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돼지 복합 호흡기 질병의 병인론 및 백신접종

Pathogenesis and Vaccination of

Porcine Respiratory Disease Complex

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Pathogenesis and Vaccination of Porcine Respiratory Disease Complex

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Abstract

Pathogenesis and Vaccination of Porcine Respiratory Disease Complex

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Porcine Respiratory Disease Complex (PRDC) has been used to describe a complex disease characterized by respiratory symptoms and poor growth in grower and finisher, between the ages of 14 and 22 weeks. PRDC is frequently caused by the interaction and synergy of viral and bacterial pathogens. Major important pathogens, involved are Porcine circovirus-2 (PCV-2), *Mycoplasma hyopneumoniae (M. hyopneumoniae)* and porcine reproductive and respiratory syndrome virus (PRRSV). The severity of PRDC microscopic lesions varies depending on the type and number of pathogens. However, moderate-to-severe interstitial pneumonia with

peribronchiolar lymphoid tissue hyperplasia and fibrosis is the most common lung lesion. In recent years, PRDC has emerged as the most economically devastating disease in the Asian swine industry, including South Korea.

PCV-2 and *M. hyopneumoniae* are two of the most commercially important disease in the swine industry. *M. hyopneumoniae* is one of the major pathogens, causing the porcine respiratory disease complex. The pathogen adheres to the epithelia cells of the trachea, bronchi, and bronchioles, causing damage on the mucosal clearance system, modulating the immune system, and making the pigs more susceptible to secondary respiratory bacterial pathogens.

PCV-2, a virus in the *Circoviridae* family, is a common virus in pigs that has been linked to porcine circovirus-associated diseases (PCVAD) like postweaning multisystemic wasting syndrome (PMWS), PRDC, porcine dermatitis and nephropathy syndrome (PDNS). Co-infection with PCV-2 and *M. hyopneumoniae* results in lower average daily weight gain and increased time to market weight, both of which bring significant economic losses.

Part I study was to reproduce severe pneumonic lesions, similar to those during naturally-occurring porcine respiratory disease complex, in pigs dually inoculated with PRRSV and *M. hyopneumoniae* at 6 weeks of age, followed by inoculation with PCV-2 virus after two weeks. Time and sequence of infection with three pathogens mirror Asian field conditions. Microscopically, interstitial pneumonia and peribronchiolar lymphoid hyperplasia are considered the most characteristic lung lesions in infected pigs. The results of the present study demonstrate that inoculation of pigs with these three pathogens can lead to severe interstitial pneumonia with peribronchial or peribronchiolar lymphoid hyperplasia and fibrosis.

Part II study was to compare three different types of combination vaccine: a trivalent vaccine containing Porcine circovirus-2a and -2b (PCV-2a/b), and *M. hyopneumoniae*, a mixable bivalent vaccine containing PCV-2a and *M. hyopneumoniae*, and a ready-to-use bivalent vaccine containing PCV-2a and *M. hyopneumoniae*. Two farms were selected on the basis of their subclinical PCV-2d infection and enzootic pneumonia. A total of 120 pigs in each farm were randomly divided into 4 groups (30 pigs per group). The trivalent-vaccinated group from both farms outperformed each bivalent-vaccinated group in terms of growth performance. Growth performance was significantly improved during the fattening periods (70-175 days of age) of the mixable bivalent-vaccinated group in comparison with the ready-to-use bivalent-vaccinated group in one farm. The trivalent-vaccinated group elicited higher levels of neutralizing antibodies and interferon-γ secreting cells (IFN-γ-SC) against PCV-2d, while simultaneously decreasing the levels of PCV-2d load in blood when compared against the mixable and ready-to-use bivalent-vaccinated groups. The trivalent-vaccinated group also elicited higher levels of IFN-γ-SC against *M. hyopneumoniae* and lower levels of *M. hyopneumoniae* loads in the larynx when compared with the mixable and ready-to-use bivalent-vaccinated groups. The results of the present study demonstrated that a trivalent vaccine containing PCV-2a/b and *M. hyopneumoniae* resulted in a better productive parameter, higher immune responses, and less blood-viral and mycoplasmal larynx-loads when compared with the mixable and ready-to-use bivalent vaccines despite the presence of ongoing farm subclinical PCV-2d infection and enzootic pneumonia. The results of this study demonstrate a strategic method and effective vaccine regimes against PRDC for swine producers and practitioners.

Keyword: Porcine circovirus-2; *Mycoplasma hyopneumoniae*; Porcine respiratory disease complex; Co-infection; Combination vaccine; Vaccine efficacy

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LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
dpi	Days post-infection
dpv	Days post-vaccination
ELISA	Enzyme-linked immune sorbent assay
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
NA	Neutralizing antibody
ORF	Open reading frame
PCV	Porcine circovirus
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PMWS	Post-weaning multisystemic wasting syndrome
PRDC	Porcine respiratory disease complex
PRRS	Porcine reproductive and respiratory syndrome
SEP	Swine Enzootic Pneumonia
SIV	Swine influenza virus

GENERAL INTRODUCTION

Porcine Respiratory Disease Complex (PRDC) is a multifactorial and complex disease caused by a combination of infectious agents and environmental factors that negatively impact pig health, resulting in decreased performance, increased mortality, and economic losses (1). The interaction and synergy of viral and bacterial pathogens is often responsible for PRDC. Porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), and Porcine circovirus-2 (PCV-2) are the most clinically significant pathogens (2).

Primary agents in pig respiratory infections include viral agents such as PRRSV, swine influenza virus (SIV), pseudorabies virus (PRV), and possibly porcine respiratory coronavirus (PRCV) and PCV-2, as well as bacterial agents such as *M. hyopneumoniae*, *Bordetella bronchiseptica*, and *Actinobacillus pleuropnemoniae*.

Pasteurella multocida is the most common opportunistic agent, but other common opportunistic agents include *Glaesserella parasuis, Streptococcus suis, Actinobacillus suis, Trueperella pyogenes, Salmonella choleraesuis, Salmonella suis, and Salmonella pyogenes* (3).

Virus, *M. hyopneumoniae*, and several opportunistic bacteria collaborate to cause respiratory disease losses on the majority of PRDC infected farms. Aside from the long-known pathogens that cause respiratory disease, several emerging and changing pathogens also play an important role in the development of PRDC.

The objective of this dissertation is to provide scientific background for effective herds control and better vaccine strategy by describing the main etiological and pathological views of PRDC by reviewing two i1nvestigations associated with PRDC pathogenesis based on experimental infection models in conventional pigs

and clinical vaccine efficacy using three different commercially available combo vaccine including PCV-2a and PCV-2a/2b and *M. hyopneumoniae.*

LITERATURE REVIEW

1. Porcine Respiratory Disease Complex (PRDC)

Porcine Respiratory Disease Complex (PRDC) is caused by a combination of infectious agents and environmental factors, affecting pig health and resulting in decreased performance, increased mortality, and economic losses (2).

According to the report from the National Animal Health Monitoring System in US, respiratory disease is the most critical problem with mortality in nursery, grower and finisher (3). Korea Animal and Plant Quarantine Agency (APQA) reported that swine pneumoniae lesions are observed in 39.9% in pigs at slaughter houses. In addition, complexed infection, more than 2 pathogens, involved, accounts for 70% (4). ①In addition, Porcine circovirus-2 (PCV-2) and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) are sure to major pathogens, causing respiratory disease, turning out 429 cases and 150 cases, respectively in 2020. The term PRDC describes swine respiratory disease, caused by various etiologies that resulted in clinical disease and poor feed conversion rate (FCR), especially in grower and finisher stages. On most PRDC infected farms, one or two viruses, *M. hyopneumoniae*, and several opportunistic bacteria collaborate to cause respiratory disease. In the respiratory infection in pig, primary agents in pigs include viral agents like porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), pseudorabies virus (PRV), and possibly porcine respiratory coronavirus (PRCV) and PCV-2 and bacterial agents like *M. hyopneumoniae, Bordetella bronchiseptica,* and *Actinobacillus pleuropneumoniae.* The most common

① The information from the courtesy of Optipharm(www.optipharm.co.kr)

opportunistic agent is *Pasteurella multocida* (*P. multocida*) but other common opportunistic agents include *Glaesserella parasuis, Streptococcus suis, Actinobacillus suis, Trueperella pyogenes. Salmonella choleraesuis.*

A retrospective study on natural cases of PRDC was conducted in Korea to check the prevalence of PRDC with various pathogens. Among the 105 pigs with PRDC, 85 cases were PCV-2, 66 cases were PRRSV, 60 cases were porcine parvovirus (PPV) and 14 cases were SIV. There were eighty co-infections and twenty-five single infections. Co-infection of PCV-2 with another bacterial pathogen is a common finding in PRDC. The most common combination was PCV-2 and *P. multocida* with 38 cases, followed by PCV-2 and *M. hyopneumoniae* with 33 cases (5).

2. Etiological Agents

2-1. Porcine circovirus-2 (PCV-2)

PCV-2 is common in most pig populations, and infections are frequently subclinical or mild. Direct contact and fomite transmission occur, with the virus being shed in feces, respiratory secretions, and urine. In swine, vertical transmission occurs, although maternal antibodies protect piglets from infection (6). Disease occurrence in pig-rearing operations with various patterns such as postweaning multisystemic wasting syndrome (PMWS), porcine nephropathy syndrome, porcine dermatitis, porcine respiratory disease complex (PRDC), reproductive failure, granulomatous enteritis, exudative epidermitis, and necrotizing lymphadenitis (7).

Porcine circovirus, family *Circoviridae*, genus *Circovirus*, is a non-enveloped RNA virus and the smallest virus capable of infecting mammals. Since its discovery in cell culture in 1974, another serotype PCV-2 has been discovered that causes disease in vivo and has been further subdivided into PCV-2a and 2b (8).

PCV-2a and 2b are frequently co-infected. In addition, PCV-2c, 2d, and 2e have also been found in various countries, and research on these subtypes is ongoing (9). PCV-2a and 2b has 90.9-94.4% similarity, in terms of nucleotide sequences and amino acid sequence similiary of ORF2, which have been proposed to cause pathogenesis differences (10, 11). The severity of disease has been shown to differ between PCV-2a and 2b infections, with 2b infection typically brings pulmonary edema, granulomatous enteritis, lymphoid necrosis and depletion, but it is unclear that viral or host factors are associated with these differences (12, 13). Some studies, however, have found no difference in pathogenicity (14, 15). A high nucleotide substitution rate allows PCV2 to evolve continuously and new PCV2 strains to

emerge. The overall prevalence of PCV2, from various samples originating from commercial pigs, was 53.8 percent (325/604). In 2016, genotype-specific PCR on pen-based oral fluid samples for nationwide PCV2 surveillance revealed the following infection patterns of PCV2 genotypes at the farm level: none (6/69), PCV2a (6/69), PCV2b (2/69), PCV2d (33/69), PCV2a/d (4/69), PCV2b/d (11/69), and PCV2a/b/d (2/69). This indicates that the PCV2d genotype shift occurred on a national scale and that coexistence of different genotypes is common in Korean pig herds (16). In addition, amongst PCV2 genotype, PCV2d generates the most severe lymphoid lesion, coinfected with *M. hyopneumoniae* (17).

The most common clinical signs of PMWS include progressive weight loss (wasting) and chronic pneumonia (tachypnea and dyspnea). Morbidity within a group is usually low (5 to 50%). However, mortality among affected pigs is often very high. Icterus, paleness, and diarrhea are less commonly reported. PCV-2 has also been associated with gastric ulcers and with porcine dermatitis and nephropathy syndrome (18). Pigs in the late-nursery to mid-finishing phase (6 to 18 weeks of age) are most commonly affected. It can be very difficult clinically to distinguish PMWS from PRRSV and secondary infections. PRRSV and PCV-2 are frequently detected together in cases of PMWS (19).

2-2. Porcine reproductive and respiratory syndrome virus (PRRSV)

PRRSV is a swine pathogen that is widely spread and could cause both reproductive and respiratory disease. In the late 1980s, PRRS was first identified as acute outbreaks of reproductive failure.

PRRSV is a single-stranded RNA virus with a small envelope. The virion contains an infectious RNA genome of approximately 15 kilobases in a proteinaceous

nucleocapsid surrounded by the envelope containing five or six structural proteins. There are two main strains: one from Europe (Lelystad Virus, LV) and another from North America (VR-2332) with numerous subspecies of varying virulence. They are classified as high- or low-virulent strains based on the pulmonary changes they cause (20).

Some farms may be subclinically infected with PRRSVs depending on strain, dose, and immune status, while others experience severe reproductive and/or respiratory disease (21). High fevers, anorexia, dyspnea, tachypnea, conjunctivitis, and failure to thrive can occur in newborn and nursery pigs (22). Coughing is not a symptom of a simple PRRSV infection. Grower and finisher infected with PRRSV develop respiratory disease that ranges from no detectable symptoms to fatal pneumonia, depending on the strain of PRRSV and the type of coinfections (23). PRRSV infection destroys and impairs the function of pulmonary alveolar macrophages and pulmonary intravascular macrophages, damages the mucociliary apparatus, alters T-cell subpopulations, and may impair the function of antigen-presenting cells (APCs) such as macrophages (24, 25). The infection of macrophages by PRRSV has a significant impact on the pig's respiratory immune system. Despite macrophage infection and lysis, there is no evidence of reduced lymphocyte response to antigens, and enhanced antibody responses to experimentally administered antigens have been described (26). These findings contradict field reports from producers and veterinarians who report an increase in secondary infections associated with PRRSV disease outbreaks. These opposing viewpoints suggest that PRRSV is immunomodulatory in nature.

There is clinical evidence that PRRSV is associated with outbreaks of other pathogens, and PRRSV is the most common virus isolated from cases of PRDC (27).

Experimentally, studies found that PRRSV-infected pigs showed an increased septicemia and mortality when challenged with *S. suis* and increased pulmonary infections with *B. bronchiseptica* (28). There is also evidence of PRRSV interaction with other respiratory viruses such as PRCV and SIV in pigs, and alteration in the typical disease response to pathogens such as *G. parasuis* (29). Thus, the mechanisms that enable PRRSV infection to increase the incidence of secondary infections as observed in the field have been frustrating to demonstrate experimentally. These results demonstrate the truly complex nature of mixed respiratory disease in pigs. Even though PRRSV itself may not result in the dramatic increase in susceptibility to secondary infections that would be predicted by the field evidence, it may tip the balance in combination with other respiratory pathogens or adverse environmental conditions.

There is clinical evidence that PRRSV is linked to other pathogen outbreaks, and PRRSV is the most common virus isolated from PRDC cases. Experiments revealed that PRRSV-infected pigs had higher septicemia and mortality when challenged with *S. suis*, as well as higher pulmonary infections with *B. bronchiseptica* (28, 30). There is also evidence of PRRSV interaction with other respiratory viruses like PRCV and SIV in pigs, as well as a change in the typical disease response to pathogens like *G. parasuis* (31). Thus, it has been difficult to demonstrate experimentally the mechanisms that allow PRRSV infection to increase the incidence of secondary infections as observed in the field. These findings highlight the truly complex nature of mixed respiratory disease in pigs. Even if PRRSV alone does not cause the dramatic increase in susceptibility to secondary infections predicted by the field evidence.

2-3. Swine Influenza Virus (SIV)

Influenza A viruses, along with PRRSV, PCV-2, *M. hyopneumoniae*, and *A. pleuropneumoniae*, are important causes of acute respiratory disease in pigs (32). Swine influenza is caused by type A influenza viruses, which belong to the *Orthomyxoviridae* family. The antigenic and genetic properties of the surface proteins hemagglutinin (H) and neuraminidase (N) are used to classify subtypes. There are 15 different hemagglutinins (H1 to H15) and 9 different neuraminidases (N1 to N9). H1N1 subtype is common in North America, Europe, and Asia (33). Prior to 1998, subtype H1N1 swine influenza was almost entirely responsible for swine influenza in the United States. Beginning in mid-1998, subtype H3N2 quickly spread throughout the United States (3).

SIV is most commonly transmitted between pigs via nasopharyngeal secretions. The virus attaches to the cilia, and viral replication begins in the upper respiratory epithelium. The infection spreads to the bronchi and bronchioles, causing cilia loss, mucus extrusion, neutrophil and macrophage exudation, and necrosis and metaplasia of the airway epithelium (34). When the virus infects the alveolar epithelium, endothelium, and macrophages, the alveoli are flooded with serofibrinous exudate (35).

SIV in swine, typical infection lasts 6 to 7 days, and clinical symptoms such as fever, respiratory distress, and weakness subside within a few days. Infection is usually mild and rarely fatal (1). However, this disease can have a significant economic impact due to reproductive failure in sows and weight loss in growing pigs caused by the fever. Three SIV virus subtypes (H1N1, H3N2, and H1N2) are currently circulating in swine around the world (33). However, the origins and antigenic properties of these subtypes vary from region to region around the world.

SIV causes multifocal to diffuse pneumonia with dark red-tan, mottled areas that affect 20 to 100% of lung tissue. Lesions are frequently cranioventral in location. The lungs are frequently congested, and the airways are filled with blood-tinged foam. Mediastinal and tracheobronchial lymph nodes are frequently enlarged and hyperemic. Microscopic examination reveals necrotizing bronchitis and bronchiolitis, alveolar septal infiltration with mixed inflammatory cells, type 2 pneumocyte hypertrophy and hyperplasia, and proteinaceous fluid and mixed inflammatory cell filling of airways and alveolar spaces.

SIV-specific, antibody-producing cells have been found in both the upper and lower respiratory tracts, with immunoglobulin A appearing to be the predominant isotype. Because there appears to be little cross-immunity between the different subtypes of SIV, pigs can contract any of them, all of which can potentially cause respiratory disease (36).

2-4. *Mycoplasma hyopneumoniae*

Swine enzootic pneumonia (SEP), a chronic respiratory disease primarily affecting finishing pigs, is caused by *M. hyopneumoniae* (37). *M. hyopneumoniae* colonization of the airways causes ciliostasis, clumping, and loss of cilia, as well as loss of epithelial cells and bronchial goblet cells. As a result, the ability of the mucociliary apparatus to function and clear the airways of debris and invading pathogens is significantly reduced (38).

M. hyopneumoniae is the organism that is not easy to grow. thus, the concrete diagnosis is to be based on interpretation of lesions alone or in conjunction with additional tests to detect the mycoplasma in the lungs (38). Through immunohistochemistry, immunofluorescence, or polymerase chain reaction (PCR).

Most cases of porcine enzootic pneumonia have mild to moderate bronchopneumonic lesions and thus mortality is low but with co-infection of secondary pathogens such as *P. multocida*, *B. bronchiseptica, G. parasuis, A. plureopnemoniae, M. hyorhinis* (39).

Mycoplasmal pneumonia is a high-morbidity, low-mortality disease characterized by a persistent, chronic cough. Incubation period lasts 10 to 16 days, and because the disease spreads slowly, evidence of disease in the herd is often difficult to notice until pigs reach 3 to 6 months old (40). *M. hyopneumoniae* lesions begin as bronchointerstitial pneumonia and progress to suppurative or mucopurulent bronchopneumonia once secondary pathogens are present and it is often seen at necropsy (41). Gross lesions are mainly observed in limited area of cranial lobe in general but it involves more than 50% of cranioventral portions of the lungs. The color of lungs changes, depending on the disease stage, lung color is dark red in the early stages of the disease but the color turns into homogeneous pale-gray color in the later stages. Exudate from airways can be easily expressed on the cut surface, and the exudate aspect changes from purulent to mucopurulent to mucoid, along with the stage of the lesions and secondary infections (39). Microscopic lesions are distinguished by an infiltration of macrophages and neutrophils into the bronchi, bronchioles, and alveoli, as well as significant bronchus-associated lymphoid tissue (BALT) hyperplasia over time.

2-5. *Bordetella bronchiseptica*

Bordetella bronchiseptica is the most common cause of rhinitis in pigs, which can manifest as mild rhinitis, non-progressive atrophic rhinitis when *B. bronchiseptica* is the only etiologic agent, or progressive atrophic rhinitis when *B. bronchiseptica*

infects with toxigenic *P. multocida* (42).

B. bronchiseptica is a Gram-negative, small coccobacillus. The bacterium grows in aerobic conditions, preferentially at 35℃ to 37℃. *B. bronchispetica* is a non-fermentative species that can move, which is aided by peritrichous flagella (43). Colonies of *B. bronchiseptica* grow in 2-3 days on commonly used agar media. they grow faster than other species of the *Bordetella genus* and are more resistant to changing physical and chemical conditions (43). Some virulence factors play an important role in *B. bronchiseptica* pathogenicity. Fimbriae, for example, are linked to bacterial attachment and colonization of nasal epithelium cells. Dermonecrototoxin is required for the induction of clinical signs in swine (44). Iron is associated with *Bordetella bronchiseptica* metabolism and determines the bacterium's ability to colonize and proliferate (45). The disease progresses over time to atrophic rhinitis, in which the boney trabeculae of the turbinates are replaced by fibrous connective tissue due to the action of the dermonecrotic toxin (DNT) (46). The disease is nonprogressive when *B. bronchiseptica* is the sole etiologic agent, affecting primarily the ventral scrolls and turbinates and taking weeks to resolve; however, when *B. bronchiseptica* interacts with toxigenic strains of *P. multocida*, the disease is more severe. *P. multocida* toxin (PMT) and DNT work together to cause more severe turbinate atrophy, which can result in septal deviation (46). Rhinitis manifests clinically as sneezing, nasal discharge, and ocular discharge. At necropsy, atrophy of the turbinates can be seen as atrophic rhinitis progresses, and coinfection with *P. multocida* can result in brachygnathia, lateral deviation of the snout and/or epistaxis (47). Mucosal inflammatory lesions with loss of cilia and turbinate atrophy are examples of upper respiratory tract lesions (48). *B. bronchiseptica* also causes neonatal pneumonia and secondary pneumonia in older pigs. When red, consolidated

areas in the lung are visible in primary *Bordetella pneumonia*, the severity of pneumonic lesions peaks between 10 and 14 days post infection (dpi) (49).

2-6. *Pastrella multocida*

P. multocida is an ornormal and opportunistic bacteria of the oral, nasopharyngeal, and upper respiratory tracts (50), as well as the causative pathogen of infections, bringing significant economic losses (51). *P. multocida* is linked to progressive atrophic rhinitis (PAR) in pigs and, along with other respiratory pathogens, contributes to PRDC (52, 53). In China, the prevalence of *P. multocida* was reported to be 8.0 percent in diseased pigs with pneumonia or PAR, and in Korea, the prevalence ranged from 10.3 to 15.6 percent in pigs with pneumonia. Furthermore, *P. multocida* accounts for 15.6 percent of all isolated respiratory pathogens in the United States (52, 53, 54).

P. multocida is divided into three sub-species (*multocida, septica, and gallicida*) and 13 biovars (1 to 10 and 12 to 14) with carbohydrate fermentation and the production of the ornithine decarboxylase (ODC) enzyme (55). The majority of swine isolates are subspecies multocida assigned as biovars 2 or 3 (50, 56). Five capsular types are classified, based on capsular antigens (A, B, D, E, and F). *P. multocida* and capsular types A, B, D, and F have been identified in swine (50, 57). Types A and D are the most commonly isolated and cultured from pneumonia and progressive atrophic rhinitis, respectively, while types B and F are hardly found in pigs (52). Several previous researches, conducted in Korea demonstrate that type A is more common in porcine pneumonia (54), but there is little information available about subspecies, biovars, and other capsular types of *P. multocida* isolates in Korea.

Clinical signs and lesions are usually superimposed on those of a primary agent,

and usually involve a chronic intermittent cough, labored breathing, and failure to grow. Lung consolidation with a cranial-ventral distribution that is well-defined in red to gray color is common. Pleuritis, pleural adhesions, and/or abscesses are also possibilities. A lobular purulent bronchopneumonia can be seen under the microscope.

3. Current vaccine practice for PRDC

3-1. General vaccination principle

Vaccination is expected to be the most cost-effective tool for controlling PRDC. The basic principles of vaccinating pigs are the same as those are for other species. Thus, for diseases that pose a risk to growing piglets, injectable vaccines should be administered as soon as maternal antibody titers have declined. This is generally considered to be between three and six weeks of age. Depending on the vaccine manufacturer's recommendations, these piglets may need to be boosted again two to four weeks later (58). Because maternal antibodies do not interfere with mucosal immunity, oral or intranasal vaccines can be given much earlier.

There are numerous infections that pose a significant risk to newborn piglets. These are controlled by vaccinating pregnant sows and promoting the production of colostrum antibodies.

Following a diagnosis, veterinarians could recommend appropriate vaccines based on antibody titer and antigen load in the blood, which reflected the respective disease outbreak pattern in farms. Vaccines are available in a variety of forms, including antiviral and antibacterial vaccines. Vaccines are also given to sows or piglets, depending on the disease. Vaccines for piglets include *Mycoplasma, Actinobacillus pluropnemoniae, Glasser's disease, Swine erysipelas*, Circovirus, Pseudorabies, PRRS, and Influenza, while vaccines for sows include atrophic rhinitis, Clostrial dysentery, PPV, PRRS, Porcine epidemic diarrhea, and Rotavirus.

3-2. Vaccine for PCV-2

Based on the knowledge accumulated so far, PCV-2 has a significant economic impact when uncontrolled (59). Several genotypes of the small DNA virus PCV-2 are linked to a variety of swine diseases, including PMWS, PRDC, reproductive failure, granulomatous enteritis, necrotizing lymphadenitis, exudative epidermitis, and congenital tremors. PCV-2 typically infects pigs aged 6 to 12 weeks (20).

There are four PCV-2 genotypes spreading in the United States such as PCV-2a, 2b, 2d, and 2e, but their prevalence is changing over time. Until 2005, 2a was the only known PCV-2 genotype, but 2b quickly overtook it. A mutant 2b was discovered in 2012 (60). It differed from the nominate 2b only by one amino acid in open reading frame 2. (ORF2). This highly virulent mutant PCV-2b spread quickly. It quickly displaced the nominate 2b genotype as the dominant genotype and is thought to be present in one-third of US herds (61). It is now referred to as PCV-2d. In addition, PCV-2 is combined with PRRSV, SIV or *M. hyopneumoniae*, the disease becomes especially severe (1).

In the market, there are currently several commercial vaccines available. Among them, these are inactivated whole virus vaccines approved for use in sows and gilts. Other vaccines for piglets have been developed. The PCV-2 capsid protein is expressed in a baculovirus system in two subunit vaccines. An inactivated chimeric vaccine is also available, in which the capsid gene from nonpathogenic PCV-1 replaces the same gene in PCV-2. Another chimeric vaccine contains antigens from both PCV-2a and 2b. All of these vaccines induce both neutralizing antibodies and cell-mediated immunity against PCV-2a while also providing cross-protection against PCV-2b and 2d. PCV-2 vaccines can also be combined with Mycoplasma and PRRS vaccines. These vaccines lower viremia and disease pathology while

increasing daily weight gain (63, 64).

3-3. Vaccine for *Mycoplasma hyopneumoniae*

Pneumonia caused by *M. hyopneumoniae* is one of the most contagious and economically significant chronic respiratory disease affecting the commercial swine industry. It damages the respiratory tract, causing secondary bacterial and viral infections in animals, such as porcine pleuropneumonia (64). When combined with immunosuppressive viruses like PCV-2 and PRRSV, this infection is especially dangerous. Management and vaccine are effective tools to control enzootic pneumonia. The most common type of vaccine is an inactivated whole bacterial vaccine.

The production of IgA antibodies, which prevent *M. hyopneumoniae* from adhering to the mucosal surface, is thought to play an important role in protection (40). As a result, there has been a lot of interest in developing adjuvant and delivery systems that can select mucosal IgA antibodies (65).

These bacterins can be administered to piglets, as well as introduced growers and breeders. They help to prevent or lessen the severity of lung lesions while also increasing daily weight gain. In China, a modified live vaccine (Mhp-168 strain) that has been attenuated by more than 300 serial passages in vitro is used, and it may provide superior protection (66). There is also a bacterin available to treat *Mycoplasma hyorhinis*, which causes arthritis, pericarditis, and peritonitis in pigs.

3-4. Bivalent vaccine for *Mycoplasma hyopneumoniae* and Porcine circovirus-2

PCV-2 and *M. hyopneumoniae* are the most frequent pathogens, that are involved in PRDC cases, causing a negative impact on swine health and thus result in

significant economic losses in farms. Swine farmers including Korean swine farmers are using PCV-2 and *M. hyopneumoniae* bivalent vaccines to control PRDC (67, 68).

Vaccination is an important control measure for these infections, and its use is widely used to prevent the associated losses (69, 70). In terms of PCV-2, while the best results have been obtained when pigs are vaccinated at 6 weeks of age, the conventional PCV-2 vaccination is performed at 3 to 4 weeks of age (70). Piglets are thought to be first exposed to *M. hyopneumoniae* during the lactation period, so vaccination against this pathogen should also be performed in the first few weeks of life (67). Thus, using vaccines that are protective for both PCV-2 and *M. hyopneumoniae* at the same time is epidemiologically justified for the following reasons ; first, their efficacy is equivalent to that obtained when they are administered separately (71); second, this strategy offers benefits to swine producers such as reducing labor costs, vaccination application, and the risk of pathogen transmission through needles; and third, the reduced need for animal manipulation and injection improves piglet welfare. This has led to a demand for single-dose bivalent vaccines containing PCV-2 and *M. hyopneumoniae* (72).

There are a few ready-to-use vaccines for both pathogens on the market, but the questions about their performance in the field are not fully understood.

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PART I. Experimental reproduction of porcine respiratory disease complex in pigs inoculated porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* and followed by inoculation with Porcine circovirus-2

ABSTRACT

The aim of this study was to reproduce severe pneumonic lesions, similar to those during naturally-occurring porcine respiratory disease complex, in pigs dually inoculated with porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* at 6 weeks of age, followed by inoculation with Porcine circovirus-2 at two weeks after. Time and sequence of infection with three pathogens mirror Asian field conditions. Microscopically, interstitial pneumonia and peribronchiolar lymphoid hyperplasia are considered the most characteristic lung lesions in infected pigs. The results of the present study demonstrate that inoculation of pigs with these three pathogens can lead to severe interstitial pneumonia with peribronchial or peribronchiolar lymphoid hyperplasia and fibrosis.

KEY WORD: Porcine Respiratory Disease Complex, *Mycoplasma hyopneumoniae*, Porcine circovirus-2, porcine reproductive and respiratory syndrome virus

INTRODUCTION

The term 'Porcine Respiratory Disease Complex (PRDC)' has been used to describe the complicated disease characterized by respiratory symptoms and poor growth in growing and finishing pigs, typically approximately 14 to 22 weeks of age (1, 2, 3). PRDC commonly occurs due to the interaction and synergy of both viral and bacterial pathogens. Among those, porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, and Porcine circovirus-2 (PCV-2) are considered to be the most clinically important pathogens (2). The microscopic lesions of PRDC can vary in severity depending on the types and numbers of pathogens. However, the most common lung lesions include moderate-to-severe interstitial pneumonia with peribronchiolar lymphoid tissue hyperplasia and fibrosis (1, 3).

In recent years, PRDC has become the most economically disastrous disease in the Asian swine industry. In Korean farms, pigs show severe PRDC signs at 11 to 16 weeks following infection with PRRSV and *M. hyopneumoniae* at 5 to 7 weeks and by PCV-2 infection at 7 to 9 weeks based on analysis of diagnostic cases and a serological survey (C. Chae, personal observation). Although PRDC has long been associated with PRRSV, *M. hyopneumoniae*, and PCV-2, to date, there is no experimental reproduction of PRDC by infecting pigs with these three pathogens. The objective of this study was to reproduce a PRDC model which mimics naturally occurring PRDC by sequential infection with PRRSV / *M. hyopneumoniae*, and PCV-2.

MATERIALS AND METHODS

Animals

A total of 36 colostrum-fed, cross-bred, conventional piglets were purchased at 18 days of age from a PRRSV- and *M. hyopneumoniae*-free commercial farm based on serological testing of the breeding herd, and long term clinical and slaughter history. At 21 days of age, pigs were sero-negative for PRRSV (IDEXX PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA), *M. hyopneumoniae* (*M. hyo*. Ab test, IDEXX Laboratories Inc.), and PCV-2 (PCV-2 Ab Mono Blocking, Synbiotics, Lyon, France). In addition, negative results were also obtained for PCV-2 and PRRSV from sera samples and for *M. hyopneumoniae* from nasal swabs by real-time polymerase chain reaction (PCR) (4, 5, 6).

Pathogens

PRRSV strain SNUVR090851 (type 2 genotype, lineage 1, GenBank JN315685), *M. hyopneumoniae* strain SNU98703, and PCV-2 strain SNUVR000463 (type 2b genotype, GenBank KF871068) were used as inocula. Co-infection with PCV-2 strain SNUVR000463 and *M. hyopneumoniae* strain SNU98703 induced severe pneumonia in lungs and lymphoid depletion in the lymph node in infected pigs (7). Similarly, co-infection with the identical PCV-2 strain SNUVR000463 and PRRSV strain SNUVR090851 also induced similar symptoms as did the previous co-infection (8).

Experimental design

A total of 36 pigs were randomly divided into 2 groups (infected or control, 18 pigs per group) using random number generation function (Excel, Microsoft Corporation, Redmond, WA, USA). At 0 days post-inoculation (dpi, 42 days of age), the pigs in infected group were inoculated with PRRSV and *M. hyopneumoniae*. For inoculation, a 5 hours interval was chosen after PRRSV inoculation before inoculating with *M. hyopneumoniae* to avoid mixture of two pathogens which may decrease infectivity. Pigs were intranasally administered a 3 ml inoculation of PRRSV containing 1.2 x 10^5 50% tissue culture infective dose ($TCID_{50}$)/ml. Five hours after PRRSV inoculation, pigs were anesthetized with a mixture of 2.2 mg/kg xylazine hydrochloride (Rompun, Bayer Korea Ltd., Seoul, Korea) and 2.2 mg/kg tiletamine hydrochloride and 2.2 mg/kg zolazepam hydrochloride (Zoletil 50, Virbac, Carros, France) by intramuscular injection, and were inoculated intratracheally with 7 ml of *M. hyopneumoniae* culture medium containing 10^7 color changing units (CCU)/ml as previously described (9, 10). At 14 dpi (56 days of age), pigs in infected group were intranasally administered a 3 ml inoculation of PCV-2 containing 1.2 x 10^5 $TCID_{50}$/ml. The pigs in control group received the same amount of phosphate buffered saline (PBS, 0.01M, pH 7.4) with the same inoculation methods at 0 and 14 dpi.

All pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 35 dpi as previously described (11). Tissues (lung, superficial inguinal lymph node, liver, and kidney) were collected from each pig at necropsy. Tissues were fixed for 24 hours in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use, and Ethics

Committee.

In situ hybridization of PRRSV

In situ hybridization was used the lung tissues in order to detect specific nucleic acids for PRRSV and *M. hyopneumoniae* (12, 13).

Immunohistochemistry of PCV-2

Immunohistochemistry was used the lung tissues in order to detect specific antigens for PCV-2 (14). Tissue sections were deparaffinized in xylene, rehydrated through graded alcohols, and air-dried. Endogenous alkaline phosphatase was quenched with 20% glacial acetic acid solution for 2 min at 4 ℃. All slides were then incubated with normal mouse serum in phosphate-buffered saline (PBS) (0.1 M, pH 7.4) for 30 min at room temperature to saturate nonspecific protein-binding sites. A monoclonal mouse and monoclonal mouse anti-PCV-2 antibody (Dr. Gordon M Allen, Veterinary Sciences Division, Belfast, UK) were used at a dilution of 1:250 in PBS containing 0.1% Tween 20. Monoclonal antibodies were coated on the slides and incubated for 1 hr at room temperature. After 3 washes with 0.1% Tween 20 in PBS (0.01 M, pH 7.4), sections were flooded and incubated for 1 hr. at 37 ℃ with alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G antibody diluted 1:200 in PBS (0.01 M, pH 7.4) containing 0.1% Tween 20. The slides were washed with 0.1% Tween 20 in PBS 3 times. Then, sections were equilibrated with Tris buffer (0.1 M, pH 8.2) for 5 min at room temperature. The final reaction was produced by immersing the sections in a solution of red substrate for 20 min at room temperature.

RESULTS

Clinical signs

Clinical signs in infected pigs were characterized mainly by labored breathing, lethargy, coughing, and occasionally sneezing. The majority of the infected pigs had rough hair coats. Respiratory signs were not observed in uninfected pigs. Grossly, lungs failed to collapse upon removal from the chest cavity.

Macroscopic lesions

The most severely affected lungs were mottled tan or diffusely tan rubbery and, were firmer and heavier than normal (Fig. 1). The infected pigs also had overall enlarged superficial inguinal lymph nodes.

Microscopic lesions

Microscopically, interstitial pneumonia and peribronchiolar lymphoid hyperplasia is considered the most characteristic lung lesion in infected pigs. Alveolar septa were markedly thickened by type 2 pneumocyte hypertrophy and hyperplasia, septal infiltration with macrophages, fewer lymphocytes and plasma cells (Fig. 2A). Many alveolar septa were diffusely lined by hypertrophied type 2 pneumocytes. Alveolar spaces were filled with macrophages, necrotic macrophages, and occasionally, multinucleated cells, and proteinaceous fluid. No intracytoplasmic grape-like inclusion bodies of PCV-2 were not observed in macrophages and multinucleated giant cells. Lungs from infected pigs had moderate-to-severe peribronchiolar and perivascular lymphohistiocytic cuffing and nodular formation (Fig. 2A). Lung had

lymphohistiocytic inflammation in the lamina propria of airways, and mixed inflammation in the lumina of the airway. Lymphoid lesions were characterized by mild-to-severe lymphoid depletion and mild-to-severe histiocytic-to-granulomatous inflammation. Moderate to marked multifocal peribronchial and peribronchiolar fibrosis was also observed in infected pigs. Lymph nodes were depleted of mature lymphocytes and contained pyknotic basophilic nucleic and adjacent karyorrhectic debris, germinal centers were reduced or absent. Macrophages in depleted follicle often contained clusters of grape-like round basophilic to amphophilic intracytoplasmic inclusions of PCV-2.

In situ Hybridization

Hybridization signals for PRRSV and *M. hyopneumoniae* nucleic acid were detected in all 18 infected pigs. Immunohistochemical signals for PCV-2 antigen were also detected in all 18 infected pigs. In general, PRRSV-positive cells were detected mainly in the alveolar septa. The positive cells generally had large oval nuclei and abundant cytoplasm (Fig. 3A). A hybridization signal of *M. hyopneumoniae* was seen in the luminal surface of bronchial and bronchiolar lining epithelial cells (Fig. 3B).

Immunohistochemistry

PCV-2-positive cells were detected mainly in the alveolar septa. The positive cells generally had large oval nuclei and abundant cytoplasm (Fig. 3C).

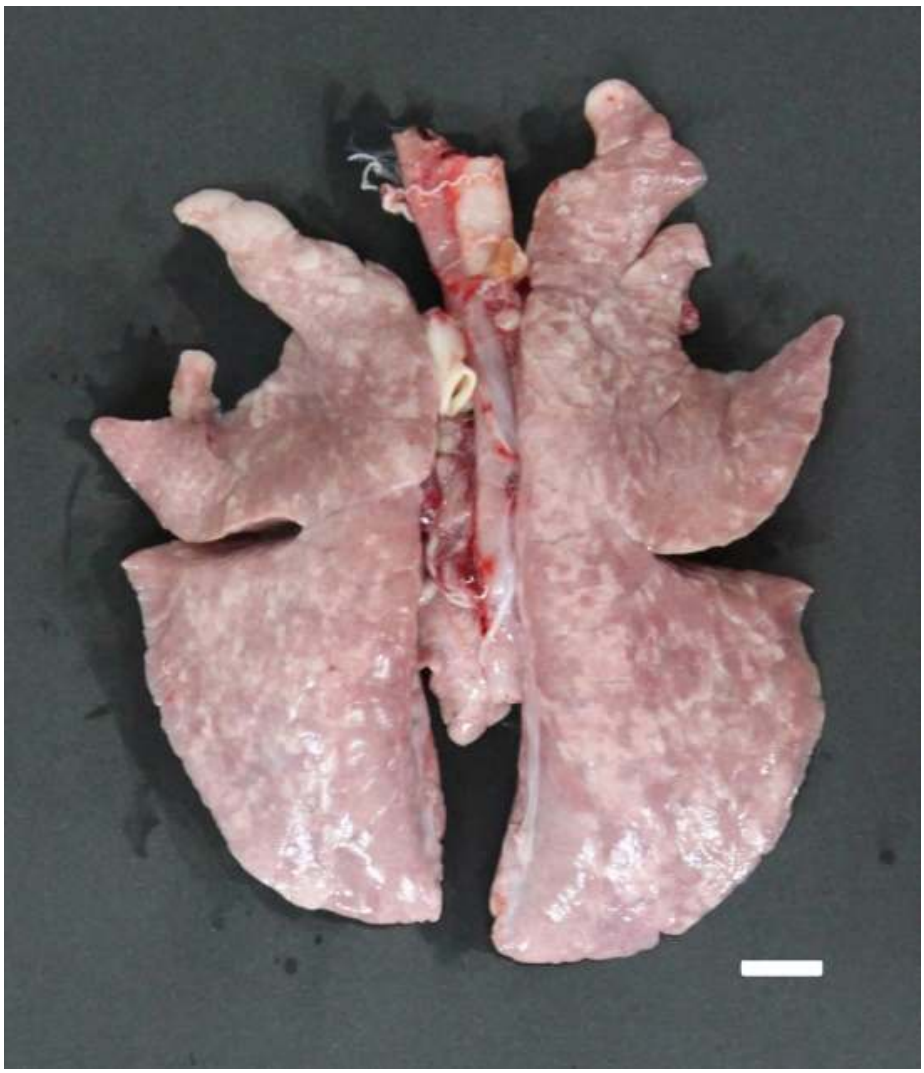

Figure 1. Photomicrograph of lung showing severe mottled-tan consolidation. Bar=50 μm.

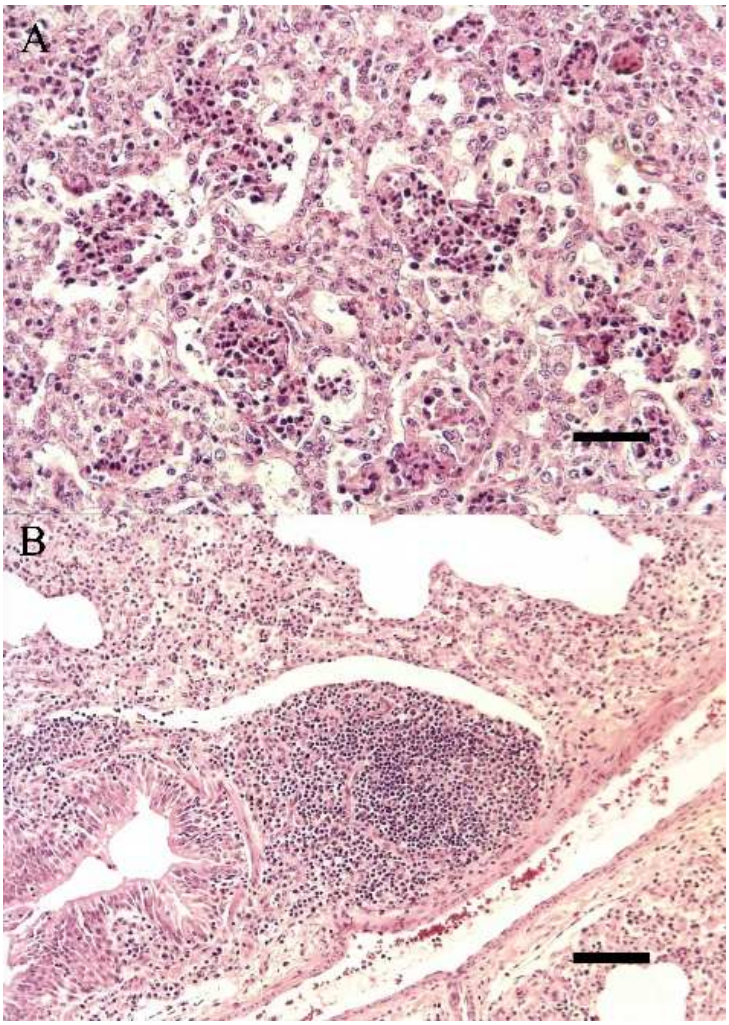

Figure 2. Phototomicrograph of section of lung showing severe markedly thickened by type 2 pneumocyte hypertrophy and hyperplasia in alveolar septa, and severe infiltration with macrophages, necrotic macrophages, and multinucleated cells in alveolar spaces. Hematoxylin and eosin (H&E) staining. Bar=50 µm (A). Photomicrograph of section of lung showing moderate-to-severe peribronchiolar lymphohistiocytic cuffing and nodular formation. H&E staining. Bar=50 µm (B).

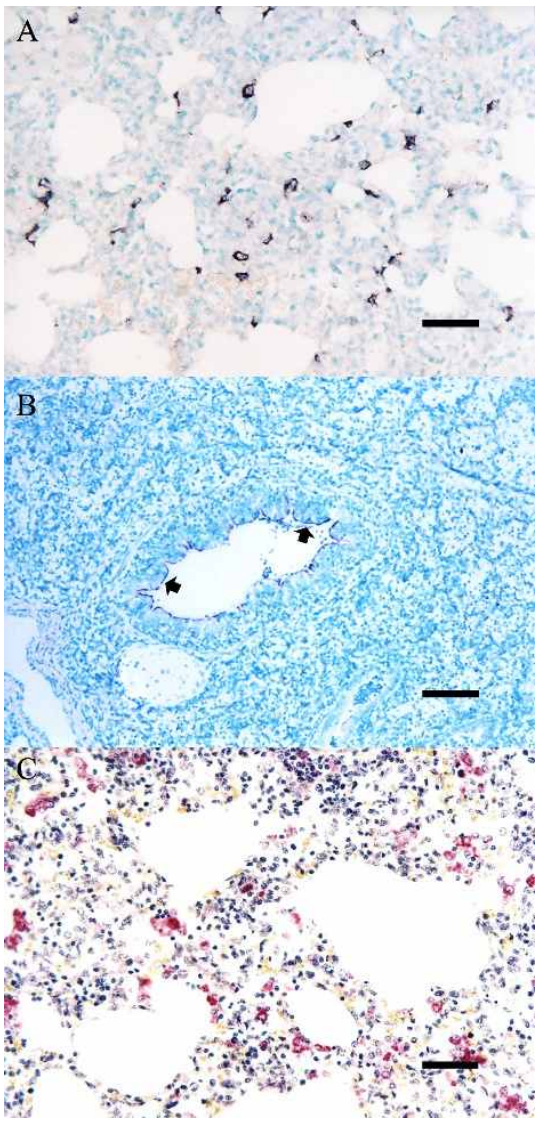

Fig. 3. Photomicrograph of section of lung showing porcine reproductive and respiratory syndrome virus nucleic acids in alveolar septa. *In situ* hybridization, Bar=50 µm (A). Photomicrograph of section of lung showing *Mycoplasma hyopneumoniae* nucleic acids (arrows) in surface of epithelial lining cells in bronchiole. *In situ* hybridization, Bar=50 µm (B). Photomicrograph of section of lung showing Porcine circovirus-2 antigens in alveolar septa. Immunohistochemistry, Bar=50 µm (C)

DISCUSSION

In this study, the order of infection with PRRSV, *M. hyopneumoniae*, and PCV-2 was designed on the basis of natural infection patterns in pig farms and the severity of lung lesions due to interaction of these three pathogens. Time and order of infection with these three pathogens mirrored Asian field conditions. In most pig-rearing Asian countries including Korea, pigs are typically infected with PRRSV and *M. hyopneumoniae* at around 5-7 weeks of age followed by PCV-2 infection at 7-9 weeks of age and with clinical signs appearing around 11-16 weeks of age. The severity of the lung lesions caused by these three pathogens differs depending on the order of infection. Studies have shown that co-infection of pigs with PRRSV and *M. hyopneumoniae*, causes more severe histopathological lung lesions compared to sequential infection (15). In contrast, sequential infection with *M. hyopneumoniae* and PCV-2, causes more severe histopathological lung lesions compared to co-infection (16, 17).

The results of the present study demonstrate that 6-week-old pigs dually inoculated with PRRSV and *M. hyopneumoniae*, followed by the inoculation with PCV-2 two weeks after (i.e. at 8 weeks of age) exhibit severe pneumonic lesions mimicking those of naturally-occurring PRDC (3, 18). The most striking and consistent pathologic lesions are interstitial pneumonia with peribronchial or peribronchiolar lymphoid hyperplasia and fibrosis. Because PRRSV and PCV-2 are primarily infected with interstitial macrophages in alveolar septa, both viruses are involved in causing interstitial pneumonia. In contrast, *M. hyopneumoniae* is involved in causing peribronchial and peribronchiolar hyperplasia because it is

primarily detected in surface of epithelial lining cells.

This is the first experimental reproduction of PRDC by infection with the three most common pathogens known to be associated with this disease. The PRDC model infected with PRRSV, *M. hyopneumoniae*, and PCV-2 would be useful in evaluating the efficacy of vaccines in terms of PRDC prevention. Further studies are needed to determine the interaction among the three pathogens to cause PRDC.

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PART II. Comparative growth performance of three types of combination vaccines containing Porcine circovirus-2 and *Mycoplasma hyopneumoniae* under field conditions

ABSTRACT

This field trial compared three different types of combination vaccine: a trivalent vaccine containing Porcine circovirus-2a and -2b (PCV-2a/b), and *Mycoplasma hyopneumoniae*, a mixable bivalent vaccine containing PCV-2a and *M. hyopneumoniae*, and a ready-to-use bivalent vaccine containing PCV-2a and *M. hyopneumoniae*. Two farms were selected on the basis of their subclinical PCV-2d infection and enzootic pneumonia. A total of 120 pigs in each farm were randomly divided into 4 groups (30 pigs per group). The trivalent-vaccinated group from both farms outperformed each bivalent-vaccinated group in terms of growth performance. Growth performance was significantly improved during the fattening periods (70-175 days of age) of the mixable bivalent-vaccinated group in comparison with the ready-to-use bivalent-vaccinated group in one farm. The trivalent-vaccinated group elicited higher levels of neutralizing antibodies and interferon-γ secreting cells (IFN-γ-SC) against PCV-2d, while simultaneously decreasing the levels of PCV-2d load in blood when compared against the mixable and ready-to-use bivalent-vaccinated groups. The trivalent-vaccinated group also elicited higher levels of IFN-γ-SC against *M. hyopneumoniae* and lower levels of *M. hyopneumoniae* loads in the larynx when compared with the mixable and ready-to-use bivalent-vaccinated groups. The results of the present study demonstrated that a trivalent vaccine containing PCV-2a/b and *M. hyopneumoniae* resulted in a better productive parameter, higher immune responses, and less blood-viral and mycoplasmal larynx-loads when compared with the mixable and ready-to-use bivalent vaccines despite the presence

of ongoing farm subclinical PCV-2d infection and enzootic pneumonia.

Keywords: enzootic pneumonia, *Mycoplasma hyopneumoniae*, Porcine circovirus-2, subclinical PCV-2 infection, combined vaccine

INTRODUCTION

Porcine circovirus-2 (PCV-2) and *Mycoplasma hyopneumoniae* are two dominate economic pathogens that cause costly diseases in the global pork industry. PCV-2 infection can produce overt clinical diseases, porcine circovirus-associated diseases (PCVAD), and cause subclinical PCV-2 infection; the latter being considered the most common form of PCV-2 infection worldwide (1). Currently, PCV-2 is further divided into at least eight genotypes, noted as 'a to h' (2). Among those, PCV-2d is the most predominant genotype in Asia and North America (3-5). *M. hyopneumoniae* infection causes enzootic pneumonia, a chronic respiratory disease which impacts growth (6). *M. hyopneumoniae* continues to frustrate swine practitioners and producers.

Complication from both subclinical PCV-2 infection and enzootic pneumonia in the field are linked to porcine reproductive disease complex (PRDC) that results in economic losses, poor growth performance, and increased animal treatment costs. Vaccination is one of the most efficient tools and is used worldwide to control PCV-2 and *M. hyopneumoniae* infections. Combination vaccines containing PCV-2 and *M. hyopneumoniae* have recently increased in demand within the Asian pork industry, as they reduce animal stress and save in labor cost.

Three different types of PCV-2 and *M. hyopneumoniae* combination vaccines are commercially available; a trivalent vaccine containing PCV-2a/b and *M. hyopneumoniae*, a mixable bivalent vaccine containing PCV-2a and *M. hyopneumoniae*, and a ready-to-use bivalent vaccine containing PCV-2a and *M. hyopneumoniae*. The trivalent vaccine is the first combination vaccine to contain

PCV-2b antigen. PCV-2b antigen is so genetically close to PCV-2d, that PCV-2d was previously known as mutant PCV-2b. Since combination vaccines containing PCV-2d and *M. hyopneumoniae* are not yet commercially available, it is necessary to compare the effect of the three different types of existing combination vaccines on growth performance under field conditions. A full-scale comparative field trial has yet to be undertaken. The objective of the study was to compare the resulting pig growth performance among three different types of combination vaccines in herds with existing subclinical PCV-2 infection and enzootic pneumonia.

MATERIALS AND METHODS

Farm history

The clinical field trial was performed on two farms, noted as Farm A (450 sows) and Farm B (400 sows) located in Chungcheung Province of Republic of Korea. Both farms were farrow-to-finish operation with an all-in-all-out production system. The porcine reproductive and respiratory syndrome virus (PRRSV) status was stable in sow population. High-parity (> 5 parity) sows were the only seropositive against PRRSV in both farms. Vaccination against PCV-2 and *M. hyopneumoniae* was not received in sows in both farms.

Two farms were selected based on their subclinical PCV-2 infection and enzootic pneumonia status. Subclinical PCV-2 infection was diagnosed on both farms (1) which was defined as follow: decreased average daily gain without overt clinical sings, absence of or minimal histopathological lesions in superficial inguinal lymph nodes, and the presence of low amounts of PCV-2 in superficial inguinal lymph node (as determined by immunohistochemistry) in three out of four suspected pigs on Farm A and two out of five suspected pigs on Farm B. Pre-trial investigations identified a PCV-2 serological profile presenting an increase in antibody titers starting around seven weeks of age, while 7–16 week-old pigs were also PCV-2 polymerase chain reaction (PCR) seropositive in the two farms. *M. hyopneumoniae* serology was positive in 12-16 week-old pigs. Furthermore, the nasal swabs of 7 week-old pigs were PCR-positive for *M. hyopneumoniae* in the two farms. Together, these results supported an active PCV-2 and *M. hyopneumoniae* infection in Farms A and B.

Table I. Field experimental design.

Group	Type of Vaccine	Name of Vaccine
VacA1 and VacB1	Ready-To-Use Trivalent	Fostera Gold PCV MH
VacA2 and VacB2	Mixable Bivalent	CircoFLEX+MycoFLEX
VacA3 and VacB3	Ready-To-Use Bivalent	Porcilis PCV M Hyo
UnVacA and UnVacB	None	None

Experimental design

To minimize sow variation, eight piglets at 21 days of ages were pulled from each of 15 sows and uniformly divided into four groups (two per groups). A total of 120 pigs in each farm was used in this comparative field trial and were randomly assigned into one (30 pigs per group, male = 15 and female = 15) of four groups using the random number generator function (Excel, Microsoft Corporation, Redmond, WA, USA) (**Table 1**). Pigs in the VacA1 and VacB1 groups were received a 2.0 mL of trivalent vaccine (Fostera Gold PCV MH, Serial No: 395164A, Expiration date: 10-Dec-2021, Zoetis, Parsippany, NJ, USA) by intramuscular route in the neck muscle at 21 days of age. Pigs in the VacA2 and VacB2 groups were received a 2.0 mL of freshly mixed bivalent vaccine (FLEXcombo, Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri, USA) by intramuscular route in the neck muscle at 21 days of age. FLEXcombo vaccine was prepared by mixing equal volumes of Ingelvac CircoFLEX (Serial No. 3091337B, Expiration date: 15-Apr-2021) and Ingelvac MycoFLEX (Serial No. 2730677A, Expiration date: 10-Dec-2021) prior to use according to label directions. Pigs in the VacA3 and VacB3 groups were received a 2.0 mL of bivalent vaccine (Porcilis PCV M Hyo, Lot No. C752B02, Expiration date: 23-Sep-2021, MSD Animal Health, Boxmeer, Netherlands) by intramuscular route

in the neck muscle at 21 days of age. Pigs in the UnVacA and UnVacB groups were received intramuscularly with 2.0 mL of phosphate buffered saline (PBS, 0.01M, pH 7.4) at 21 days of age.

Blood and laryngeal swabs were collected from each treatment group at 0 (21 days old), 28 (49 days old), 49 (70 days old), 91 (112 days old) days post-vaccination (dpv). Pig were snared and restrained with a mouth gag for laryngeal swab collection, where the swabs were guided into the larynx with a laryngoscope. Once the epiglottis was in a low position, the internal walls of the laryngeal cartilages were swept with the swabs.

Clinical observations

The pigs were monitored daily for abnormal clinical signs and scored (0 to 6) weekly for severity of clinical respiratory signs with a blind system (7). Mortality and age of death were recorded. Pigs that died throughout the field trial were necropsied for the diagnosis of death.

Average daily weight gain

The pigs were weighed on 0 (21 days old), 49 (70 days old), and 154 (175 days old) dpv. The average daily weight gain (ADWG; grams/pig/day) was analyzed over two time periods: (i) between 21 and 70 days old and (ii) between 70 and 175 days old. ADWG was calculated as the difference between the body weights of two weight time points divided by the number of days between these two eight time points. Data for dead or removed pigs were included in the calculation.

Quantification of PCV-2d DNA in blood

DNA was extracted from serum samples using the commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA). Real-time PCR was performed to quantify the amount of PCV-2d genomic DNA (8).

Quantification of *M. hyopneumoniae* DNA in laryngeal swabs

DNA extracted from laryngeal swabs using the commercial kit (QIAamp DNA Mini Kit, QIAGEN). Real-time PCR was performed to quantify the amount of the *M. hyopneumoniae* genomic DNA (9).

Serology

The serum samples were tested using commercially available enzyme-linked immunosorbent assays (ELISAs) for PCV-2 (SERELISA PCV-2 Ab Mono Blocking, Synbiotics, Lyon, France) and *M. hyopneumoniae* (*M. hyo.* Ab test, IDEXX Laboratories Inc.). For ELISA results, serum samples were considered positive for antibodies against PCV-2 if the reciprocal ELISA titer was > 350, and positive for antibodies against *M. hyopneumoniae* if the sample-to-positive (S/P) ratio was ≥ 0.4, in accordance with the manufacturer's instructions for each kit. The serum samples were also tested to measure neutralizing antibodies (NA) titer against PCV-2d (10-12).

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISpot) assay was conducted to determine the frequency of PCV-2d- and *M. hyopneumoniae*-specific interferon-γ secreting cells

(IFN-γ-SC) in peripheral blood mononuclear cells (PBMC) (8, 13). The IFN-γ positive spots on the membranes were imaged, analyzed and counted using an automated ELISpot Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany). The frequency of PCV-2d and *M. hyopneumoniae*-specific IFN-γ-SC was expressed as the numbers of IFN-γ-SC per million PBMC. ELISpot assay was done in duplicate.

Pathology

Macroscopic lung lesion scores were evaluated by the percentage of the pneumonic lesions. The scoring was performed and recorded by two pathologists (Chae and one graduate student). For the entire lung (100 points were be 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) (7). Microscopic lung mycoplasmal lesions were scored (0 to 6) based on the severity of peribronchiolar lymphoid tissue hyperplasia (14). Microscopic lymphoid lesions were scored (0 to 5) based on the severity of lymphoid depletion and granulomatous inflammation (15).

Statistical analysis

Prior to statistical analysis, real-time PCR data were transformed to $\log_{10}$ values. The Shapiro-Wilk test will be utilized to test the collected data for a normal distribution. If the normality assumption was met, one-way analysis of variance (ANOVA) was performed. Result from ANOVA test which showed 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 Krustal-Wallis test was performed. Kruskal-Wallis test result with such a statistical significance was 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.

RESULTS

Clinical signs

In Farm A, the vaccinated (VacA1) group had significantly lower ($P < 0.05$) respiratory signs than that of the unvaccinated (UnVacA) group at 21 and 84 to 112 dpv. All three vaccinated (VacA1, VacA2, and VacA3) groups had significantly lower ($P < 0.05$) respiratory signs than that of the unvaccinated (UnVacA) group at 28 to 77 dpv. In Farm B, one vaccinated (VacB1) group had significantly lower ($P < 0.05$) respiratory signs than that of the unvaccinated (UnVacB) group at 14 and 91 to 133 dpv. Three vaccinated (VacB1, VacB2, and VacB3) groups had significantly lower ($P < 0.05$) respiratory signs than that of the unvaccinated (UnVacB) group at 21 to 84 dpv.

Average daily weight gain

A difference in mean body weight was not observed between vaccinated and unvaccinated animals at the time the study began (21 days of age) on either farm. In Farm A, ADWG of vaccinated animals (VacA1, VacA2, and VacA3 groups) was significantly higher ($P < 0.05$) than that of unvaccinated animals (UnVacA group) during the fattening (70 to 175 days of age) and overall (21 to 175 days) periods. ADWG of the VacA1 group was significantly higher ($P < 0.05$) than that of the VacA2 and VacA3 groups during the fattening period (70 to 175 days of age) (Figure 1A). The ADWG of vaccinated animals (VacB1, VacB2, and VacB3 groups) in Farm B was significantly higher ($P < 0.05$) than that of unvaccinated animals (UnVacB group) during the fattening period (70 to 175 days of age) and overall period (21 to 175 days). ADWG of the VacB1 and VacB2 groups was significantly higher ($P <$

0.05) than that of the VacB3 group during the fattening period (70 to 175 days of age) (Figure 1B).

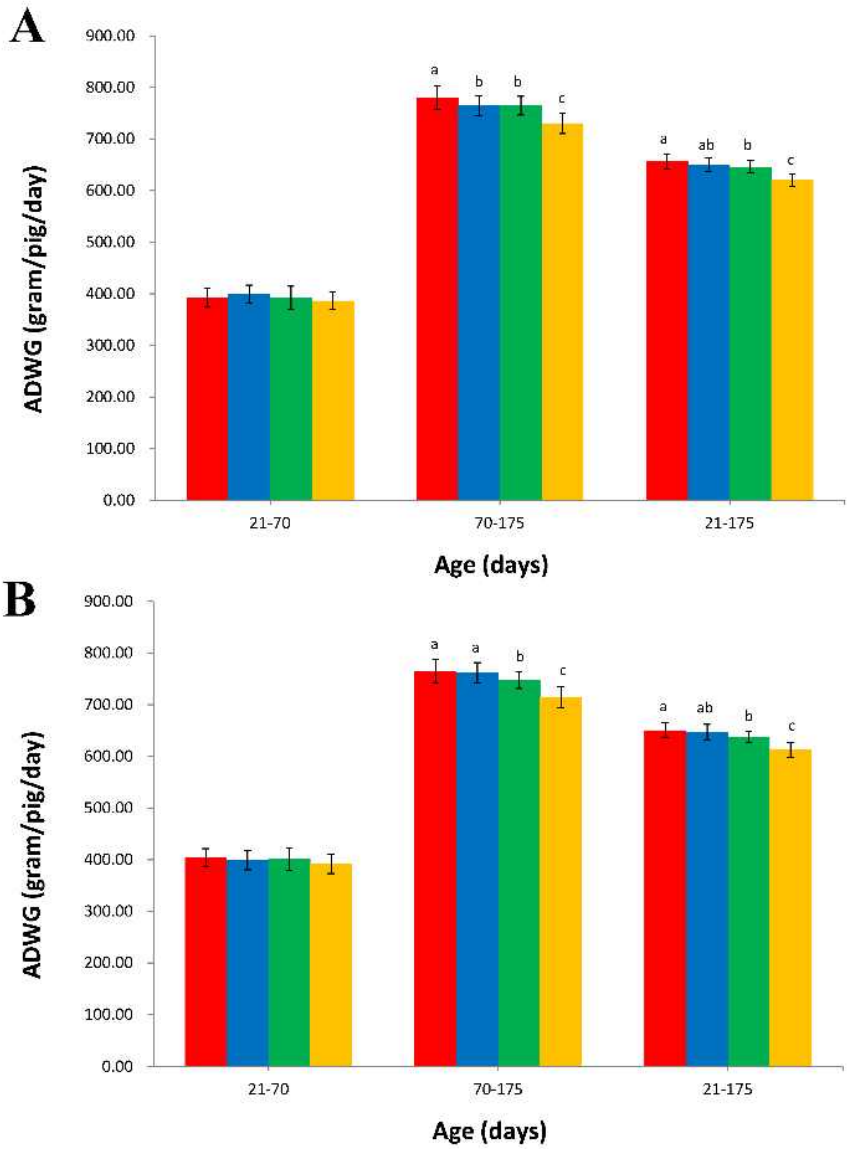


Figure 1. Average daily weight gain in Farm A (A) from VacA1 (■), VacA2 (■), VacA3 (■), and UnVacA (■), and Farm B (B) from VacB1 (■), VacB2 (■), VacB3 (■), and UnVacB (■). Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 4 groups in each of farms.

Mortality

Mortality at Farm A was reported as follows: Two pigs from the VacA1 group died at 55 and 74 days of age (respectively) of suppurative bronchopneumonia as diagnosed by a combination of *M. hyopneumoniae* that was detected with PCR, and *Trueperella pyogenes* that was isolated from the lungs. One pig from the VacA2 group died of severe watery diarrhea at 70 days of age, where bacteria was not found during isolation from the small and large intestine. One pig from the VacA2 group died at 85 days of age of bronchopneumonia as diagnosed by a combination of *M. hyopneumoniae* that was detected with PCR and *Pasteurella multocida* that was isolated from the lungs. One pigs from the VacA3 died at 73 days of age of bronchopneumonia and pleuritis as diagnosed by a combination of PCV-2d and *M. hyopneumoniae* that were detected with PCR, and *Glaesserella parasuis* that was isolated from the lungs. Three pigs from the UnVacA group died at 65, 72, and 88 days of age (respectively), of bronchopneumonia as diagnosed by a combination of PCV-2d and *M. hyopneumoniae* that were detected with PCR, and *P. multocida* that was isolated from the lungs.

Mortality at Farm B was reported as follows: One pig from the VacB1 group died at 65 days of age of unknown etiology without any pathological lesions in lung and brain. One pig from the VacB2 group died at 77 days of age of bronchopneumonia as diagnosed by a combination of PCV-2d that was detected with PCR and *G. parasuis* that was isolated from the lungs. Two pigs from the VacB3 group died at 72 days of age of bronchopneumonia as diagnosed by a combination of *M. hyopneumoniae* that was detected with PCR and *T. pyogenes* that was isolated from the lungs. One pig from the VacB3 group died at 80 days of age of bronchopneumonia and fibrinous pericarditis as diagnosed by a combination of PCV-

2d that was detected with PCR and *G. parasuis* that was isolated from the lungs and pericardium. One pig from the UnVacB group died at 74 days of age of unknown etiology without any pathological lesions in lung and brain. Two pigs from the UnVacB group died at 77 and 83 days of age by a combination of PCV-2d and *M. hyopneumoniae* that were detected with PCR, and *P. multocida* that was isolated from the lungs.

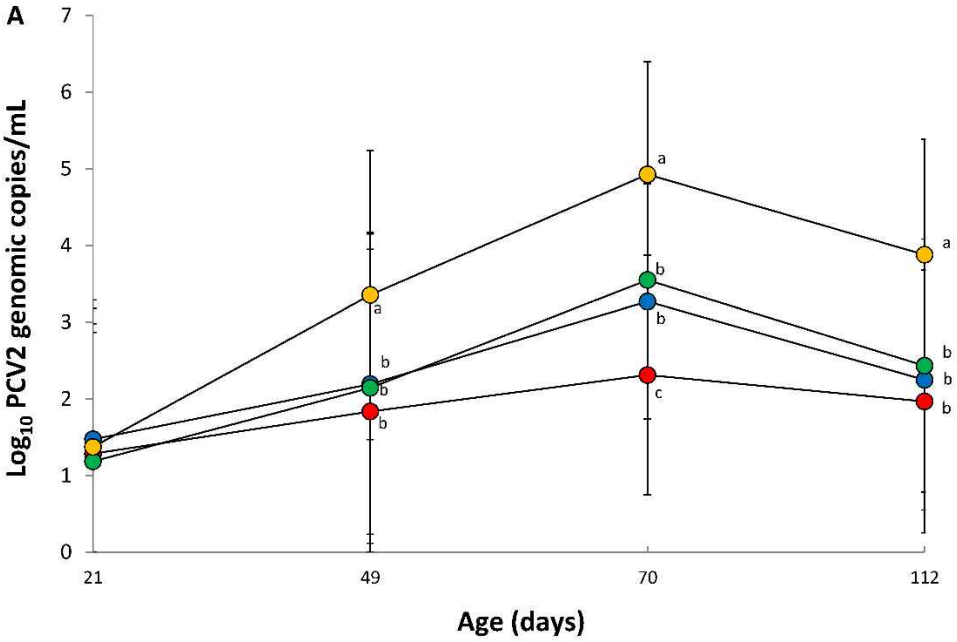
Quantification of PCV-2d in blood

In Farm A, vaccinated (VacA1, VacA2, and VacA3 groups) animals had significantly lower ($P < 0.05$) amount of PCV-2d loads in their blood than that of unvaccinated (UnVacA group) animals at 28, 49, and 91 dpv (Figure 2A). In Farm B, vaccinated (VacB1, VacB2, and VacB3 groups) animals had significantly lower ($P < 0.05$) amounts of PCV-2d loads in their blood than that of unvaccinated (UnVacB group) animals at 28, 49, and 91 dpv. The amount of PCV-2d load in blood from the VacB1 group was significantly lower ($P < 0.05$) than that of VacB3 group at 49 dpv (Figure 2B).

Quantification of *M. hyopneumoniae* in laryngeal swabs

In Farm A, vaccinated (VacA1, VacA2, and VacA3 groups) animals had significantly lower ($P < 0.05$) amounts of *M. hyopneumoniae* loads in their larynx than that of unvaccinated (UnVacA group) animals at 28, 49, and 91 dpv (Figure 2C). In Farm B, vaccinated (VacB1, VacB2, and VacB3 groups) animals had significantly lower ($P < 0.05$) amounts of *M. hyopneumoniae* loads in their larynx than that of unvaccinated (UnVacB group) animals at 28, 49, and 91 dpv. The amount of *M.*

hyopneumoniae load in the larynx from the VacB1 group were significantly lower ($P < 0.05$) than those of the VacB2 and VacB3 groups at 49 dpv (Figure 2D).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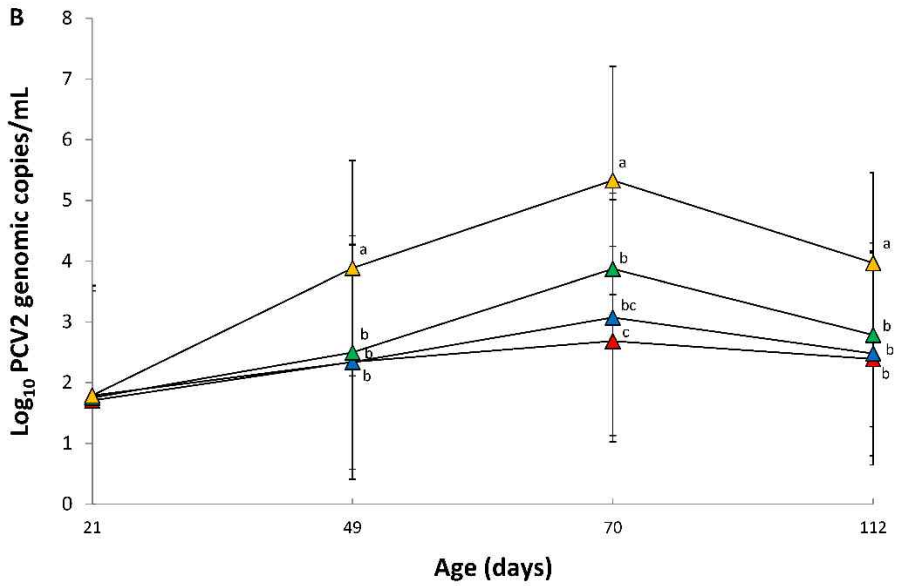
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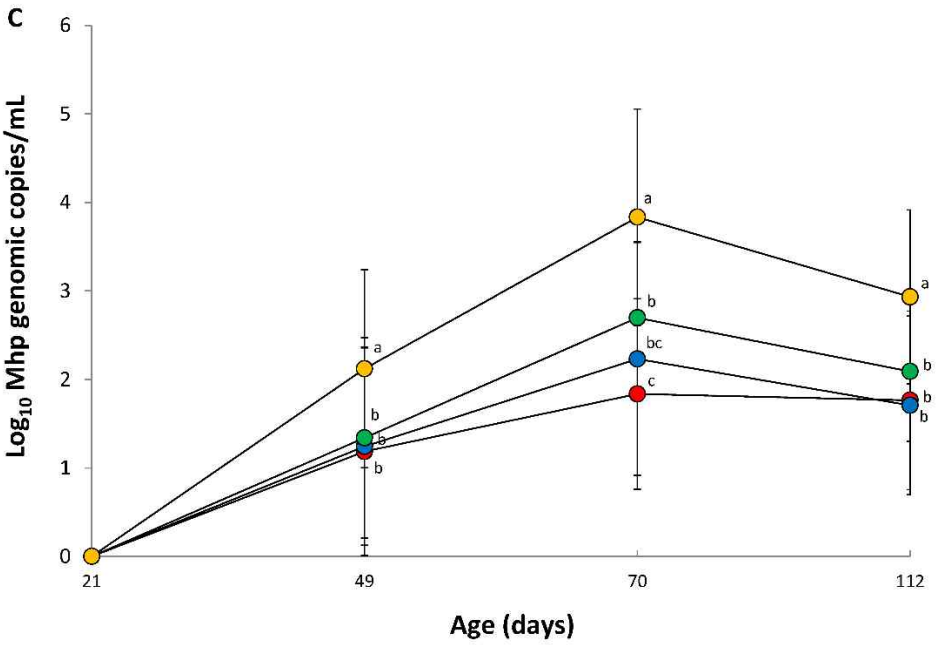
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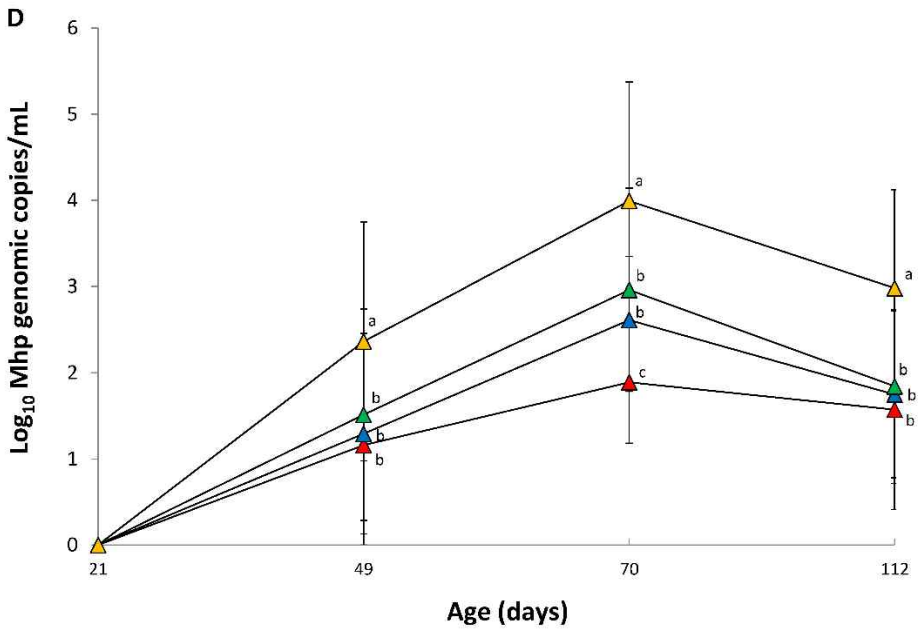
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Log10 Mhp genomic copies/mL
0
1
2
3
4
5
6
21
49
70
112
Age (days)
a
b
b
b
a
b
b
c
a
b
b
b

Figure 2. Genomic copy number of Porcine circovirus-2d (PCV-2d) and *Mycoplasma hyopneumoniae* DNA.

A – Mean values of the genomic copy number of PCV-2d in blood from VacA1 (●), VacA2 (●), VacA3 (●), and UnVacA (●) groups in Farm A.

B – Mean values of the genomic copy number of PCV-2d in blood from VacB1 (▲), VacB2 (▲), VacB3 (▲), and UnVacB (▲) groups in Farm B.

C – Mean values of the genomic copy number of *M. hyopneumoniae* in larynx from VacA1 (●), VacA2 (●), VacA3 (●), and UnVacA (●) groups in Farm A.

D – Mean values of the genomic copy number of *M. hyopneumoniae* in larynx from VacB1 (▲), VacB2 (▲), VacB3 (▲), and UnVacB (▲) groups in Farm B.
Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 4 groups in each of farms.

Immune responses against PCV-2

In Farm A, vaccinated (VacA1, VacA2, and VacA3 groups) animals had significantly higher ($P < 0.05$) PCV-2 ELISA and NA titers than that of unvaccinated (UnVacA group) animals at 28, 49, and 91 dpv. A comparison between vaccinated groups yielded that PCV-2 ELISA (Figure 3A) and NA titers (Figure 3C) from the VacA1 group were significantly higher ($P < 0.05$) than those of the VacA3 group at 91 dpv. Vaccinated (VacA1, VacA2, and VacA3 groups) animals had significantly

higher ($P < 0.05$) PCV-2d-specific IFN-γ-SC levels than unvaccinated (UnVacA group) animals at 28, 49, and 91 dpv. A comparison between vaccinated groups yielded that the VacA1 group PCV-2d-specific IFN-γ-SC level was significantly higher ($P < 0.05$) than that of VacA3 groups at 49 and 91 dpv (Figure 3E).

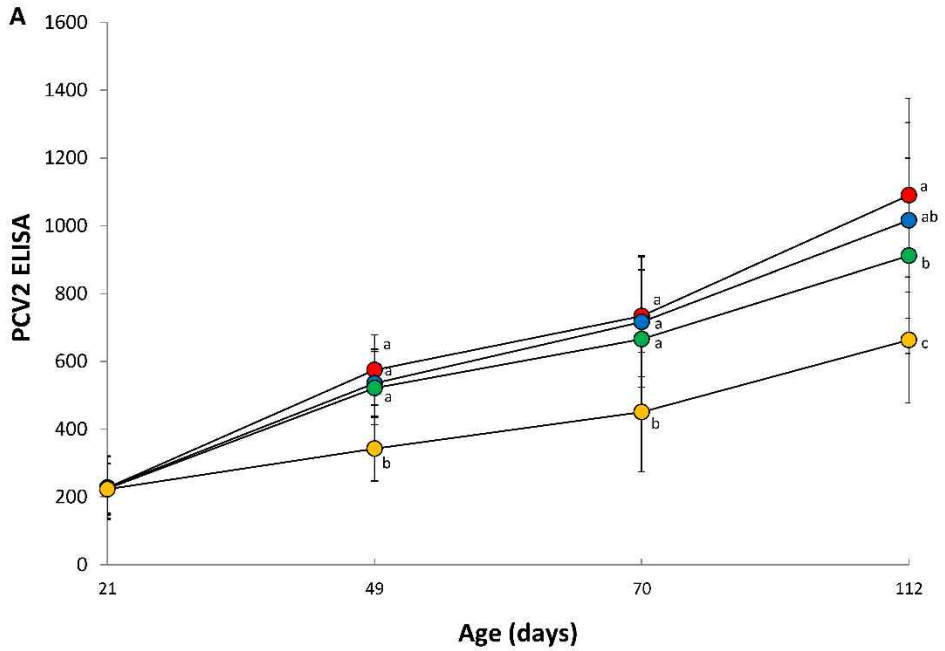
In Farm B, vaccinated (VacB1, VacB2, and VacB3 groups) animals had significantly higher ($P < 0.05$) PCV-2 ELISA (Figure 3B) and NA titers (Figure 3D) than unvaccinated (UnVacB group) animals at 28, 49, and 91 dpv. In a comparison of vaccinated groups, PCV-2 ELISA titers from the VacB1 group were significantly higher ($P < 0.05$) than those of VacB3 group at 91 dpv (Figure 3B). PCV-2 NA titers from the VacB1 group were significantly higher ($P < 0.05$) than those of the VacB3 group at 49 and 91 dpv (Figure 3D). Vaccinated (VacB1, VacB2, and VacB3 groups) animals had significantly higher ($P < 0.05$) PCV-2d-specific IFN-γ-SC levels than unvaccinated (UnVacB group) animals at 28, 49, and 91 dpv. In a comparison of vaccinated groups, the PCV-2d-specific IFN-γ-SC level from the VacB1 group was significantly higher ($P < 0.05$) than that of VacB3 groups at 28, 49, and 91 dpv (Figure 3F).

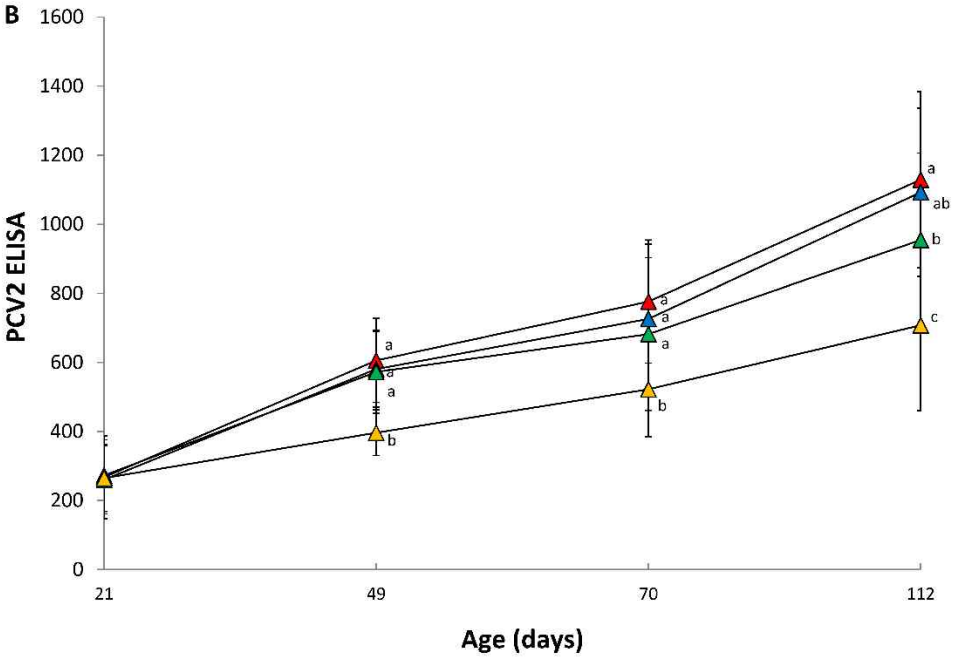
Immune responses against *M. hyopneumoniae*

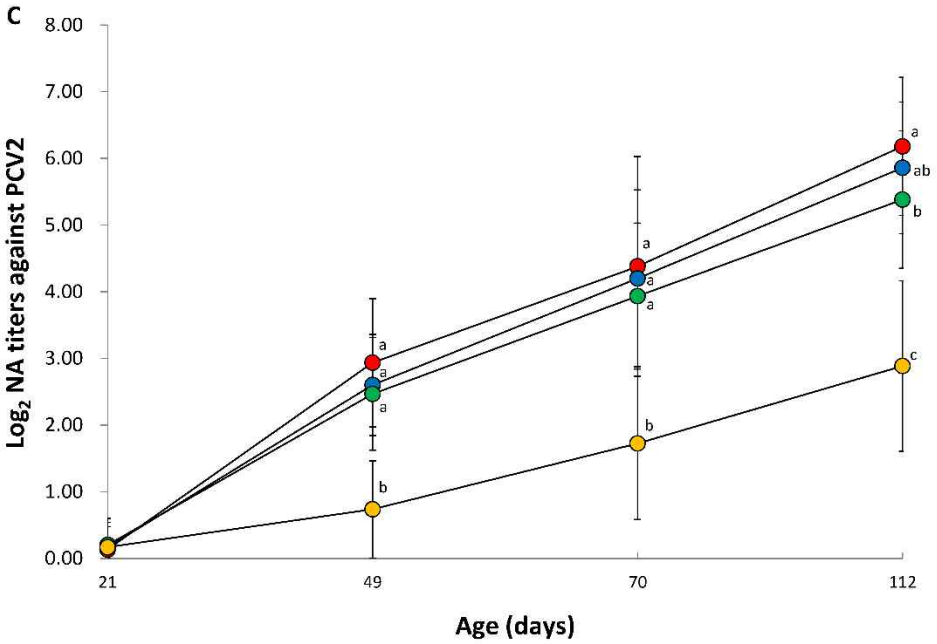
In Farm A, vaccinated (VacA1, VacA2, and VacA3 groups) animals had significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratios compared with unvaccinated (UnVacA group) animals at 28, 49, and 91 dpv. Between the vaccinated groups, *M. hyopneumoniae* ELISA S/P ratios from the VacA1 group were significantly higher ($P < 0.05$) than that of the VacA3 group at 49 and 91 dpv (Figure 4A). Vaccinated (VacA1, VacA2, and VacA3 groups) animals had significantly

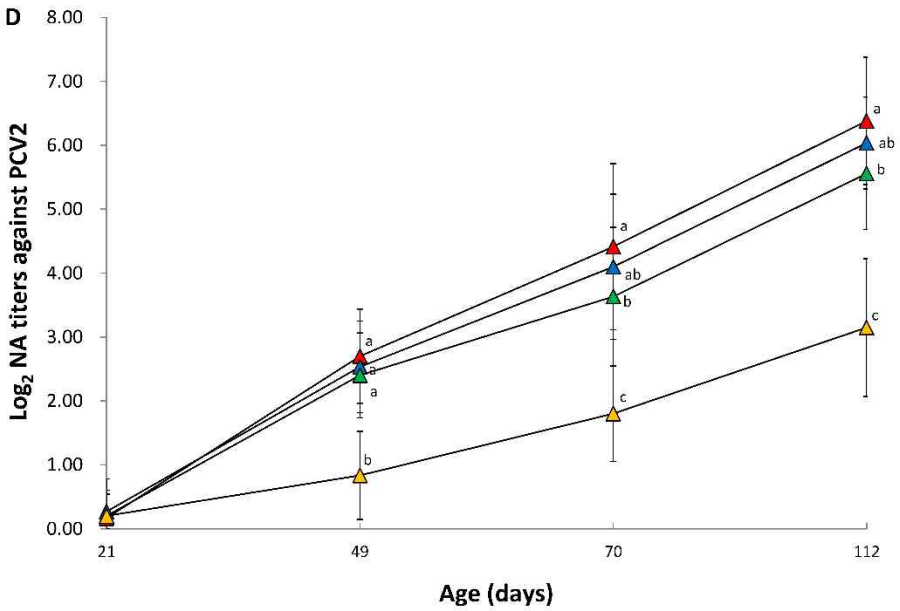
higher ($P < 0.05$) *M. hyopneumoniae*-specific IFN-γ-SC levels than unvaccinated (UnVacA group) animals at 28, 49, and 91 dpv. In a comparison of vaccinated groups, *M. hyopneumoniae*-specific IFN-γ-SC levels from the VacA1 group were significantly higher ($P < 0.05$) than that of the VacA3 group at 28 and 49 dpv (Figure 4C).

In Farm B, vaccinated (VacB1, VacB2, and VacB3 groups) animals had significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratios than unvaccinated (UnVacB group) animals at 28, 49, and 91 dpv. In a comparison of vaccinated groups, *M. hyopneumoniae* ELISA S/P ratios from the VacB1 group were significantly higher ($P < 0.05$) than those of the VacB3 group at 91 dpv (Figure 4B). Vaccinated (VacB1, VacB2, and VacB3 groups) animals had significantly higher ($P < 0.05$) *M. hyopneumoniae*-specific IFN-γ-SC levels than unvaccinated (UnVacB group) animals at 28, 49, and 91 dpv. Between vaccinated groups, the *M. hyopneumoniae*-specific IFN-γ-SC level from the VacB1 group was significantly higher ($P < 0.05$) than that of the VacB3 group at 49 dpv. (Figure 4D).


A
1600
1400
1200
1000
800
600
400
200
0
PCV2 ELISA
21
49
70
112
Age (days)
a
ab
b
c


B
1600
1400
1200
1000
800
600
400
200
0
PCV2 ELISA
21
49
70
112
Age (days)
a
ab
b
c


C
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Log2 NA titers against PCV2
21
49
70
112
Age (days)
a
a
a
b
a
a
a
b
a
ab
b
c


D
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Log2 NA titers against PCV2
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70
112
Age (days)
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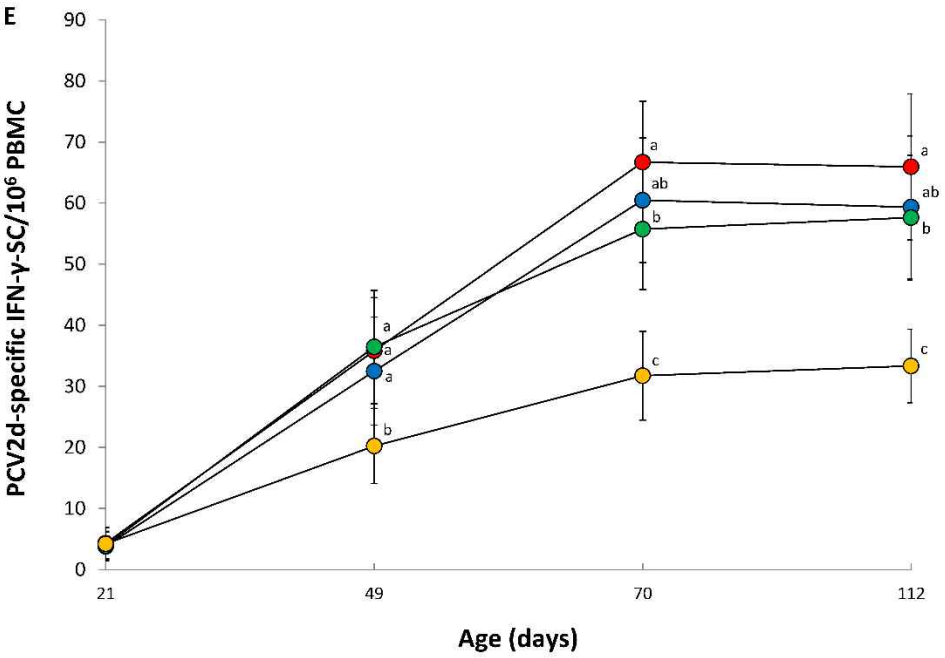
a
a
b
a
ab
b
c
a
ab
b
c


E
PCV2d-specific IFN-γ-SC/10⁶ PBMC
90
80
70
60
50
40
30
20
10
0
21
49
70
112
Age (days)
a
ab
b
a
a
a
b
c
a
ab
b
c

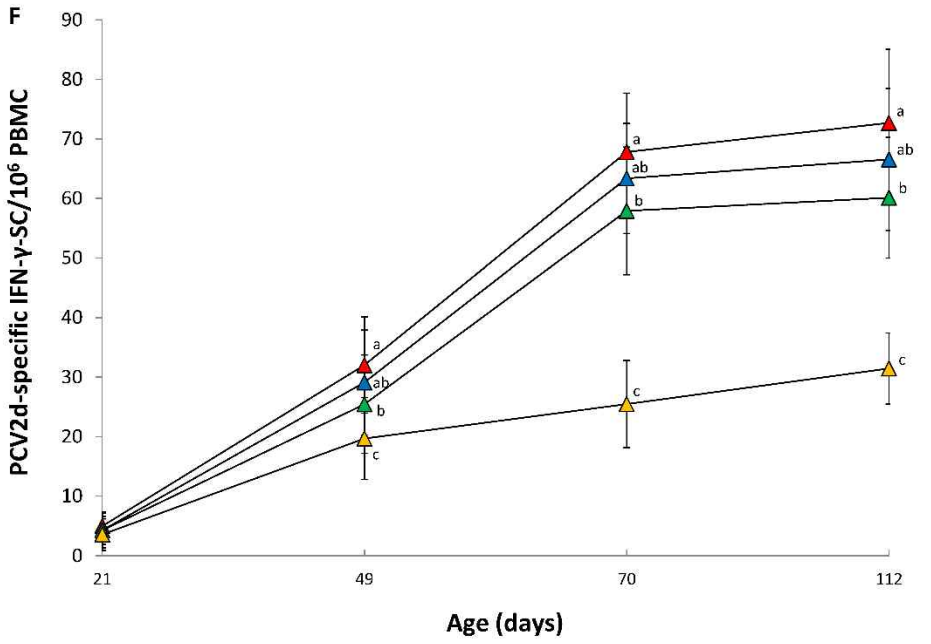

F
PCV2d-specific IFN-γ-SC/10⁶ PBMC
90
80
70
60
50
40
30
20
10
0
21
49
70
112
Age (days)
a
ab
b
a
ab
b
c
a
ab
b
c

Figure 3. Immune responses against Porcine circovirus-2d (PCV-2d).

A – Mean values of the of PCV-2 enzyme-linked immunosorbent assay (ELISA) in serum from VacA1 (●), VacA2 (●), VacA3 (●), and UnVacA (●) groups in Farm A.

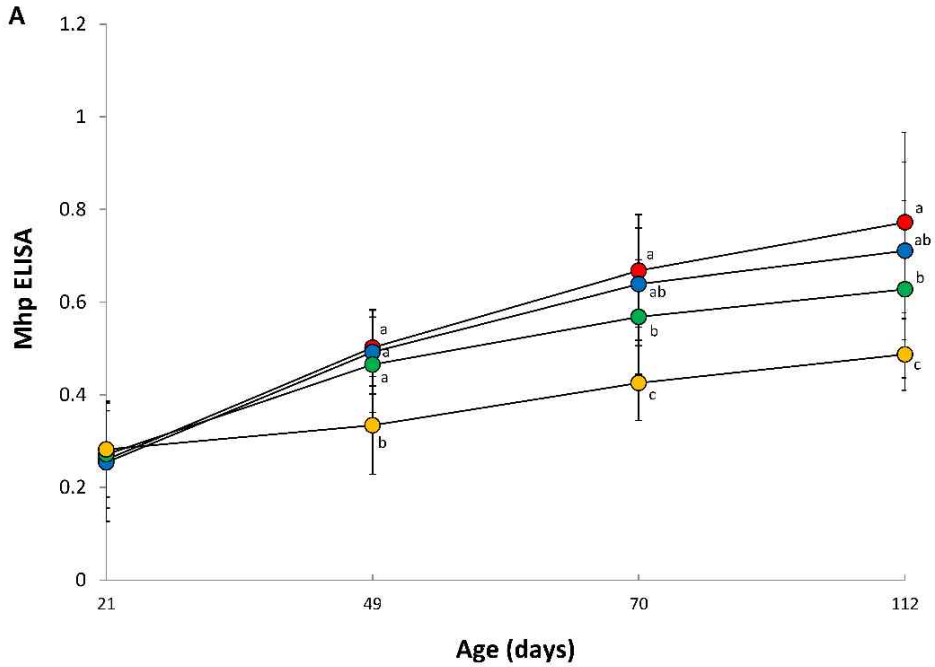
B –Mean values of the PCV-2 ELISA in serum from VacB1 (▲), VacB2 (▲), VacB3 (▲), and UnVacB (▲) groups in Farm B.

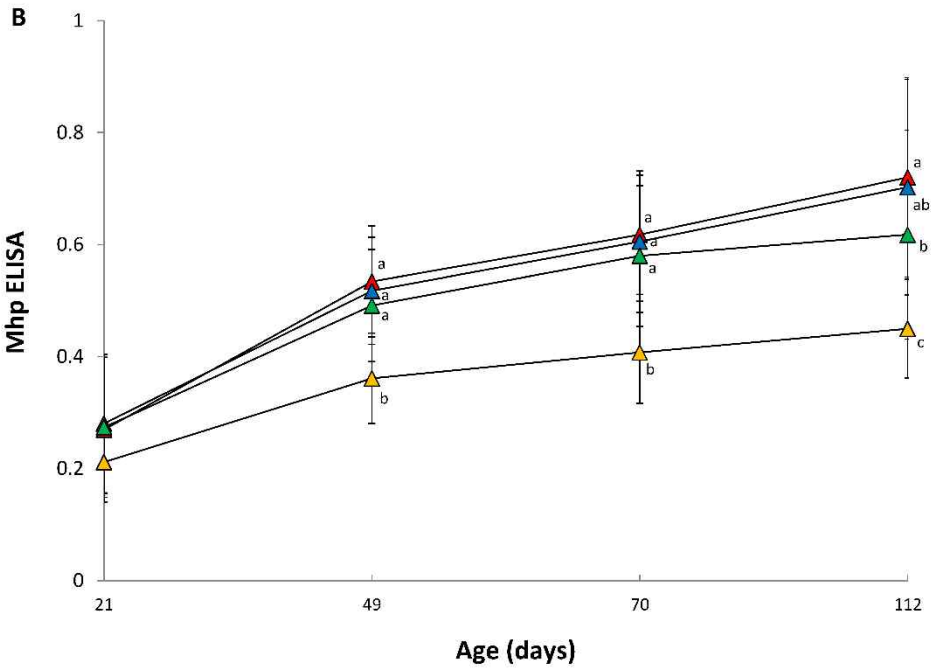
C – Mean values of the neutralizing antibody (NA) titers against PCV-2d in serum from VacA1 (●), VacA2 (●), VacA3 (●), and UnVacA (●) groups in Farm A.

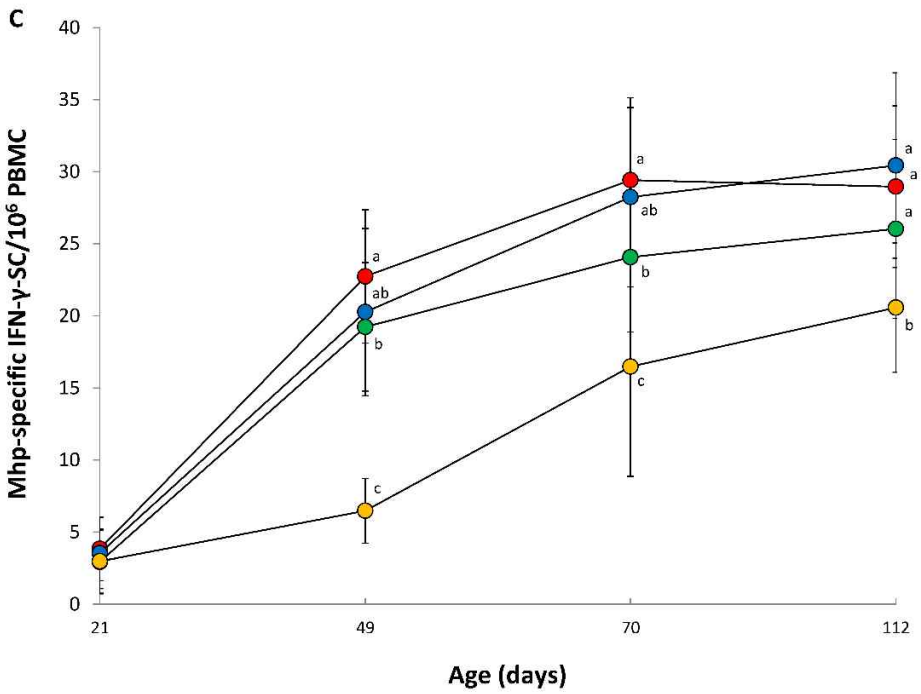
D – Mean values of the NA titers against PCV-2d in serum from VacB1 (▲), VacB2 (▲), VacB3 (▲), and UnVacB (▲) groups in Farm B.

E – Mean values of the PCV2d-specific interferon-γ secreting cells (IFN-γ-SC)/10^6 peripheral blood mononuclear cells (PBMC) from VacA1 (●), VacA2 (●), VacA3 (●), and UnVacA (●) groups in Farm A.

F – Mean values of the PCV2d-specific IFN-γ-SC/10^6 PBMC from VacB1 (▲), VacB2 (▲), VacB3 (▲), and UnVacB (▲) groups in Farm B.


A
1.2
1
0.8
0.6
0.4
0.2
0
Mhp ELISA
21
49
70
112
Age (days)
a
ab
b
c


B
1
0.8
0.6
0.4
0.2
0
Mhp ELISA
21
49
70
112
Age (days)
a
ab
b
c


C
Mhp-specific IFN-γ-SC/10⁶ PBMC
Age (days)
0
5
10
15
20
25
30
35
40
21
49
70
112
a
ab
b
c

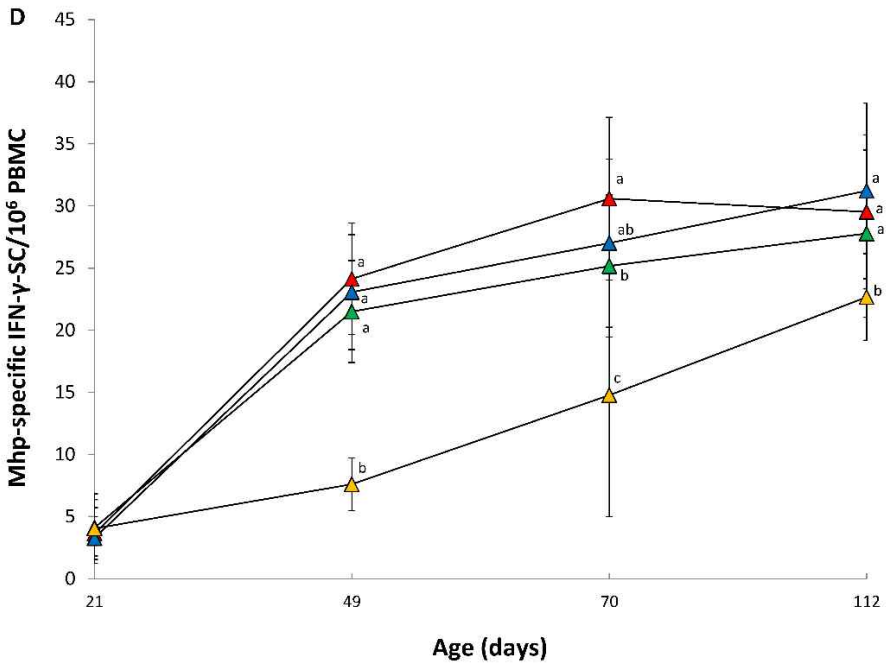

D
Mhp-specific IFN-γ-SC/10⁶ PBMC
Age (days)
0
5
10
15
20
25
30
35
40
45
21
49
70
112
a
ab
b
c

Figure 4. Immune responses against *Mycoplasma hyopneumoniae*.

A – Mean values of the *M. hyopneumponiae* enzyme-linked immunosorbent assay (ELISA) in serum from VacA1 (●), VacA2 (●), VacA3 (●), and UnVacA (●) groups in Farm A.

B –Mean values of the *M. hyopneumponiae* ELISA in serum from VacB1 (▲), VacB2 (▲), VacB3 (▲), and UnVacB (▲) groups in Farm B.

C – Mean values of the *M. hyopneumoniae*-specific interferon-γ secreting cells (IFN-γ-SC)/10^6 peripheral blood mononuclear cells (PBMC) from VacA1 (●), VacA2 (●), VacA3 (●), and UnVacA (●) groups in Farm A.

D – Mean values of the *M. hyopneumoniae*-specific IFN-γ-SC/10^6 PBMC from VacB1 (▲), VacB2 (▲), VacB3 (▲), and UnVacB (▲) groups in Farm B

Pathology

Vaccinated (VacA1, VacA2, VacA3, VacB1, VacB2, and VacB3 groups) animals at Farms A and B had significantly lower ($P < 0.05$) macroscopic and microscopic lung lesion scores when compared to the unvaccinated (UnVacA and UnVacB groups) animals at 154 dpv (**Table 2**).

Table II. Macroscopic and microscopic pathology of vaccinated and unvaccinated groups.

Farm	Group	Macroscopic Lung Lesion	Microscopic	
			Lung Lesion	Lymphoid Lesion
Farm A	VacA1	15.54 ± 6.00 [a]	0.78 ± 0.55 [a]	0.69 ± 0.49
	VacA2	16.99 ± 5.54 [a]	0.75 ± 0.56 [a]	0.76 ± 0.56
	VacA3	17.30 ± 7.73 [a]	0.86 ± 0.52 [a]	0.93 ± 0.83
	UnVacA	27.70 ± 9.79 [b]	2.12 ± 0.91 [b]	1.03 ± 0.35
Farm B	VacB1	16.72 ± 5.25 [a]	0.73 ± 0.60 [a]	0.74 ± 0.59
	VacB2	16.48 ± 4.36 [a]	0.79 ± 0.53 [a]	0.79 ± 0.58
	VacB3	18.39 ± 6.57 [a]	0.81 ± 0.50 [a]	0.84 ± 0.51
	UnVacB	30.94 ± 10.74 [b]	2.47 ± 1.04 [b]	1.03 ± 0.38

Different superscripts (a, b and c) indicate significant ($P < 0.05$) different among 4 groups in each of farms.

DISCUSSION

Two farms that represented typical Korean pig farm in terms of housing conditions and health status were selected on the basis of their history with subclinical PCV-2d infection and enzootic pneumonia. A common clinical feature between these two diseases at the farms was retardation of growth. Growth performance was therefore the most critical protective index when comparing the three combination vaccines. In the present comparative field trial, the trivalent-vaccinated group resulted in a better growth performance in pigs than the mixable and ready-to-use bivalent-vaccinated groups. The mixable bivalent vaccine was able to significantly improve the growth performance during the fattening period (70-175 days of age), compared to the ready-to-use bivalent vaccine. These results in contrast with comparative experimental challenge study (16) where statistical differences in growth performance were not observed between the same two bivalent vaccines. Several possibilities may account for the discrepancy between experimental and field conditions. Pig rearing conditions in experimental studies are different from field conditions, where pigs continue to expose and re-expose themselves to field PCV-2d and *M. hyopneumoniae* by horizontal and vertical transmission, which exacerbates the disease. Other key differences include the small sample size (number of animals used per group), short duration of study, or to the overly-regulated experimental conditions, all of which are different from commercial farms. Several key factors affect pig growth performance in field conditions such as herd characteristics, biosecurity, and husbandry practices and management. When all of these factors are combined, comparative field clinical trials are able to determine exactly what type of vaccines yield the better growth performance.

All three types of combination vaccines evaluated in this trial successfully induced the protective immunity of pigs as seen by humoral and cellular immune responses throughout the course of the study. A higher level of protective immunity results in the reduction of pathogen loads in tissues and pathological lesions, consequently protecting pigs from the pathogens. Types of protective immunity against PCV-2 such as neutralizing antibodies and IFN-γ-SC levels are well correlated with the reduction of PCV-2 loads in blood and the reduction of lymphoid lesions (11,17-19). The trivalent-vaccinated group elicited higher levels of PCV-2d neutralizing antibodies and IFN-γ-SC, and lower levels of PCV-2d load in blood compared with the mixable and ready-to-use bivalent-vaccinated groups. Although the trivalent vaccine outperformed both bivalents in terms immune response and viral load, statistical differences in lymphoid lesions were not observed between any of the three vaccinated groups or the unvaccinated groups. This may be attributed to the fact that both farms had concurrent subclinical PCV-2 infection circulating within the herds. These results may be due to subclinical PCV-2 infection, which results in mild lymphoid lesions or even the absence of lymphoid lesions altogether. Although protective immunity against *M. hyopneumoniae* is not well defined, cell-mediated immunity is used to evaluate pig protection from *M. hyopneumoniae* infection (20, 21). The trivalent vaccine outperformed the mixable and ready-to-use bivalent vaccines for *M. hyopneumoniae* protection, as indicated by the higher levels of elicited IFN-γ-SC and lower levels of *M. hyopneumoniae* load in the larynx that of the vaccinated group. A statistical difference was not observed between any of the vaccinated groups in relation to lung lesion severity, indicating that cell-mediated immunity may not be the only protective immunity.

Increased growth performance, higher immune responses, and lessened viral and

mycoplasmal loads between the trivalent and two (mixable and ready-to-use) bivalent vaccines may be attributed to the difference in antigens and adjuvant between vaccines. The trivalent vaccine was the only vaccine containing PCV-2b antigen. Combination vaccines containing PCV-2b antigen may be clinically superior to other combination vaccines that contain PCV-2a antigen in the efficient control of PCV-2d infection due to the genetic proximity between PCV-2b and PCV-2d (22). The *M. hyopneumoniae* antigen strain in the trivalent vaccine differed from that of two (mixable and ready-to-use) bivalent vaccines which both shared the same strain (22). It is therefore possible that the different stain of *M. hyopneumoniae* antigen found in the trivalent vaccine may have affected its immunogenicity and protective effect over the strain in the bivalent vaccines.

The clinical field data gathered from this study is intended to provide pig producers with growth performance clinical information. Complications of both subclinical PCV-2 infection and enzootic pneumonia appear to cause PRDC in the field. The results of the present study demonstrate that a trivalent vaccine containing PCV-2a/b and *M. hyopneumoniae* resulted in a better productive parameter and immune responses, lower viral blood load, and reduced mycoplasmal larynx load when compared with two different bivalent vaccines in the presence of ongoing herd subclinical PCV-2d infection and enzootic pneumonia.

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GENERAL CONCLUSION

PRDC is the most common and economically important disease, causing significant negative impact on the productivity of swine farms. Porcine circovirus-2 (PCV-2) and *Mycomplasma hyopneumoniae* are major pathgens, closely related with PRCD. The co-infection of pigs with PCV-2 and *M. hyopneumoniae* has contributed to the severe development of porcine respiratory disease complex, which has resulted in an increase in animal medication costs as well as economic losses. Vaccines against PCV-2 and *M. hyopneumoniae* are the two most commonly used vaccines in the world, including Korea. Because the recommended vaccination time for both PCV-2 and *M. hyopnemoniae* is rather similar, the use of a combined vaccine is preferred option, mainly due to labor cost and convenience.

Part I study, the order of infection with PRRSV, *M. hyopneumoniae*, and PCV-2 was designed on the basis of natural infection patterns in pig farms and the severity of lung lesions due to interaction of these three pathogens. Time and order of infection with these three pathogens mirrored Asian field conditions. In most pig-rearing Asian countries including Korea, pigs are typically infected with PRRSV and *M. hyopneumoniae* at around 5-7 weeks of age followed by PCV-2 infection at 7-9 weeks of age and with clinical signs appearing around 11-16 weeks of age. The severity of the lung lesions caused by these three pathogens differs depending on the order of infection. Studies have shown that co-infection of pigs with PRRSV and *M. hyopneumoniae*, causes more severe histopathological lung lesions compared to sequential infection. In contrast, sequential infection with *M. hyopneumoniae* and PCV-2, causes more severe histopathological lung lesions compared to co-infection.

The results of the present study demonstrate that 6-week-old pigs dually inoculated with PRRSV and *M. hyopneumoniae*, followed by the inoculation with PCV-2 two weeks after (i.e. at 8 weeks of age) exhibit severe pneumonic lesions mimicking those of naturally-occurring PRDC. The most striking and consistent pathologic lesions are interstitial pneumonia with peribronchial or peribronchiolar lymphoid hyperplasia and fibrosis. Because PRRSV and PCV2 are primarily infected with interstitial macrophages in alveolar septa, both viruses are involved in causing interstitial pneumonia. In contrast, *M. hyopneumoniae* is involved in causing peribronchial and peribronchiolar hyperplasia because it is primarily detected in surface of epithelial lining cells.

This is the first experimental reproduction of PRDC by infection with the three most common pathogens known to be associated with this disease. The PRDC model infected with PRRSV, *M. hyopneumoniae*, and PCV-2 would be useful in evaluating the efficacy of vaccines in terms of PRDC prevention. Further studies are needed to determine the interaction among the three pathogens to cause PRDC.

Part II study was to compare three different types of combination vaccine such as a mixed trivalent vaccine containing PCV-2a, PCV-2b and *M. hyopneumoniae* antigens, a mixable ready-to-use bivalent vaccine containing PCV-2a and *M. hyopneumoniae* antigens and a mixed bivalent vaccine containing PCV-2a and *M. hyopneumoniae* antigens. Two farms were selected on the basis of their subclinical PCV-2d infection and enzootic pneumonia. A total of 120 pigs in each farm were randomly divided into 4 groups (30 pigs per group). The trivalent-vaccinated group from both farms outperformed each bivalent-vaccinated group in terms of growth performance. Growth performance was significantly improved during the fattening periods (70-175 days of age) of the mixable bivalent-vaccinated group in comparison

with the ready-to-use bivalent-vaccinated group in one farm. The trivalent-vaccinated group elicited higher levels of neutralizing antibodies and interferon-γ secreting cells (IFN-γ-SC) against PCV-2d, while simultaneously decreasing the levels of PCV-2d load in blood when compared against the mixable and ready-to-use bivalent-vaccinated groups. The trivalent-vaccinated group also elicited higher levels of IFN-γ-SC against *M. hyopneumoniae* and lower levels of *M. hyopneumoniae* loads in the larynx when compared with the mixable and ready-to-use bivalent-vaccinated groups. The results of the present study demonstrated that a trivalent vaccine containing PCV-2a/b and *M. hyopneumoniae* resulted in a better productive parameter, higher immune responses, and less blood-viral and mycoplasmal larynx-loads when compared with the mixable and ready-to-use bivalent vaccines despite the presence of ongoing farm subclinical PCV-2d infection and enzootic pneumonia. The results of this study demonstrate a strategic method and effective vaccine regimes against PRDC for swine producers and practitioners.

국문 논문 초록

돼지 복합 호흡기 질병의 병인론 및 백신접종

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돼지 호흡기 복합 질병(PRDC)은 육성돈과 비육돈 단계에서 호흡기 증상과 성장저하로 인한 생산성 저하가 특징인 한국을 포함한 세계 양돈 산업에 경제적 피해가 큰 돼지 질병이다. 돼지 호흡기 복합 질병은 바이러스 및 세균 병원체들의 상호작용과 시너지 작용에 의해 발생하며, 대표적인 바이러스 원인체로는 돼지 생식기 호흡기 증후군 바이러스 (Porcine reproductive and respiratory virus), 돼지 써코 바이러스 2형 바이러스 (Porcine circovirus-2 virus), 돼지 인플루엔자 바이러스 (Swine influenza virus), 돼지 열병 바이러스 (Classical swine fever

virus), 돼지 오제스키병 바이러스 (Pseudorabies virus)등이 있다. 대표적인 세균 원인체로는, 마이코플라즈마 (*Mycoplasma hyopneumoniae*), 파스튜렐라균(*Pasteurella multocida*), 보데텔라균(*Bordetella bronchiseptica*), 글래써시균(*Glaesserella parasuis*), 연쇄상구균(*Streptococcus suis*), 흉막폐렴균(*Actinobacillus spp.*)등이 있다. 이 중 돼지 써코 바이러스 2형 바이러스와 마이코플라즈마는 돼지 복합 호흡기 질병에 관여하는 대표적인 병원체들이고, 한국내 대부분의 농장에서 해당 병원체들로 인한 피해를 방어하고, 최소화하기 위하여 백신 프로그램을 적용하고 있으나, 농장들은 다양한 상업화된 백신들 중에 농장에 적절한 백신의 선택을 어려워하고 있다. 따라서 본 연구에서는 돼지 복합 호흡기 질병의 실험모델 확립과 대표적인 상업화된 돼지 써코 바이러스 2형과 마이코플라즈마 백신들을 접종 후, 각각의 백신접종 실험군들간에 백신 효능과 농장 생산성 관련 지표들을 비교해 보려고 하였다.

첫번째 실험에서는 농장들에서 돼지 호흡기 복합 질병의 일반적인 감염형태인 6주령 자돈들에게 돼지 생식기 호흡기 증후군 바이러스와 마이코플라즈마를 접종하고 2주 후에 돼지 써코 바이러스 2형 바이러스를 접종하여 돼지에 실험적으로 돼지 복합 호흡기 질병을 유발시켰다. 세 가지 병원체의 감염 형태는 한국을 포함한 아시아에서의 양돈장에서의 현장 감염 상황을 반영한다. 해당실험을 통해 돼지 복합 호흡기 질병의 가장 특징적인 병변인 기관지 주변 림프구 과형성 및 섬유화를 동반한 심각한 간질성 폐렴을 유발할 수 있었다.

두번째 실험에서는 PCV-2d 무증상감염과 유행성 폐렴이 발생하고 있는 2개 돼지농장들에서 농장당 각각 120마리의 돼지를 4개 그룹으로 나누어, PCV-2a, PCV-2b, *M. hyopneumoniae* 항원들을 모두 포함한 3가 혼합백신을 적용한 실험군, 백신사용전 혼합하여 사용할 수 있는 PCV-2a 1가 백신과 *M. hyopneumoniae* 1가 백신을 혼합적용한 실험군, PCV-2a항원과 *M. hyopneumoniae*항원이 같이 포함된 2가 혼합백신을 적용한 실험군, 대조군들에 대하여, 각각의 백신들의 효능을 임상증상, 일당증체량, 폐사율, PCV-2d 바이러스 혈중농도, 기관지 스왑에서 마이코플라즈마의 농도, 면역반응, 병변 등의 지표들을 가지고 비교하였다. 3가 혼합백신 접종 그룹은 다른 실험군들에 비교하여, 성장지표, PCV-2d에 대한 더 높은 수준의 중화 항체와 인터페론 감마 분비세포들(interferon-γ secreting cells)을 유도, 혈중 PCV-2d 농도수준을 낮춘 동시에 마이코플라즈마 인터페론 감마분비세포의 증가와 후두스왑에서 마이코플라즈마 농도수준을 낮추었다. 본 연구의 결과는 양돈장들과 양돈전문가들에게 돼지 써코 바이러스 2형 바이러스와 마이코플라즈마가 연관된 돼지 복합 호흡기 질병에 대한 효과적인 백신 전략을 제시한다.

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

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