수의학박사학위논문

Field Comparison of Two Combined Vaccines of Porcine Circovirus Type 2 and *Mycoplasma hyopneumoniae* Based on Pathological and Immunological Analysis

돼지 써코바이러스 타입 2 및 돼지 유행성 폐렴 합제백신 2종의 병리학 및 면역학적 분석을 통한 야외 비교평가

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Abstract

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Monovalent vaccines for porcine circovirus type 2 (PCV2) or *Mycoplasma hyopneumoniae* had been developed and successfully applied to swine farms. Nevertheless both two pathogens are still one of the most important pathogens in swine industry. It is because PCV2 and *M. hyopneumoniae* are

prevalent among pig population and their eradication is not easy in commercial swine farms. Both pathogens are the primary agents involved in porcine respiratory disease complex (PRDC) and *M. hyopneumoniae* infection can potentiate the severity of PCV2 lesions.

Recently combined vaccines of PCV2 and *M. hyopneumoniae* have been introduced in the fields. These vaccines have been welcomed in the market because they can provide prevention of two essential diseases with less stress and also reduce labor costs. Before applying combined vaccines to fields, it is necessary to evaluate its efficacy against PCV2d genotype since in Asian countries including Korea PCV2d genotype is the major field genotype while each commercial combined type vaccine is produced based on their own PCV2 genotype. According to various studies of PCV2 monovalent vaccines, homologous genotype vaccine provides better protection in viremia than heterologous vaccine even if both vaccines prevent the development in lesions.

In chapter I, clinical study was conducted in swine farms with a new trivalent vaccine of PCV2 and *M. hyopneumoniae* which is introduced to Korea for the first time. The vaccine includes PCV2a- and PCV2b-genotype antigen and it is expected its good efficacy to PCV2d genotype because PCV2b is genetically close to PCV2d. Three farms were selected based on their history of PCV2d subclinical or clinical infection and mycoplasmal pneumonia. Vaccinated group was administered the vaccine in two ways,

vaccination of 1.0ml dose at 3 and 24 days of age or 2.0ml dose at 21 days according to the manufacturer's recommendations. Both vaccinated groups showed significantly higher average daily weight gain than unvaccinated group. Vaccination elicited neutralizing antibodies and interferon-ɣ-secreting cells (IFN-ɣ-SC), which reduced PCV2 viremia and lymphoid lesions and in similar way, it elicited IFN-ɣ-SC, which reduced the amount of *M. hyopneumoniae* in laryngeal swab and the severity of lung lesion. This study demonstrated the trivalent vaccine was efficacious in protection of PCV2d and *M. hyopneumoniae* in swine farms.

In chapter II, comparative field study was conducted between the new trivalent vaccine and a bivalent vaccine containing PCV2a and *M. hyopneumoniae* antigen. The defining difference between these two vaccines is the inclusion or absence of PCV2b antigen. As the result of calculating T cell epitope contents comparison scores between each vaccine and PCV2d field strain, trivalent vaccine showed better coverage than bivalent vaccine as expected, since PCV2b is genetically close to PCV2d. In the field comparative study, trivalent vaccine having PCV2a/2b and *M. hyopneumoniae* antigen was administered in one-dose or two-dose and bivalent vaccine having PCV2a and *M. hyopneumoniae* antigen was administered in one-dose. Trivalent vaccine groups showed significantly better growth performance than bivalent vaccine group and also reduced the amount of PCV2d loads in the blood and feces, and *M. hyopneumoniae* load

in the larynx when compared with the bivalent vaccine group. Between the one- and two-dose trivalent vaccine group, there were no statistical differences in growth performance, serology, amount of PCV2d loads in the blood and feces, amount of *M. hyopneumoniae* load in larynx, and pathological lesions. This study showed the efficacy of each combined vaccine in protecting PCV2d and *M. hyopneumoniae* can be different and it is related to the accordance of genetic type of PCV2 antigen with field strain.

Keywords: porcine circovirus type 2; *Mycoplasma hyopneumoniae*; porcine respiratory disease complex; combined vaccine of PCV2 and *M. hyopneumoniae*

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TABLE OF CONTENTS

LIST OF TABLES

LITERATURE REVIEW

CHAPTER I.

CHAPTER II.

LIST OF FIGURES

CHAPTER I.

LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
CD	Colostrum-deprived
CF	Colostrum-fed
Cp	Capsid protein
CVPC	Cranioventral pulmonary consolidation
dpi	days post-inoculation
dpv	days post-vaccination
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Ennzyme-linked immunospot assay
EP	Enzootic pneumonia
EpiCC	T cell epitope contents comparison
GN	Gnotobiotic
IFN-ɣ-SC	Interferone-ɣ secreting cells
IL	Interleukin
MDA	Maternally-derived antibody
MH	*Mycoplasma hyopneumoniae*

NA	Neutralizing antibody
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCVAD	Porcine circovirus associated disease
PCV2	Porcine cirocovirus type 2
PDNS	Porcine dermatitis and nephropathy syndrome
PK	Porcine kidney
PMWS	Postweaning multisystemic wasting syndrome
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
QIA	Animal and plant quarantine agency
Rep	Replication-associated protein
SPF	Specific pathogen free
ssDNA	single-stranded DNA

GENERAL INTRODUCTION

Porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* are economically important pathogens in swine industry. Regarding economic impact of PCV2, England swine industry was affected about £52.6 million only in 2008 before vaccine was introduced [1]. From the research comparing *M. hyopneumoniae* positive and negative flow groups, groups from positive sow farms showed lower average daily gain (36g/day difference, 4.2% reduction) and 1.26% higher mortality than *M. hyopneumoniae* eliminated groups [2]. Considering higher treatment cost and poorer productivity, *M. hyopneumoniae* positive farm spent average US$7.00 per pig marketed more.

These two pathogens are still the major agents related with pneumonia of pigs. Korea Animal and Plant Quarantine Agency investigated respiratory disease status of finishers from 60 farms in 2021. From pathogen analysis of 303 samples, PCV2, PCV3, *M. hyopneumoniae* and *Mycoplasma hyorhinis* were the most related with severity of lung lesion [3].

PCV2 and *M. hyopneumoniae* are one of the most common pathogens detected in pigs with porcine respiratory disease complex (PRDC) [4, 5]. From research of PRDC cases in Korea, 85 pigs among 105 pigs were positive for PCV2 and 33 cases were co-infection of PCV2 and *M.*

hyopneumoniae [5]. PCV2 and *M. hyopneumoniae* dual challenge model (sequential) was established for investigating the interactions between two pathogens. From the results, *M. hyopneumoniae* potentiated the severity of PCV2-associated lung and lymphoid lesions and increased the incidence of Porcine circovirus associated disease (PCVAD) in pigs [6].

As prevention of these two pathogens are essential in swine farms, commercial vaccines have been developed and used widely in fields. Recently combined vaccines of PCV2 and *M. hyopneumoniae* are introduced in the market and its usage keeps increasing. It is necessary to evaluate efficacy of combined vaccines in fields because the efficacy can be different according to vaccine's PCV2 genotype match to field strain.

In this dissertation, a new trivalent vaccine of PCV2a/2b and *M. hyopneumoniae* were evaluated its efficacy in field conditions affected PCV2d which is a major genotype of PCV2 in Korea and Asian region. In addition to field trials, the trivalent vaccine was compared with a bivalent vaccine of PCV2a and *M. hyopneumoniae* in a farm affected PCV2d.

LITERATURE REVIEW

1. Porcine Circovirus 2

1.1. Historical review

In 1998, researchers reported porcine circovirus-like virus from pigs with postweaning multisystemic wasting syndrome (PMWS) in Canada, US and France [7, 8]. This novel virus seemed to be identical in morphology to PCV, porcine kidney (PK)-15 contaminant virus which was not considered to cause clinical signs in pigs and was also detected as PCV in immunohistochemistry and In situ hybridization results of PMWS pig's tissue [7, 8]. However, the PCV-like virus was antigenically distinct from PCV by monoclonal antibody reaction and had limited antigenic homology with the original PCV [8].

After genomic analysis of PMWS pigs from US, France and Canada, researchers found its nucleotide sequence identity was below 80% with PCV [9]. Investigating two major open reading frames (ORFs), nucleotide homology of ORF1 was 83% and ORF2 was relatively low, 67% [10]. From antigenic and genomic differences between these viruses, it was proposed the new circovirus should be referred to as PCV2 and the PK-15 contaminant PCV as PCV1 [9].

Since PCV2 was reported from PMWS pigs in North America and France, PCV2 was isolated from PMWS pigs in more European countries. In 2000, PCV2 was first reported in Korea, also [11]. In 2000, PCV2 was detected from pigs with porcine dermatitis and nephropathy syndrome (PDNS) which was considered to be related with PMWS incidence [12]. PCV2 was also identified in aborted piglets and it was detected in multiple organs like myocardium, liver, lung and kidney of fetus [13]. PCV2 is considered to be transmitted vertically in uterine and it can cause reproductive disease.

1.2. Etiology

Species PCV2 belongs to the family Circoviridae which are non-enveloped viruses with circular, single-stranded DNA (ssDNA) genomes. Family Circoviridae is divided into two genera, Circovirus and Cyclovirus. PCV1, PCV2 and PCV3 belong to genus Circovirus [14, 15].

From 3D results calculated after cryomicroscopy, PCV2 has icosahedral structure containing 60 capsid protein molecules arranged in 12 pentamer units and its overall diameter is about 20.5nm [16].

The genome size of PCV2 is 1766-1769 nucleotides and PCV1 is 1758-1760 nucleotides. PCV3 genome contains 1999-2001 nucleotides [17-19]. By genomic sequence, PCV1 is less than 80% identical to PCV2 and 45.5% identical to PCV3 [20, 21]. Comparing PCV2 with PCV3, 46.8% nucleotides

are identical [21]. PCV4 shows 50.3% genomic identity with PCV1, 51.5% with PCV2 and 43.2% with PCV3 [22]. Circovirus has two major open reading frames (ORFs) and a conserved nonanucleotide motif marking the origin of replication. Replication-associated protein (Rep) gene is encoded on the virion strand and capsid protein (Cp) gene is encoded on the complementary strand of a dsDNA replicative form [15].

Using metagenomic sequencing method, PCV3 was discovered from tissue samples of porcine dermatitis nephropathy syndrome (PDNS) sows, aborted fetus, myocarditis and multi-systemic inflammation pigs. The replicase proteins of PCV3 shares 48% amino acid identity to the Rep of PCV2 and the Cp of PCV3 shares 24% and 26% aa-identity to those of PCV1 and 2, respectively [23, 24].

1.3. Genotypes of PCV2

To define different phylogenetic groups of PCV2, scientists suggested using ORF2 gene to perform genotyping for PCV2. As the frequency distribution of pairwise distances among 196 ORF2 PCV2 sequences showed the lowest frequency at 0.035 between peaks, 0.035 was suggested as genetic distance cut-off value differentiating genotypes. Using the proposed methodology, PCV2 genotypes were defined as three; PCV2a, PCV2b and PCV2c in 2008 [25, 26].

Further developed method for PCV2 genotyping were proposed in 2018 because of the growing difficulty for classifying consistently current and novel viral sequences. The proposed method was genotyping with three conditions; intra-genotype p-distance below 0.013, bootstrap higher than 70% and at least 15 available sequences. By analyzing 4586 ORF2 sequences, 8 genotypes of PCV2 were classified as PCV2a to PCV2h. PCV2b was the major genotype with 45.84% in total sequences and PCV2d was the major genotype with 45.08% in sequences from Asia [19, 27]. In 2016, genotype-specific PCR on pen-based oral fluid samples was carried out in nationwide scale in Korea. From the research, PCV2d was detected as the major genotype in 72% farms (50/69) and 25% farms (17/69) was infected PCV2d with PCV2a/b genotype [28].

1.4. Epidemiology

PCV2 is widespread in swine population and can be found in every kind of swine farms, from the SPF farm to back yard farm. In both PMWS affected and non-affected farms, PCV2 seroprevalence data showed almost 100% seropositive in European countries [20, 29]. From the PCV2 surveillance using pen-based oral fluid samples in Korea, 78.2% samples showed PCR positive result [28].

PCV2 were found in wild boars which showed PMWS signs and its genetic

sequence of ORF2 had 98.7% homology with a reference PCV2 isolate [30]. From the PCV2 genotype surveillance of wild boar isolates in South Korea, PCV2d and PCV2b was 80.2% and 16.5% each [31].

Horizontal transmission of PCV2 was demonstrated with experiments to contact naive pigs with infected pigs. Cesarean-derived colostrum-deprived pigs which was inoculated PCV2 showed clinical signs and pathological lesions of PCV2 infection. After commingling the infected pigs with naive pigs, all naive pigs showed seroconversion within 3 weeks [32]. In another study, SPF or conventional pigs were inoculated with a tissue homogenate of pigs which showed clinical signs of PCV2 infection. SPF pigs were commingled with these pigs after inoculation and 10 of the 11 SPF pigs were developed pyrexia and growth retardation [33].

Vertical transmission of PCV2 was demonstrated by inoculating PCV2 intranasally to pregnant sows at 3 weeks before farrowing date. The infected sows showed abortion and premature farrowing and PCV2 antigen and DNA were detected by immunohistochemistry and In situ hybridization in multiple organs of stillborn and liveborn piglets [34]. In Korea, a retrospective study of abortion cases showed 13.1% PCV2 positive by PCR and From North America prevalence study, PCV2 DNA was 39.9% positive in serum of pre-suckle piglets [35, 36].

In PCV2 shedding experiments, PCV2 DNA was detected until 21 days post-inoculation (dpi) from blood, fecal, tonsillar swabs of colostrum deprived

SPF piglets inoculated PCV2 oronasally [37]. In another experiments, PCV2 shedding was shown until 70 dpi by detecting PCV2 DNA in oropharyngeal and nasal swabs and fecal samples of inoculated piglets [38]. In experiments of monitoring PCV2 DNA quantitatively, PCV2 DNA was detected from all samples by 69 dpi without difference in amounts and the peak was reached by 16 dpi with a significant decrease after 35 dpi [39].

Naturally and experimentally infected boars can shed PCV2 in their semen. After intranasal inoculation of four boars with PCV2, PCV2 DNA was detected as soon as 5 dpi in the semen of two infected boars and intermittently thereafter in the semen of all four infected boars. PCV2 DNA was positive at 47 dpi in the semen of two infected boars [40].

1.5. Pathogenesis

Initial target organ of PCV2 is lymphoid tissues in pigs [41]. PCV2 is detected in the cytoplasm of monocyte, pulmonary macrophages and monocyte-derived macrophages. But viral replication is not observed in pulmonary macrophages. PCV2 may bypass degradation in the monocytic cell and remain undetected by the immune system [42]. Study of quantification of PCV2 DNA and capsid mRNA in peripheral blood mononuclear cells (PBMCs) suggest that PCV2 replicates in lymphocytes, particularly T lymphocytes [43].

PCV2 infection is needed for the clinical sign expression of PMWS [14]. From the results of experimental infections of gnotobiotic (GN), colostrum-deprived (CD) and colostrum-fed (CF) pigs with PCV2 alone, clinical PMWS has been produced and it is now accepted that PCV2 is the causal agent of PMWS. The severity of disease can be increased if GN and/or CD pigs are co-infected with other agents or immunostimulated [44].

PCV2 infection and replication in lymphoid tissue can destroy lymphoid follicles, leading to lymphoid depletions. Destruction of lymphoid follicles and leukopenia associated with PCV2 infection can lead pigs to immunosuppression status [41]. The cells of PMWS lesions were characterized using histological and immunohistochemical method [45]. B and T lymphocytes reduction or loss was the most relevant changes. The numbers of macrophages were increased and partial loss and redistribution of antigen presenting cells were observed. Depletion of T lymphocytes primarily involved $CD4^+$ cells and $CD8^+$ cells [46].

PCV2 inoculation was performed in fetuses at either 57, 75 or 92 gestational days and in piglets at 1 day of age. During fetal life, viral antigens were detected in cardiomyocytes, hepatocytes and macrophages and infected cell numbers decreased with increasing fetal age. Postnatally, macrophages were the only target cell type [47].

From the research of direct intra-fetal inoculation at 57 gestational days, fetal death occurred with mummification [48]. Pregnant sows showed

abortion and premature farrow when inoculated with intranasal route and sows inseminated with PCV2a or PCV2b-contaminated semen showed failure of pregnancy or fetus mummification [49, 50].

1.6. Clinical signs and lesions

1.6.1. PMWS

PMWS is also called as PCV2 systemic disease (PCV2-SD) [51]. PMWS most commonly affects pigs of 2-4 months of age. Morbidity and mortality are variable depending on the farms. The usual rates are 4-30% and 70-80%, respectively [29].

Clinical signs of PMWS are wasting, dyspnea, diarrhea, pallor of skin and occasionally icterus [52]. On PMWS-affected farms, other diseases like Aujeszky's disease, porcine reproductive and respiratory syndrome (PRRS), Porcine Parvovirus (PPV) infection, Glasser's disease, streptococcal meningitis, salmonellosis, post-weaning colibacillosis, non-specific diarrhea, hepatosis dietetica and bacterial pneumonia are more commonly found [14].

The most obvious lesion of PMWS is the enlargement of lymph node [53]. This feature is found mainly at inguinal, submandibular, mesenteric and mediastinal lymph node [54]. However, these lesions are not always present and lymph node of normal size to atrophic are usually seen in more advanced phase of PMWS.

Lymphocyte depletion is observed within lymphoid follicles or in the paracortical zones. Large histiocytic cells and giant multinucleate cells infiltration is observed in subcapsular sinuses of lymphoid tissue. Multinucleate giant cells may also appear in lymph follicles and in parafollicular zones [53].

Interstitial pneumonia is the most usual lung lesion observed in PMWS pigs. Lung may be enlarged, non-collapsed and rubbery in consistency. Interstitial edema and catarrhal-purulent bronchopneumonia are often observed [14, 54].

Lympho-histiocytic inflammatory infiltration in portal zones, single cell necrosis of hepatocytes, swelling and vacuolation of hepatocyte cytoplasm and karyomegaly can be seen in liver of PMWS pigs. In some cases, severe lesions with generalized perilobular fibrosis, disorganization of liver plates and massive loss of hepatocytes are observed and these lesions are associated with icterus [54].

1.6.2. PDNS

PDNS is a vascular disease affecting nursery and growing pigs and less commonly breeding animals [55]. The prevalence of the disease in affected herds is usually less than 1%. Mortality among pigs of 3-months-old was nearly 100%, while one-third of the affected pigs aged between 1.5 and 3 months die [56].

In the acute phase of the disease, the most obvious sign is round to

irregular, red to purple macules of the skin [55]. With time, the lesions become covered by dark crusts and fade gradually, sometimes leaving scars. The lesions distribute typically at the perineal area of hindquarters, limbs, dependent part of the abdomen and thorax, and the ears.

The cause of death in PDNS-affected pigs is an acute renal failure, with usually marked increase of creatinine and urea level in serum [56].

Red-to-dark macules and papules of skin is associated with necrotizing vasculitis of dermal and hypodermal capillaries and arterioles, and extensive hemorrhages [14]. Necrotizing vasculitis is prominent in the skin, kidney pelvis, mesenterium and spleen.

Pigs affected PDNS have bilaterally enlarged pale kidneys which have subcapsular petechiae affecting the renal cortex [56]. Diffuse fibrinous glomerulitis is the most striking microscopic lesion. A moderate to severe non-purulent interstitial nephritis with dilation of renal tubules is also seen [14].

1.6.3. Reproductive disease

PCV2 infection can be etiological cause of mummification and stillbirths, a high neonatal mortality rate and piglets with congenital tremors or hind leg ataxia [57]. Newly established pig herd or PCV2 seronegative herds can be affected PCV2 reproductive disease while most of breeding herds are not suffering from the clinical disease due to the fact that seroprevalence of

PCV2 in adult pigs is high [51].

From stillborn and non-viable neonatal piglets, severe, diffuse myocarditis can be observed and PCV2 antigens are found in liver, lung and kidney [58].

2. *Mycoplasma hyopneumoniae*

2.1. *Mycoplasma hyopneumoniae*

M. hyopneumoniae causes a chronic respiratory disease in pigs known as enzootic pneumoniae (EP) and plays a primary role in the porcine respiratory disease complex (PRDC) [59].

Mycoplasmas are the smallest cells and have small genomes with a limited number of genes resulting in a lack of biosynthetic pathways [60]. *M. hyopneuminae* grows slowly compared with other porcine mycoplasmas. 2-3 days after inoculation of the media under 5-10% carbon dioxide atmosphere, barely visible colonies can be found in solid agar medium [59].

M. hyopneuminae is genetically diverse and it was proved by various method like restriction enzyme digestion, arbitrarily primed PCR and amplified-fragment length polymorphism [61-63]. The genetic differences between isolates seems to be linked to their virulence. By randomly amplified polymorphic DNA, only virulent isolates had specific base pair band [64].

Nose-to-nose contact between infected and susceptible pigs is the most common route of *M. hyopeumoniae* transmission. *M. hyopeumoniae* can be detected in nasal swab samples of experimentally infected pigs or naturally infected pigs by nested PCR method [65]. First exposure of piglets to *M. hyopeumoniae* is via nose-to-nose contact from sows. Gilts have relatively

high *M. hyopeumoniae* colonization than older sows, which means piglets of lower parity sows can be exposed more to *M. hyopeumoniae* [66]. But the relation between parity of sows and colonization of piglets still remains unclear [67].

M. hyopeumoniae infects pigs for long periods. Experimentally infected pigs transmitted *M. hyopeumoniae* to sentinels up to 200 dpi and its DNA was detected up to 214 dpi [68].

2.2. Pathogenesis

M. hyopeumoniae induce ciliostasis and loss of cilia in tracheal rings by adhesion along the ciliated respiratory epithelium of pigs [69]. This results in clearance reduction of debris and invading pathogens. As a results of this, upper respiratory commensal bacteria such as *Pasteurella multocida*, *Streptococcus suis*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, and others are able to infect in the alveoli as secondary pathogens [59].

Adhesion of *M. hyopeumoniae* is related with adhesin P97/P102 paralogue families and P159 [70-72]. Most of the proteins from P97/P102 paralogue families and P159 are processed and cleaved extensively. This leads to a dynamic surface topography of *M. hyopeumoniae* which could be involved in host evasion and modulation of the immune response [73].

M. hyopeumoniae infections may suppress phagocytic responses of alveolar macrophages when pigs were exposed with a secondary pathogen [74]. *M. hyopeumoniae* also induces macrophages to produce proinflammatory cytokines like interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor [59]. Production of proinflammatory cytokines leads to inflammation and tissue injury in the lung.

The immunosuppressive effect of *M. hyopeumoniae* was suggested by evaluating lymphocyte transformation [75]. According to the strains, *M. hyopeumoniae* reduced lymphocyte transformation by 50-98.7%, which means *M. hyopeumoniae* have immunosuppressive effect on the cell-mediated immune response.

M. hyopeumoniae alone typically causes a mild chronic pneumonia. When infected with other pathogens, respiratory disease often becomes severe. In enzootic pneumonia, secondary infection of upper respiratory bacteria causes more severe pneumonia. *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Glaesserella parasuis*, *Trueperella pyogens*, streptococci or staphylococci are commonly found in enzootic pneumonia cases [73].

M. hyopeumoniae interacts with viral respiratory pathogens and it can be developed to PRDC [59]. *M. hyopeumoniae* potentiates PRRSV-induced disease and lesions [76]. The presence of PRRSV also result in increased acute mycoplasmal pneumonia. The interacts between *M. hyopeumoniae* and

PCV2 was studied in 2004 [6]. *M. hyopeumoniae* potenciated the severity of PCV2-associated lung and lymphoid lesions, increased the amount and prolonged the presence of PCV2-antigen, and also increased the incidence of PMWS in pigs.

2.3. Clinical signs and lesions

The main clinical sign of *M. hyopeumoniae* infection is chronic, dry non-productive cough which can be inconsistent and variable in intensity [77]. In most cases, onset is insidious, slowly spreading among herds [59]. More severe clinical signs like fever, decreased appetite, labored breathing or prostration can be developed due to secondary pathogens. When *M. hyopeumoniae* infects naive herds, the disease may be more severe, increasing the morbidity up to 100%.

Gross lesion of infected lung consists of purple to grey areas of pulmonary consolidation, mainly located bilaterally in the apical, intermediate, accessory and the cranial parts of the diaphragmatic lobes [78]. In case of secondary bacterial infections, lungs can be affected in higher portion and lung lesions are firm and heavy with mucopurulent exudate in the airways [59].

Microscopically, the pneumonia is characterized by perivascular and peribronchiolar lymphoid hyperplasia, pneumocyte type II hyperplasia and edema fluid in the alveolar spaces with neutrophils, macrophages and plasma

cells [79].

Evaluation of the *M. hyopeumoniae* pneumonia in herds is assessed at slaughter normally. Lung lesions in pigs affected by enzootic pneumonia consists of cranioventral pulmonary consolidation (CVPC) and several lung scoring methods are in place for the evaluation of CVPC. Two-dimensional approaches are normally based on evaluation of affected area while three-dimensional approaches are based on evaluation of affected lung weight [78].

3. Porcine Respiratory Disease Complex

The term porcine respiratory disease complex (PRDC) was used to describe pneumonia of multiple pathogens causing clinical disease and failure to gain weight later in the finishing stage but nowadays it has also been used to describe pneumonia of mixed pathogens that occur in swine of any age [80]. Since PRDC is not caused by a single pathogen but rather is a multifactorial disease, the pathogens isolated from pigs vary between and within production units. The most common pathogens detected in PRDC pigs are as follows; PRRSV, *M. hyopneumoniae*, Swine influenza virus, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Glaesserella parasuis*, *Streptococcus suis*, PCV2, Pseudorabies virus [4].

Primary pathogens, such as respiratory epithelium-damaging viruses (influenza) predispose the pig to secondary infection by lowering the local and systemic defense mechanisms of the host. Primary pathogens are viruses or mycoplasmas, and secondary pathogens are bacteria [81].

From a retrospective study of PRDC cases in Korea, PCV2 was the most prevalent virus in lung tissue from PRDC pigs and 55% of cases was co-infection of PCV2 and PRRSV. PCV2 and *M. hyopneumoniae* co-infection cases were 31%. Among PCV2 infection cases, 56% was co-infection of PCV2 and bacterial pathogens [5].

M. hyopneumoniae also plays a important role in the ways of interacting with essential viruses like PCV2 or PRRSV in PRDC pigs. *M. hyopneumoniae* infected pigs with minimal to nondetectable mycopalsmal penumonia lesions manifested significantly increased PRRSV-induced pneumonia lesions compared to pigs infected with PRRSV only [76].

Since PCV2 and *M. hyopneumoniae*, these two pathogens are prevalent and critical in PRDC cases, prevention strategy is essential in swine industry. Commerical monovalent vaccines for each pathogen had been developed and applied to swine farms and nowadays combined type vaccines of PCV2 and *M. hyopneumoniae* are introduced to the field.

4. PCV2 Vaccine

Commercial PCV2 vaccines for growing pigs and breeding herds became available worldwide since 2004 [82]. The first vaccine on the market was CIRCOVAC® (Ceva) which is an inactivated type for sows and gilts. Ingelvac CircoFLEX® (Boehringer Ingelheim) and Porcilis PCV®/Circumvent® (MSD) are recombinant vaccines for growing pigs. These vaccines are subunit vaccines based on ORF2 proteins expressed by baculovirus systems. In early days, PCV2 commercial vaccines were produced based on genotype PCV2a strains and still these vaccines are the major products in PCV2 vaccine market.

In swine industry, the PCV2a genotype had been replaced by the PCV2b genotype since 2005 and nowadays PCV2d genotype is the most prevalent in North America [83]. In Korea, PCV2d genotype was also found to be prevalent genotype [28]. From the studies of PCV2 challenge after vaccination, homologous vaccination (matched genotype) may offer better protection than heterologous vaccination (non-matched genotype) [83]. In 2013, PCVAD outbreaks were reported in US farms which pigs were vaccinated with PCV2a based vaccine and PCV2d genotype was identified in these cases [84]. In Korea, there also was a report of PCV2a based vaccine failure to PCV2d infection cases [85].

PCV2 vaccination in pigs around 3 weeks age has been proved its efficacy against PCV2 infections. The vaccinated pigs showed higher mean daily weight gain, decreased mortality and cull rates, reduced viremia, shedding and viral load in tissues than unvaccinated pigs [86, 87].

In sows, vaccination showed improved reproductive parameters of breeding stock [82]. Sow vaccination also can be related with mortality of piglets since sow PCV2 viremia and antibody titer is related with piglet mortality in PMWS affected farms [88].

Vaccination both sows and piglets also showed its efficacy in PMWS outbreaks [89]. Repeated PCV2 vaccination in sows and high levels of maternally derived antibodies did not interfere with immunity in their piglets after vaccination [90].

PCV2 vaccine efficacy is considered to be based on anti-PCV2 antibodies, either from sow vaccination or piglet vaccination [82]. But low antibody responses after vaccination do not mean its lack of protection because when animals are not seroconverted, they were all protected against the pathogenic PCV2 challenge [91]. Cell-mediated immunity is also considered to be import for PCV2 protection. From analyzing IFN-γ-SC specific to PCV2 and Cap protein, cell-mediated immunity development after piglet vaccination was demonstrated [87].

5. *Mycoplasma hyopneumoniae* Vaccine

Commercial vaccines for *M. hyopeumoniae* are widely applied worldwide and they mostly consist of inactivated, adjuvanted whole-cell preparations [92]. *M. hyopeumoniae* vaccination improves daily weight gain, feed conversion ratio and reduces medication costs, prevalence of pneumonia lesions and severity of pneumonia lesions [93]. And vaccination also reduces *M. hyopeumoniae* prevalence at upper respiratory tract sites [94]. Vaccination of piglets is commonly used because infection of *M. hyopeumoniae* may already occur during the first weeks of life [92]. Regarding sow vaccination, piglets from sows vaccinated did not showed the differences in *M. hyopeumoniae* colonization, but the piglets had a significant lower mean of EP-compatible lung lesions than piglets from non-vaccinated sows when they are at 23 weeks of age [95].

M. hyopeumoniae vaccination induces both systemic and mucosal cellular and humoral immune responses [96]. Vaccination increases mucosal IgG, IgM and IgA and serum antibodies. $CD8^+$ T cells are found higher than $CD4^+$ T cells in most tissues and IL-10 secreting cells are detected more in vaccinated animals. Lower $CD4^+$ T cells and IL-10 induction seem to be the reason why the vaccinated animals develop less severe clinical symptoms. Higher IL-12 induction and IFN-ɣ secreting cells indicate that the vaccine

induces cellular immune responses. However, vaccine induced antibody levels do not be related to disease protection [97].

M. hyopeumoniae vaccination reduced the potentiation of PRRSV-induced pneumonia by *M. hyopeumoniae* and this may be because the vaccine prevents TNF-α increase in the lung due to *M. hyopeumoniae* challenge [92, 98]. And infection or vaccination with PRRSV decreased the efficacy of *M. hyopeumoniae* vaccination [98].

6. Combined Vaccine of PCV2 and *Mycoplasma hyopneumoniae*

Since PCV2 and *M. hyopeumoniae* infections are highly prevalent and economically important pathogens, commercial PCV2 and *M. hyopeumoniae* vaccines are widely used in swine industry [99]. Each vaccination did not show any protective effect to the disease development of the other pathogen in dually infected pigs, so it is necessary to vaccinate pigs with both PCV2 and *M. hyopeumoniae* for the control of co-infection with PCV2 and *M. hyopeumoniae* [99, 100].

Recently combined vaccines of PCV2 and *M. hyopeumoniae* are used globally in swine farms. Bivalent or trivalent combined vaccine can provide obvious benefit in term of labour than monovalent vaccines which need two injections [101]. In Korea, combination vaccines containing PCV2 and *M. hyopeumoniae* are being used for almost half of yearly produced piglets [102]. Commercial combined vaccines are being produced by several companies and each vaccine has differentiation by the genotype of PCV2 antigen. Porcillis® PCV M Hyo (MSD Animal Health) is PCV2a-based bivalent vaccine of PCV2 and *M. hyopeumoniae* and Circo/MycoGard® (Pharmgate Animal Health) is PCV2b-based bivalent vaccine. Fostera® Gold PCV MH (Zoetis) is PCV2a- and PCV2b-based trivalent vaccine of PCV2 and *M. hyopeumoniae.* Features of major commercial PCV2 monovalent

vaccines and combined vaccines of PCV2 and *M. hyopneumoniae* are compared in the table 1.

From the genetic distance study of inter-genotype of PCV2, PCV2b and PCV2h genotype strains were the most close genotypes to PCV2d genotype which is a major genotype in the fields nowadays [27]. From the study of T cell epitope contents comparison between various PCV2 vaccines and PCV2d genotype, bivalent PCV2 whole virus vaccine having PCV2a and PCV2b antigen showed higher scores than PCV2a-based subunit vaccine and PCV2a or PCV2b-based whole virus vaccine [103]. By these laboratory analysis, it is expected that trivalent vaccine having PCV2a/2b and *M. hyopneumoniae* antigen will show good efficacy to PCV2d genotype infection.

Field studies of combined vaccines and comparative studies between combined vaccines are needed to evaluate its efficacy against PCV2d, major PCV2 genotype of fields. Comparative field study between PCV2a-based bivalent vaccine and PCV2b-based bivalent has been reported recently [104]. From the study, both bivalent PCV2a- and PCV2b-based vaccines showed good efficacy against subclinical PCV2d infection and enzootic pneumonia. Comparative study between PCV2a-based bivalent vaccine and PCV2a/2b-based trivalent vaccine has not been carried yet, so it is necessary to evaluate vaccines comparatively in the field condition.

PCV2 and *M. hyopneumoniae* antigen	Commercial name	Company	PCV2 genotype	Vaccine type
Monovalent (PCV2 only)	Ingelvac CircoFLEX®	Boehringer Ingelheim	PCV2a	Subunit vaccine by baculovirus
Monovalent (PCV2 only)	CIRCOVAC®	Ceva	PCV2a	Inactivated vaccine
Monovalent (PCV2 only)	Porcilis PCV®/Circumvent®	MSD	PCV2a	Subunit vaccine by baculovirus
Bivalent (PCV2/MH)	Porcillis® PCV M Hyo	MSD	PCV2a	Subunit vaccine by baculovirus
Bivalent (PCV2/MH)	Circo/MycoGard®	Pharmgate Animal Helath	PCV2b	Subunit vaccine by baculovirus
Trivalent (PCV2/MH)	Fostera® Gold PCV MH	Zoetis	PCV2a/2b	Chimeric whole virus vaccine

Table 1. Features of major commercial PCV2 monovalent vaccines and combined vaccines of PCV2 and *M. hyopneumoniae*. The shadowed products in the cells are subject products of this study.

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Chapter I

A field efficacy trial of a trivalent vaccine containing porcine circovirus type 2a and 2b, and *Mycoplasma hyopneumoniae* in three herds

Abstract

This field trial was designed to evaluate the efficacy of a new trivalent vaccine containing porcine circovirus type 2a and 2b (PCV2a/b), and *Mycoplasma hyopneumoniae* at three independent locations. Three farms were selected based on their history of PCV2 and *M. hyopneumoniae* co-infection. Each farm housed a total of 60, three-day-old pigs that were randomly allocated to one of three treatment groups. Pigs were administered the trivalent vaccine intramuscularly with either a 1.0 mL dose at 3 and 24 days of age, or with a 2.0 mL dose at 21 days of age in accordance with the manufacturer's recommendations. Clinically, the average daily weight gain of the one-dose and two-dose vaccinated groups within all three farms were significantly higher ($p<0.05$) than those of unvaccinated animals during the growing (70 to 112 days of age), finishing (112 to 175 days of age), and overall (3 to 175 days of age) stages of production. One-dose and two-dose vaccinated animals elicited neutralizing antibodies and interferon-ɣ secreting cells (IFN-ɣ-SC), which reduced the amount of PCV2 in terms of blood load and reduced the severity of lymphoid lesions when compared with unvaccinated animals. Similarly, one-dose and two-dose vaccinated animals elicited IFN-ɣ-SC, which reduced the amount of *M. hyopneumoniae* in terms of laryngeal load and reduced the severity of lung lesions. The intramuscular administration of either one-dose and two-dose of trivalent vaccine was not significant different in any of the evaluated parameters. The results of field

trial demonstrated that the trivalent vaccine was efficacious in the protection of swine herds where PCV2d and *M. hyopneumoniaen* were in active circulation.

Keywords: enzootic pneumonia, *Mycoplasma hyopneumoniae*, porcine circovirus type 2, porcine circovirus-associated diseases, vaccine

1. Introduction

Porcine circovirus type 2 (PCV2) is a very small, circular, single-stranded DNA virus. It is a primary etiologic agent of 'porcine circovirus associated disease' (PCVAD) [1, 2]. Since introduction of efficacious PCV2 vaccines, subclinical infection is currently the most common form of PCV2 infection worldwide [3]. The only observable disease manifestation associated with subclinical PCV2 infection is a decrease in average daily gain [4-6]. PCV2 is currently further divided into eight genotypes, designated as 'a to h' [7]. The second global genotype shift from PCV2b to PCV2d in 2014 [8] marked the worldwide spread of PCV2d, launching it as the most prevalent PCV2 genotype in Asia and North America [9-14].

Mycoplasma hyopneumoniae lacks a cell wall, has a very small amount of genetic material, and is one of the smallest bacteria in nature [15]. Enzootic pneumonia, caused by *M. hyopneumoniae*, is one of the most prevalent diseases affecting swine production and inflicts significant economic losses due to the resulting reduced growth rate and feed conversion efficiency [16].

Co-infection of PCV2 with *M. hyopneumoniae* causes of major worldwide economic losses within the swine industry. Vaccination against PCV2 and *M. hyopneumoniae* are therefore routinely and widely used in the Asian pig industry. A new trivalent vaccine containing PCV2a and 2b (PCV2a/b) along with *M. hyopneumoniae* (registered as Fostera® Gold PCV MH in the USA and Asia/CircoMax® Myco in Europe, Zoetis, Parsippany, NJ, USA) has been

introduced into the Asian market. The trivalent vaccine is of particular interest because it contains PCV2b, which is genetically close to PCV2d. Although PCV2a-based vaccines may protect pigs against PCV2d [17-20], vaccine failure has also been reported in PCV2a-vaccinated herds [21-23]. The objective of this study was to determine the efficacy in relation to growth performance of a new trivalent vaccine containing PCV2a/b and *M. hyopneumoniae* in pig farms suffering from concurrent circulation of PCV2d and *M. hyopneumoniae.*

2. Materials and methods

2.1 Farm history

The clinical field trial was conducted on three farms from June to December of 2020. Farms were labeled as "A, B, and C" and were 380-sow, 260-sow, and 430-sow (respectively) farrow-to-finish swine operations with an all-in-all-out production system. The status of porcine reproductive and respiratory syndrome virus (PRRSV) on all 3 farms was stable; with no active PRRSV circulation (high-parity sows were the only seropositive animals in the herd). All replacement gilts used in the three farms tested seronegative for *M. hyopneumoniae* and were vaccinated one for PCV2 on arrival. Sows from three farms were not immunized for either PCV2 or *M. hyopneumoniae*. All piglets received vaccinations for PCV2 and *M.*

hyopneumoniae at 3 weeks of age, classical swine fever virus and *Erysipelothrix rhusiopathiae* at 6 weeks of age, and foot and mouth disease virus at 8 and 12 weeks of age. Pigs were weaned at 21 days of age.

Each farm consistently suffered pig loss over several months due to growth retardation and respiratory disease in the late post-weaning and growing stages. Clinical signs first appeared at approximately 7 to 10 weeks of age and reached peak mortality (approximately 1-3%=farm A, 1-2%=farm B, and 2-5%=farm C) between 10 to 15 weeks of age.

Farms A and B were selected based on their subclinical PCV2 infection and enzootic pneumonia. Previous diagnoses fulfilled the definition of subclinical PCV2 infection [3] to include decreased average daily gain without overt clinical signs, absence of or minimal histopathological lesions in superficial inguinal lymph nodes, and a low amount of PCV2 antigen presence in superficial inguinal lymph nodes as determined by immunohistochemistry in 3 out of 5 suspected pigs on the two farms. PCV2d was detected in serum from 3 pigs with each of these two farms, where $\log_{10}$ DNA copies/mL ranged from 2.35 to 3.23 from farm A and 2.45 to 3.32 from farm B. These values were consistent with the definition of subclinical PCV2 infection [24, 25]. A lung examination was performed at the slaughterhouse, and was suggestive of enzootic pneumonia with craniovental bronchopneumonia lesions in 60% of the 30 pigs had. Farm C was selected based on its clinical history of PCVAD and enzootic pneumonia. Previous diagnoses fulfilled the

definition of PCVAD [24] to include clinical signs (i.e., retardation of growth), histopathological findings (i.e., lymphoid depletion and lymphoid granulomatous inflammation with intracytoplasmic inclusion bodies), along with PCV2 antigen presence in lymphoid lesions as determined by immunohistochemistry in 4 out of 5 suspected animals on the farm. PCV2d was detected in serum from 3 pigs that ranged 4.35 to 5.18 $\log_{10}$ DNA copies/mL which was consistent with defined PCVAD [25, 26]. A lung examination was performed at the slaughterhouse, which confirmed that 20% of the 30 pigs had mycoplasmal pneumonia lesions

2.2 Study design

The results of this field study will be sent for registration and therefore strictly adhered to the guidelines of the Republic of Korea's Animal, Plant & Fisheries Quarantine & Inspection Agency (QIA, http://www.qia.go.kr). QIA protocols mandate that a total of 20 pigs were assigned to each study group. Study design considerations included randomization, personnel blinding, and that animals were both weight-matched and sex-matched under a controlled clinical field trial format. To minimize sow variation, either six or nine, three-day-old pigs were randomly selected from seven total sows. If six (or nine), three-day-old pigs were pulled from a sow, two (or three) pigs were assigned to each of three uniform study groups. A total of 180 pigs were used for the entire study. Sixty pigs per farm were

randomly divided into 3 groups within each farm (20 pigs per group; 10 = male and 10 = female) using the random number generator function (Excel, Microsoft Corporation, Redmond, WA, USA) (Table 1).

The pigs in the VacA1, VacB1, and VacC1 groups were injected intramuscularly in the right side of the neck at study day 18 (21 days of age) with 2.0 mL of the trivalent vaccine containing PCV2a/b and *M. hyopneumoniae* (Fostera® Gold PCV MH, Zoetis). Each farm received a different serial of the vaccine as follows: Farm A=Serial No: 395164A, Expiration date: 10-Dec-2021, Farm B=Serial No: 394687A, Expiration date: 10-Dec-2021, and Farm C=Serial No: 413369A, Expiration date: 03-Feb-2022. Pigs in the VacA2, VacB2, and VacC2 groups were injected intramuscularly in the right side of the neck at study days 0 (3 days of age) and 21 (24 days of age) with 1.0 mL of the trivalent vaccine. Pigs in the UnVacA, UnVacB, and UnVacC groups were injected intramuscularly in the right side of the neck at study days 0 (3 days of age) and 21 (24 days of age) with 1.0 mL of phosphate buffered saline (PBS, 0.01M, pH 7.4).

At 28 days of age, pigs from the vaccinated and unvaccinated groups were commingled and randomly assigned into 6 pens (10 pigs per pen) using the random number generator function (Excel, Microsoft Corporation). All pens were identical in design with equipment including free access to water and feed. Five pigs from each group were randomly selected and euthanized for necropsy at 112 days of age. The rest of pigs from each group were

euthanized for necropsy at 175 days of age. Pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution as previously described [27]. Lung, liver, tonsil, kidney, spleen, small and large intestine, and superficial inguinal lymph node tissues were collected from each pig at the time of necropsy. Tissues were fixed for 24 hours in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. The protocol for this field study was approved by the Seoul National University Institutional Animal Care and Use Committee (approval number SNU-191017-10).

Table 1. Experimental design

Farm	Group	No. of pigs	Vaccination (dosage)
A	VacA1	20	D 18 (21 days of age; 2 ml)
	VacA2	20	D 0 (3 days of age; 1 ml), D 21 (24 days of age; 1 ml)
	UnVacA	20	None
B	VacB1	20	D 18 (21 days of age; 2 ml)
	VacB2	20	D 0 (3 days of age; 1 ml), D 21 (24 days of age; 1 ml)
	UnVacB	20	None
C	VacC1	20	D 18 (21 days of age; 2 ml)
	VacC2	20	D 0 (3 days of age; 1 ml), D 21 (24 days of age; 1 ml)
	UnVacC	20	None

2.3 Sampling collection

Blood and laryngeal swabs were collected at study days 0 (3 days of age), 18 (21 days of age), 46 (49 days of age), 67 (70 days of age), and 109 (112 days of age). Pigs were snared and restrained with a mouth gag for laryngeal swab collection. Swabs were guided with a laryngoscope down into the larynx. The internal walls of the laryngeal cartilages were then swept with the swabs once the larynx was visualized and the epiglottis was in a low position as previously described [28].

2.4 Mortality

Pigs that died were subjected to gross pathological examination within 24 h at local veterinary practitioners. All major organs such as brain, lung, superficial inguinal lymph node, small and large intestine, liver, kidney, and tonsils were collected from each pig submitted to the diagnostic laboratory. Polymerase chain reaction assays were used in order to detect specific nucleic acids for PCV2, PRRSV, swine influenza virus, and *M. hyopneumoniae* [29-32]. All other bacterial isolation, and identifications were carried out by using routine methods.

2.5 Clinical observations

Pig physical condition was monitored daily, and pigs were scored weekly for clinical signs as previously described [33]. Briefly, scoring was defined as follows: 0 (normal), 1 (rough haircoat), 2 (rough haircoat and dyspnea), 4 (severe dyspnea and abdominal breathing), 5 (severe dyspnea and abdominal breathing, and hesitation of movement) and 6 (death). Scoring observers were blinded to vaccination status.

2.6 Growth performance

Pigs were weighed at study days 0 (3 days of age), 18 (21 days of age), 67 (70 days of age), 109 (112 days of age), and 172 (175 days of age). Average daily gain (ADG=gram/pig/day) was determined for study day 0 to 18, study day 18 to 67, study day 67 to 109, and study day 109 to 172. The ADG during these various 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.

2.7 PCV2 DNA in blood

A commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA) was used to extract DNA from serum samples for PCV2d. The number of genomic DNA copies for PCV2a, PCV2b, and PCV2d was then quantified by real-time PCR [34, 35]. To construct a standard curve, real-time PCR was

performed in quadruplicate in two different assays: (i) 10-fold serial dilutions of the PCV2 plasmid were used as the standard, with concentrations ranging from 10^{10} to 10^{2} copies/mL, and (ii) 10-fold serial dilutions of PCV2 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 PCV2 plasmid was prepared as described previously [34]. Culture supernatants of PCV1-free PK-15 cells were used as negative control.

2.8 *M. hyopneumoniae* DNA in laryngeal swabs

DNA was extracted from laryngeal swabs using the commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA) to quantify the *M. hyopneumoniae* genomic DNA copy numbers by real-time PCR as previously described [36]. The forward and reverse primers (5′-TTG ACT GCT ATC TTT GCA CGA TAA G-3′ and 5′- ACA ATA ATT GCT GAC CGT GGC-3′) and probe (5′-FAM-TGT CCA CTG CTG CAA ATA TTC GAT TTC TTG AA-TAMRA-3′) were used to detect *M. hyopneumoniae* [36].

To construct a standard curve, real-time PCR was performed in quadruplicate in 10-fold serial dilution of chromosomal DNA from *M. hyopneumoniaes* strain SNU98703, with concentrations ranging from 10ng/μL to 1fg/μL. One femtogram of chromosomal DNA from *M. hyopneumoniae* is considered to be approximately one genome equivalent [37]. A positive and

negative control was included in each run using chromosomal DNA from *M. hyopneumoniaes* strain SNU98703 and double distilled water, respectively, as the template.

2.9 Serology

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

Serum samples were tested for serum virus neutralization using PCV2d strain (SNUVR202002, GenBank no. MW821481) [38, 39]. Serum samples were heat-inactivated at 56°C for 30 minutes prior to performing the test. The neutralization titer with this assay was calculated as the reciprocal of the highest dilution of the serum that was able to 80% block PCV2-infection in PK-15 cells. Thus the lowest dilution contained 25% serum (1:1 dilution of serum + equal volume of PCV2d stock), thereby the detection limit of

this assay was $2\log_2$.

2.10 Enzyme-linked immunospot

An enzyme-linked immunospot (ELISpot) assay was conducted to measure the numbers of PCV2d- and *M. hyopneumoniae*-specific interferon-ɣ secreting cells (IFN-ɣ-SC) [35, 40]. Briefly, 100ml containing 2 x 10^6 peripheral blood mononuclear cells (PBMC) in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., SelectScience, Bath, UK) were seeded unto plates precoated overnight with anti-porcine IFN-ɣ monoclonal antibody (5μg/ml, MABTECH, Mariemont, OH, USA) and incubated with PCV2d (20mg/ml), *M. hyopneumopniae* (4mg/ml), phytohemagglutinin (10mg/ml, Roche Diagnostics GmbH, Mannheim, Germany) as a positive control, or PBS as a negative control for 20h at 37°C in a 5% humidified CO_2 atmosphere. The wells were washed five times with PBS (200ml per well) and thereafter, the procedure followed manufacturer's instructions using commercial ELISpot assay kit (MABTECH). The spots on the membranes were read by an automated ELISpot reader (AID ELISpot Reader, AID GmbH, Strassberg, Germany). The results were expressed as the number of responding cells/million PBMC.

2.11 Pathology

Two pathologists at the Seoul National University scored the severity of macroscopic lung lesions in order to estimate the percentage of the lung affected by pneumonia [41, 42]. Two blinded veterinary pathologists then examined the collected pulmonary and lymphoid tissue sections. Pulmonary lesions were scored the severity of peribronchiolar lymphoid tissue hyperplasia by mycoplasmal pneumonia lesions ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse) [42]. Severity of lymphoid lesion severity was scored from 0 to 5 (0, normal; 1, mild lymphoid depletion; 2, mild to moderate lymphoid depletion and histiocytic replacement; 3, moderate diffuse lymphoid depletion and histiocytic replacement; 4, moderate to severe lymphoid depletion and histiocytic replacement; 5, severe lymphoid depletion and histiocytic replacement) [43].

2.12 Immunohistochemistry

Immunohistochemistry for PCV2 was performed as previously described [44]. Nine sections (3 sections from 3 different blocks) of the same lymph node of each pig were used for the morphometric analyses of immunohistochemistry. Quantitative data was analyzed from the prepared immunohistochemistry slides using the NIH Image J 1.45s Program

(http://imagej.nih.gov/ij/download.html). PCV2 analysis was conducted by the random selection of 10 microscopic areas, where the number of positive cells per unit area (0.95 mm^2) was determined as previously described [45]. The mean values were also calculated.

2.13 Statistical analysis

All real-time PCR data and neutralizing antibody titers were transformed to log_{10} and log_2, respectively, values prior to statistical analysis. The Shapiro-Wilk test evaluated data for normal distribution. One-way analysis of variance (ANOVA) was used to examine differences in variables with normal distribution (ADWG, growth performance, PCV2 DNA, *M. hyopneumoniae* DNA, PCV2 ELISA IgG titer, PCV2 neutralizing antibody titer, *M. hyopneumoniae* ELISA S/P ratio, and number of IFN-ɣ-SC). Kruskal-Wallis test was used for variables without a normal distribution (clinical signs, neutralizing antibody titers against PCV2d, macroscopic and microscopic lung lesion scores) for 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. 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. Results were reported in p value where a value of $p<0.05$ was considered to be

significant.

3. Results

3.1 Mortality

The overall mortality rate is summarized in Table 2. Diagnostic results indicated that mortality at all farms was primarily related to co-infection with PCV2 and *M. hyopneumoniae* in unvaccinated animals. Mortality at Farm A was reported as follows: One pig from the VacA1 group died of streptococcal meningitis caused by *Streptococcus suis* at study day 80 (83 days old). Two pigs from the VacA2 group died of streptococcal meningitis caused by *S. suis* at study day 72 (75 days old) and suppurative bronchopneumonia caused by *Staphylococcus aureus* at study day 91 (94 days old), respectively. Two unvaccinated pigs died of enzootic pneumonia caused by *M. hyopneumoniae* and *Pasteurella multocida* at study day 64 and 91 (67 and 94 days old), respectively, and one unvaccinated pig died of PCVAD and Glasser's disease caused by *Glaesseralla parasuis* at study day 113 (116 days old).

Mortality at Farm B was reported as follows: One pig from the VacB2 group died of pneumonic pasteurellosis caused by *P. multocida* at study day 57 (60 days old). Two unvaccinated pigs died of severe respiratory disease caused by PCV2d and *M. hyopneumoniae* at study day 82 (85 days old) and

enzootic pneumonia caused by *M. hyopneumoniae* and *P. multocida* at study day 102 (105 days old), respectively. One unvaccinated pig died of lymphoid depletion caused by PCV2d and fibrinous pleuritis and pericarditis caused by *G. parasuis* at study day 93 (96 days old).

Mortality at Farm C was reported as follows: One pig from the VacC1 group died of suppurative bronchopneumonia caused by *S. aureus* at study day 50 (53 days old). Two unvaccinated pigs died of enzootic pneumonia caused by *M. hyopneumoniae* and *S. aureus* at study day 61 and 68 (64 and 71 days old), respectively. Two additional unvaccinated pigs died of severe respiratory disease caused by PCV2d and *M. hyopneumoniae* at study day 65 and 84 (68 and 87 days old), respectively.

Table 2. Body weight (mean ± standard deviation) of vaccinated in pig vaccinated for trivalent vaccine containing PCV2a/b and *M. hyopneumoniae* or unvaccinated pigs on 3 swine farms

Farm	Group	Body weight (kg)			Mortality
		D 0 (3 days of age)	D 18 (21 days of age)	D 172 (175 days of age)	
A	VacA1	2.45 ± 0.16	5.83 ± 0.55	106.61 ± 1.54*	5%
	VacA2	2.53 ± 0.21	5.99 ± 0.42	106.06 ± 2.06*	10%
	UnVacA	2.56 ± 0.16	5.84 ± 0.48	100.60 ± 1.73	10%
B	VacB1	2.50 ± 0.24	5.56 ± 0.24	106.21 ± 2.16*	0%
	VacB2	2.50 ± 0.22	5.67 ± 0.22	107.25 ± 1.68*	5%
	UnVacB	2.62 ± 0.16	5.74 ± 0.16	100.28 ± 2.33	15%
C	VacC1	2.57 ± 0.23	5.91 ± 0.52	104.68 ± 1.40*	5%
	VacC2	2.57 ± 0.28	5.82 ± 0.28	104.17 ± 1.95*	0%
	UnVacC	2.72 ± 0.14	5.89 ± 0.43	99.18 ± 1.68	20%

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

3.2 Clinical signs

Vaccinated pigs (VacA1 and VacA2) from farm A had significantly lower ($p<0.05$) clinical sign scores when compared with unvaccinated animals (UnVacA) at study days 60 to 74 (Figures 1a and 1b). Farm B vaccinates (VacB1 and VacB2) also had significantly lower ($p<0.05$) clinical sign scores when compared with unvaccinated animals (UnVacB), but study days 46 to 81 (Figures 1a and 1b). On farm C, vaccinated pigs (VacC1 and VacC2) had significantly lower ($p<0.05$) clinical sign scores when compared with unvaccinated animals (UnVacC) at study days 39 to 116 (Figures 1a and 1b). A difference in respiratory signs was not observed between one-dose and two-dose vaccinated groups in any of the three farms.

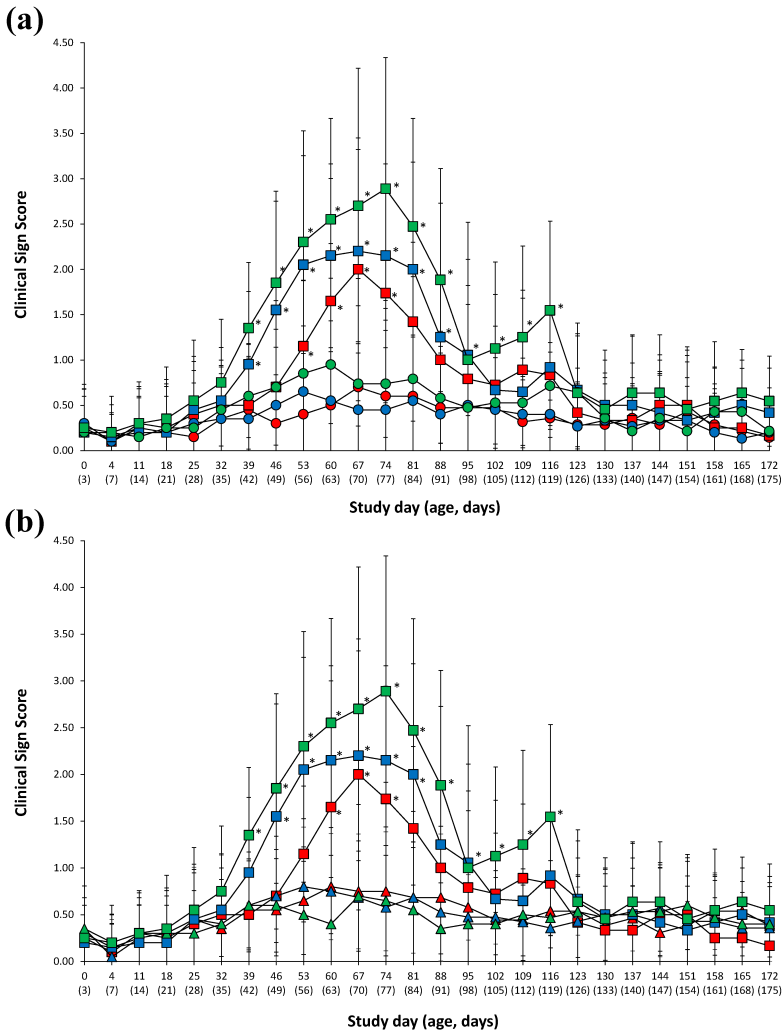

Figure 1. Clinical sign scores. (a) Clinical sign scores (means ± standard deviation) from VacA1 (●), VacB1 (●), VacC1 (●), UnVacA (■), UnVacB (■), and UnVacC (■) groups. (b) Clinical sign scores (means ± standard deviation) from VacA2 (▲), VacB2 (▲), VacC2 (▲), UnVacA (■), UnVacB (■), and UnVacC (■) groups. *Significant difference ($p<0.05$) between vaccinated and unvaccinated group within the same farm

3.3 Growth performance

The body weight of pigs at study days 0 (3 days of age) and 21 (24 days of age) did not differ significantly between vaccinated and unvaccinated group at the time of vaccination on all 3 farms. Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) had a significantly higher ($p<0.05$) body weight when compared with unvaccinated pigs in all farms (A-C) at study day 172 (175 day of age) (Table 2).

Vaccinated pigs from all farms (A-C) had significantly higher ($p<0.05$) ADG at study days 67 to 109 (70 to 112 days old) and 109 to 172 (112 to 175 days old) when compared with unvaccinated pigs from the same farm. Overall (study days 0 to 172), the difference between vaccinated and unvaccinated groups was significant ($p<0.05$) on all farms (Table 3). There were no significant differences in the ADG between one-dose and two-dose vaccinated groups on all farms.

Table 3. Average daily gain (ADG; mean ± standard deviation) in pig vaccinated for trivalent vaccine containing PCV2a/b and *M. hyopneumoniae* or unvaccinated pigs on 3 swine farms

Farm	Group	ADG (gram/day/pig)			
		D 0-18 (3-21 days old)	D 18-109 (21-112 days old)	D 109-172 (112-175 days old)	D 0-172 (3-175 days old)
A	VacA1	187.50 ± 31.42	588.31 ± 20.01	794.22 ± 34.13*	605.73 ± 8.45*
	VacA2	192.22 ± 27.07	573.55 ± 18.13	785.96 ± 33.13*	601.92 ± 11.83*
	UnVacA	182.50 ± 28.34	536.30 ± 22.31	753.70 ± 22.53	569.77 ± 10.01
B	VacB1	169.72 ± 36.41	572.27 ± 18.57*	795.87 ± 33.20*	602.79 ± 12.44*
	VacB2	176.39 ± 19.63	576.26 ± 13.94*	802.04 ± 21.53*	608.76 ± 9.75*
	UnVacB	173.61 ± 25.63	531.02 ± 23.98	746.96 ± 34.62	567.97 ± 13.08
C	VacC1	185.56 ± 32.79	560.94 ± 15.53*	785.15 ± 20.54*	593.90 ± 8.31*
	VacC2	180.83 ± 18.88	556.14 ± 13.45*	785.93 ± 33.31*	590.74 ± 10.57*
	UnVacC	176.11 ± 24.48	518.82 ± 24.11	740.26 ± 35.76	560.73 ± 9.39

*Significant difference ($p<0.05$) between vaccinated and unvaccinated group within the same farm

3.4 PCV2 viremia

Vaccinated pigs (VacA1, VacA2, VacB1, and VacB2) from farms A and B had a significantly lower ($p<0.05$) number of genomic copies of PCV2d in their blood when compared with unvaccinated pigs (UnVacA and UnVacB) at study days 46, 67, and 109. Farm C vaccinates (VacC1 and VacC2) also had a significantly lower ($p<0.05$) number of genomic copies of PCV2d in their blood when compared with unvaccinated pigs (UnVacC) at study days 67 and 109. Two-dose vaccinated pigs (VacC2) from farm C had a significantly lower ($p<0.05$) number of genomic copies of PCV2d in their blood when compared with unvaccinated pigs (UnVacC) at study day 46 (Figures 2a and 2b).

The one-dose and two-dose vaccinated pigs from farms A, B, and C had comparable number of genomic copies of PCV2d DNA throughout the entire field trials with no significant farm-to-farm differences between the three sites. Genomic copies of PCV2a and PCV2b DNA were not detected in any pigs from three farms throughout the entire field study.

3.5 *M. hyopneumoniae* DNA in laryngeal swab

Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) from three farms had a significantly lower ($p<0.05$) number of genomic copies of *M. hyopneumoniae* in their laryngeal swabs when compared with

unvaccinated pigs (UnVacA, UnVacB, and UnVacC) at study days 67 and 109 (Figures 2c and 2d).

The one-dose and two-dose vaccinated pigs from three farms had comparable number of genomic copy of *M. hyopneumoniae* DNA in their laryngeal swabs throughout the entire field trials, and significant differences were not found between groups on the three farms.

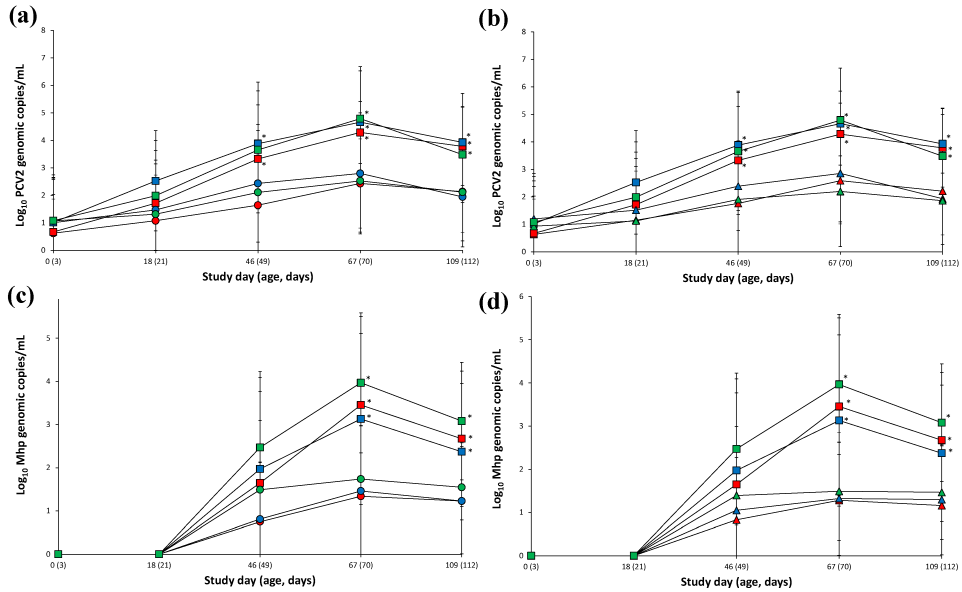

Figure 2. Real-time PCR results for porcine circovirus type 2d (PCV2d) and *Mycoplasma hyopneumoniae*. (a) Number of PCV2d genomic copies (means ± standard deviation) in the blood from VacA1 (●), VacB1 (●), VacC1 (●), UnVacA (■), UnVacB (■), and UnVacC (■) groups. (b) Number of PCV2d genomic copies (means ± standard deviation) in the blood from VacA2 (▲), VacB2 (▲), VacC2 (▲), UnVacA (■), UnVacB (■), and UnVacC (■) groups. (c) Number of *M. hyopneumoniae* genomic copies in laryngeal swabs from VacA1 (●), VacB1 (●), VacC1 (●), UnVacA (■), UnVacB (■), and UnVacC (■) groups. (d) Number of *M. hyopneumoniae* genomic copies in laryngeal swabs in two-dose vaccinated (Vac2) and unvaccinated (UnVac) groups. *Significant difference ($p<0.05$) between vaccinated and unvaccinated group within the same farm

3.6 Immune responses against PCV2

Two-dose vaccinated pigs (VacA2, VacB2, and VacC2) from three farms had a significantly higher ($p<0.05$) PCV2 ELISA IgG titer at study day 18 when compared with one-dose vaccinated (VacA1, VacB1, and VacC1) and unvaccinated (UnVacA, UnVacB, and UnVacC) pigs. Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) from three farms had a significantly higher ($p<0.05$) PCV2 ELISA IgG titer at study days 46, 67, and 109 when compared with unvaccinated pigs (Figure 3a). Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) from three farms had a significantly higher ($p<0.05$) PCV2 neutralizing antibody titer at study days 46, 67, and 109 when compared with unvaccinated pigs (Figure 3b).

Two-dose vaccinated animals (VacA2, VacB2, and VacC2) from three farms had a significantly higher ($p<0.05$) number of PCV2d-specific IFN-ɣ-SC at study day 18 when compared with one-dose vaccinated (VacA1, VacB1, and VacC1) and unvaccinated (UnVacA, UnVacB, and UnVacC) pigs. Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) from three farms had a significantly higher ($p<0.05$) number of PCV2d-specific IFN-ɣ-SC at study days 46, 67, and 109 when compared with unvaccinated pigs (Figure 3c).

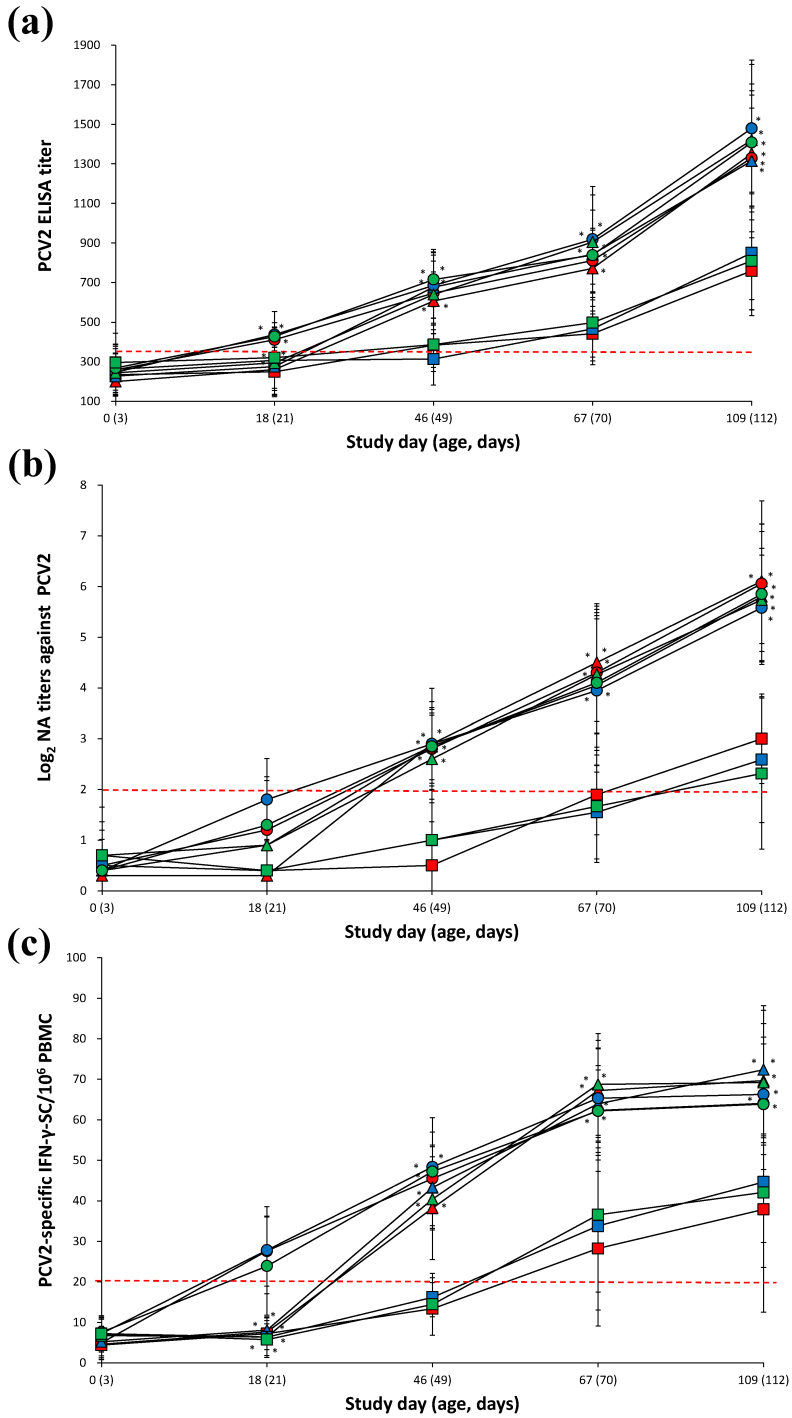
(a)
PCV2 ELISA titer
1900
1700
1500
1300
1100
900
700
500
300
100
0 (3)
18 (21)
46 (49)
67 (70)
109 (112)
Study day (age, days)
(b)
Log2 NA titers against PCV2
8
7
6
5
4
3
2
1
0
0 (3)
18 (21)
46 (49)
67 (70)
109 (112)
Study day (age, days)
(c)
PCV2-specific IFN-γ-SC/10^6 PBMC
100
90
80
70
60
50
40
30
20
10
0
0 (3)
18 (21)
46 (49)
67 (70)
109 (112)
Study day (age, days)

Figure 3. Immune responses against porcine circovirus type 2 (PCV2). (a) ELISA titer (means ± standard deviation) in serum samples. (b) Neutralizing antibody (NA) titers against PCV2d in serum samples. (c) ELISpot assay for PCV2d-specific interferon-ɣ secreting cells (IFN-ɣ-SC) in peripheral blood mononuclear cells (PBMC) from VacA1 (▲), VacB1 (▲), VacC1 (▲), VacA2 (●), VacB2 (●), VacC2 (●), UnVacA (■), UnVacB (■), and UnVacC (■) groups. Red dotted line is cuff-off (ELISA titer >350 titer, NA titer > 2 $\log_2$, and ELISpot number of PCV2d-specific IFN-ɣ-SC > 20 cells/10^6 PBMC). *Significant difference (p<0.05) between vaccinated and unvaccinated group within the same farm

3.7 Immune responses against *M. hyopneumoniae*

Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) from three farms had a significantly higher ($p<0.05$) *M. hyopneumoniae* ELISA S:P ratio at study days 46, 67 and 109 when compared with unvaccinated pigs (UnVacA, UnVacC, and UnVacC) (Figure 4a).

Two-dose vaccinated pigs (VacA2 and VacB2) from farms A and B had a significantly higher ($p<0.05$) number of *M. hyopneumoniae*-specific IFN-ɣ-SC at study day 18 when compared with the unvaccinated pigs. Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) from three farms had a significantly higher ($p<0.05$) number of *M. hyopneumoniae*-specific IFN-ɣ-SC at study days 46 and 67 when compared with unvaccinated pigs (UnVacA, UnVacC, and UnVacC) (Figure 4b).

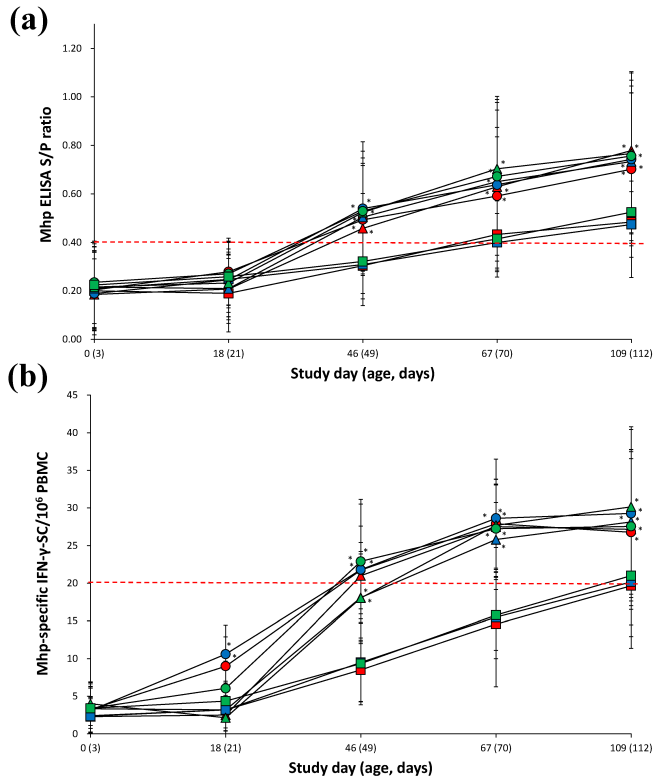

Figure 4. Immune responses against *Mycoplasma hyopneumoniae*. (a) ELISA sample-to-positive (S/P) ratio (means ± standard deviation) in serum samples. (b) ELISpot assay for *M. hyopneumoniae* (Mhp)-specific interferon-ɣ secreting cells (IFN-ɣ-SC) in peripheral blood mononuclear cells (PBMC) from VacA1 (▲), VacB1 (▲), VacC1 (▲), VacA2 (●), VacB2 (●), VacC2 (●), UnVacA (■), UnVacB (■), and UnVacC (■) groups. Red dotted line is cuff-off (ELISA S/P ratio ≥ 0.4 and ELISpot number of *M. hyopneumoniae*-specific IFN-ɣ-SC > 20 cells/10^6 PBMC). *Significant difference (p<0.05) between vaccinated and unvaccinated group within the same farm

3.8 Pathology

Mycoplasmal lung lesions were characterized by various degree of peribronchiolar lymphoid tissue hyperplasia. PCV2-associated lesions in lymphoid tissues were characterized by lymphoid depletion and histiocytic-to-granulomatous inflammation with/without low-to-moderate numbers of multinucleated giant cells. Vaccinated pigs (VacA1, VacA2, VacB1, VacB2, VacC1, and VacC2) from three farms had significantly lower ($p<0.05$) macroscopic lung lesion score, microscopic lung and lymphoid lesion scores, and number of lymphoid PCV2-positive cells when compared to unvaccinated pigs (UnVacA, UnVacB, and UnVacC) at study day 109 (Table 4 and 5). On farm C, vaccinated pigs (VacC1 and VacC2) had significantly lower ($p<0.05$) microscopic lung and lymphoid lesion scores, and number of lymphoid PCV2-positive cells when compared to unvaccinated pigs (UnVacA, UnVacB, and UnVacC) at study day 172 (Table 4 and 5). There were no significant differences in overall scores for microscopic lung and lymphoid lesions, and the numbers of lymphoid PCV2-positive cells between one-dose and two-dose vaccination regimens.

Table 4. Lung lesion scores (means ± standard deviation)

Farm	Group	Macroscopic Lesions		Microscopic Lesions	
		D 109 (112 days old)	D 172 (175 days old)	D 109 (112 days old)	D 172 (175 days old)
A	VacA1	22.5 ± 9.8*	17.8 ± 1.7	0.80 ± 0.18*	0.52 ± 0.30
	VacA2	24.8 ± 9.3*	17.3 ± 3.3	0.88 ± 0.24*	0.72 ± 0.16
	UnVacA	47.2 ± 11.0	25.6 ± 10.4	2.64 ± 0.56	0.96 ± 0.45
B	VacB1	24.7 ± 5.5*	18.9 ± 5.9	1.00 ± 0.33*	0.80 ± 0.25
	VacB2	25.2 ± 4.1*	18.8 ± 6.3	1.12 ± 0.50*	0.72 ± 0.16
	UnVacB	45.4 ± 10.5	27.1 ± 6.4	2.40 ± 0.44	1.12 ± 0.52
C	VacC1	26.4 ± 3.8*	22.3 ± 8.0*	1.28 ± 0.27*	0.96 ± 0.08*
	VacC2	25.5 ± 7.9*	23.7 ± 5.7*	1.08 ± 0.56*	0.92 ± 0.32*
	UnVacC	52.1 ± 6.4	34.6 ± 4.3*	3.80 ± 0.28	2.12 ± 0.37

*Significant difference ($p<0.05$) between vaccinated and unvaccinated group within the same farm

Table 5. Lymphoid lesion scores and PCV2-positive cells (means ± standard deviation)

Farm	Group	Microscopic Lesions		No. of PCV2-positive cells	
		D 109 (112 days old)	D 172 (175 days old)	D 109 (112 days old)	D 172 (175 days old)
A	VacA1	0.96 ± 0.29*	0.64 ± 0.20	4.33 ± 0.63*	2.80 ± 0.91
	VacA2	0.84 ± 0.39*	0.76 ± 0.37	4.93 ± 0.90*	3.13 ± 1.05
	UnVacA	2.04 ± 0.41	1.04 ± 0.43	10.87 ± 1.24	4.07 ± 1.08
B	VacB1	1.04 ± 0.20*	0.60 ± 0.33	5.33 ± 0.76*	3.13 ± 0.75
	VacB2	0.92 ± 0.32*	0.56 ± 0.27	5.80 ± 0.34	3.47 ± 1.05
	UnVacB	2.20 ± 0.36	1.00 ± 0.42	11.60 ± 2.00	4.80 ± 0.86
C	VacC1	1.16 ± 0.32*	0.92 ± 0.20*	5.93 ± 0.33*	3.60 ± 0.93*
	VacC2	1.12 ± 0.20*	0.96 ± 0.08*	6.20 ± 0.50*	3.73 ± 0.98*
	UnVacC	3.04 ± 0.46	1.40 ± 0.28	14.07 ± 1.39	5.93 ± 1.18

*Significant difference ($p<0.05$) between vaccinated and unvaccinated group within the same farm

4. Discussion

The common sign of PCV2 and *M. hyopneumoniae* co-infection is growth retardation. Vaccination against these two pathogens is needed and widely used to improve pig growth performance. Therefore, growth performance was selected as the most critical index in the efficacy evaluation of a trivalent vaccine under field conditions. The pigs vaccinated with the trivalent vaccine demonstrated improved growth performance suggesting that the vaccine may have contributed to the favorable outcome in the farm A and B herds with subclinical PCV2 infection. Overt clinical signs of PCVAD were not observed on either of these two farms.. Such field observations have also been reported in other pig rearing countries such as Canada [16], the UK [4], Spain [46], Germany [47], and Switzerland [6]. Swine practitioners and producers are therefore aware of the costly impact that subclinical PCV2 infection has in swine herds. This may directly impact the decision of producers to vaccinate animals even in the absence of overt clinical signs of PCVAD.

The trivalent vaccine containing PCV2a/b and *M. hyopneumoniae* evaluated in the field trials elicited protective immunity against PCV2d and *M. hyopneumoniae*. Protective immunity in the forms of PCV2-specific neutralizing antibodies and IFN-ɣ-SC reduced the amount of PCV2 viral blood-load and reduced the severity of lymphoid lesions [48-51]. For the aspect of immune responses to *M. hyopneumoniae*, humoral immunity has

not been associated with protection [52] but cell-mediated immunity plays a role to protect pigs from *M. hyopneumoniae* infection [53]. Trivalent vaccine was successful in inducing a measurable cellular immune response and reducing the severity of mycoplasmal lung lesions. Significant differences were not observed between the one-dose and two-dose vaccinated groups in relation to the induction of detectable immune response against PCV2 and *M. hyopneumoniae*, the reduction of genomic copies of PCV2 in blood and *M. hyopneumoniae* in laryngeal swabs, and the reduction of pulmonary and lymphoid lesion severity. The trivalent vaccine administered as either one or two doses therefore elicited detectable immune response and provided protection against PCV2 and *M. hyopneumoniae* infection.

Pathological evaluation was also critical in evaluating the protective index as lesion reduction is related to growth performance in both PCV2 and *M. hyopneumoniae* infection [54-57]. No differences in lymphoid lesions or lymphoid PCV2 antigen-positive cells were observed between vaccinated and unvaccinated animals at study day 172 (175 days old) in the two farms (farms A and B) with subclinical PCV2 infection. The minimal or mild lymphoid lesion severity and low number of PCV2 antigen-positive cells of farm A and B pigs were consistent with definition of subclinical infection [3]. Unlike the two farms (A and B) with a history of subclinical PCV2, on farm C with a history of PCVAD statistical differences in lymphoid lesions and lymphoid PCV2-positive cells were observed between vaccinated and unvaccinated animals at study day 172 (175 days old). Mycoplasmal

pneumonic lesions and laryngeal swab load from pigs at study day 109 (112 days old) were significantly reduced in the vaccinated group when compared to the unvaccinated group in all three farms. Mycoplasmal pneumonia resolved in finishing pigs by study day 172 (175 days old) in farms A and B (which had subclinical PCV2 infection and enzootic pneumonia). Co-infection of pigs with PCV2 and *M. hyopneumoniae* causes PRDC and exacerbates lung lesion severity. This was observed in the Farm C finishing pigs at study day 172 (175 days old), where the effect of vaccination on the reduction of lung lesion severity was proven. Commercial farm pigs used field trials such as this are continuously exposed and re-exposed to the prevalent field PCV2d and *M. hyopneumoniae* by horizontal transmission. Natural co-infections as well as other intrinsic and extrinsic factors also exacerbate disease in these less-controlled commercial settings. A true evaluation of the direct effect of vaccination on pathological outcomes would require a controlled experimental challenge study.

Piglets also face potential interference from maternally-derived antibodies (MDA) present at the time of vaccination. In general, early vaccination against PCV2 and *M. hyopneumoniae* was proven as effective in piglets less than one week of age regardless of MDA presence [58, 59]. This field study did not evaluate the effect of MDA on vaccine efficacy. Additionally studies are necessary to explore this theory and ultimately determine the effect of MDA on trivalent vaccine efficacy under well-controlled experimental conditions.

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Chapter II

Comparative Evaluation of Growth Performance between Bivalent and Trivalent Vaccines Containing Porcine Circovirus Type 2 (PCV2) and *Mycoplasma hyopneumoniae* in a Herd with Subclinical PCV2d Infection and Enzootic Pneumonia

Abstract

The present field trial compared two combined vaccines of porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae*, each administered in herd with subclinical PCV2d infection and enzootic pneumonia. One vaccine was a bivalent containing PCV2a and *M. hyopneumoniae* and the other was a trivalent vaccine containing PCV2a and 2b (PCV2a/b), and *M. hyopneumoniae*. The defining difference between these two vaccines was the inclusion or absence of PCV2b antigen. A total of 480, 21day-old pigs were randomly allocated to one of four treatment groups (120 pigs per group, male = 60 and female = 60). These groups included; one-dose trivalent-vaccinated, two-dose trivalent-vaccinated, one-dose bivalent-vaccinated, and unvaccinated. The one- and two-dose trivalent vaccinated pigs exhibited significantly better growth performance when compared with those vaccinated with the bivalent vaccine. The one- and two-dose trivalent vaccinated pigs also reduced the amount of PCV2d loads in the blood and feces, and resulted in a lower *M. hyopneumoniae* load in the larynx when compared with one-dose bivalent vaccinated pigs. Statistical differences were not observed between the one- and two-dose trivalent-vaccinated groups in terms of growth performance, serology, amount of PCV2d loads in the blood and feces, amount of *M. hyopneumoniae* load in larynx, and pathological lesions. The results of the present study will provide swine practitioners and producer with comparative clinical field data to select the proper vaccine and

vaccination regiment for herds suffering from subclinical PCV2d infection and enzootic pneumonia.

Keywords: *Mycoplasma hyopneumoniae*; porcine circovirus type 2; trivalent vaccine

1. Introduction

Porcine circovirus type 2 (PCV2), a member of the family Circoviridae, is a common virus of pigs found throughout the world and is recognized as one of the most economically threatening pathogens to the global pork industry [1]. PCV2 may not be a new virus but it still remains a constant challenge due to the wide range of syndromes and diseases that is causes. Postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure, and enteric manifestations are all examples of porcine circovirus-associated diseases (PCVAD). Although PCV2 has been classified as a well-controlled pathogen since 2018, due to the wide use of vaccines, most farms still experience subclinical PCV2 infection [2]. Currently, PCV2 is classified into at least eight genotypes that are designated consecutively based on the time of first identification with lower case letters, "a to h" [3]. The "d" genotype (PCV2d) is considered the most prevalent and predominant genotype in Asia and North America, today [4-6]. *Mycoplasma hyopneumoniae* is prevalent and highly contagious in the majority of swine herds throughout the global pig industry. *M. hyopneumoniae* causes mycoplasmal pneumonia, which is characterized by a chronic, non-productive cough with high morbidity and low mortality. Enzootic pneumonia that is caused by *M. hyopneumoniae* when combined with opportunistic bacteria, such as *Pasteurella multocida*, continues to be a

significant chronic respiratory disease [7]. Enzootic pneumonia leads to decreased average daily gain and an increased number of days to market weight, both of which result in significant economic losses [7].

PCV2 and *M. hyopneumoniae* are economically important pathogens and the primary agents involved in the PRDC found within global pig production systems. Vaccination for PCV2 and *M. hyopneumoniae* is one of the most effective strategies in the control of both pathogens, especially Asian pork industry [8]. Korean swine farms currently use combination vaccines containing PCV2 and *M. hyopneumoniae* for more than 50% of their pigs (http://www.kahpha.or.kr (accessed on 29 April 2021)). Combined vaccination is consequently considered part of routine management practices.

Recently, a new trivalent vaccine containing PCV2a and 2b (PCV2a/b), and *M. hyopneumoniae* (Fostera® Gold PCV MH/CircoMax® Myco, Zoetis, Parsippany, NJ, USA) was introduced into the global market (http://www.zoetisus.com (accessed on 29 April 2021)). The PCV2b antigen of the trivalent vaccine is of particular interest as it is genetically closely related to PCV2d (formerly referred to as mutant PCV2b), which is currently the predominant PCV2 genotype in Asian pig populations [4-6]. Although PCV2a-based vaccines can provide cross-protection against PCV2d under experimental conditions [9-12], the emergence of PCV2d has still been linked to PCVAD outbreaks within these PCV2a-vaccinated herds [13-15]. In an additional comparative experimental study, PCV2b-based vaccines may be less effective than PCV2a-based vaccines at protecting against the PCV2d

genotype [16]. Nevertheless, comparative field trial between a bivalent vaccine containing PCV2a and *M. hyopneumoniae* and a trivalent vaccine containing PCV2a/b and *M. hyopneumoniae* has yet to be undertaken. The objective of this study was to compare a bivalent and trivalent vaccine, with an emphasis on the evaluation of growth performance in herds in the presence of subclinical PCV2d infection and enzootic pneumonia.

2. Material and methods

2.1. Farm History

The clinical field trial was conducted on a 1200-sow, farrow-to-finish swine farm that implemented an all-in-all-out production system. The farm was selected based on its history of subclinical PCV2d infection and enzootic pneumonia. The status of porcine reproductive and respiratory syndrome virus (PRRSV) at the farm was stable; with no active PRRSV circulation (high-parity sows were the only seropositive animals in the herd). No PRRS modified-live virus vaccine was administered for at least one year in sows and piglets. Piglets were vaccinated for PCV2 (Ingelvac CircoFLEX®, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA) and *M. hyopneumoniae* (Ingelvac MycoFLEX®, Boehringer Ingelheim Vetmedica Inc.) at 3 weeks of age. Submitted cases met the definition of subclinical PCV2 infection [17] based on 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 3 out of 5 suspected pigs. In addition, *M. hyopneumoniae* infection was determined in all three, 68 day-old pigs by display of severe dry coughing, histopathological peribronchiolar lymphoid tissue hyperplasia, and the detection of *M. hyopneumoniae* in lung samples by real-time PCR [18]. A pilot survey was implemented to assess the circulation of PCV2 and *M. hyopneumoniae* in the herd. Pre-trial investigations identified a PCV2 serological profile that presented an increase in antibody titers starting around 7 weeks of age. Pigs that were 7-15 weeks of age tested positive for PCV2 in their blood by PCR methodology. *M. hyopneumoniae* serology tested as partially positive in 7 week-old pigs, and completely positive in 10 week-old pigs. Together, these results show early and prolonged PCV2 and *M. hyopneumoniae* infections were circulating within the herd.

2.2. Experimental Design

To minimize sow variation, eight, 21 day-old pigs were randomly selected using the random number generator function (Excel, Microsoft Corporation, Redmond, WA, USA) from each sow and assigned evenly (two pigs per sow) to each of the four groups. A total of 480 pigs was randomly divided into 4 groups (120 pigs per group; male = 60 and female = 60) using the same software and function (Table 1). The pigs in the VacA1 group were intramuscularly vaccinated with a 2.0 mL dose of the trivalent vaccine

(Fostera® Gold PCV MH, Serial No: 413369A, Expiration date: 03 February 2022, Zoetis, Parsippany, NJ, USA) at 21 days of age. The pigs in the VacA2 group were intramuscularly vaccinated with a 1.0 mL dose of the trivalent vaccine (Fostera® Gold PCV MH) at 21 and 42 days of age, respectively. Pigs in the VacB group were intramuscularly vaccinated with a 2.0 mL dose of the bivalent vaccine (Porcilis® PCV M Hyo, Lot No. C746B02, Expiration date: 09 September 2021, MSD Animal Health, Boxmeer, Netherlands) at 21 days of age. Pigs in the UnVac group were injected intramuscularly with 2.0 mL of phosphate buffered saline (PBS, 0.01 M, pH 7.4) at 21 days of age. Pigs were comingled and randomly assigned into 48 pens within the same building. 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.

Whole blood, and fecal and laryngeal swabs were collected at 0 (21 days old), 28 (49 days old), 49 (70 days old), 91 (112 days old) days post-vaccination (dpv). Pigs were snared and restrained with a mouth gag for laryngeal swab collection. Swabs were guided with a laryngoscope down into the larynx. The internal walls of the laryngeal cartilages were then swept with the swabs once the larynx was visualized and the epiglottis was in a low position [19].

This study was conducted according to the guidelines of the Seoul National University Institutional Animal Care and Use Committee approved protocol SNU-200914-2.

Table 1. Field experimental design

Groups	No. of Pigs	Vaccine	Dosage	Age (Day)
VacA1	120	Fostera® Gold PCV MH	One (2.0 mL)	21
VacA2	120	Fostera® Gold PCV MH	Two (1.0 mL)	21, 42
VacB	120	Porcilis® PCV MHyo	One (2.0 mL)	21
UnVac	120	Phosphate buffered saline	One (2.0 mL)	21

2.3. Clinical Observations

The pigs were monitored daily for abnormal clinical signs and scored weekly using scores ranging from 0 (normal) to 6 (death) [20]. Observers were blinded to vaccination and type of vaccine status. Mortality rate was calculated as the number of pigs that died divided by the number of pigs initially assigned to that group within batch. Pigs that died or were culled throughout the study was necropsied. Evaluation of injection site reaction including palpation was performed 24 h post-vaccination.

2.4. Average Daily Weight Gain

The live weight of each pig was measured at 0 (21 days old), 49 (70 days old), and 154 (175 days old) days post-vaccination. The average daily weight gain (ADWG; gram/pig/day) was analyzed over two time periods: (i) between 21 and 70 days old and (ii) between 70 and 175 days old. 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 pigs were included in the calculation.

2.5. T Cell Epitope Contents Comparison Analysis

PCV2d strain (SNUVR202002, GenBank no. MW821481) was isolated in inguinal lymph node from 68 day-old pig in submitted diagnostic case. The relatedness between vaccine sequences and field strain was analyzed by T cell epitope contents comparison (EpiCC) analysis as previously described

[21]. To quantify vaccine T cell epitope coverage, the shared EpiCC score of each vaccine-field strain comparison was divided by that field strain's baseline EpiCC and expressed as a percentage.

2.6. Quantification of PCV2d DNA in Blood and Feces

DNA was extracted from serum and fecal samples using the commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA) to quantify PCV2d genomic DNA copy numbers by real-time PCR [22].

2.7. Quantification of *M. hyopneumoniae* DNA in Laryngeal Swabs

DNA was extracted from laryngeal swabs using the commercial kit (QIAamp DNA Mini Kit, QIAGEN) to quantify the *M. hyopneumoniae* genomic DNA copy numbers by real-time PCR [18].

2.8. Serology

The serum samples were tested using the commercially available enzyme-linked immunosorbent assay (ELISA) kits for *M. hyopneumoniae* (M. hyo. Ab test, IDEXX Laboratories Inc. Inc., Westbrook, ME, USA) and PCV2 (SERELISA PCV2 Ab Mono Blocking, Synbiotics, Lyon, France). Serum samples were considered positive for *M. hyopneumoniae* antibody if the sample-to-positive (S/P) ratio was $\geq$0.4, and positive for anti-PCV2 antibodies if the reciprocal ELISA titer was >350, in accordance with the manufacturer's instructions for each kit.

2.9. Pathology

The severity of macroscopic lung lesions was scored to estimate the percentage of the lung affected by pneumonia. The scoring was done by two pathologists (Chae and one graduate student) at the Seoul National University (Seoul, Republic of Korea). For the entire lung (100 points were assigned 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) [20]. Two blinded veterinary pathologists then examined the collected lung and lymphoid tissue sections and scored the severity of peribronchiolar lymphoid tissue hyperplasia by mycoplasmal pneumonia lesions (0 to 6) [23]. Lymphoid lesion severity was scored (0 to 5) based on lymphoid depletion and granulomatous inflammation [24].

2.10. Statistical Analysis

Prior to statistical analysis, real-time PCR data were transformed to $\log_{10}$ values. Statistical analyses were performed IBM SPSS Statistics for Windows version 23.0 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test will be utilized to test the collected data for a normal distribution. One-way analysis of variance (ANOVA) was used to examine whether there are statistically significant differences at each time point within different groups. A one-way ANOVA test result with such a statistical significance was be further evaluated by conduction a post-hoc test for a pairwise

comparison with Tukey's adjustment. If the normality assumption was not met, the Kruskal-Wallis test was be performed. Results from Kruskal-Wallis test which showed statistical significance were further evaluated with the Mann-Whitney test to include Tukey's adjustment to compare the differences among the groups. Results were reported in p-value where a value of $p<0.05$ was considered to be significant.

3. Results

3.1. Clinical Signs

Respiratory signs, such as dyspnea and tachypnea, were significantly lower ($p<0.05$) in vaccinated animals (VacA1, VacA2, and VacB groups) than those in unvaccinated animals (UnVac group) at 21 to 126 dpv. A comparison between vaccinated groups determined that respiratory signs, such as dyspnea and tachypnea in the VacA1 group were significantly lower ($p<0.05$) than those in the VacB group at 63 and 98 dpv.

3.2. Average Daily Weight Gain

A difference in mean body weight was not observed between vaccinated (VacA1, VacA2, and VacB groups) and unvaccinated (UnVac group) animals at the time the study began (21 days of age). The ADWG of vaccinated animals (VacA1, VacA2, and VacB groups) was significantly higher ($p<0.05$) than that of unvaccinated animals (UnVac group) during the fattening period

(70 to 175 days of age) and overall period (21 to 175 days). In a comparison of vaccinated groups, the ADWG of the VacA1 group was significantly higher ($p<0.05$) than that of the VacB group during the fattening period (70 to 175 days of age). The ADWG of the VacA1 and VacA2 groups was significantly higher ($p<0.05$) than that of the VacB group during the overall period (21 to 175 days of age) (Table 2).

Table 2. Growth performance with average daily weight gain (ADWG) and pathology between vaccinated and unvaccinated animals

	Age (Days)	Groups			
		VacA1	VacA2	VacB	UnVac
ADWG (gram/pig/day)	21-70	399.90±25.44	401.89±24.05	395.51±24.20	393.57±31.13
	70-175	775.62±20.65[a]	772.73±18.45[a,b]	765.23±22.73[b]	715.74±26.26[c]
	21-175	656.06±11.85[a]	654.74±11.38[a]	647.65±14.17[b]	613.46±14.33[c]
Body weight	21	5.57±0.32	5.56±0.33	5.51±0.35	5.50±0.36
	175	106.60±1.82[a]	106.39±1.71[a]	105.25±2.15[b]	99.96±2.19[c]
Macroscopic lung lesions	175	17.82±6.90[a]	18.21±7.85[a]	19.70±8.21[a]	28.60±10.67[b]
Microscopic lung lesions	175	0.73±0.56[a]	0.78±0.60[a]	0.88±0.65[a]	2.04±0.93[b]
Microscopic lymphoid lesions	175	0.69±0.59	0.73±0.61	0.86±0.58	1.07±0.37

[a,b,c] Different superscripts indicate significant ($p<0.05$) difference among 4 groups.

3.3. Mortality

Diagnostic results indicated that mortality was primarily related to co-infection with PCV2 and *M. hyopneumoniae* in unvaccinated animals. In the VacA1 group, one pig died of unknown hemorrhagic diarrhea at 63 days of age. Two additional pigs from the VacA1 group died of bronchopneumonia as determined by a combination of *M. hyopneumoniae* as detected with PCR, and *P. multocida* that was isolated from the lungs at 72 and 75 days of age. In the VacA2 group, three pigs died of bronchopneumonia, as determined by a combination of PCV2d that was detected with PCR, and *Glaesserella parasuis* that was isolated from the lungs at 52, 70, and 78 days of age. Three pigs in the VacB group died of bronchopneumonia, as determined by a combination of *M. hyopneumoniae* that was detected with PCR, and *Trueperella pyogenes* that was isolated from the lungs at 60, 80, and 82 days of age. Two additional VacB pigs died of bronchopneumonia, as determined by a combination of PCV2d that was detected with PCR and *P. multocida* that was isolated from the lungs at 72 days of age. In the UnVac group, one pigs died of salmonellosis, as determined by *Salmonella typhimurium* that was isolated from the large intestine at 52 days of age. Four UnVac group pigs died of bronchopneumonia, as determined by a combination of PCV2d and *M. hyopneumoniae* that were detected with PCR, and *G. parasuis* that was isolated from the lungs at 64, 75, 88, and 110 days of age. Three additional UnVac group pigs died of bronchopneumonia from a combination of *M.*

hyopneumoniae that was detected with PCR, and *P. multocida* and *T. pyogenes* that were isolated from the lungs at 72 (2 pigs) and 80 days of age.

3.4. T Cell Epitope Content Comparison Analysis

Shared EpiCC score was higher in the trivalent vaccine compared to the bivalent vaccine. T cell epitope coverage of bivalent vaccine against field PCV2 strain (SNUVR202002) was 62% and of trivalent vaccine against the same field PCV2d strain was 83%. This represented 33% improvement of an epitope coverage (Table 3).

Table 3. Summary of T cell epitope contents comparison (EpiCC) scores between porcine circovirus type 2 (PCV2) vaccine and field strain

	ORF2 of PCV2 of Vaccines		
	Monovalent [a]	Bivalent [b]	Trivalent [c]
Vaccine baseline [d]	6.83	6.50	8.66
Average baseline (sd) [e]		10.49 (0.16)	
EpiCC [f]	6.83	6.50	8.66
Coverage [g]	65.36%	62.22%	82.82%

[a] Monovalent (Ingelvac CircoFLEX®) vaccine used in the farms. [b] Bivalent (Porcilis® PCV M Hyo) vaccine used in this study. [c] Trivalent (Fostera® Gold PCV MH) vaccine used in this study. [d] EpiCC score calculated for the vaccine compared to itself. [e] Average baseline EpiCC score (and standard deviation) of full-length field strain. [f] EpiCC score of the vaccine compared to full-length field strain. [g] Coverage of each field strain's baseline EpiCC score expressed as a percentage.

3.5. Quantification of PCV2d DNA in Blood and Feces

The amount of PCV2d DNA loads in blood from vaccinated animals (VacA1, VacA2, and VacB groups) were significantly lower ($p<0.05$) than that of unvaccinated animals (UnVac group) at 28, 49, and 91 dpv. PCV2d DNA loads in blood from the VacA1 and VacA2 groups were significantly lower ($p<0.05$) than that of the VacB group at 49 dpv (Figure 1A). The amount of PCV2d DNA loads in feces from vaccinated animals (VacA1, VacA2, and VacB groups) were significantly lower ($p<0.05$) than that of unvaccinated animals (UnVac group) at 28, 49, and 91 dpv (Figure 1B).

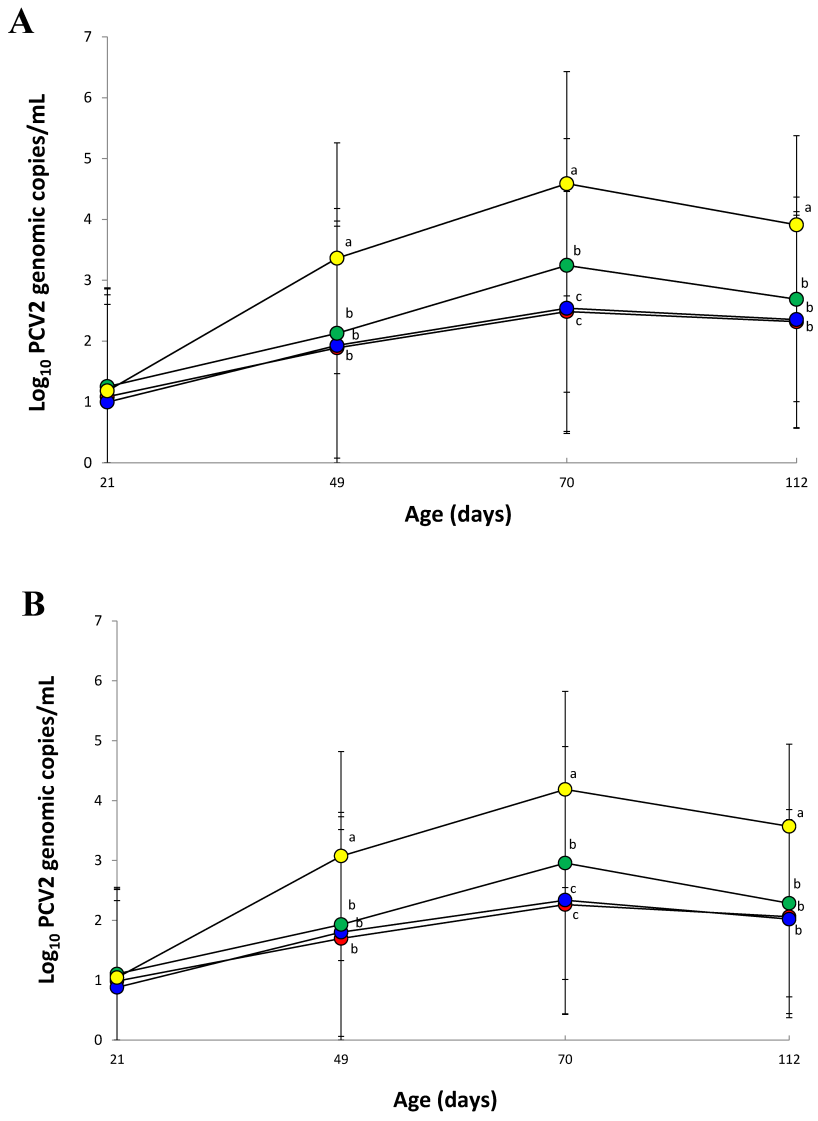

Figure 1. Mean values of the genomic copy number of PCV2d DNA in serum (A) and feces (B) from VacA1 (●), VacA2 (●), VacB (●), and UnVac (●). Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($p<0.05$) different among 4 groups.

3.6. Quantification of *M. hyopneumoniae* DNA in Laryngeal Swabs

The amount of *M. hyopneumoniae* DNA loads in laryngeal swabs from vaccinated animals (VacA1, VacA2, and VacB groups) were significantly lower ($p<0.05$) than that of unvaccinated animals (UnVac group) at 28, 49, and 91 dpv. In comparison of vaccinated groups, the amount of *M. hyopneumoniae* DNA loads in laryngeal swabs from the VacA1 group was significantly lower ($p<0.05$) than that of the VacB group at 49 dpv (Figure 2).

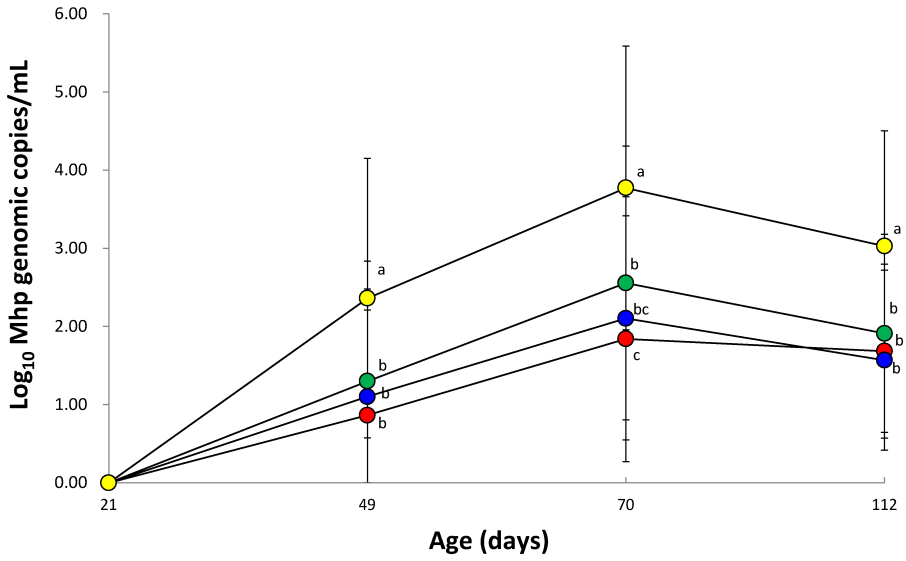

Figure 2. Mean values of the genomic copy number of *Mycoplasma hyopneumoniae* DNA in laryngeal swab from VacA1 (●), VacA2 (●), VacB (●), and UnVac (●). Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($p<0.05$) different among 4 groups.

3.7. Immune Responses against PCV2

Vaccinated animals from VacA1, VacA2, and VacB groups produced significantly higher ($p<0.05$) PCV2 ELISA titers at 28, 49, and 91 dpv than that of unvaccinated animals from the UnVac group. In comparison of vaccinated groups, the PCV2 ELISA titers of the VacA1 and VacA2 groups was significantly higher ($p<0.05$) than that of the VacB group at 49 and 91 dpv (Figrue 3A).

3.8. Immune Responses against *M. hyopneumoniae*

Vaccinated animal from VacA1, VacA2, and VacB groups produced significantly higher ($p<0.05$) *M. hyopneumoniae* ELISA S/P ratios at 28, 49, and 91 dpv than that of unvaccinated animal from the UnVac group. In comparison of vaccinated groups, the *M. hyopneumoniae* ELISA S/P ratios of the VacA1 and VacA2 groups was significantly higher ($p<0.05$) than that of the VacB group at 91 dpv (Figure 3B).

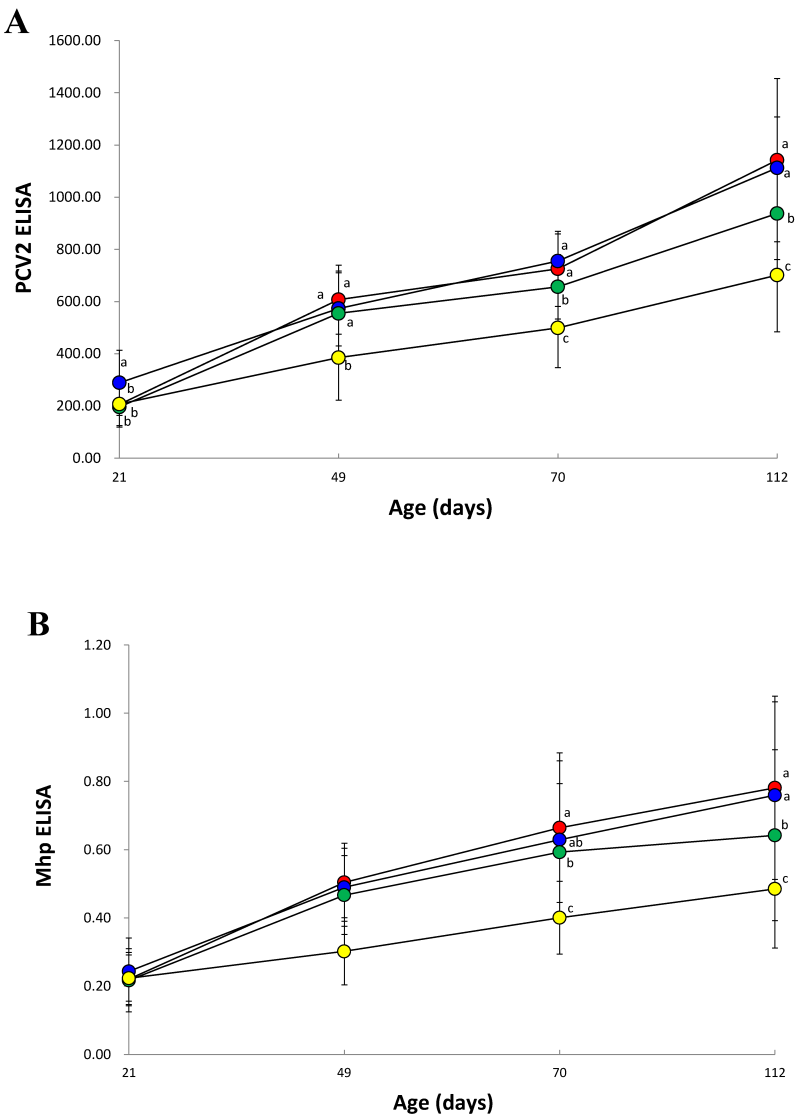

Figure 3. Mean values of the anti-PCV2 antibodies (A) and anti-*Mycoplasma hyopneumoniae* antibodies (B) from VacA1 (●), VacA2 (●), VacB (●), and UnVac (●). Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($p<0.05$) different among 4 groups.

3.9. Pathology

Vaccinated (VacA1, VacA2, and VacB) groups had significantly lower (p<0.05) macroscopic and microscopic lung lesion scores when compared to unvaccinated (UnVac) group at 154 dpv (Table 2).

4. Discussion

The present field trial is used to compare two different combination vaccines; a bivalent vaccine containing PCV2a and *M. hyopneumoniae*, and a trivalent vaccine containing PCV2a/b and *M. hyopneumoniae*. Under the field conditions of the present study, where subclinical PCV2d infection and enzootic pneumonia was circulating within the farm, pigs vaccinated with the trivalent vaccine exhibited significantly better growth performance when compared with the bivalent vaccine. There were no statistical differences in growth performance between one-dose and two-dose trivalent-vaccinated groups. The economic benefit of trivalent-vaccinated groups over bivalent-vaccinated group was evaluated by differences on market weight at the time of slaughter . Trivalent-vaccinated groups improved significantly (p<0.05) body weight by 1.245 kg/pig (106.495 kg in combined trivalent vaccinated group vs. 105.25 kg from bivalent-unvaccinated group), leading to an increase of revenue by 2.98 US dollars (exchange rate; US $1.00 = 1169.40 Korean Won) per pig.

The improved growth performance of the trivalent-vaccinated groups over

the bivalent-vaccinated group may be attributed to the different epitope determinant between vaccine and field PCV2 strain; T cell epitope coverage of bivalent vaccine against field PCV2d strain was 62% and of trivalent vaccine against the same field PCV2d strain is 83%. Therefore, trivalent PCV2a/b and *M. hyopneumoniae* vaccine strains provide better protection against field PCV2d strain compared to bivalent vaccine containing PCV2a vaccine strain only. These results agree with previous EpiCC analysis, in which combination of PCV2a and PCV2b vaccine shared on average more T cell epitope content with strains from all the different genotypes than monovalent PCV2a vaccines [21]. PCV2a-based vaccines provide partial cross-protection against PCV2d under experimental conditions [9-12]. Several differences exist between these experimental challenge conditions of the study from those of commercial pig farms. Pigs in commercial farms are continuously exposed and re-exposed to the prevalent field PCV2d virus by horizontal transmission. Natural confections as well as other intrinsic and extrinsic factors also exacerbate disease in less-controlled commercial setting. Under field conditions, the levels of cross-protection provided by PCV2a-based vaccines against PCV2d have been questioned, due to reports of PCV2d identification in PCV2a-vaccinated herds [13-15]. In this comparative field trial, trivalent vaccination reduced the amounts of PCV2d loads in the blood and feces when compared to bivalent vaccination. The reduction of PCV2 viremia is well correlated with protection against PCV2 infection [25-27]. The present results indicate that the trivalent vaccination

provided better protection against PCV2d when compared to bivalent vaccination against PCV2d subclinical infection under field conditions.

The strains of the *M. hyopneumoniae* antigen and adjuvant formulation differed between the two combination vaccines. In particular, adjuvant formulation is known to affect the immunogenicity and protective effect of inactivated whole-cell *M. hyopneumoniae* bacterins [28]. Trivalent vaccination reduced the amount of *M. hyopneumoniae* load in larynx when compared to the bivalent vaccine. Although correlation between the reduction of *M. hyopneumoniae* in the larynx and vaccine protection is not well known, reducing the amount of *M. hyopneumoniae* loads in the larynx are more likely to reduce horizontal transmission to neighboring pigs.

Regardless of vaccine type, vaccinated animals had a significantly greater reduction in mycoplasmal lung lesions compared to unvaccinated animals. These results are consistent with previous studies, where vaccination of pigs with *M. hyopneumoniae* reduces pneumonic lung lesions in field trials [29-31]. A significant difference in lymphoid lesions was not observed between the vaccinated and unvaccinated groups. This may be attributed to the subclinical PCV2 infection on the farm where the field clinical trial was conducted, as PCV2-associated lymphoid lesions are typically mild in pigs with subclinical PCV2 infection [17].

This is the first comparative field trial that evaluated the differences between a bivalent PCV2a and *M. hyopneumoniae* vaccine and trivalent PCV2a/b and *M. hyopneumoniae* vaccine. Broader coverage resulting from

the PCV2 vaccine's two genotypes provides additional insurance against the evolving PCV2 virus in the field. It is clinically meaningful to conduct comparative field clinical trial on farm with subclinical PCV2d infection and enzootic pneumonia.

5. Conclusions

This is the first comparative field trial that evaluated the differences between a bivalent and trivalent vaccine containing PCV2a and *M. hyopneumoniae* and a trivalent vaccine containing PCV2a/b and *M. hyopneumoniae*. Pigs vaccinated with the trivalent vaccine exhibited significantly better growth performance when compared with the bivalent vaccine. No statistical differences in growth performance were observed between one-dose and two-dose trivalent-vaccinated groups. The improved growth performance of the trivalent-vaccinated groups over the bivalent-vaccinated group may be attributed to the different epitope determinant between vaccine and field PCV2 strain; T cell epitope coverage of bivalent vaccine against field PCV2d strain is 62% and of trivalent vaccine against the same field PCV2d strain is 83%. It is clinically meaningful to conduct comparative field clinical trial on farm with PCV2d subclinical infection and enzootic pneumonia.

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GENERAL CONCLUSION

PCV2 genotype had been sifted from PCV2a to PCV2b since 2005 and nowadays PCV2d is the most prevalent in all over the world including Korea. According to the studies of PCV2 challenge after vaccination, homologous vaccination resulted better protection than heterologous vaccination. In addition to it, there were some reports of PCV2a genotype vaccine failure to PCV2d infection cases. For this reason, field evaluation of commercial vaccines being produced based on various PCV2 genotype would be needed for the veterinarians or swine producers.

A new trivalent combined vaccine of PCV2a/b and *M. hyopeumoniae* were introduced in the market. This vaccine has recombinant antigen from PCV2b genotype besides PCV2a genotype and for this reason, this trivalent vaccine is expected to have more wide coverage against field PCV2, including PCV2d genotype. Field trial was conducted in three swine farms affected PCV2d and *M. hyopeumoniae* to evaluate efficacy of the vaccine. Growth performance is one of the most critical indexes for evaluating vaccine's efficacy in case of PCV2 and *M. hyopeumoniae* infection and this trivalent vaccine showed significantly improved growth performance in both subclinical and clinical infection of PCV2d genotype. In the aspect of protective immunity, the trivalent vaccine elicited PCV2-specific neutralizing

antibodies and IFN-ɣ-SC and also induced a measurable cellular immune response to *M. hyopeumoniae* infection. In pathological evaluation, statistical differences in lymphoid lesions and lymphoid PCV2-positive cells were observed in 175 days pigs only in PCVAD farm. Mycoplasmal pneumonic lesions and laryngeal swab load from 112 days old pigs were significantly reduced in the vaccinated group in all three farms. Overall, the trivalent vaccine provided protection against PCV2d and *M. hyopeumoniae* in field conditions.

Preexisting bivalent combined vaccine of PCV2a and *M. hyopeumoniae* is also considered to be effective to PCV2d infection, so it is necessary to evaluate the new trivalent combined vaccine and the bivalent vaccine in their efficacy comparatively against PCV2d genotype infection. In T cell EpiCC anlysis between the vaccines and PCV2d strain, coverage of the bivalent vaccine against field PCV2d strain was 62% and the trivalent vaccine was 83%, which is 33% improved coverage. In a farm with subclinical PCV2d and *M. hyopeumoniae* infection, the trivalent vaccine exhibited significantly better growth performance than the bivalent vaccine. In analysis of PCV2d loads in the blood and feces, the trivalent vaccine reduced the amounts of PCV2d DNA when compared with the bivalent vaccine. And the trivalent vaccine reduced the amount of *M. hyopeumoniae* load in larynx when compared with the bivalent vaccine, which can contribute decrease of horizontal transmission. In pathological evaluation, both vaccines reduced mycoplasmal lung lesions significantly compared to

unvaccinated group and there were no differences in lymphoid lesions between the vaccinated and unvaccinated groups. Overall, trivalent vaccine of PCV2a/b and *M. hyopeumoniae* provided better protection than bivalent vaccine of PCV2a and *M. hyopeumoniae* in field conditions.

This study first demonstrated efficacy of the new trivalent combined vaccine in PCV2d and *M. hyopneumoniae* infected field condition. And it was also demonstrated that the trivalent combined vaccine showed better protection than a major bivalent combined vaccine against PCV2d and *M. hyopneumoniae* co-infection by both theoretical analysis and field comparative study.

국문 논문 초록

돼지 써코바이러스 타입 2 및 돼지 유행성 폐렴 합제백신 2종의 병리학 및 면역학적 분석을 통한 야외 비교평가

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돼지 써코바이러스 타입 2 (PCV2) 혹은 *Mycoplasma hyopneumoniae* 단일 백신은 개발이 완료되어 돼지 농장에 성공적으로 적용되어왔다. 하지만 이 두 병원체는 아직도 양돈산업에서 가장 중요한 병원체들 중 하나인데, 그것은 PCV2와 *M. hyopneumoniae*가 돈군에서 매우 흔하게 존재하는 반면 일반농장에서의 박멸은 쉽지 않기 때문이다. 두 병원체 모두 돼지호흡기복합감염증 (PRDC)의 주요 원인체이며 특히 *M. hyopneumoniae*는 PCV2 병변을 더욱 심화시키기도

한다.

최근에는 PCV2와 *M. hyopneumoniae* 합제백신들이 현장에 도입되고 있다. 이러한 합제백신은 돼지에 더 적은 스트레스로 두 핵심질병에 대한 방어력을 제공하며 동시에 노동력 절감의 효과도 있어 시장의 환영을 받고 있다. 합제백신을 적용하기에 앞서 PCV2d 유전형에 대한 효력을 평가해야 할 필요가 있는데, 이는 현재 한국을 포함한 아시아 국가들에서 PCV2d 유전형이 야외에서의 주요한 유전형인 반면, 상용화된 합제백신의 경우 제조사의 고유한 PCV2 유전형에 기초하여 생산되기 때문이다. PCV2 단일백신에 대한 여러 연구에 따르면 야외 바이러스와 이종 유전형의 백신은 동종 유전형의 백신과 마찬가지로 병변 형성 예방효과는 나타내지만 바이러스 혈증 억제에 대해서는 효과가 덜한 것으로 나타난 바 있다.

첫 번째 연구에서는 한국에 처음으로 도입되는 3가 백신 형태의 PCV2 및 *M. hyopneumoniae* 합제백신을 돼지 농장들에서 임상시험 하였다. 이 백신은 PCV2a 및 PCV2b 유전형 항원을 포함하고 있는데, PCV2b는 유전적으로 PCV2d와 가까워 PCV2d에 대해 좋은 효력을 보일 것으로 예상되었다. PCV2d 준임상형 혹은 임상형 병력과 마이코플라스마성 폐렴 병력이 있는 세 농장을 선정하였다. 시험군은 두 가지 방법으로 백신접종을 하였는데, 제조사의 용법에 따라 1.0ml을 3일령과 24일령에 2회 혹은 2.0ml을 21일령에 1회 접종하였다. 두 시험군 모두 대조군에 비하여 일당증체량이 유의미하게 높았다. 백신은 PCV2 중화항체 및 PCV2 특이적인 인터페론 감마 분비세포를 유도하였는데 이로 인하여 바이러스 혈증과 림프조직병변이 감소되었다. 비슷하게 *M. hyopneumoniae* 특이적인 인터페론 감마 분비세포가 유도되었고 이는 후두부의

M. hyopneumoniae 검출량과 폐병변 정도를 감소시켰다. 이와같이 본 연구는 삼가 합제백신이 돼지 농장에서 PCV2d와 *M. hyopneumoniae* 방어에 효과적이라는 것을 증명하였다.

두 번째 연구에서는 이러한 3가 합제백신과 PCV2a 및 *M. hyopneumoniae* 항원을 가지고 있는 2가 합제백신을 비교하여 야외 임상시험을 실시하였다. 이 두 백신의 주요한 차이점이라면 PCV2b 항원의 유무라고 할 수 있다. 두 백신을 각각 야외 PCV2d 바이러스와 T세포 항원결정기를 비교하여 점수화 해 본 결과 3가 합제백신이 2가 합제백신보다 더 좋은 점수를 나타내었는데, 이것은 PCV2b가 유전적으로 PCV2d와 근접하기 때문인 것으로 판단된다. 야외 비교임상시험에서, PCV2a/b 및 *M. hyopneumoniae* 항원을 가진 3가 합제백신의 경우 1회 접종 및 2회 접종을 실시하였으며, PCV2a 및 *M. hyopneumoniae* 항원을 가진 2가 합제백신의 경우 1회 접종을 실시하였다. 3가 합제백신은 2가 합제백신보다 혈액과 분변에서의 PCV2d 검출량을 더 감소시켰고, 후두에서의 *M. hyopneumoniae* 검출량 역시 감소시켰다. 3가 합제백신의 1회 및 2회 접종 그룹간 비교시 증체성적, 혈액학적 데이터, 혈액과 분변에서의 PCV2d 검출량, 후두에서의 *M. hyopneumoniae* 검출량, 병리학적 병변 모두 유의미한 차이는 없었다. 이와같이 본 연구는 합제백신별로 PCV2d와 *M. hyopneumoniae*를 방어함에 있어서 효능이 다를 수 있음을 실증하였고, 이러한 결과는 백신과 야외주의 PCV2 유전형 일치 정도와 관련이 있음을 보여주었다.

주요어: 돼지 써코바이러스 타입 2; *Mycoplasma hyopneumoniae*; 돼지호흡기복

합감염증; PCV2와 *Mycoplasma hyopneumoniae* 합제백신

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