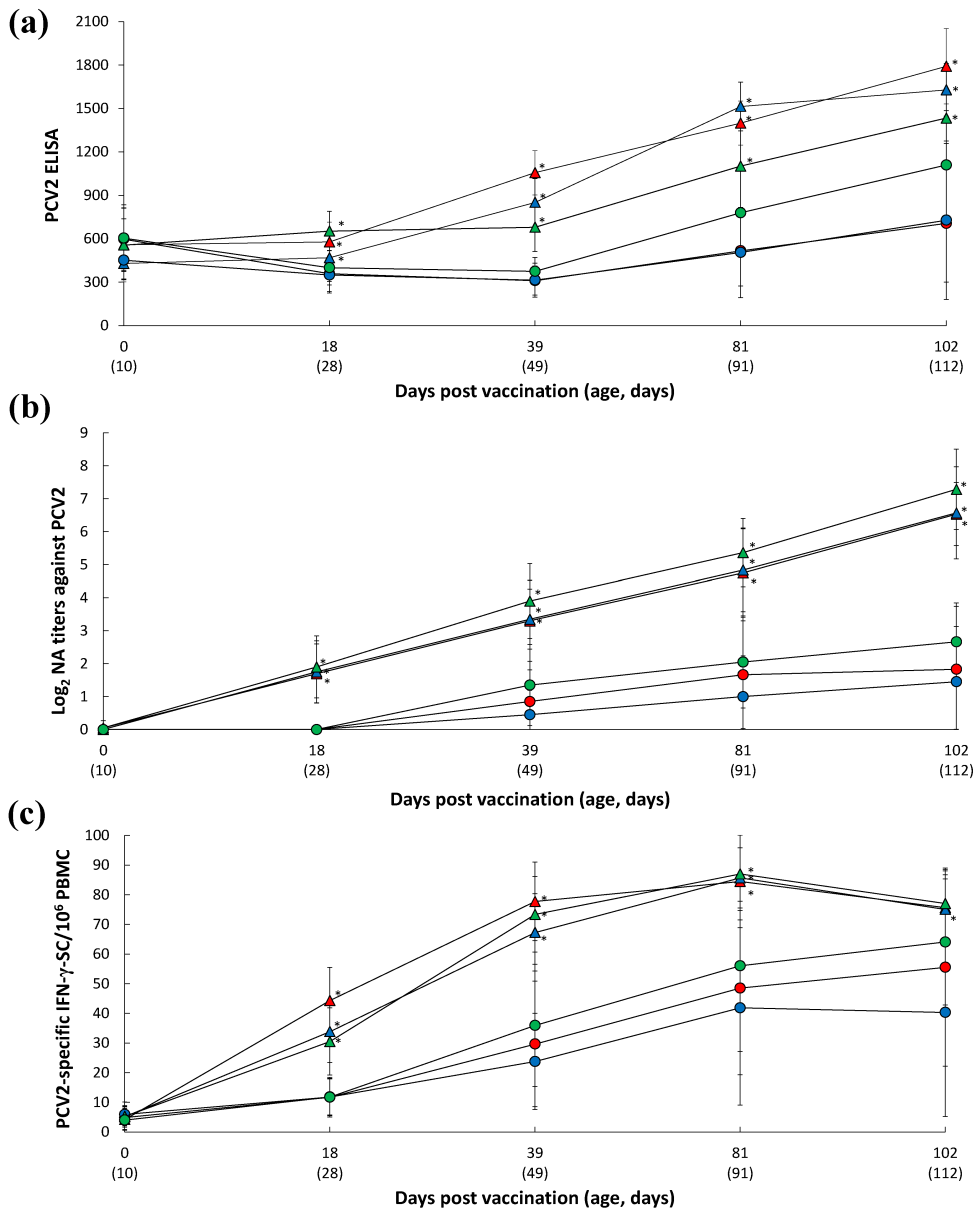

FIGURE 3 Mean values of the PCV2 ELISA titers (a), the PCV2d-specific neutralizing antibodies (b), and PCV2d-specific IFN-g-SC/10^6 PBMC (c) from VacA (▲) and UnVacA (●), VacB (▲) and UnVacB (●), and VacC (▲) and UnVacC (●) groups. Variation is expressed as the standard deviation. *Significant difference ($p < 0.05$) between vaccinated and unvaccinated group within the same farm.

Immunological responses against *M. hyopneumoniae*

At the time of vaccination, (10 days of age; 0 dpv), anti-*M. hyopneumoniae* antibodies were not detected in any of the groups or farms. Vaccinated animals from Farms A and C had significantly higher ($p < 0.05$) *M. hyopneumoniae* ELISA S/P ratios at 39 and 81 dpv (respectively) when compared with unvaccinated animals. Farm B vaccinated animals had a significantly higher ($p < 0.05$) *M. hyopneumoniae* ELISA S/P ratios at 18 and 39 dpv when compared with unvaccinated animals. The mean number of *M. hyopneumoniae*-specific IFN-ɣ-SC remained at basal levels (< 20 cells/10^6 PBMC) in both groups until 18 dpv (Fig. 4a). The mean number of *M. hyopneumoniae*-specific IFN-ɣ-SC was significantly higher ($p < 0.05$) in the vaccinated groups when compared with the unvaccinated groups from 18 to 81 dpv (Farm A) and from 39 to 81 dpv (Farms B and C) (Fig. 4b).

Pathology

The results of lung and lymphoid lesion score are summarized in Table 1. Vaccinated pigs from all three farms had a significantly lower severity ($p < 0.05$) of *M. hyopneumoniae*-induced pneumonia and PCV2-associated lymphoid lesions when compared with unvaccinated pigs. Vaccinated pigs on three farms also had significantly lower numbers of PCV2 antigen-positive cells within lymphoid lesions when compared with unvaccinated pigs.

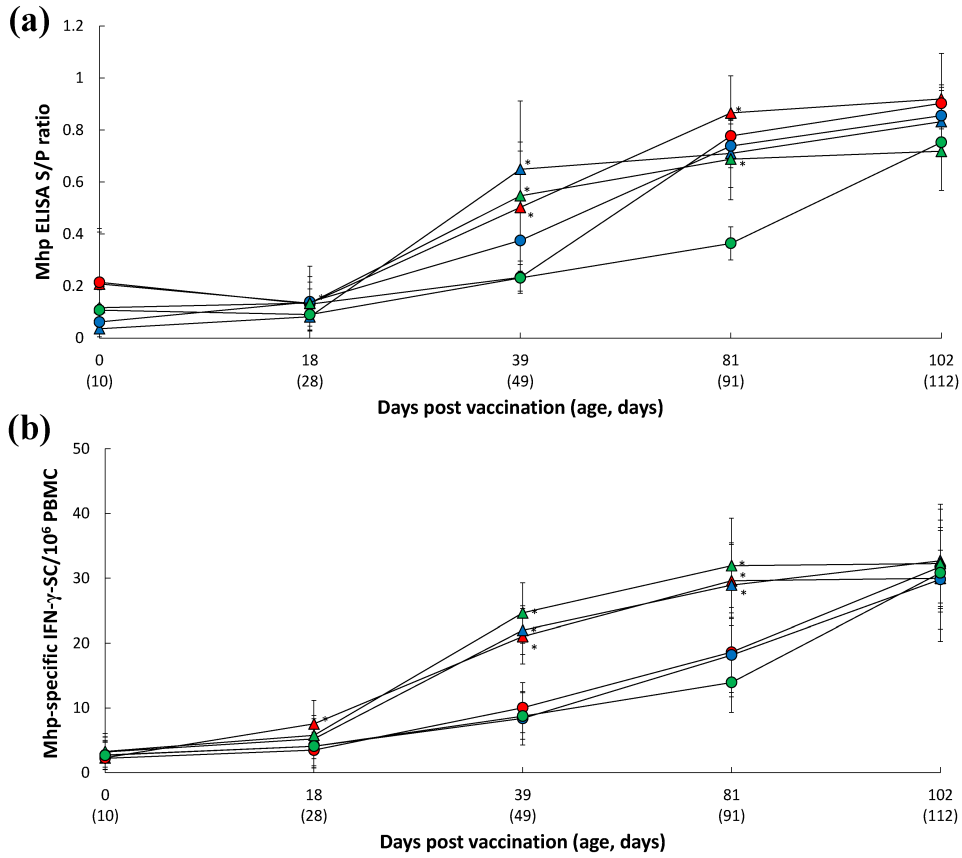

FIGURE 4 Mean values of the *M. hyopneumoniae* ELISA S/P ratio (a) and *M. hyopneumoniae*-specific IFN-g-SC/10^6 PBMC (b) from VacA (▲) and UnVacA (●), VacB (▲) and UnVacB (●), and VacC (▲) and UnVacC (●) groups. Variation is expressed as the standard deviation. *Significant difference ($p < 0.05$) between vaccinated and unvaccinated group within the same farm.

Discussion

In this field trial, vaccination against PCV2b and *M. hyopneumoniae* resulted in a statistically significant improvement in growth performance when compared with unvaccinated pigs in all three farms, regardless of whether the farm was experiencing a subclinical PCV2 and clinical *M. hyopneumoniae* concurrent infection (Farms A and C), or concurrent PCVAD and clinical *M. hyopneumoniae* infection (Farm B). Although Farm B received a PCV2a-vaccine, PCVAD was not eradicated. Commercial PCV2a-based vaccines have proven to provide cross-protection against PCV2d [4, 22 - 24], yet vaccine failure (contradicting this cross-protection) due to PCV2d infection has also been reported in PCV2a-vaccinated herds [25 - 27]. Therefore, vaccines containing PCV2b may provide better protection against PCV2d, which is genetically closed to PCV2b. On the other hands, despite vaccination with *M. hyopneumoniae* on the 3 farms, clinical signs and mycoplasmal lung lesions were still observed in pigs at the time of slaughter. Similarly, bivalent-vaccinated pig groups from all three farms exhibited some degree of clinical signs and mycoplasmal lung lesions at the time of slaughter. These results indicate that vaccination alone is not sufficient in protecting pigs from *M. hyopneumoniae*.

The most common age of piglet PCV2 vaccination occurs either at weaning or immediately after weaning (around 3 - 4 weeks of age). In contrast, the evaluated bivalent vaccine containing PCV2b and

M. hyopneumoniae recommended administration to the piglets at 10 days old. This younger-aged piglet was still immunologically mature enough to elicit active immunization after vaccination, as the onset of active immunity has been proven to begin as early as in 5-day-old piglets, post-vaccination [28]. Vaccination at such a young age may result in additional complications, however, as the piglets face potential interference of maternally derived antibodies (MDA) present at the time of vaccination. The role of PCV2 MDA plays in active immunity of piglets after vaccination is a somewhat controversial issue. It has been stated that PCV2 MDA can affect the age of PCV2 infection [29] while contradictory evidence exists that demonstrated that high PCV2 MDA titers ($\geq$ 10 log2) could interfere with piglets' active seroconversion after vaccination [30]. In the present study, piglets with high ELISA (> 9 log2) or NA (> 7 log2) titers seemed to show interference with the development of the humoral immune response after vaccination. Most of the piglets (> 80% in three Farms) had lower ELISA (< 9 log2) or NA (< 7 log2) titers at the time of vaccination (data not shown). The data presented in this study supports that the bivalent vaccine can elicit PCV2-specific NA and IFN-ɣ-SC even in the presence of MDA. It is therefore concluded that a negative MDA effect on active immunization after vaccination could not have impacted the efficacy of the bivalent vaccine.

Optimal timing of vaccination against *M. hyopneumoniae* also continues to be debated because of the interference of MDA. *M. hyo-*

pneumoniae may infect pigs within the first three weeks of life [31, 32]. Similarly, *M. hyopneumoniae* is frequently detected in laryngeal swabs from suckling piglets in Korea (personal observation Dr. Chae). Therefore, an earlier vaccination against *M. hyopneumoniae* at less than 21 days old may be necessary in order to vaccinate piglets to prevent the onset of a natural infection. A number of studies have been conducted to evaluate early piglet vaccination. One experimental challenge study proved that vaccination of pigs at 7 days old with *M. hyopneumoniae* was effective in reducing lung lesions even in the presence of MDA at a titer considerably higher than what is typically seen in the field [33, 34]. Moreover, protective immunity against *M. hyopneumoniae* is primarily cell-mediated [35, 36] and the present field trial proved that the bivalent vaccine elicited *M. hyopneumoniae*-specific IFN-ɣ-SC even in the presence of MDA. Therefore, a MDA effect on active immunization after vaccination is less likely to have an impact on the efficacy of the bivalent vaccine.

The bivalent vaccine is able to elicit protective immunity against PCV2 and *M. hyopneumoniae*. PCV2-specific NA and IFN-ɣ-SC are key needed component to reduce PCV2 viremia and lymphoid lesions [30, 37, 39]. PCV2 viremia and lymphoid lesions results were significantly lower in the vaccinated groups when compared with controls in all three trial farms. Similarly, *M. hyopneumoniae*-specific IFN-ɣ-SC plays a crucial role to reduce nasal shedding of *M. hyopneumoniae* and mycoplasmal lung lesions [16, 38]. In the presented field trials, vaccination resulted in the reduction of nasal

shedding of *M. hyopneumoniae* and mycoplasmal lung lesions when compared with controls.

Vaccination against PCV2 and *M. hyopneumoniae* has becomes standard practice, and almost 100 % and 80 % of the Korean herds are vaccinated against PCV2 and *M. hyopneumoniae*, respectively [24]. Use of a bivalent vaccine reduces the number of injections the pig receives, is more convenient than handling multiple products, and increases administration efficiency. This bivalent vaccine containing PCV2b and *M. hyopneumoniae* provide good efficacy against PCV2d and *M. hyopneumoniae* on farms with concurrent subclinical PCV2 and clinical *M. hyopneumoniae* infection, and concurrent PCVAD and clinical *M. hyopneumoniae* infection.

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GENERAL CONCLUSION

The objective of the dissertation was to evaluate the efficacy of the single-dose bivalent vaccine of PCV2b and *M. hyopneumoniae* through the experimental dual challenge and the field evaluation. PCV2 and *M. hyopneumoniae* coinfection is characterized by poor growth performance, however two studies showed significant improvement of growth performance and higher ADWG in vaccinated pig groups than unvaccinated pig groups.

In the experimental dual challenge, the level of neutralizing antibodies showed the correlation with the PCV2b viremia reduction, while frequent of IFN-γ-SC did not. Cell-mediated immunity has proven to play a key role in controlling *M. hyopneumoniae* infection. In this study, the level of IFN-γ-SC showed the correlation with a significant reduction in the amount of *M. hyopneumoniae* nasal shedding.

In field evaluation, although vaccinated pig groups showed better results than unvaccinated ones, vaccinated pig groups from three farms exhibited some degree of clinical sings and mycoplasmal lung lesions at the time of slaughter. These results may indicate that vaccination alone is not sufficient in protecting pigs from *M. hyopneumoniae.* The most common age of piglet PCV2 vaccination is around 3 weeks of age, however this new single-dose bivalent vaccine containing PCV2b and *M. hyopneumoniae* is recommended administration to the piglet from 10 days old. In this study, the data

support that the bivalent vaccine can elicit PCV2-specific neutralizing antibodies and IFN-γ-SC even in the presence of MDA. It is considered that a negative MDA effect on active immunization after vaccination could not have impacted the efficacy of the bivalent vaccine. PCV2 viremia and lymphoid lesions results were significantly lower in the vaccinated groups when compared with controls in all three trial farms. The protective immunity against *M. hyopneumoniae* is primarily cell-mediated and the present field trial proved that the bivalent vaccine elicited *M. hyopneumoniae*-specific IFN-γ-SC even in the presence of MDA. Vaccinated pig groups showed lower nasal shedding and mycoplasmal lung lesions than unvaccinated groups in all three farms.

These studies demonstrated that use of the new single-dose bivalent vaccine containing PCV2b and *M. hyopneumoniae* induced high levels of protective immunity, reduced PCV2 viremia and lymphoid lesions, and reduced *M. hyopneumoniae* nasal shedding and lung lesions. In conclusion, this vaccine can reduce the number of injections the pig receives, as well as provide good efficacy against PCV2 and *M. hyopneumoniae*.

국문 논문 초록

돼지써코바이러스 2b형과 마이코플라즈마 복합백신의 병리학적 및 면역학적 효능 평가 연구

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한국을 포함한 돼지 산업이 발달한 나라에서 돼지써코바이러스 2형(PCV2)과 *M. hyopneumoniae*는 복합감염을 일으켜 돼지호흡기복합감염증(PRDC)과 같은 원인이 되어 돼지 산업에 경제적으로 피해를 입히는 매우 중요한 병원체들이다. 따라서 두 병원체에 대한 백신을 접종하므로써 질병을 통제하고 농장의 피해를 줄이고자 노력해 왔다. 두 병원체에 대한 백신은 그 효과가 입증이 되어왔고, 여러 회사에서 백신을 공급해 왔다. 주사 횟수를 줄이기 위한 노력으로 두가지 병원체를 항원으로 사용하는 복합백신이나 각각의 백신을 섞어 사용할 수 있는 백신이 공급되고 있다. PCV2 백신의 경우 대부분 PCV2a를 기반으로 만들어졌다. 하지만 'mutant PCV2b'라고 불렸던 PCV2d의 발생이 증가하고 있다. 최근

에 PCV2b와 *M. hyopneumoniae*를 포함하는 2가 백신이 개발이 되어 한국에 소개가 되었다. 이 백신은 두개의 병원체가 함께 포함되어 있으며 접종시기 또한 기존의 백신이 3주령에 접종하는 것과 달리 10일령부터 접종이 가능하고, 접종 용량 또한 1ml이기 때문에 백신에 대한 효과와 효능을 평가할 필요가 있었다. 따라서 본 연구에서는 이 새로운 2가 백신에 대한 PCV2와 *M. hyopneuomoniae*를 동시 감염시키는 공격접종과 필드평가를 통해 백신의 효과와 효능을 검증해 보고자 하였다.

Chapter I에서 백신의 효능을 평가하기 위해 24두의 돼지를 백신접종/공격감염(Vac/Ch), 백신비접종/공격감염(UnVac/Ch) 그리고 백신비접종/비공격감염(UnVac/UnCh) 군으로 나누어, 10일령(공격감염 25일 전)에 Vac/Ch군과 UnVac/Ch 군에 PCV2와 *M. hyopneumoniae*를 5시간의 간격을 두고 동시에 감염을 시키는 모델로 실험을 진행하였다. 10일령(공격감염 25일 전)부터 56일령(공격감염 21일 후)까지 진행되었으며, 공격감염 21일 째에 안락사를 시킨 후 폐병변과 임파절 병변을 확인하여 평가하였다. 그 결과 Vac/Ch군이 UnVac/Ch군에 비하여 실험이 진행되는 동안 호흡기 증상이 현저히 낮았으며, 체중의 증가는 Vac/Ch군과 UnVac/UnCh군이 UnVac/Ch군에 비하여 월등한 평균일당증체(ADG)를 보였다. 공격감염 25일전, 14일전, 당일, 7일 후, 14일 후 그리고 21일 후에 실시한 채혈검사와 비강스왑 검사에서는 Vac/Ch군이 UnVac/Ch군에 비하여 PCV2의 바이러스혈증과 *M. hyopneumonia*의 비강내 검출 수준이 현저히 낮았다. 체액성 면역과 세포성 면역 형성 지표 역시 Vac/Ch군이 UnVac/Ch군에 비해 현저히 높게 형성되었음을 확인하였다. *M. hyopneumoniae*에 의한 폐병변과 PCV2에 의한 임파절 병변에서도 Vac/Ch군의 돼지들이 UnVac/Ch군의 돼지들에 비해 현저히 낮았다.

Chapter II에서는 필드평가를 위해 PCV2의 준임상 증상을 보이고, *M. hyopneumoniae* 임상증상을 보이는 3개의 농장에서 백신의 권장 방법에 따라 10일령 돼지에 1ml을 근육 접종하여 식염수를 접종한 대조군과 비교하는 실험을 175일령까지 진행하였다. 백신의 효과를 평가하기 위하여 매주 임상 증상을 관찰하여 기록하였으며, 각 그룹의 돼지들을 실험 개시일(10일령)과, 70일, 112일 그리고 175일에 체중을 측정하여, 구간별 평균일당증체량(ADWG)를 계산하였다. 효능을 평가하기 위하여 백신접종 당일, 18일 후, 39일 후, 81일 후 그리고 102일 후에 채혈 및 비강스왑을 통해 PCV2d의 바이러스혈증과 *M. hyopneumoniae*의 비강배출 정도를 측정하였고, 체액성 면역과 세포성 면역에 대한 지표를 측정하여 비교하였다. 그 결과, 백신을 접종한 군에서 백신을 접종하지 않은 군에 비해 임상 스코어가 유의성 있게 낮았으며, ADWG는 높아 사육 성적이 좋았다. 백신접종군은 혈중내 PCV2d 레벨과 *M. hyopneumoniae*의 비강배출의 정도가 비 백신접종군에 비해 현저히 낮았으며, 면역반응은 체액성 면역 및 세포성 면역에 대한 지표가 현저히 높았다.

이 두가지의 실험으로 PCV2b와 *M. hyopneumoniae*로 구성된 새로운 2가 백신을 10일령에 접종하더라도 준임상형 PCV2와 임상형 *M. hyopneumoniae*에 대한 효과가 있다는 것과, 각각의 병원체에 대한 면역 반응도 잘 이루어진다는 것이 확인되었다. 또한 PCV2d가 유행하는 한국의 현장에서 예방 목적으로 사용할 수 있다는 결론을 내릴 수 있었다.

주요어: PCV2; *Mycoplasma hyopneumoniae*; 2가 백신; 공격접종; 돼지 호흡기복합감염증

학번: 2020-20725