수의학박사학위논문

Interaction of *Mycoplasma hyopneumoniae*, Porcine Circovirus type 2, and Porcine Reproductive and Respiratory Syndrome Virus

마이코플라즈마, 써코바이러스, 돼지 생식기 호흡기 증후군 바이러스의 상호작용 연구

2016 년 2 월

서울대학교 대학원

수의학과, 수의병인생물학 및 예방수의학전공

박 수 진

Abstract

Interaction of *Mycoplasma hyopneumoniae*, Porcine Circovirus type 2, and Porcine Reproductive and Respiratory Syndrome Virus

(Supervisor: Chanhee Chae, D.V.M. Ph.D.)

Su-Jin Park

Department of Veterinary Pathology and

Preventative Medicine (Pathology)

Graduate School of Seoul National University

Porcine Respiratory Disease Complex (PRDC) is the most serious concern for swine producers in Korea and other countries. The most common viral agents involved in PRDC include Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Classical Swine Fever (CSF), Swine Influenza Virus (SIV), Pseudorabies Virus (PRV), and Porcine Circovirus type 2 (PCV2). Bacterial Pathogens associated with PRDC

include *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica*, and *Haemophilus parasuis*, *Streptococcus suis* and the *Actinobacillus spp*. It is important to know that the interactions between pathogens can be a major factor in determining severity of the disease. The successful control of PRDC is based on the accurate diagnosis of the problem pathogens present on a herd basis. And it has been found that timing of intervention strategies, whether antibiotics or vaccines, is increasingly important on a herd basis. The objective of this thesis is to determine the effects of those pathogens and vaccine efficacy throughout experimental model and challenge test.

Part I is with PCV2 and *Mycoplasma hyopneumoniae* vaccinations on disease severity in an experimental PCV2-*M. hyopneumoniae* dual challenge model. Vaccine effectiveness was evaluated using microbiological (PCV2 viremia and *M. hyopneumoniae* nasal shedding), immunological (neutralizing antibodies and IFN-γ-secreting cells), and pathological (gross lung lesions, histopathologic pulmonary and lymphoid lesions, and the presence of PCV2 antigen and *M. hyopneumoniae* DNA within the lesions) evaluations. Although *M. hyopneumoniae* potentiates the severity of PCV2-associated lesions and lesion-associated PCV2 antigen level in dually challenged pigs, vaccination against *M. hyopneumoniae* alone did not reduce PCV2 viremia, PCV2-induced lesions, or PCV2 antigen in dually challenged pigs. In addition, vaccination against PCV2 did not reduce the nasal shedding of *M. hyopneumoniae*, the *M. hyopneumoniae*-induced pulmonary lesions or the lesion-associated *M.*

hyopneumoniae DNA in dually challenged pigs. Dual challenge with PCV2 and *M. hyopneumoniae* did not interfere with the induction of active immunity induced by a previous single vaccination for either PCV2 or *M. hyopneumoniae*. The results of this study demonstrated that (i) vaccination against *M. hyopneumoniae* alone did not decrease the potentiation of PCV2-induced lesions by *M. hyopneumoniae* and (ii) vaccination against PCV2 alone decreased the potentiation of PCV2-induced lesions by *M. hyopneumoniae* in dually challenged pigs.

Part II is to determine the effects of *Mycoplasma hyopneumoniae* and/or PRRSV vaccination on dually infected pigs. In total, 72 pigs were randomly divided into nine groups (eight pigs per group), as follows: five vaccinated and challenged groups, three non-vaccinated and challenged groups, and a negative control group. Single-dose vaccination against *M. hyopneumoniae* alone decreased the levels of PRRSV viremia and PRRSV-induced pulmonary lesions, whereas single-dose vaccination against PRRSV alone did not decrease nasal shedding of *M. hyopneumoniae* and mycoplasma-induced pulmonary lesions in the dually infected pigs. *M. hyopneumoniae* challenge impaired the protective cell-mediated immunity induced by the PRRSV vaccine, whereas PRRSV challenge did not impair the protective cell-mediated immunity induced by the *M. hyopneumoniae* vaccine. The present study provides swine practitioners and producers with efficient vaccination regimes; vaccination against *M. hyopneumoniae* is the first step in protecting pigs against co-infection with *M. hyopneumoniae* and PRRSV.

Keywords:

Mycoplasma hyopneumoniae; Porcine Circovirus type 2; Porcine reproductive and respiratory syndrome virus; Porcine respiratory disease complex; Vaccine

Student number: 2014-30548

CONTENTS

PART II. Interaction between single-dose *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus vaccines on dually infected pigs

LIST OF TABLES

PART I

PART II

LIST OF FIGURES

PART I.

PART II.

LIST OF ABBREVIATIONS

BAL	Bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
CDCD	Cesarean-derived, colostrum-deprived
CMI	Cell mediated immunity
dpc	Days post-challenge
DPI	Days post-inoculation
ELISA	Enzyme-linked immune-sorbent assay
EP	Enzootic pneumonia
IFN-γ-SCs	Interferon gamma secreting cells
IHC	Immunohistochemistry
IL	Interleukin
ISH	*in situ* hybridization
NA	Neutralizing antibody
MLV	Modified live virus
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCV	Porcine circovirus
PCVAD	Porcine circovirus associated diseases
PDNS	Porcine dermatitis and nephropathy syndrome
PMWS	Postweaning multisystemic wasting syndrome
PRDC	Porcine respiratory disease complex
PRRS	Porcine reproductive and respiratory syndrome
Th	Helper T lymphocyte
$TCID_{50}$	Median tissue culture infective dose

GENERAL INTRODUCTION

Porcine Respiratory Disease Complex (PRDC) results from a complex of infectious agents and environmental factors, affecting the health of the pig and resulting in reduced performance, increased mortality and economic losses. In 2015, Korea Animal and Plant Quarantine Agency (QIA) Animal Disease Diagnosis Result of 2015 2nd Quarter Report described that PRDC showed the most high percentage of growth compare to the last year [20]. A retrospective study was performed on natural cases of PRDC to determine the association and prevalence of PRDC with various co-existing pathogens in Korea. Among the 105 pigs with PRDC, 85 were positive for Porcine circovirus 2 (PCV2), 66 were positive for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), 60 were positive for Porcine parvovirus (PPV), and 14 were positive for Swine influenza virus (SIV). There were 80 co-infections and 25 single infections. A co-infection of PCV2 with another additional bacterial pathogen is frequently diagnosed in PRDC. The combination of PCV2 and *Pasteurella multocida* (38 cases) was most prevalent followed by PCV2 and *Mycoplasma hyopneumoniae* (33 cases). The consistent presence of PCV2, but lower prevalence of other viral and bacterial pathogens in all pigs examined with PRDC, has led us to speculate that PCV2 plays an important role in PRDC [11].

Also Van Alstine described that *M. hyopneumoniae* and PRRSV are two major primary pathogens and that are commonly isolated from pigs suffering from PRDC [35].

Those *M. hyopneumoniae*, PCV2 and PRRS have a greater impact in co-infections compared to other pathogens like Pseudorabies Virus, SIV or *Bordetella bronchiseptica*. This is because those pathogens are modulating the respiratory immune system, PRRSV infects macrophages, PCV2 infects lymphocytes and *M. hyopneumoniae* non-specifically attracts macrophages and lymphocytes. Infections of these pathogens when combined with other pathogens increase respiratory disease can also become even more problematic [33].

Proper management of PRDC begins with the effective diagnosis. Available diagnostic tools are virus isolation, immunohistochemistry, *in situ* hybridization, and polymerase chain reaction (PCR). Veterinarians and producers can make a decision about which pathogens are important and where in the production system to most appropriately do so. This may involve the sow herd, pig flow changes, weaning age changes, ventilation modifications, pulse medication, and/or vaccination. Cross sectional necropsies and serological profiles allow for defining where in the production system to implement vaccination and medication. Now quality vaccines are available for the most important primary viral (PRRSV, SIV, PRV) and mycoplasmal pathogens which can play a major role in most of the respiratory disease outbreaks today. Strategically administered high quality vaccines are helpful to establish uniform immunity and reduce the risk of respiratory disease outbreaks in highly susceptible populations of pigs [29].

The target of the following literature review and 2 studies (Part I and II) are to describe and identify (i) the 3 major causative agents of PRDC which is *M. hyopneumoniae*, PCV2 and PRRS, and later to cover (ii) Interaction between those 3 agents together

with the understanding of Immune system, (iii) Vaccine efficacy and strategy as a long term management tool for the farms.

LITERATURE REVIEW

1. Major Causative Agents of Porcine Respiratory Disease Complex

1-1. *Mycoplasma hyopneumoniae*

M. hyopneumoniae is the principal etiological agent of Swine enzootic pneumonia (SEP), a chronic respiratory disease that affects mainly finishing pigs. Colonization of the airways by *M. hyopneumoniae* results in ciliostasis, clumping and loss of cilia and loss of epithelial cells and bronchial goblet cells. This results in a significant reduction in the ability mucociliary apparatus to function and clear the airways of debris and invading pathogens [7]. Although many efforts to control *M. hyopneumoniae* infection, significant economic losses in pig production worldwide due to SEP continue. *M. hyopneumoniae* is typically introduced into pig herds by the purchase of subclinically infected animals or, less frequently, through airborne transmission over short distances. In herds, it is transmitted by direct contact from infected sows to their offspring or between pen mates. The 'gold standard' technique used to diagnose *M. hyopneumoniae* infection, bacteriological culture is seldom used routinely. Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) detection methods, in addition to post-mortem inspection in the form of abattoir surveillance or field necropsy, are the techniques most frequently used to investigate the potential involvement of *M. hyopneumoniae* in porcine respiratory disease. Such techniques have been used to monitor the incidence of *M. hyopneumoniae* infection in herds both clinically and sub-clinically affected by SEP, in

vaccinated and non-vaccinated herds and under different production and management conditions. Differences in the clinical course of SEP at farm level and in the efficacy of *M. hyopneumoniae* vaccination suggest that the transmission and virulence characteristics of different field isolates of *M. hyopneumoniae* may vary [27].

1-2. Porcine circovirus type 2 (PCV2)

PCV2 is a member of the family Circoviridae, a recently established virus family composed of small, non-enveloped viruses, with a circular, single-stranded DNA genome. PCV2, which is found all over the world in the domestic pig and probably the wild boar, has been recently associated with a number of disease syndromes, which have been collectively named porcine circovirus associated diseases (PCVAD). Postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS) and reproductive disorders are the most relevant ones. Among them, only PMWS is considered to have a severe impact on domestic swine production. PMWS mainly affects nursery and/or fattening pigs; wasting is considered the most representative clinical sign in this disease. Diagnosis of this disease is confirmed by histopathological examination of lymphoid tissues and detection of a moderate to high amount of PCV2 in damaged tissues. Since PMWS is considered a multifactorial disease in which other factors in addition to PCV2 are needed in most cases to trigger the clinical disease, effective control measures have focused on the understanding of the co-factors involved in individual farms and the control or elimination of these triggers. PDNS, an immuno-complex disease characterized by

fibrino-necrotizing glomerulonephritis and systemic necrotizing vasculitis, has been linked to PCV2, but a definitive proof of this association is still lacking. PCV2-associated reproductive disease seems to occur very sporadically under field conditions, but it has been characterized by late-term abortions and stillbirths, extensive fibrosing and/or necrotizing myocarditis in fetuses and the presence of moderate to high amounts of PCV2 in these lesions [23, 25].

For diagnosis, immunohistochemistry and in situ hybridization, but not polymerase chain reaction or virus isolation. A hallmark of microscopic lesions of PMWS is granulomatous inflammation in the lymph nodes, liver, spleen, tonsil, thymus, and Peyer's patches. Large, multiple, basophilic or amphophilic grape-like intracytoplasmic inclusion bodies are often seen in the cytoplasm of macrophages and multinucleated giant cells [6]. Significant differences in PCV2 load were observed between animals with severe, moderate and mild PMWS lesions, although variability within each group was high, probably due to heterogeneity in disease progression. It was suggested that high viral load is a major feature of PMWS affected pigs. With this fact, Liu et al. described a competitive PCR (cPCR) assay for monitoring PCV DNA in serum samples from piglets. The cPCR was based on competitive coamplification of a 502- or 506-bp region of the PCV1 or PCV2 ORF2, respectively, with a known concentration of competitor DNA, which produced a 761- or 765-bp fragment, respectively. The cPCR was validated by quantification of a known amount of PCV2 wild-type plasmids. They also used the technique to determine PCV genome copy numbers in infected cells. Finally they measured PCV2 NA loads in clinical samples. More than 50% of clinically

healthy piglets could harbor both types of PCV. While PCV1 was detected in only 3 of 16 pigs with PMWS, all the sick piglets contained PCV2. A comparison of the PCV2 DNA loads of healthy and sick animals revealed a significant difference, indicating that the development of PMWS may require a certain amount of PCV2 [14].

1-3. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

PRRSV belongs to a group of RNA viruses that establish persistent infections. A proposed strategy for evading immunity during persistent PRRSV infection is by preventing the induction of IFN activity in pigs and/or by blocking the activation of antiviral proteins in permissive cells. IFN-γ mRNA expression was observed in the lymph nodes and lungs of pigs infected with wild-type PRRSV strain SDSU-23983. Pretreatment of MARC-145 cells with IFN-γ inhibited wild-type (SDSU-23983 P6) and culture-adapted (SDSU-23983 P136) PRRS viruses in a dose-dependent manner and at relatively low concentrations. The effect of IFN-γ on virus replication included reductions in the number of infected cells, virus yield, and RNA content in single cells. Virus replication was partially restored by the addition of 2-aminopurine (2-AP), an inhibitor of dsRNA inducible protein kinase (PKR). The addition of 2-AP also restored the viral RNA content per cell to near normal levels, suggesting that inhibition of viral RNA synthesis was through PKR. The principal difference between P6 and P136 isolates was the recovery of P136 replication with lower concentrations of 2-AP. Immunostaining with anti-PKR antibody showed a redistribution of PKR from the

cytoplasm into nucleoli of infected cells [22]. IFN-γ is known to inhibit PRRSV replication and this was identified with the study report by Bautista and Molitor, showing the effect of IFN-γ on the replication of PRRSV in macrophages. Pretreatment with IFN-γ profoundly affected PRRSV replication in porcine macrophages evaluated by reduction in titer and percentage of positive cells. The effect of IFN-γ on PRRSV replication was both dose-dependent and related to the time of exposure. The mechanism of action was not due to blocking virus attachment. The inhibitory effect on PRRSV replication in macrophages suggests that IFN-γ may play an important role in protection [2].

2. Immune System and major PRDC pathogens

2-1. *Mycoplasma hyopneumoniae* and immune system

The complex, chronic pathogenesis of *M. hyopneumoniae* mediated respiratory disease appears dependent on the alteration or modulation of the respiratory immune response. Immunopathology changes are a major component of mycoplasmal pneumonia. Pulmonary alveolar macrophages infected with both *M. hyopneumoniae* and APP were shown to have reduced phagocytic capability [4]. Rodriguez et al. described *M. hyopneumoniae* infection histologically by infiltration of mononuclear cells in airways and prominent hyperplasia of the bronchus-associated lymphoid tissue (BALT). To gain further insight into the pathogenesis of *M. hyopneumoniae* infection, cytokine expression in the lung, with particular attention to the BALT, was examined

immunohistochemically in pigs naturally infected with *M. hyopneumoniae*. An increase in proinflammatory and immuneregulatory cytokines (especially IL-2, IL-4 and tumor necrosis factor [TNF]-alpha, and to a lesser extent IL-1 [alpha and beta] and IL-6) was detected in the BALT, which showed intense lymphoid hyperplasia. IL-1beta and TNF-alpha were also detected in the bronchoalveolar exudate of infected pigs, and IL-6 and IL-8 were demonstrated in mononuclear cells of the alveolar septa. The results showed that in *M. hyopneumoniae* infection, macrophage and lymphocyte activation results in the expression of a number of cytokines capable of inducing lung lesions and lymphoreticular hyperplasia of the BALT [21]. These proinflammatory cytokines increases the inflammation in the lung, which further reduces the respiratory immune system's ability to control other pathogens in the respiratory tract. The effect of immunosuppression on *Mycoplasma hyopneumoniae* infection was evaluated by comparing data from infected, thymectomized, and antithymocyte serum-treated pigs (group 1) with data from infected (group 2) and non-infected (group 3) healthy pigs. After groups 1 and 2 pigs were inoculated intranasally with *M. hyopneumoniae*, mycoplasmas tended to multiply slightly more in the lungs and bronchial lymph nodes of group 1 pigs than that of group 2 pigs. Organisms were also isolated from the spleen of 1 of 3 group 1 pigs. Pneumonia developed in group 2 pigs and was characterized by massive peribronchial, peribronchiolar, and perivascular lymphoid hyperplasia and exudate consisting mainly of polymorphonuclear leukocytes in the alveoli and lumina of the bronchioles and bronchi. In group 1 pigs, perivascular and peribronchiolar cuffings by lymphocytes were less prominent, and the extent of intraluminal exudate

was severe and widespread. Bronchial lymph nodes from group 2 pigs had marked hyperplasia of germinal centers and paracortical areas. In group 1 pigs, germinal centers were hyperplastic, whereas in the paracortical areas, depletion of lymphocytes was evident. Through this study, they confirmed that cell-mediated immune mechanisms are important in the development of pneumonic lesions in enzootic pneumonia of pigs [30].

2-2. Porcine circovirus and immune system

The interaction between PCV2 and the pig immune system has been suggested to be a determinant event for the pathogenesis of PMWS. To gain insight into the host immune mechanisms developed upon PCV2 infection, early innate and adaptive immune responses were examined in 1-week-old, caesarean-derived, colostrum-deprived (CDCD) piglets using a subclinical infection model of PCV2 in combination with lipopolysaccharide (LPS) as a potential immunostimulation factor. The use of LPS did not show any significant effect on the course of PCV2 infection, nor did in the evolution of the immunological parameters evaluated. Ex vivo responses were detected as early as 1 DPI and consisted of an elevation of the plasmatic levels of IL-8 in PCV2-inoculated pigs followed by an increase on plasmatic IFN-alpha at day 5 PI. Regarding IL-10, only one PCV2-inoculated pig was positive (7 DPI); this pig was the only one in which viremia persisted until the end of the study. In vitro cytokine determination showed that, regardless of the treatment administrated to the pigs, an IL-10 release was observed when peripheral blood mononuclear cells (PBMC) cultures were stimulated

with PCV2. Seroconversion to PCV2 measured by an immunoperoxidase monolayer assay (IPMA) occurred between 7 and 14 days PI, whereas NA did not appear until day 29 PI. PCV2 DNA was first detected in serum at day 7 PI, reaching the peak of viremia between days 14 and 21 PI, followed by a drop in viral load that was found coincident with the appearance of PCV2-IFN-γ-SC and NA. Results from this study suggested that viral clearance might be mediated by the development of PCV2-IFN-γ-SC in contribution to the PCV2-specific NA [9].

2-3. Porcine reproductive and respiratory syndrome virus and immune system

The immune response to PRRSV begins with an attenuated innate antiviral response in the cytoplasm of an infected macrophage. IFN and inflammatory cytokine responses are weak [1]. The down regulation of IFN-alpha production facilitates PRRS replication since IFN-alpha mediates inhibition of PRRS virus replication. PRRSV also blocks IFN-alpha production after super infection with TGEV, a strong inducer of IFN-alpha. This weak innate response may compromise the subsequent initiation and elaboration of antigen specific adaptive immunity. In addition, suppression of innate antiviral immune mechanisms may increase the risk of secondary infections. It was reported that PRRSV-specific IFN-γ-SCs plays key roles in protective cell-mediated immunity against PRRSV infection. Infection of swine with virulent PRRSV induced a rapid, robust antibody response that comprised predominantly non-neutralizing antibodies and waned after approximately 3 months. In contrast, the initial onset of virus-specific IFN-γ-SCs in the pig lymphocyte population remained at a fairly low

level during this period and then increased gradually in frequency, plateauing at 6 months post-infection. A similar polarization of the host humoral and cellular immune responses was also observed in pigs immunized with a PRRS-modified live virus (MLV) vaccine [17].

3. Interaction between *M. hyopneumoniae*, PCV2, and PRRSV

3-1. Interaction between *Mycoplasma hyopneumoniae* and PCV2

Opriessnig et al. investigated the interactions between *M. hyopneumoniae* and PCV2 and established a model for studying the pathogenesis of and testing intervention strategies for the control of PCV2-associated PRDC. Pigs were randomly assigned to four groups. Group 1 served as controls, group 2 was inoculated with *M. hyopneumoniae*, group 3 was dual infected with *M. hyopneumoniae* and PCV2, and group 4 was inoculated with PCV2. Pigs were challenged with *M. hyopneumoniae* at 4 weeks of age followed by PCV2 at 6 weeks of age. Dual-infected pigs had moderate dyspnea, lethargy, and reduced weight gain. The overall severity of macroscopic lung lesions, PCV2-associated microscopic lesions in lung and lymphoid tissues, and the amount of PCV2-antigen associated with these lesions were significantly higher in dual-infected pigs compared with all other groups. 23.5% of dual-infected pigs had decreased growth rate and severe lymphoid depletion and granulomatous lymphadenitis associated with high amounts of PCV2-antigen consistent with PMWS. PCV2-antigen in lung tissue was most often associated with *M. hyopneumoniae*-

induced peribronchial lymphoid hyperplasia, suggesting that this is an important site for PCV2 replication in the lung. Author founded that *M. hyopneumoniae* potentiates the severity of PCV2-associated lung and lymphoid lesions, increases the amount and prolongs the presence of PCV2-antigen, and increases the incidence of PMWS in pigs [19].

Kekarainen et al. suggested that PMWS-affected pigs are immunosuppressed and, therefore, more prone to develop co-infections. They elucidated that PCV2 downregulates in vitro the immune cell functions during recall antigen responses [10]. Kyriakis et al. designed to investigate that non-specific immuno-modulation can influence the clinical and pathological expression of PMWS in pigs naturally infected with PCV2. Eighty-four pigs on a commercial pig farm were allocated to three groups of 28 pigs each, during an outbreak of PMWS. The pigs in the first group received an intramuscular injection of a vaccine against *M. hyopneumoniae* at each of 7 and 28 days of age, followed by an intramuscular injection of normal saline at 42 days of age. The animals in the second group received, by intramuscular injection, normal saline at 7 days of age followed by a non-specific immuno-modulating drug (Baypamun, Bayer, Leverkusen, Germany) at each of 28 and 42 days of age. The pigs in the third (control) group received an intramuscular injection of normal saline on each of 7, 28 and 42 days of age. The trial was concluded when the pigs had reached the age of 73 days. Clinical signs characteristic of PMWS developed in 42.9% of pigs inoculated with RespiSure (Zoetis, Madison, NJ, USA) and in 50% of pigs treated with Baypamun; six pigs from each of these groups died. Moderate to severe gross and histopathological

lesions of PMWS, associated with abundant PCV2 antigen, were seen in a wide range of tissues of pigs from these groups at the end of the trial. In contrast, only 10.7% of pigs in the control group developed clinical signs and only one died. Mild to moderate lesions and scant PCV2 antigen were occasionally observed in tissues of control pigs at the end of the trial. The result demonstrated that non-specific stimulation of the immune system by a vaccine or an immuno-modulator drug can potentiate viral replication and increase the severity of clinical signs during an outbreak of PMWS [12].

On the other hand, Sibila et al. assessed the effect of simultaneous experimental inoculation of PCV2 (intranasal delivery) and *M. hyopneumoniae* (transtracheal delivery) into conventional, seropositive 6-week-old piglets. And no significant clinical signs and in mean body weight and rectal temperature were observed between the groups. Mild microscopic lesions similar to those reported for PMWS were observed in two PCV2 pigs and in one PCV2 + *M. hyopneumoniae* animal. *M. hyopneumoniae*-compatible lung lesions were observed in 21/24 pigs inoculated with *M. hyopneumoniae* (10 from the *M. hyopneumoniae* group and 11 from the PCV2 + *M. hyopneumoniae* group). PCV2 was detected by in-situ hybridization in 3/12 PCV2 and in 4/12 PCV2 + *M. hyopneumoniae* animals. No significant differences in PCV2 load (serum and nasal and fecal swabs), duration of viremia or antibody titer were detected between PCV2-inoculated groups. No significant differences in *M. hyopneumoniae* load in nasal swabs, percentage of *M. hyopneumoniae*-seropositive pigs and mean lung score was detected between *M. hyopneumoniae*-inoculated groups. Under the conditions of the present study, concurrent inoculation of PCV2 and *M.*

hyopneumoniae did not result in potentiation of clinical signs and lesions attributed to either infection [26].

3-2. Interaction between *Mycoplasma hyopneumoniae* and PRRSV

PRRSV and *M. hyopneumoniae* are frequently isolated pathogens from pigs with respiratory disease. Thacker et al. demonstrated through an experimental model that showing a *mycoplasma* species acting to potentiate a viral pneumonia. *M. hyopneumoniae*, which produces a chronic, lymphohistiocytic bronchopneumonia in pigs, was found to potentiate the severity and the duration of a virus-induced pneumonia in pigs. Pigs were inoculated with *M. hyopneumoniae* 21 days prior to, simultaneously with, or 10 days after inoculation with PRRSV, which induces an acute interstitial pneumonia in pigs. PRRSV-induced clinical respiratory disease and macroscopic and microscopic pneumonic lesions were more severe and persistent in *M. hyopneumoniae*-infected pigs. At 28 or 38 days after PRRSV inoculation, *M. hyopneumoniae*-infected pigs still exhibited lesions typical of PRRSV-induced pneumonia, whereas the lungs of pigs which had received only PRRSV were essentially normal. On the basis of macroscopic lung lesions, it appears that PRRSV infection did not influence the severity of *M. hyopneumoniae* infection, although microscopic lesions typical of *M. hyopneumoniae* were more severe in PRRSV-infected pigs. These results indicate that *M. hyopneumoniae* infection potentiates PRRSV-induced disease and lesions. Most importantly, *M. hyopneumoniae*-infected pigs with minimal to nondetectable mycoplasmal pneumonia lesions manifested

significantly increased PRRSV-induced pneumonia lesions compared to pigs infected with PRRSV only. This discovery is important with respect to the control of respiratory disease in pigs and has implications in elucidating the potential contribution of mycoplasmas in the pathogenesis of viral infections of other species, including humans [32]. To investigate the efficacy and level of immune response induced by a *M. hyopneumoniae* bacterin in pigs previously vaccinated with a MLV PRRS vaccine and subsequently challenged with *M. hyopneumoniae*. Pigs were vaccinated once with MLV PRRS vaccine (Day 0), twice with *M. hyopneumoniae* vaccine (Days 8 and 22), or both, and challenged with *M. hyopneumoniae* on Day 36. Necropsies were performed on Days 35 and 61-63. Efficacy of *M. hyopneumoniae* vaccine was determined by reduction in the percentage of lung affected by *M. hyopneumoniae* pneumonia. Serum antibodies to PRRSV and *M. hyopneumoniae*, antibodies to *M. hyopneumoniae* in bronchoalveolar lavage (BAL), and production of *M. hyopneumoniae* specific IFN-γ-SCs in tissues and peripheral blood were measured on Days 0 and 14 and at necropsy. Immunological assays included ELISAs for PRRSV and *M. hyopneumoniae* antibodies and an enzyme-linked immunospot assay for *M. hyopneumoniae*-specific IFN-γ-SCs. Mycoplasma vaccine efficacy was not decreased by prior administration of PRRS vaccine. Serum *M. hyopneumoniae* antibody levels in pigs receiving both vaccines and challenged were numerically but not significantly higher than those in pigs receiving only *M. hyopneumoniae* vaccine and challenged. Pigs receiving both vaccines had significantly lower PRRSV sample:positive (S:P) ratios, but remained ELISA-positive (S:P>0.4). Levels of *M. hyopneumoniae* -specific

IgG and IgA in BAL Were significantly higher in both groups vaccinated for *M. hyopneumoniae* and challenged. Administration of a PRRS vaccine to *M. hyopneumoniae* -free pigs prior to vaccination with *M. hyopneumoniae* vaccine did not interfere with vaccine efficacy or immune responses to *M. hyopneumoniae* infection [3].

The effect of a single-dose *M. hyopneumoniae* vaccine was studied in growing pigs. Each of 24 vaccinated cohorts of approximately 1200 pigs reared in separate barns was matched by time, farm site, and sex with unvaccinated cohorts. Pigs were naturally exposed to *M. hyopneumoniae* and PRRSV. Daily weight gain was 42g per pig per day higher and mortality rate was 15.2/1000 pigs lower for vaccinated cohorts. Age at PRRSV onset was associated with mortality rate, but did not modify vaccine effects. *M. hyopneumoniae* vaccination was effective in promoting growth in spite of concurrent PRRSV infection [18].

Several studies found that infection with *M. hyopneumoniae* increased the duration and severity of respiratory disease induced by PRRSV. The purpose of this experiment was to determine whether vaccination against *M. hyopneumoniae* and/or PRRSV decreased the enhancement of PRRSV-induced pneumonia. Both *M. hyopneumoniae* bacterin and PRRSV vaccine decreased the severity of clinical respiratory disease. Infection or vaccination with PRRSV appeared to decrease the efficacy of the *M. hyopneumoniae* bacterin. Vaccination with *M. hyopneumoniae* bacterin decreased the potentiation of PRRSV-induced pneumonia observed in the dual infected pigs. However, PRRSV vaccination in combination with *M. hyopneumoniae* bacterin eliminated this benefit

and the amount of pneumonia induced by PRRSV increased. PRRSV vaccine alone did not decrease the potentiation of PRRSV pneumonia by *M. hyopneumoniae* [31].

There also was a study determine if PRRSV infection altered the severity of acute *M. hyopneumoniae* infection in young pigs. Twenty five, 3-week-old male pigs were randomly assigned by litter and weight to one of 3 groups. Groups 1 (PRRSV only, n=5) and 2 (PRRSV + *M. hyopneumoniae*, n=10) were inoculated intranasally with PRRSV (IN-5 isolate, 10(5) $TCID_{50}$) and viremia in all pigs was confirmed by virus isolation from serum 3 days later. Group 3 (*M. hyopneumoniae* only, n=10) was inoculated at the same time with virus free culture media. Seven days after virus inoculation, Groups 2 and 3 were inoculated intratracheally with *M. hyopneumoniae* (strain P-5722-3, 10(7) CCU). All pigs were euthanized and necropsied 28 days later, when maximum lesions of mycoplasmosis occurs. Pigs in group 1 did not cough and had no gross lung lesions, but were still viremic at necropsy. *M. hyopneumoniae* was isolated from all pigs in groups 2 and 3, but differences were not significant. Similarly, there were no differences in average days coughing (8.9 +/- 2.8 v 11.2 +/- 4.5, P=0.17), grossly pneumonic lung (16.5% v 17%, P=0.91), or microscopic lung lesion scores (10.1 +/- 2.6 v 11.1 +/- 1.9, P=0.35) between pigs in groups 2 and 3. Under these experimental conditions, PRRSV infection did not increase the severity of experimental *M. hyopneumoniae* infection in young pigs [36].

3-3. Immune responses in co-infections

Concurrent infection with porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) is known as one of the major causes for porcine respiratory disease complex (PRDC). Dual infection with PCV2 and PRRSV is consistently to have more severe clinical presentations and pulmonary lesions than infection with PCV2 alone or PRRSV alone. However, it is not known if dual infections with PCV2 and PRRSV in different infection order may lead to different clinical symptoms in the host. To mimic the possible field conditions, swine alveolar macrophages (AMs) were inoculated with PCV2 and PRRSV in vitro simultaneously or with one virus 18 h earlier than the other. The cell viability, cytopathic effects, antigen-containing rates, phagocytotic and microbial killing capabilities, cytokine profiles (IL-8, TNF-α, and IFN-α) and FasL transcripts were determined, analyzed, and compared to prove the hypothesis. A marked reduction in PRRSV antigen-containing rate, cytopathic effect, and TNF-α expression level was revealed in AMs inoculated with PCV2 and PRRSV simultaneously and in AMs inoculated with PCV2 first then PRRSV 18 h later, but not in AMs inoculated with PRRSV first then PCV2 18 h later. Transient decrease in phagocytosis but constant reduction in microbicidal capability in AMs in the group inoculated with PCV2 alone and constant decrease in phagocytosis and microbicidal capability in AMs in all PRRSV-inoculated groups were noted. The levels of IL-8, TNF-α, IFN-α, and FasL transcripts in AMs in all groups with dual inoculation of PCV2 and PRRSV were significantly increased regardless of the infection orders as compared with infection by PCV2 alone or PRRSV alone. Swine

AMs infected with PCV2 first then PRRSV later or infected with PCV2 and PRRSV simultaneously displayed marked reduction in PRRSV antigen-containing rate, cytopathic effect, and TNF-α expression level. The different inoculation orders of PCV2 and PRRSV in AMs leading to different results in viral antigen positivity, cytopathology, and cytokine profile may explain, at least partially, the underlying mechanism of the enhanced pulmonary lesions in PRDC exerted by dual infection with PCV2 and PRRSV and the variable clinical manifestations of PRDC-affected pigs in the field [16]. Proinflammatory cytokines are believed to play an important role in porcine respiratory disease by coordinating and activating the adaptive immune response, which enables the host to eliminate offending pathogens. However, if cytokine levels become excessive, tissue damage and even death of the host can occur. Therefore, determination of both the presence and the quantity of proinflammatory cytokines can lead to an increased understanding of the pathogenesis of disease and the corresponding host's immune response. Induction of the proinflammatory cytokines interleukin-1 (IL-1) (α and β), IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor alpha (TNF-α) in pulmonary alveolar macrophages (PAMs) was assessed following experimental infection with porcine reproductive and respiratory syndrome virus (PRRSV) and/or *M. hyopneumoniae* by using in vivo and in vitro models. The in vivo model consisted of pigs infected with PRRSV and/or *M. hyopneumoniae* and necropsied at 10, 28, or 42 days post-infection. Pigs infected with both pathogens had a greater percentage of macroscopic lung lesions, increased clinical disease, and slower viral clearance than pigs infected with either pathogen alone. The pigs infected with

both PRRSV and *M. hyopneumoniae* had significantly increased levels of mRNA for many proinflammatory cytokines in PAMs collected by bronchoalveolar lavage (BAL) at all necropsy dates compared to those in uninfected control pigs. Increased levels of IL-1β, IL-8, IL-10, and TNF-α proteins in BAL fluid, as measured by enzyme-linked immunosorbent assay, confirmed the increased cytokine induction induced by the pathogens. An in vitro model consisted of *M. hyopneumoniae*-inoculated tracheal ring explants cultured with PRRSV-infected PAMs. PAMs were harvested at 6 or 15 h post-infection with either or both pathogens. The in vitro study detected increased IL-10 and IL-12 mRNA levels in PAMs infected with PRRSV at all time periods. In addition, IL-10 protein levels were significantly elevated in the culture supernatants in the presence of *M. hyopneumoniae*-inoculated tracheal ring explants. The increased production of proinflammatory cytokines in vivo and in vitro associated with concurrent *M. hyopneumoniae* and PRRSV infection may play a role in the increased rates of pneumonia associated with PRRSV infection. The increased levels of IL-10 may be a possible mechanism that PRRSV and *M. hyopneumoniae* use to exacerbate the severity and duration of pneumonia induced by PRRSV and modulate the respiratory immune response [15].

Zhang et al. determined cytokine and chemokine mRNA expression profiles in tracheobronchial lymph nodes from pigs singularly infected with porcine circovirus type 2 (PCV2), *M. hyopneumoniae*, or co-infected with both. Twenty-eight pigs were randomly assigned to one of four groups: (group1) negative controls, (group 2) inoculated with *M. hyopneumoniae* (group 3) inoculated with *M. hyopneumoniae* and

PCV2, and (group 4) inoculated with PCV2. *M. hyopneumoniae* infection significantly stimulated innate cytokines, IL-1B and IL-8. PCV2 infection significantly stimulated expression of IFNG, IL-8, NOS2A and chemokines CCL-2, CCL-5, and CXCL-10. IFNB, IL-1B and IL-12 were slightly increased with PCV2 infection and IFNA and IL-4 were significantly downregulated. Compared to negative control group, co-infection resulted in a significant increase in expression of IFNG, IL-1B, IL-8, CCL-5, CXCL-10, and weak stimulation of IFNB, IL-6 and IL-10; IL-13 and IFNA were significantly downregulated. Overall, *M. hyopneumoniae* potentiated PCV2 infection by increasing IFNG and IL-10 mRNA expression levels. The increase of IFNG and chemokines and decrease of IFNA in PCV2 injected and Co-injected pigs were correlated with increased severity of lymphoid lesions and the presence of PCV2 antigen. In summary, this work provided evidence that the increased severity of lesions in PCV2 and *M. hyopneumoniae* co-infected pigs was associated mainly with the presence of PCV2 antigen and alterations of cytokine mRNA expression profiles [37].

4. Vaccine Efficacy

4-1. *Mycoplasma hyopneumoniae* vaccine efficacy

M. hyopneumoniae vaccination is the most cost-effective strategy for the control and prevention of the disease. To evaluate the protective efficacy of the vaccination of *M. hyopneumoniae* (J strain) in the molecular size range 70 to 85 kDa (F3 antigen) in combination with adjuvants, the study was done with pigs challenged with *M. hyopneumoniae*. A vaccine efficacy experiment with assessment of serum and respiratory tract antibody responses. F3 antigens were emulsified with five different adjuvants. To groups of three pigs per vaccine, four vaccines were given by intramuscular injection, and two vaccines, including one of those given intramuscularly, were given by intraperitoneal injection. Compared to six unvaccinated pigs, animals vaccinated with F3 antigen displayed significantly reduced pneumonia (54% reduction in mean lung score) following experimental challenge. Analysis of post-vaccination, pre-challenge IgG and IgA ELISA antibody absorbances in serum and respiratory tract washings revealed no correlation with lung score. Six weeks after challenge, pigs previously vaccinated intramuscularly mostly demonstrated greater IgG and IgA responses in respiratory tract washings, and greater IgG serum antibody responses, than those vaccinated by intraperitoneal injection. Pigs vaccinated with *M. hyopneumoniae* antigens in the molecular size range of 70 to 85 kDa showed a significant reduction in lung lesions compared with unvaccinated control animals after experimental challenge. IgG and IgA antibody concentrations in serum and respiratory tract washings after vaccination do not provide a useful prognostic indicator of

protection from enzootic pneumonia [8].

Also there have been several studies to evaluate immune responses induced by administration of *M. hyopneumoniae* vaccine to pigs. Pigs with 60 healthy 7- to 10-day-old cross-bred boars were assigned to 1 of 4 pig groups: vaccinated, challenged; vaccinated, nonchallenged; nonvaccinated, challenged; nonvaccinated, nonchallenged. Vaccinated pigs received IM injections of a mycoplasma bacterin on days 0 and 14, whereas nonvaccinated pigs received saline solution. Pigs in the challenged groups were inoculated intratracheally with *M hyopneumoniae* on day 42. Pigs were euthanatized and necropsied 41, 44, 48, and 70 days after the first vaccination, and proportion of lung surface with pneumonic lesions was determined. Percentage of lymphocyte subpopulations and number of IFN-γ secreting lymphocytes in blood and tissues, cytokine and antibody concentrations in BAL fluid, and serum antibody concentrations were determined. Vaccination against and infection with *M .hyopneumoniae* induced a local mucosal immune response in the respiratory tract of pigs. Proportion of lung surface with pneumonic lesions in vaccinated challenged pigs was reduced on day 70, compared with nonvaccinated challenged pigs. Vaccination stimulated the production of *M hyopneumoniae*-specific IFN-γ secreting blood lymphocytes. Tumor necrosis factor-alpha concentration in BAL fluid on day 70 was increased in nonvaccinated challenged pigs, compared with vaccinated challenged pigs.

Vaccination against *M hyopneumoniae* induced local, mucosal, humoral, and cellular immune responses. Moreover, vaccination reduced the severity of lung lesions in

challenged pigs, suggesting that mucosal antibodies, mediation of the inflammatory response, and cell-mediated immune responses are important for control of mycoplasmal pneumonia in pigs [34].

But despite efforts to control *M. hyopneumoniae* infection, significant economic losses in pig production continue to occur. Recent study with genome-based vaccine showed the potential to help understand the biology and pathogenesis of *M. hyopneumoniae*, and contribute to the development of more effective vaccines and diagnostic tests. Simionatto et al. reviewed the characteristics of *M. hyopneumoniae* related to pathogenesis and control measures. They focused special emphasis on vaccination strategies that have been proposed with the use of reverse vaccinology approaches [28].

4-2. Porcine circovirus type 2 Vaccine Efficacy

PCV2 vaccines were initially developed to control PMWS, but they are now also used against other PCVAD. To identify the effective vaccine, it is important for the users to focus on the types of commercial vaccines, the criteria of vaccine efficacy, the clinical, virological, immunological and pathological efficacy and the use of PCV2 vaccines against different clinical manifestations of PCVAD in their farm [5].

Seo et al. described the efficacy of the reformulated inactivated chimeric PCV1-2 vaccine under field conditions. Three farms were selected based on their history of PMWS. On each farm, a total of 50 3-week-old pigs were randomly allocated to one of two treatment groups: (i) vaccinated at 3 weeks of age and (ii) non-vaccinated. Clinical

examination indicated that vaccinated animals displayed an improved average daily weight gain and a reduced time to market. Virological examination indicated that vaccinated animals displayed a reduced PCV2 load in the blood and nasal swabs compared to non-vaccinated animals. Pathological examination indicated that vaccination of pigs against PCV2 effectively reduced the number of PMWS-associated microscopic lesions and the PCV2 load in lymphoid tissues compared to non-vaccinated animals in the 3 herds. Immunological examination indicated that vaccinated animals induced PCV2-specific NA and IFN-γ-SCs. A reduction in the PCV2 load in the blood coincided with the appearance of both PCV2-specific NA and IFN-γ-SCs in the vaccinated animals. The number of $CD4^+$ cells was decreased in non-vaccinated animals compared to vaccinated animals. The reformulated inactivated chimeric PCV1-2 vaccine seems to be very effective in controlling PCV2 infection based on clinical, virological, pathological, and immunological evaluations under field conditions [24].

4-3. Porcine reproductive and respiratory syndrome virus Vaccine efficacy

The efficacy of two different types of commercial vaccines against PRRSV (Euro-type) was evaluated based on clinical parameters upon challenge as well as post-challenge virological profiles (viremia and viral load in tissues upon necropsy, measured in both cases by quantitative real time PCR). In an attempt to establish correlates of protective immunity, two commonly proposed parameters predictive of immunity were measured: (1) serologic responses (ELISA and neutralizing antibodies), (2) frequency of γ-IFN-

producing cells in peripheral blood mononuclear cell fraction. The vaccines compared consisted of two commercially available products that are regularly marketed in Spain: one modified live virus and one killed vaccine. The efficacy assay was carried out by vaccinating twice 3 weeks apart groups of 5 and-a-half month-old female swine and then challenging them with a European type 1 PRRSV strain (Lelystad). The results obtained indicate that the modified live virus vaccine was the only type of vaccine capable of establishing protective immunity, as measured by viral load in blood and tissues. The killed vaccine, in spite of this product evoking a spontaneous IFN-γ response and post-challenge titers of virus-neutralizing antibody, evoked no measurable protective immunity. In the case of the modified live vaccine, the protection exhibited did not appear to be based on humoral but rather on cell-mediated immunity [38].

The lack of heterologous protection by PRRSV vaccines is currently a major problem in the field. Heterologous protection by PRRS vaccines depends on the ability of the vaccine to induce an IFN-γ response. One mechanism by which the virus evades the immune system is by activating regulatory T cells (T(regs)), resulting in induction of interleukin 10 rather than IFN-γ. Our hypothesis that current PRRS vaccines do not differ from pathogenic strains in the ability to induce T(regs) was tested by inoculating three groups of pigs with two pathogenic viruses and an attenuated vaccine strain and evaluating the number of T(regs) in peripheral blood mononuclear cells. Before inoculation, the pigs, although vaccinated became infected naturally with *M. hyopneumoniae* before shipment to our research facility. Our results show that the

PRRSV vaccine strain and parent strain are equally able to induce T(regs) in pigs naturally infected with *M. hyopneumoniae*. Pigs in the vaccine and PRRSV groups had higher lung lesion scores than pigs in the control groups. The results suggest that the exacerbation *M. hyopneumoniae* respiratory disease may be due to the ability of PRRSV vaccination and viral infection to induce regulatory T cells [13].

References

[1] Albina E, Carrat C, Charley B., 1998. Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. Journal of Interferon & Cytokine Research 18, 485-490.

[2] Bautista, E.M., Molitor, T.W., 1999. IFN γ inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Archives of Virology 144, 1191-1200.

[3] Boettcher, T.B., Thacker, B.J., Halbur, P.G., Waters, W.R., Nutsch, R., Thacker, E.L., 2002. Vaccine efficacy and immune response to *Mycoplasma hyopneumoniae* challenge in pigs vaccinated against porcine reproductive and respiratory syndrome virus and M hyopneumoniae. Journal of Swine Health and Production 10, 259-264.

[4] Caruso JP, Ross RF., 1990. Effects of *Mycoplasma hyopneumoniae* and Actinobacillus (Haemophilus) pleuropneumoniae infections on alveolar macrophage functions in swine. American Journal of Veterinary Research 51, 227-231.

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

[6] Chae C., 2004. Postweaning multisystemic wasting syndrome: a review of

aetiology, diagnosis and pathology. The Veterinary Journal 168, 41-49.

[7] DeBey MC, Jacobson CD, Ross RF, 1992. Histochemical and morphologic changes of porcine airway epithelial cells in response to infection with *Mycoplasma hyopneumoniae*. American Journal of Veterinary Research 53, 1705-1710.

[8] Djordjevic SP, Eamens GJ, Romalis LF, Nicholls PJ, Taylor V, Chin J., 1997. Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing a denatured membrane antigen pool and adjuvant. Australian Veterinary Journal 75, 504-511.

[9] Fort M, Fernandes LT, Nofrarias M, Diaz I, Sibila M, Pujols J, et al., 2009. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. Veterinary Immunology and Immunopathology 129, 101-107.

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

[11] Kim J, Choi C, Chae C., 2003. Pathogenesis of postweaning multisystemic wasting syndrome reproduced by co-infection with Korean isolates of porcine

circovirus 2 and porcine parvovirus. Journal of Comparative Pathology 128, 52-59.

[12] Kyriakis SC, Saoulidis K, Lekkas S, Miliotis CC, Papoutsis PA, Kennedy S., 2002. The effect of immune-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. Journal of Comparative Pathology 126, 38-46.

[13] LeRoith, T., Hammond, S., Todd, S.M., Ni, Y., Cecere, T., Pelzer, K.D., 2011. A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with *Mycoplasma hyopneumoniae*. Veterinary Immunology and Immunopathology 140, 312-316.

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

[15] Roongroje Thanawongnuwech, Brad Thacker, Patrick Halbur, Eileen L. Thacker, 2004. Increased Production of Proinflammatory Cytokines following Infection with Porcine Reproductive and Respiratory Syndrome Virus and Mycoplasma hyopneumoniae. Clinical and Diagnostic Laboratory Immunology 11, 901-908.

[16] Yi-Chieh Tsai, Hui-Wen Chang, Chian-Ren Jeng, Tsang-Long Lin, Chun-Ming

Lin, Cho-Hua Wan, Victor Fei Pang., 2012. The effect of infection order of porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus on dually infected swine alveolar macrophages. BMC Veterinary Research 8, 174.

[17] Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2003. Gradual development of the interferon-g response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309, 18-31.

[18] Moreau, I.A., Miller, G.Y., Bahnson, P.B., 2004. Effects of *Mycoplasma hyopneumoniae* vaccine on pigs naturally infected with *M. hyopneumoniae* and porcine reproductive and respiratory syndrome virus. Vaccine 22, 2328-2333.

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

[20] QIA report 11-1541745-000008-01, 2015. Infectious Animal Disease Central Preventative Association Documents 2nd Quarter edition.

[21] Rodriguez F, Ramirez GA, Sarradell J, Andrada M, Lorenzo H., 2004.

Immunohistochemical labeling of cytokines in lung lesions of pigs naturally infected with *Mycoplasma hyopneumoniae*. Journal of Comparative Pathology 130, 306-312.

[22] Rowland, R.R., Robinson, B., Stefanick, J., Kim, T.S., Guanghua, L., Lawson, S.R., Benfield, D.A., 2001. Inhibition of porcine reproductive and respiratory syndrome virus by interferon-gamma and recovery of virus replication with 2-aminopurine. Archives of Virology 146, 539-555.

[23] Segalés J1, Allan GM, Domingo M., 2005. Porcine circovirus diseases. Animal Health Research Reviews 6,119-142.

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

[25] Segalés J1, Domingo M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. The Veterinary quarterly 24, 109-124.

[26] Sibila M, Fort M, Nofrarias M, Perez de Rozas A, Galindo-Cardiel I, Mateu E, et al., 2012. Simultaneous porcine circovirus type 2 and *Mycoplasma hyopneumoniae* co-inoculation does not potentiate disease in conventional pigs. Journal of Comparative Pathology 147, 285-295.

[27] Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F., Segales, J., 2009. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. Veterinary Journal 181, 221-231.

[28] Simionatto S, Marchioro SB, Maes D, Dellagostin OA., 2013, *Mycoplasma hyopneumnoniae*: From disease to vaccine development. Veterinary microbiology 165, 234-242.

[29] Susan L., Brockmeier, Patrick G. Halbur, Eileen L. Thacker., 2002 . Procine Respiratory Disease Complex . NCBI Bookshelf, Brogen KA, Guthmiller KM, editors. Polymicrobial Disease. Washington (DC)

[30] Tajima M, Yagihashi T, Nunoya T, Takeuchi A, Ohashi F., 1984. *Mycoplasma hyopneumoniae* infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. American Journal of Veterinary Research 45, 1928-1932.

[31] Thacker E.L., Thacker B.J., Kuhn M, Hawkins P.A., Waters W.R., 2000. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. American Journal of Veterinary Research 61, 1384-1389.

[32] Thacker, E.L., Halbur, P.G., Ross, R.F., Thanawongnuwech, R., Thacker, B.J.,

1999. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. Journal of Clinical Microbiology 37, 620-627.

[33] Thacker E.L., 2008. Porcine Respiratory Disease Complex. Proceedings, The 15th Congress of FAVA-OIE Joint Symposium on Emerging Diseases, October, Bankok, Thaland, S77, 27-30

[34] Thacker, E.L., Thacker, B.J., Young, T.F., Halbur, P.G., 2000. Effect of vaccination on the potentiation of porcine reproductive and respiratory syndrome (PRRSV)-induced pneumonia by *Mycoplasma hyopneumoniae*. Vaccine 18, 1244-1252.

[35] Van Alstine, W.G., 2012. Respiratory system. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, 10th edition. Wiley-Blackwell Publishing, Ames, IA, USA, 348-362.

[36] Van Alstine, W.G., Stevenson, G.W., Kanitz, C.L., 1996. Porcine reproductive and respiratory syndrome virus does not exacerabate *Mycoplasma hyopneumoniae* infection in young pigs. Veterinary Microbiology 49, 297-303.

[37] Zhang H, Lunney JK, Baker RB, Opriessnig T., 2011. Cytokine and chemokine mRNA expression profiles in tracheobronchial lymph nodes from pigs singularly

infected or coinfected with porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae.* Veterinary Immunology and Immunopathology 140, 152-158.

[38] Zuckermann, F.A., Garcia, E.A., Luque, I.D., Christopher-Hennings, J., Doster, A., Brito, M., Osorio, F., 2007. Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of γ-IFN-producing cells and virological parameters of protection upon challenge. Veterinary Microbiology 123, 69-85.

PART I.

Interaction of porcine circovirus type 2 and *Mycoplasma hyopneumoniae* vaccines on dually infected pigs

Seo HW, Park SJ, Park C, Chae C. 2014, Vaccine, 32, 2480-2486

Abstract

The objective of this study was to determine the effects of porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* vaccinations on disease severity in an experimental PCV2-*M. hyopneumoniae* dual challenge model. Vaccine effectiveness was evaluated using microbiological (PCV2 viremia and *M. hyopneumoniae* nasal shedding), immunological (neutralizing antibodies and IFN-γ-secreting cells), and pathological (gross lung lesions, histopathologic pulmonary and lymphoid lesions, and the presence of PCV2 antigen and *M. hyopneumoniae* DNA within the lesions) evaluations. Although *M. hyopneumoniae* potentiates the severity of PCV2-associated lesions and lesion-associated PCV2 antigen in dually challenged pigs, vaccination against *M. hyopneumoniae* alone did not reduce PCV2 viremia, PCV2-induced lesions, or PCV2 antigen in dually challenged pigs. In addition, vaccination against PCV2 did not reduce the nasal shedding of *M. hyopneumoniae*, the *M. hyopneumoniae*-induced pulmonary lesions or the lesion-associated *M. hyopneumoniae* DNA in dually challenged pigs. Dual challenge with PCV2 and *M. hyopneumoniae* did not interfere with the induction of active immunity induced by a previous single vaccination for either PCV2 or *M. hyopneumoniae*. The results of this study demonstrated that (i) vaccination against *M. hyopneumoniae* alone did not decrease the potentiation of PCV2-induced lesions by *M. hyopneumoniae* and (ii) vaccination against PCV2 alone decreased the potentiation of PCV2-induced lesions by *M. hyopneumoniae* in dually challenged pigs.

1. Introduction

Porcine circovirus type 2 (PCV2) is the primary etiological agent for several diseases and syndromes, which are collectively referred to as porcine circovirus-associated disease (PCVAD) [1]. Among these conditions, postweaning multisystemic wasting syndrome (PMWS) and porcine respiratory disease complex (PRDC) are the most important. *Mycoplasma hyopneumoniae* is the primary pathogen causing enzootic pneumonia, which is characterized by a dry, non-productive cough, reduced growth rate and poor feed conversion efficiency [2]. Co-infection with PCV2 and *M. hyopneumoniae* plays a primary role in the PRDC and continues to have a major economic impact on the global swine industry [3].

Several studies based on experimental dual infection have been conducted to better understand the interaction between PCV2 and *M. hyopneumoniae* [4-6]. In a sequential challenge model, *M. hyopneumoniae* potentiated the severity of PCV2-associated lung and lymphoid lesions, and increased the incidence of PMWS in pigs that were first inoculated with *M. hyopneumoniae* and then inoculated with PCV2 2 weeks later [4]. In contrast, in a concurrent infection model, pigs that were inoculated with both *M. hyopneumoniae* and PCV2 at 6 weeks of age did not produce the synergistic clinical outcomes observed when using the sequential challenge model [5].

Since dual infection of pigs with *M. hyopneumoniae* and PCV2 results in increased severity of PCV2-induced lesions and incidence of PMWS using the sequential challenge model [4], one possible way to minimize the effect of the *M. hyopneumoniae*-associated enhancement of PCV2 replication may be the use of a *M.*

hyopneumoniae-based vaccine. Surprisingly, however, it have been reported that *M. hyopneumoniae* vaccination alone actually increased the incidence of PMWS under experimental and field conditions [7,8]. These unexpected results make difficult to understand the interaction between *M. hyopneumoniae* vaccination and incidence of PMWS. Hence, it is necessary to conduct experimental studies to elucidate the effects of a single vaccination for either PCV2 or *M. hyopneumoniae* on dually infected pigs. Currently, commercial PCV2 and *M. hyopneumoniae* vaccines are widely used in swine production worldwide. Therefore, the objective of this study was to determine the effects of single PCV2 and/or *M. hyopneumoniae* vaccinations on pigs in an experimental PCV2 and *M. hyopneumoniae* dual challenge model.

2. Materials and Methods

2.1. Commercial vaccines

The inactivated chimeric PCV1-2 vaccine (Fostera PCV, Zoetis, Madison, NJ, USA) and the inactivated *M. hyopneumoniae* vaccine (RespiSure-One, Zoetis) were used in this study. Vaccines were administered according to the manufacturer's instructions (1 dose via the intramuscular route).

2.2. Animals

A total of 88 colostrum-fed, cross-bred, conventional piglets were weaned and purchased at 18 days of age from a porcine reproductive and respiratory syndrome virus (PRRSV)- and *M. hyopneumoniae*-free commercial farm based on serological testing of breeding herd, and long term clinical and slaughter history. Pigs were all negative for PCV2, PRRSV, and *M. hyopneumoniae* according to routine serological testing. PCV2 and PRRSV were not detected in the sera samples by the real-time polymerase chain reaction (PCR) [9,10]. *M. hyopneumoniae* was not detected in the nasal swab samples by real-time PCR [11].

2.3. Experimental design

A total of 88 pigs were randomly divided into 11 groups (8 pigs per group): 5 vaccinated challenged (VC), 3 unvaccinated challenged (UVC), 2 vaccinated unchallenged (VUC), and 1 unvaccinated unchallenged group (Table 1). At 7 days of age [-42 days post challenge (dpc)], pigs in groups 1, 2, 5, and 9 were injected

intramuscularly in the right side of the neck with 2.0 ml of the *M. hyopneumoniae* vaccine (RespiSure-One, Zoetis). At 21 days of age (-28 dpc), pigs in groups 3, 4, 5, and 10 were injected intramuscularly in the left side of the neck with 2.0 ml of the PCV2 vaccine (Fostera PCV, Zoetis). An equal volume of phosphate buffered saline (PBS) (2.0 ml) was injected in the same anatomic location in the positive and negative control pigs (groups 6, 7, 8, and 11) at 7 and 21 days of age.

At 35 days of age (-14 dpc), pigs in the VC (groups 1, 2, 4, and 5) and UVC (groups 6 and 8) were intratracheally administered with a 10 ml dose of frozen lung homogenate of *M. hyopneumoniae* strain SNU98703 (1:100 dilution in Friis medium) at a final concentration of 10^4-10^5 color changing units (CCU)/ml as previously described [12].

At 49 days of age (0 dpc), pigs in the VC (group 2, 3, 4, and 5) and UVC (groups 7 and 8) groups were intranasally administered with a 3 ml dose of PCV2b [strain SNUVR000463 (GenBank no. KF871068), 5th passage] containing 1.2 x 10^5 50% tissue culture infective dose [$TCID_{50}$]/ml (Table 1).

Table 1. Study design with vaccination and challenge statuses of *Mycoplasma hyopneumoniae* and PCV2[a].

Group	Vaccination		Challenge	
	M. hyopneumoniae (-42 dpc)	PCV2 (-28 dpc)	*M. hyopneumoniae* (-14 dpc)	PCV2 (0 dpc)
1	+	-	+	-
2	+	-	+	+
3	-	+	-	+
4	-	+	+	+
5	+	+	+	+
6	-	-	+	-
7	-	-	-	+
8	-	-	+	+
9	+	-	-	-
10	-	+	-	-
11	-	-	-	-

[a]There were eight animals in each groups, and necropsy was performed at 28 days post challenge (dpc) in all cases.

Blood samples and nasal swabs were collected at -42, -28, -14, 0, 7, 14, 21, and 28 dpc. Pigs from each group were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 28 dpc as previously described [13]. Tissues were collected from each pig at necropsy. All of the methods were approved by the

Seoul National University Institutional Animal Care and Use Committee.

2.4. Quantification of PCV2 DNA in blood

DNA was extracted from serum samples using the QIAamp DNA Mini Kit (QIAGEN Ltd, Crawley, UK) to quantify PCV2 genomic DNA copy numbers by real-time PCR [8].

2.5. Quantification of M. hyopneumoniae DNA in nasal swabs

DNA was extracted from nasal swabs using the QIAamp DNA Mini Kit (QIAGEN Ltd, Crawley, UK) to quantify the *M. hyopneumoniae* genomic DNA copy numbers by real-time PCR with primers based on the putative ABC transporter [11].

2.6. Serology

The serum samples were tested for antibodies to PCV2 and *M. hyopneumoniae* using the commercial PCV2 ELISA (Synbiotics, Lyon, France) and *M. hyopneumoniae* ELISA (IDEXX Laboratories Inc, Westbrook, ME, USA). Serum virus neutralization (SVN) test for PCV2 was performed as previously described [14].

2.7. Enzyme-linked immunospot assay

PCV2 and *M. hyopneumoniae* antigens were prepared as previously described [14, 15]. The numbers of PCV2- and *M. hyopneumoniae*-specific IFN-γ-SCs were determined in peripheral blood mononuclear cells (PBMCs) as previously described [17,18].

2.8. In situ hybridization and immunohistochemistry

In situ hybridization for *M. hyopneumoniae* and immunohistochemistry for PCV2 was performed as previously described [15, 19, 20].

2.9. Gross lung lesion scores

The total extent of gross lung lesions was estimated and calculated as previously described [21]. The frequency distribution of the lung lesion scores for each lung lobe was calculated by treatment. The percentage of total lung with lesions was calculated using the following formula: 100 × [(0.10 × left cranial) + (0.1 × left middle) + (0.25 × left caudal) + (0.10 × right cranial) + (0.10 × right middle) + (0.25 × right caudal) + (0.10 × accessory)].

2.10. Morphometric analyses.

The morphometric analyses of in situ hybridization for *M. hyopneumoniae* and immunohistochemistry for PCV2 was performed as previously described [4, 20]. For the morphometric analyses of the microscopic pulmonary and lymph lesion scores, tissue sections were blindly examined by two veterinary pathologists [4, 22].

2.11. Statistical analyses

Summary statistics were calculated for all of the groups to assess the overall quality of the data, including normality. The continuous data for the quantification of PCV2 and *M. hyopneumoniae* DNA, the PCV2 and *M. hyopneumoniae* serology, IFN-γ-SCs, and

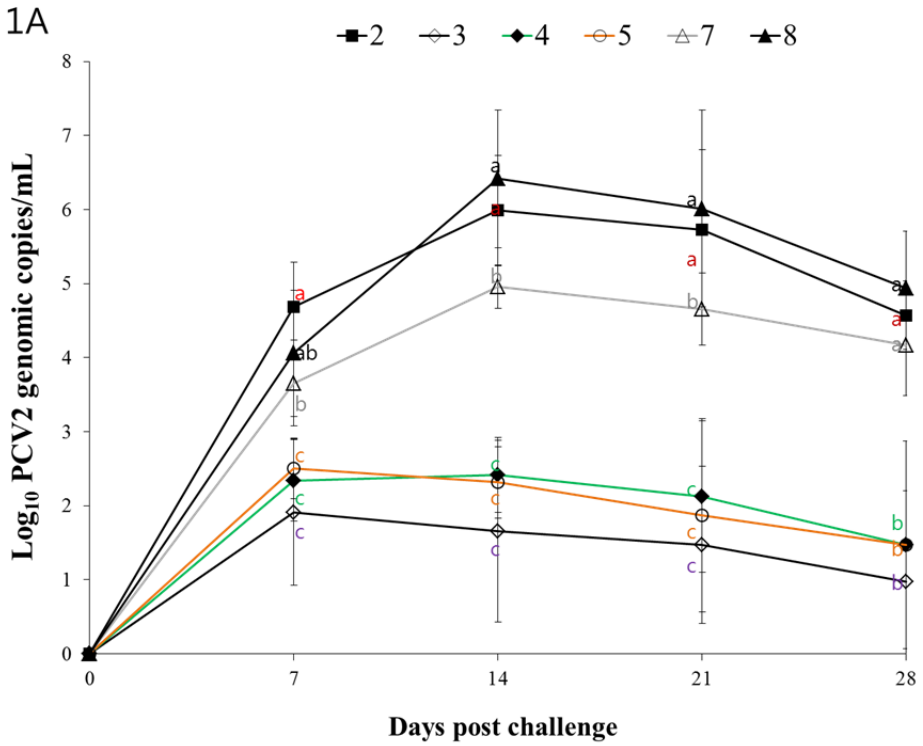
PCV2 antigen by immunohistochemistry, and *M. hyopneumoniae* DNA by in situ hybridization were analyzed using an ANOVA for each time point. When a one-way ANOVA revealed a significance of $P < 0.05$, the Bonferroni adjustment procedure was used to determine the significance of individual between group differences. Discrete data (gross lung lesions, histopathological lung and lymphoid lesions, PCV2 antigen scores, and *M. hyopneumoniae* DNA scores) were analyzed by the Chi-square and Fisher's exact tests. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Quantification of PCV2 DNA in blood

At the time of challenge, no genomic copies of PCV2 were detected in any of the sera samples from all 11 groups of pigs. Pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2) had a significantly higher number of genomic copies of PCV2 in their sera than did pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), and pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5) at 7, 14, 21, and 28 dpc ($P < 0.05$). The rest of the results are summarized in **Fig. 1A**. Throughout the experiment, no genomic copies of PCV2 were detected in any of the sera samples from pigs that were not challenged with PCV2 (groups 1, 6, 9, 10, and 11).

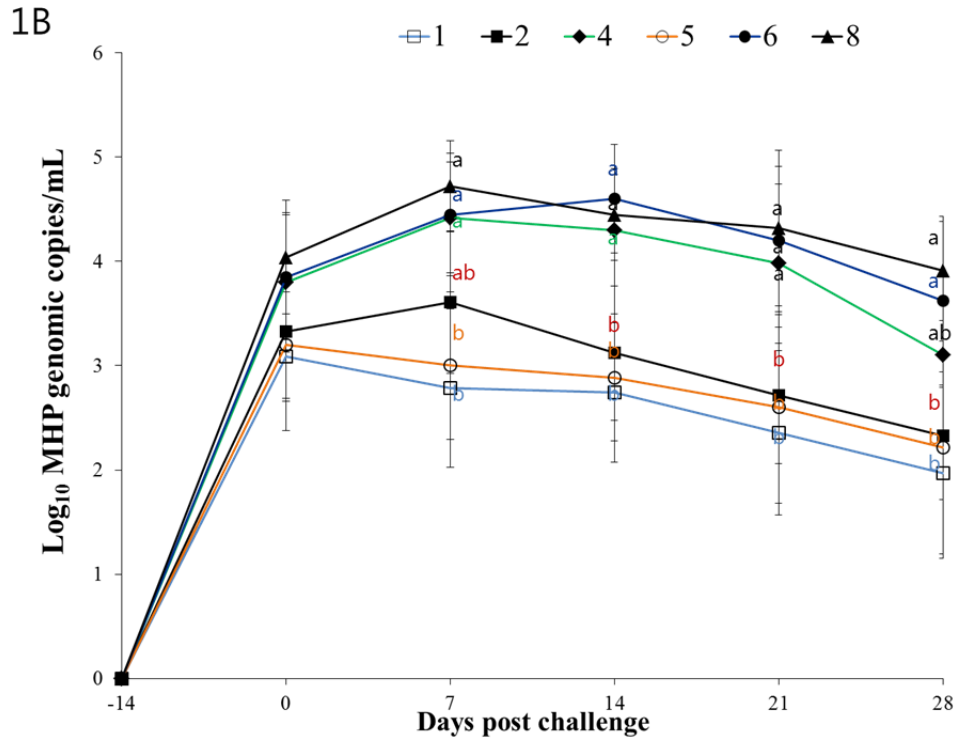
Fig. 1A. Mean values of the genomic copy numbers of PCV2 DNA in the serum samples.; Pigs that received the *M. hyopneumoniae* vaccine followed by a challenge with only *M. hyopneumoniae* (group 1, □), pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, ■), pigs that received the PCV2 vaccine followed by a PCV2-only challenge (group 3, ◇), pigs that received the PCV2 vaccine followed by a dual challenge (group 4, ◆), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5, ○), unvaccinated pigs challenged only with *M. hyopneumoniae* (group 6, ●), unvaccinated pigs challenged only with PCV2 (group 7, ∆), and pigs that received a dual challenge (group 8, ▲). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.



3.2. Quantification of M. hyopneumoniae DNA in nasal swabs

At the time of challenge, no genomic copies of *M. hyopneumoniae* were detected in any of the sera samples from all 11 groups of pigs. Unvaccinated pigs challenged with *M. hyopneumoniae* alone (group 6) and dually challenged pigs (group 8) had a significantly higher number of genomic copies of *M. hyopneumoniae* in their nasal swabs than did pigs that received the *M. hyopneumoniae* vaccine followed by a challenge with either *M. hyopneumoniae* alone (group 1) or a dual challenge (group 2), and pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5) at 14, 21, and 28 dpc ($P < 0.05$). The rest of the results are summarized in **Fig. 1B**. Throughout the experiment, no genomic copies of *M. hyopneumoniae* were detected in any of the sera samples from pigs that were not challenged with *M. hyopneumoniae* (groups 3, 7, 9, 10, and 11).

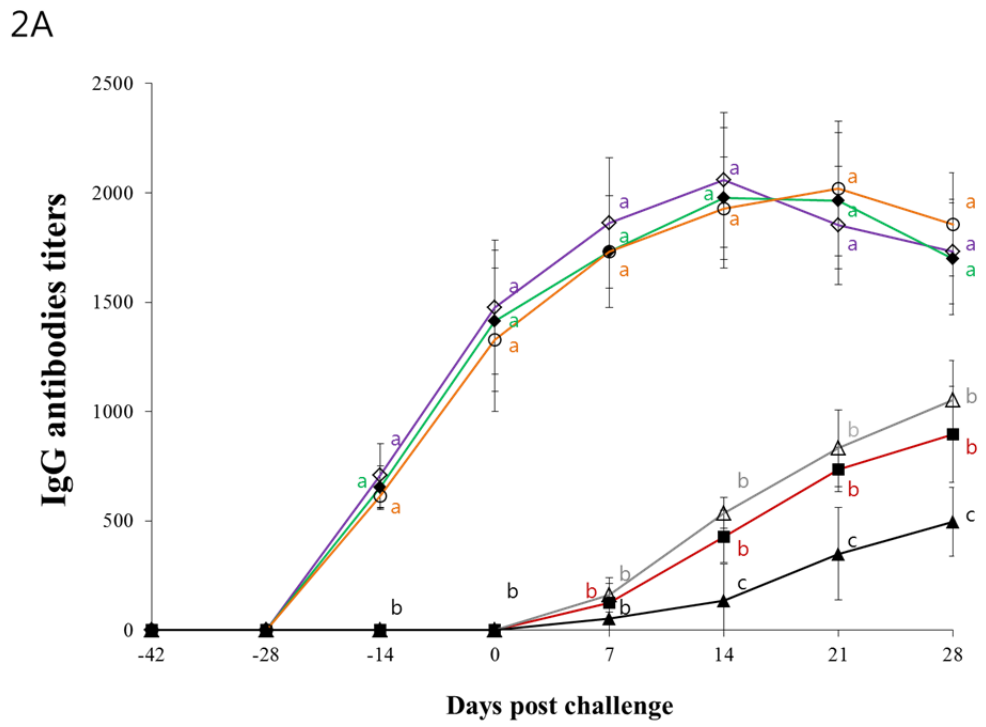
Fig. 1B. Mean values of the genomic copy numbers of *M. hyopneumoniae* DNA in the nasal swabs in the different groups; Pigs that received the *M. hyopneumoniae* vaccine followed by a challenge with only *M. hyopneumoniae* (group 1, □), pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, ■), pigs that received the PCV2 vaccine followed by a PCV2-only challenge (group 3, ◇), pigs that received the PCV2 vaccine followed by a dual challenge (group 4, ◆), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5, ○), unvaccinated pigs challenged only with *M. hyopneumoniae* (group 6, ●), unvaccinated pigs challenged only with PCV2 (group 7, ∆), and pigs that received a dual challenge (group 8, ▲). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.



3.3. Anti-PCV2 IgG antibodies

The results of anti-PCV2 IgG antibodies are summarized in **Fig. 2A**. Pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5), and pigs that received the PCV2 vaccine only (group 10) had significantly higher anti-PCV2 IgG antibody levels than did non-PCV2-vaccinated pigs that were challenged with PCV2 (groups 2, 7, and 8) from 0 to 28 dpc ($P < 0.001$). No anti-PCV2 IgG antibodies were detected in pigs from groups 9 and 11.

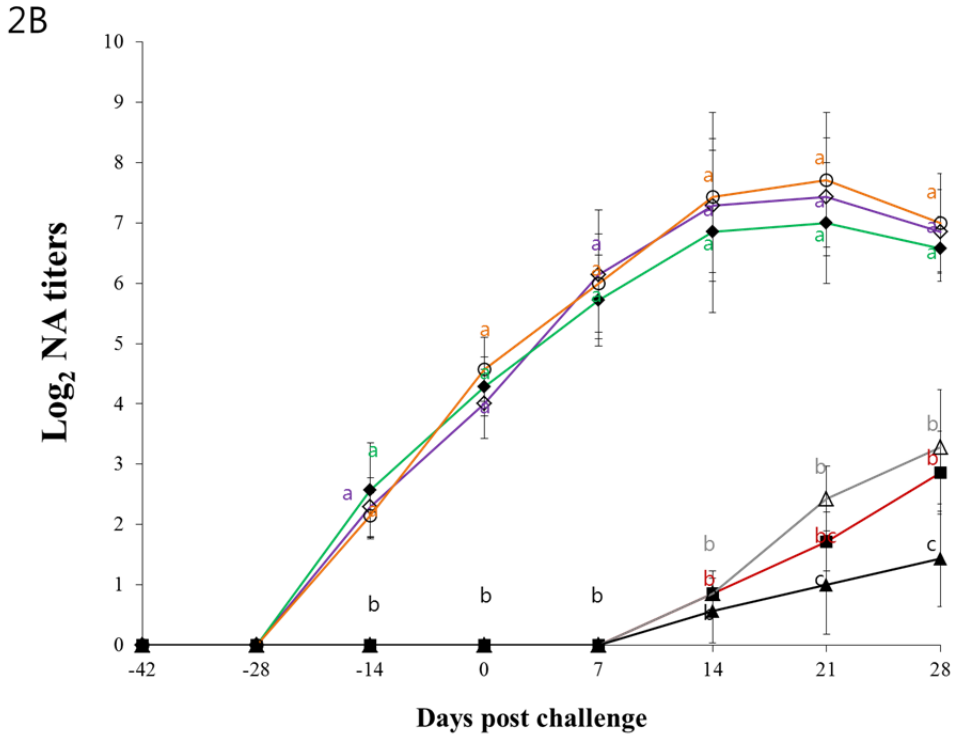
Fig. 2A. Mean values of the anti-PCV2 IgG antibody levels.; Pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, ■), pigs that received the PCV2 vaccine followed by a PCV2-only challenge (group 3, ◇), pigs that received the PCV2 vaccine followed by a dual challenge (group 4, ◆), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5, ○), unvaccinated pigs challenged only with PCV2 (group 7, △), and pigs that received a dual challenge (group 8, ▲). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.



3.4. PCV2-specific neutralizing antibodies

The results of PCV2-specific neutralizing antibodies (NA) are summarized in **Fig. 2B**. Pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), and pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5), and pigs that received the PCV2 vaccine only (group 10) had significantly higher PCV2-specific NA titers than did non-PCV2-vaccinated pigs that were challenged with PCV2 (groups 2, 7, and 8) at 14, 21 and 28 dpc ($P < 0.05$). No PCV2-specific NA titers were detected in pigs from groups 9 and 11.

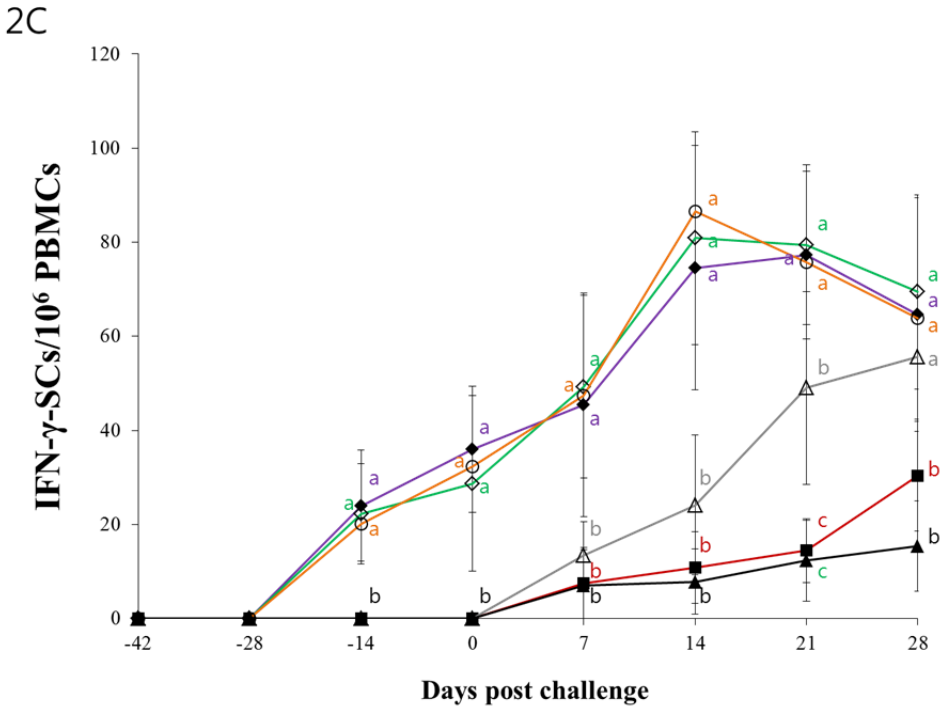
Fig. 2B. Mean values of the PCV2-specific serum neutralizing antibodies (NA) titers.; Pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, ■), pigs that received the PCV2 vaccine followed by a PCV2-only challenge (group 3, ◇), pigs that received the PCV2 vaccine followed by a dual challenge (group 4, ◆), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5, ○), unvaccinated pigs challenged only with PCV2 (group 7, ∆), and pigs that received a dual challenge (group 8, ▲). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.



3.5. PCV2-specific interferon-gamma-secreting cells

The results of PCV2-specific IFN-γ-SCs are summarized in **Fig. 2C.** Pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), and pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5) had a significantly higher numbers of PCV2-specific IFN-γ-SCs than did non-PCV2-vaccinated pigs that were challenged with PCV2 (groups 2, 7, and 8) at -14, 0, 7, 14, 21, and 28 dpc ($P < 0.05$). No PCV2-specific IFN-γ-SCs were detected in pigs from groups 9 and 11.

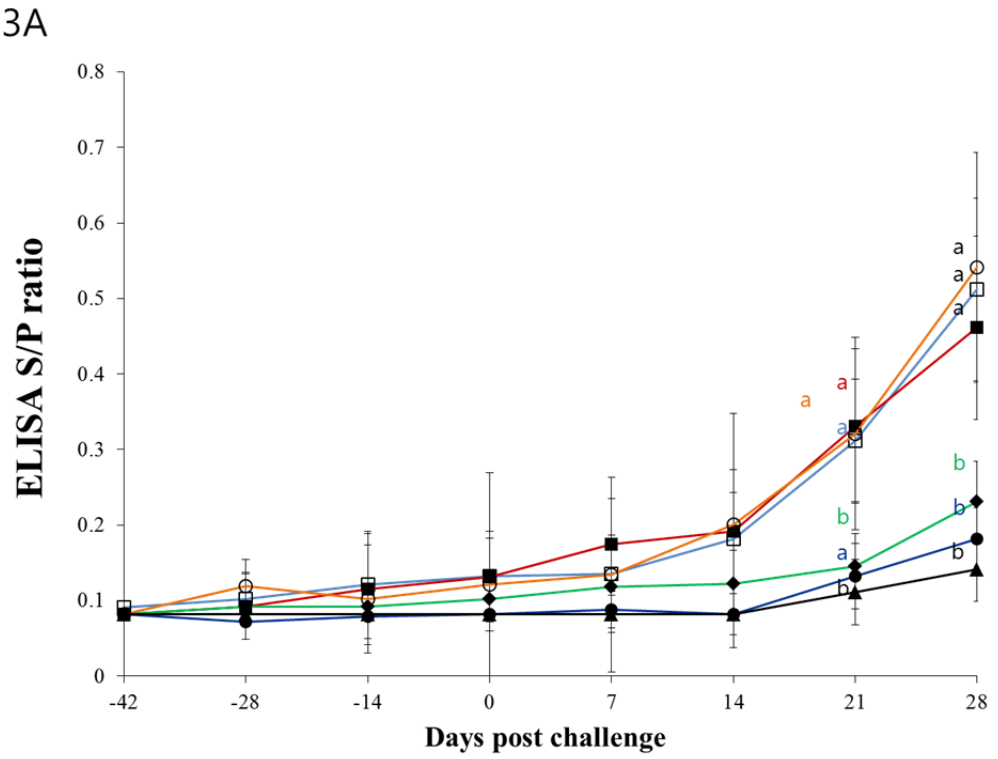
Fig. 2C. Mean number of PCV2-specific IFN-γ-SCs in the different groups; Pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, ■), pigs that received the PCV2 vaccine followed by a PCV2-only challenge (group 3, ◇), pigs that received the PCV2 vaccine followed by a dual challenge (group 4, ◆), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5, ○), unvaccinated pigs challenged only with PCV2 (group 7, Δ), and pigs that received a dual challenge (group 8, ▲). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.



3.6. Anti-M. hyopneumoniae IgG antibodies

The results of anti-*M. hyopneumoniae* IgG antibodies are summarized in **Fig. 3A**. Each pig in all 11 groups was seronegative for *M. hyopneumoniae* at 21 dpc. Pigs that received the *M. hyopneumoniae* vaccine followed by a challenge with either *M. hyopneumoniae* alone (group 1) or a dual challenge (group 2), and pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5) had significantly higher anti-*M. hyopneumoniae* IgG antibody levels than did pigs in groups 4, 6, and 8 at 21 and 28 dpc ($P < 0.05$). No anti-*M. hyopneumoniae* IgG antibodies were detected in pigs from groups 10 and 11.

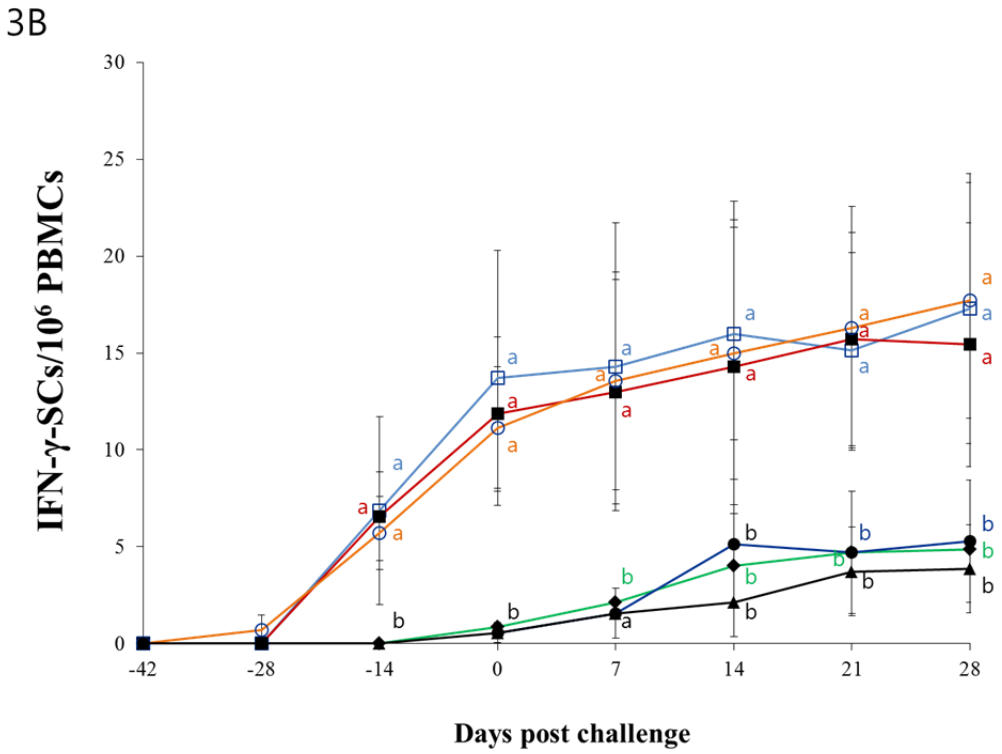
Fig. 3A. Mean values of the anti-*M. hyopneumoniae* IgG antibody levels.; Pigs that received the *M. hyopneumoniae* vaccine followed by a challenge with only *M. hyopneumoniae* (group 1, □), pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, ■), pigs that received the PCV2 vaccine followed by a dual challenge (group 4, ◆), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5, ○), unvaccinated pigs challenged only with *M. hyopneumoniae* (group 6, ●), unvaccinated pigs that received a dual challenge (group 8, ▲). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.



3.7. M. hyopneumoniae-specific interferon-γ-secreting cells

The results of *M. hyopneumoniae*-specific IFN-γ-SCs are summarized in **Fig 3B**. Pigs that received the *M. hyopneumoniae* vaccine followed by a challenge with either *M. hyopneumoniae* alone (group 1) or by a dual challenge (group 2), and pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5) had a significantly a higher numbers of *M. hyopneumoniae*-specific IFN-γ-SCs than did non-*M. hyopneumoniae*-vaccinated pigs that were challenged with *M. hyopneumoniae* (groups 4, 6, and 8) at from -14 to 28 dpc ($P < 0.05$). Pigs that received the *M. hyopneumoniae* vaccine only (group 9) had a significantly a higher numbers of *M. hyopneumoniae*-specific IFN-γ-SCs than did non-*M. hyopneumoniae*-vaccinated pigs that were challenged with *M. hyopneumoniae* (groups 4, 6, and 8) at -14, 0, and 7 dpc. No *M. hyopneumoniae*-specific IFN-γ-SCs were detected in pigs from groups 10 and 11.

Fig. 3B. Mean number of *M. hyopneumoniae*-specific IFN-γ-SCs in the different groups; Pigs that received the *M. hyopneumoniae* vaccine followed by a challenge with only *M. hyopneumoniae* (group 1, □), pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, ■), pigs that received the PCV2 vaccine followed by a dual challenge (group 4, ◆), pigs that received both the *M. hyopneumoniae* and PCV2 vaccines followed by a dual challenge (group 5, ○), unvaccinated pigs challenged only with *M. hyopneumoniae* (group 6, ●), unvaccinated pigs that received a dual challenge (group 8, ▲). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference between groups.



3.8. Lung lesion scores

Lung lesion scores are summarized in **Table 2**. Gross lung lesions were observed in the vaccinated challenged (groups 1, 2, 3, 4, and 5) and unvaccinated challenged (groups 6, 7, and 8) groups, including varying degrees of red-to-purple consolidation in the lung tissues. Pigs that received the PCV2 vaccine followed by a dual challenge (group 4) had significantly ($P < 0.05$) higher gross lung lesion scores than did pigs in the other 4 groups (1, 3, 5, and 7). No gross lung lesions were observed in pigs in the vaccinated unchallenged (groups 9 and 10) and negative control (group 11) groups.

Table 2. Scores (mean±standard deviation) of gross lung lesions, histopathologic lesions, *M. hyopneumoniae* (Mhp) DNA, and PCV2 antigen in different groups at 28 days post challenge (dpc).

Groups	Gross lung lesions	Histopathology			PCV2 Antigen		Mhp DNA
		Lung		Lymph node	Lung	Lymph node	Lung
		Mycoplasmal pneumonia*	Interstitial pneumonia				
1	3.69±1.44^{a}	0.43±0.53^{a}	0.29±0.48^{ab}	0^{a}	0^{a}	0^{a}	0.29±0.48^{a}
2	4.50±1.93^{ab}	0.71±0.65^{a}	0.86±0.72^{bc}	1.71±0.49^{cd}	11.43±4.76^{c}	20.14±7.10^{d}	0.43±0.53^{a}
3	0.88±0.83^{a}	0.14±0.37^{a}	0.43±0.53^{ab}	0.43±0.53^{ab}	2.57±3.26^{ab}	3.00±3.91^{ab}	0^{a}
4	11.875±6.69^{cd}	2.00±0.57^{b}	1.29±0.95^{c}	0.57±0.78^{ab}	6.43±5.47^{bc}	6.14±4.70^{b}	1.29±0.75^{b}
5	3.25±1.58^{a}	0.57±0.53^{a}	0.57±0.53^{abc}	0.57±0.53^{ab}	3.14±3.29^{ab}	4.29±4.53^{ab}	0.43±0.53^{b}
6	10.13±2.75^{bc}	2.29±0.48^{b}	1.28±0.48^{c}	0^{a}	0^{a}	0^{a}	1.57±0.78^{b}
7	1.0±1.20^{a}	0.43±0.53^{a}	0.71±0.76^{bc}	1.00±0.58^{bc}	10.43±4.28^{c}	14.29±5.88^{c}	0^{a}
8	16.75±7.83^{d}	2.57±0.53^{b}	2.29±0.49^{d}	2.43±0.53^{d}	19.85±7.86^{d}	24.57±8.06^{d}	1.86±1.06^{b}
9	0.25±0.46^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
10	0.31±0.59^{a}	0^{a}	0.25±0.48^{a}	0^{a}	0^{a}	0^{a}	0^{a}
11	0.38±0.52^{a}	0.14±0.37^{a}	0.14±0.37^{a}	0^{a}	0^{a}	0^{a}	0^{a}

*peribronchiolar and perivascular lymphoid tissue hyperplasia.

Different letters (a, b, c, and d) indicate that the groups are significantly ($P < 0.05$) different from each other.

3.9. Histopathologic lesion scores

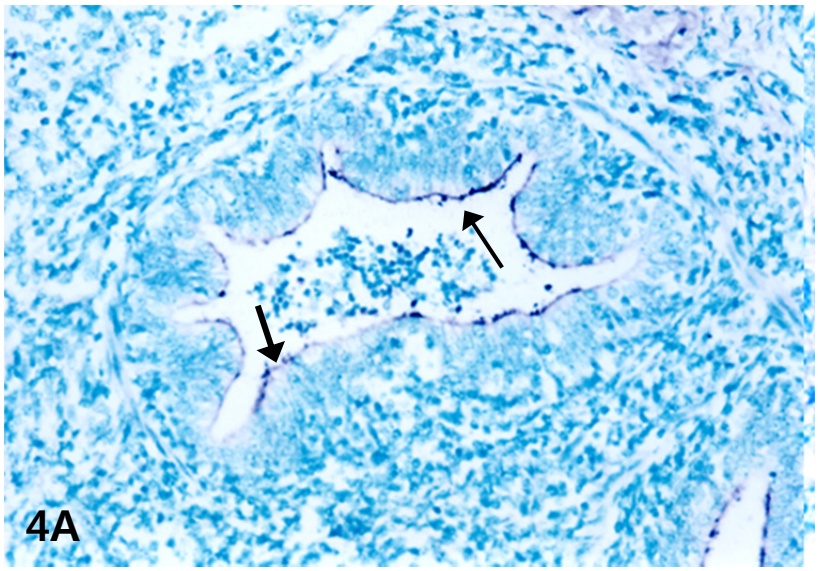
The results of lymphoid and pulmonary lesion scores are summarized in **Table 2.** The typical granulomatous inflammatory reaction and lymphoid depletion that is associated with PCV2 infection in pigs and is consistent with the histopathologic lesions in PCVAD were observed in the lymph nodes from dually challenged pigs (group 8). Pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2) had significantly ($P < 0.05$) higher lymphoid lesion scores than did pigs in the other 4 groups (3, 4, 5 and 6).

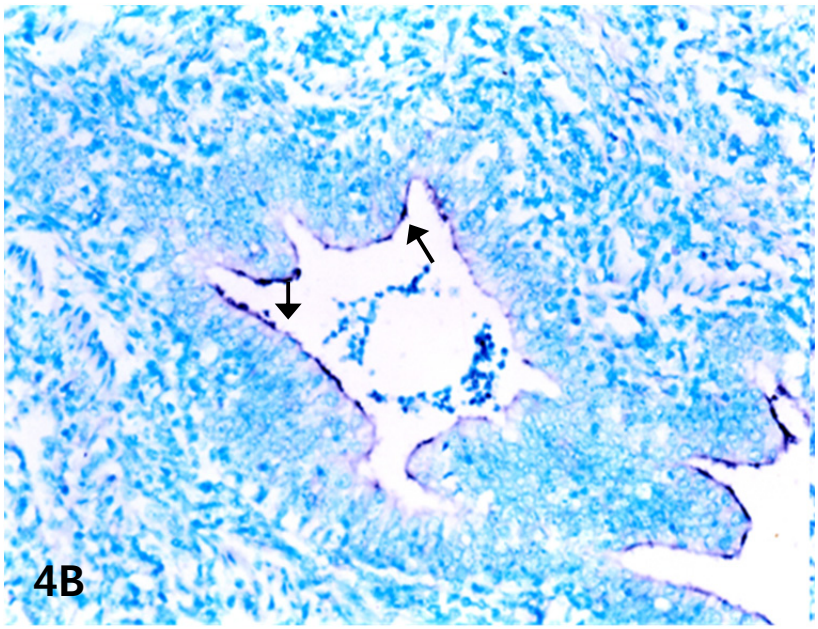
The pulmonary histopathologic lesions were characterized by moderate peribronchiolar lymphoid tissue hyperplasia and thickened alveolar septa (interstitial pneumonia) in the dually challenged pigs (group 8). Pigs that received the PCV2 vaccine followed by a dual challenge (group 4) had significantly ($P < 0.05$) higher lesion scores for peribronchiolar lymphoid tissue hyperplasia than did pigs in the other 4 groups (1, 3, 5, and 7) (**Table 2**).

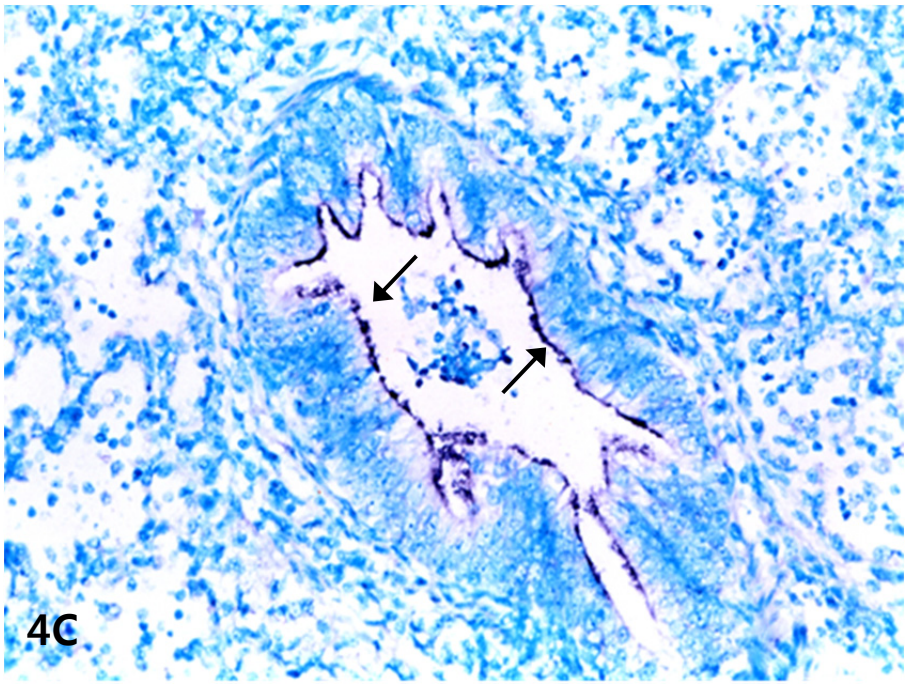
3.10. Immunohistochemistry of PCV2 antigen

The results of immunohistochemistry of PCV2 antigen are summarized in **Table 2**. PCV2 antigen was detected in lymph nodes and lungs from PCV2-challenged pigs [groups 2 (**Fig. 4A**), 3, 4, 5, 7 (**Fig. 4B**), and 8 (**Fig. 4C**)]. Pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2) had a significantly ($P < 0.05$) higher number of PCV2-positive cells per unit tissue in their lymph nodes than did pigs in groups 3, 4, 5, and 7, as well as a higher number of PCV2-positive cells per unit tissue in their lungs than did pigs in groups 3 and 5 (**Table 2**).

Fig. 4. Immunohistochemistry of PCV2. Positive signals (arrows) were detected in the macrophages in the lungs from different groups; pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge (group 2, **4A**), unvaccinated pigs challenged only with PCV2 (group 7, **4B**), and pigs that received a dual challenge (group 8, **4C**).



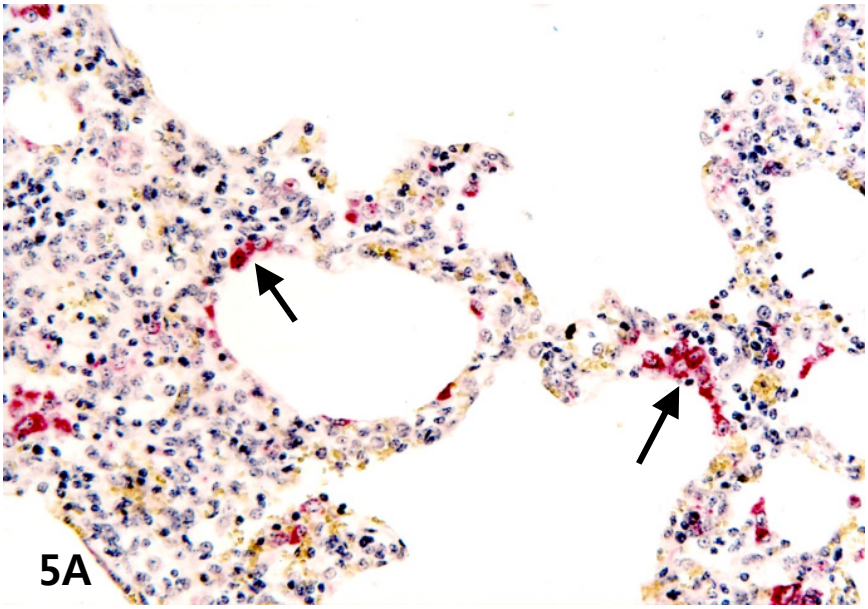


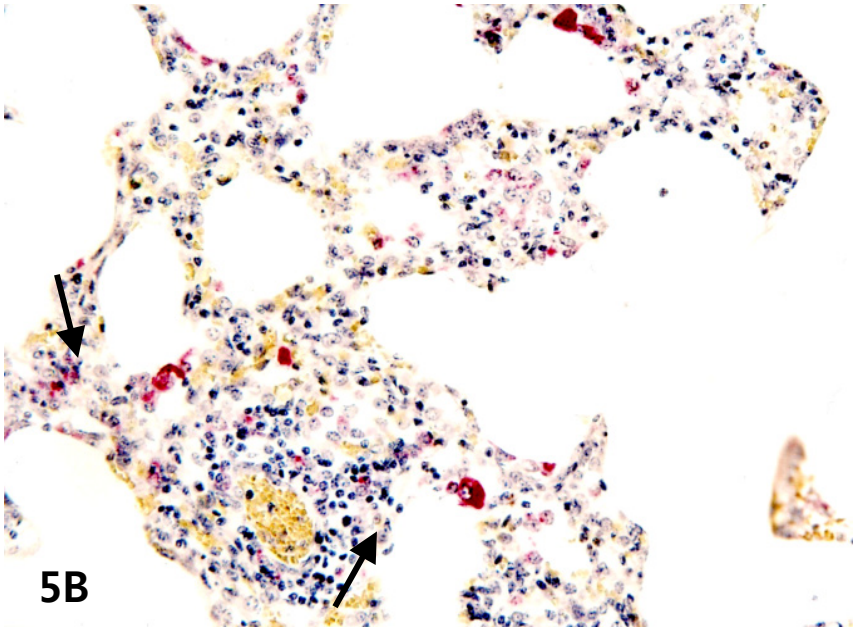

4C

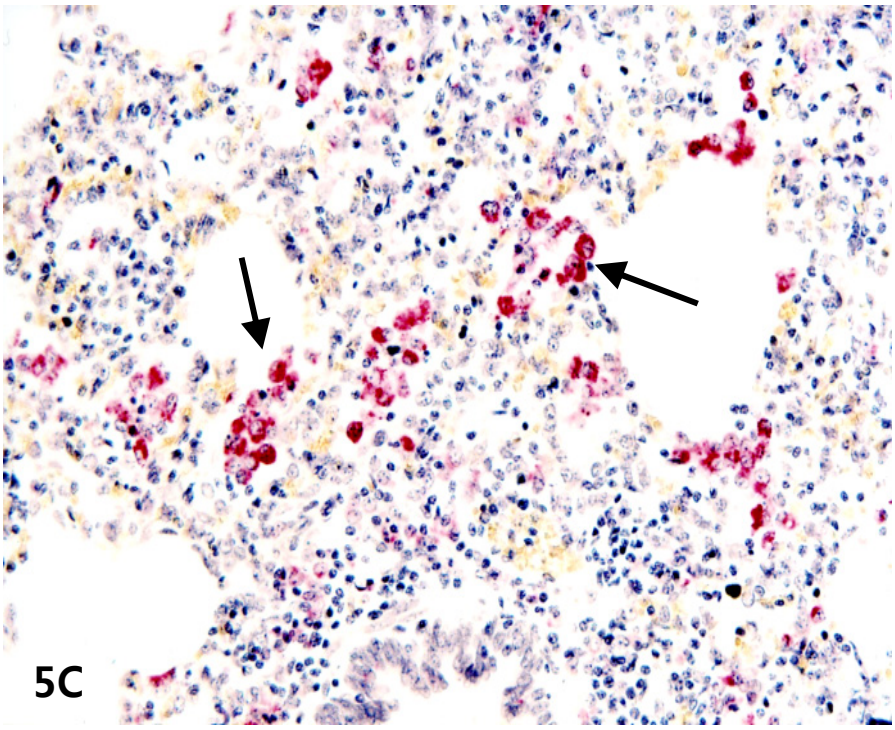
3.11. In situ hybridization of M. hyopneumoniae DNA

The results of in situ hybridization of *M. hyopneumoniae* DNA are summarized in **Table 2.** *M. hyopneumoniae* DNA was associated with the surface of epithelial cells of bronchi and bronchioli in lungs from pigs challenged with *M. hyopneumoniae* [groups 1, 2, 4 (**Fig. 5A**), 5, 6 (**Fig. 5B**), and 8 (**Fig. 5C**)]. Unvaccinated dually challenged pigs (group 8), unvaccinated pigs challenged only with *M. hyopneumoniae* (group 6), and pigs that received the PCV2 vaccine followed by a dual challenge (group 4) had a significantly ($P < 0.05$) higher number of *M. hyopneumoniae*-positive cells per unit tissue in their lungs than did pigs in groups 1, 2, and 5 (**Table 2**).

Fig. 5. In situ hybridization of *M. hyopneumoniae*. Positive signals (arrows) were detected in the surface of bronchiolar epithelium in the lungs from different groups; pigs that received the PCV2 vaccine followed by a dual challenge (group 4, **5A**), unvaccinated pigs challenged only with *M. hyopneumoniae* (group 6, **5B**), and pigs that received a dual challenge (group 8, **5C**).






5C

4. Discussion

In this study, dually challenged pigs had significantly increased PCV2-viremia, more severe PCV2-induced pulmonary and lymphoid lesions, and higher levels of lesion-associated PCV2 antigen than did pigs that were challenged only with PCV2. These results are in agreement with previous findings where *M. hyopneumoniae* potentiates the severity of PCV2-associated lesions and PCV2 antigen levels within the lesions in pigs [4]. In contrast, dually challenged pigs did not significantly potentiate the nasal shedding of *M. hyopneumoniae* or the *M. hyopneumoniae*-induced pulmonary lesions compared with pigs that were challenged only with *M. hyopneumoniae*. Enhancement of PCV2 replication by *M. hyopneumoniae* is clinically significant because the clinical signs of PCVAD are dependent on the levels of PCV2 viremia [23, 24]. Although it may vary from laboratory to laboratory based on the standards used for quantification, the PCV2 load in the blood, as quantified by real-time PCR, is used to categorize PCV2-infected pigs as subclinically infected ($< 10^6$ DNA copies/mL), suspected (10^6-10^7 DNA copies/mL) and PCVAD-positive ($>10^7$ DNA copies/mL) [25-27].

Given that *M. hyopneumoniae* exacerbates PCV2-induced disease, one possible way to minimize the effect of the *M. hyopneumoniae*-associated enhancement of PCV2 replication may be the use of a *M. hyopneumoniae*-based vaccine for pigs in a PCV2/*M. hyopneumoniae*-co-infected herd. Interestingly, however, in this study, vaccination with *M. hyopneumoniae* alone did not reduce the PCV2 viremia or the PCV2-associated lymphoid lesions in pigs that were dually challenged. Although our experimental condition cannot be generalized as such to co-infected herds, a vaccination with *M.*

hyopneumoniae alone may not be sufficient for reducing PCV2 viremia, PCV2-associated lung and lymphoid lesions, or lesion-associated PCV2 antigen in pigs from PCV2/*M. hyopneumoniae*-co-infected herds. Moreover, the types of strains used, the infection doses, the timing of infection, the types of vaccines may also largely influence the outcome.

The induction of protective immunity by a PCV2 vaccination results in reduced PCV2 viremia [24, 28-30]. In this study, PCV2 vaccination elicits PCV2-specific NA and IFN-γ-SCs, even in pigs that received the PCV2 vaccine followed by a dual challenge. These results provide swine producers and practitioners with clinically significant information. *M. hyopneumoniae* is highly prevalent (ranging between 38% and 100%) in almost all swine production areas worldwide [2] and has a modulating effect on the immune system [31]. Nevertheless, the efficacy of PCV2 vaccines may not be affected by a subsequent infection with *M. hyopneumoniae*.

Vaccination is still considered the most effective tool for controlling infection by *M. hyopneumoniae* although mycoplasmal infections can be controlled by other practices such as improved management, pig flow, biosecurity measures, and housing conditions, as well as the use of antibiotics [2, 32]. Cell-mediated immunity induced by vaccination is important for the control of mycoplasmal pneumonia in pigs [18, 33]. In this study, a single vaccination against *M. hyopneumoniae* induced *M. hyopneumoniae*-specific IFN-γ-SCs, even in pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge. Although PCV2 is able to hamper the development of immune responses by suppressing Th1 responses [34], the efficacy of *M. hyopneumoniae*

vaccines do not appear to be affected by subsequent infection with PCV2 and *M. hyopneumoniae*. These results suggest *M. hyopneumoniae* vaccines may be effective under field conditions where PCV2 is widespread in the swine population.

There are two different co-infection models (sequential vs. concurrent infection) to determine the interaction between PCV2 and *M. hyopneumoniae* [4, 5]. Under Korean field conditions, *M. hyopneumoniae* from nasal swabs is most commonly detected in pigs that are 4-7 weeks old while PCV2 from sera samples is most commonly detected in pigs that are 6-9 weeks old, based on diagnostic samples from Seoul National University (C. Chae, personal observation). Therefore, the sequential infection model rather than the concurrent infection model more closely mimics the Korean field situations. The results of this study provide swine producers and practitioners with efficient vaccination regimens for controlling PCV2 and *M. hyopneumoniae* infections in co-infected swine herds.

References

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

[2] Simionatto S, Marchioro SB, Maes D, Dellagostin OA., 2013. *Mycoplasma hyopneumnoniae*: From disease to vaccine development. Veterinary microbiology 165, 234-242.

[3] Kim J, Chung HK, Chae C., 2003. Association of porcine circovirus 2 with porcine respiratory disease complex. The Veterinary Journal 166, 251-256.

[4] Opriessnig T, Thacker EL, Yu S, Fenaux M, Meng X-J, Halbur PG., 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. Veterinary Pathology 41, 624-640.

[5] Sibila M, Fort M, Nofrarias M, Perez de Rozas A, Galindo-Cardiel I, Mateu E, et al., 2012. Simultaneous porcine circovirus type 2 and *Mycoplasma hyopneumoniae* co-inoculation does not potentiate disease in conventional pigs. Journal of Comparative Pathology 147, 285-295.

[6] Zhang H, Lunney JK, Baker RB, Opriessnig T., 2011. Cytokine and chemokine mRNA expression profiles in tracheobronchial lymph nodes from pigs singularly infected or coinfected with porcine circovirus type 2 and *Mycoplasma hyopneumoniae*. Veterinary Immunology and Immunopathology 140, 152-158.

[7] Allan GM, McNeilly F, McNair I, O'Connor M, Meehan B, Gilpin D, et al., 2001. Neonatal vaccination for *Mycoplasma hyopneumoniae* and post-weaning multisystemic wasting syndrome: a field trial. Pig Journal 48, 34-41.

[8] Kyriakis SC, Saoulidis K, Lekkas S, Miliotis CC, Papoutsis PA, Kennedy S., 2002. The effect of immune-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. Journal of Comparative Pathology 126, 38-46.

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

[10] Wasilk A, Callahan JD, Christopher-Hennings J, Gay TA, Fang Y, Dammen M, et al. 2004. Detection of U.S., Lelystad, and European-like porcine reproductive and respiratory syndrome viruses and relative quantitation in boar semen and serum

samples by real-time PCR. Journal of Clinical Microbiology 42, 4453-4461.

[11] Dubosson CR, Conzelmann C, Miserez R, Boerlin P, Frey J, Zimmermann W, et al. 2004. Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples. Veterinary microbiology 102, 55-65.

[12] Kim D, Kim CH, Han K, Seo HW, Oh Y, Park C, et al., 2011. Comparative efficacy of commercial *Mycoplasma hyopneumoniae* and porcine circovirus 2 (PCV2) vaccines in pigs experimentally infected with *M. hyopneumoniae* and PCV2. Vaccine 29, 3206-3212.

[13] Beaver BV, Reed W, Leary S, McKiernan B, Bain F, Schultz R, et al., 2001. 2000 Report of the AVMA panel on euthanasia. Journal of the American Veterinary Medical Association 218, 669-696.

[14] Pogranichnyy RM, Yoon KJ, Harms PA, Swenson SL, Zimmerman JJ, Sorden SD., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunology 13, 143-153.

[15] Park C, Oh Y, Seo HW, Han K, Chae C., 2013. Comparative effects of vaccination against porcine circovirus type 2 (PCV2) and porcine reproductive and

respiratory syndrome virus (PRRSV) in a PCV2-PRRSV challenge model. Clinical and Vaccine Immunology 20, 369-376.

[16] Bandrick M, Pieters M, Pijoan C, Molitor TW., 2008. Passive transfer of maternal *Mycoplasma hyopneumoniae*-specific cellular immunity to piglet. Clinical and Vaccine Immunology 15, 540-543.

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

[18] Thacker EL, Thacker BJ, Kuhn M, Hawkins PA, Waters WR., 2000. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. American Journal of Veterinary Research 61, 1384-1389.

[19] Kwon D, Chae C., 1999. Detection and localization of *Mycoplasma hyopneumoniae* DNA in lungs from naturally infected pigs by in situ hybridization using a digoxigenin-labeled probe. Veterinary Pathology 36, 308-313.

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

[21] Straw BE, Backstrom L, Leman AD., 1986. Examination of swine at slaughter. Part II. Findings at slaughter and their significance. Compendium on Continuing Education for the Practising Veterinarian 8, S106-122.

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

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

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

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

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

[27] Opriessnig T, Patterson AR, Madson DM, Pal N, Halbur PG., 2009. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine 27, 1002-1007.

[28] Fort M, Fernandes LT, Nofrarias M, Diaz I, Sibila M, Pujols J, et al., 2009. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. Veterinary Immunology and Immunopathology 129, 101-107.

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

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

[31] Rodriguez F, Ramirez GA, Sarradell J, Andrada M, Lorenzo H., 2004. Immunohistochemical labeling of cytokines in lung lesions of pigs naturally infected with *Mycoplasma hyopneumoniae*. Journal of Comparative Pathology 130, 306-312.

[32] Mateusen B, Maes D, Van Goubergen M, Verdonck M, de Kruif A., 2002. Effectiveness of treatment with linocomycin hydrochloride and/or vaccination against *Mycoplasma hyopneumoniae* for controlling chronic respiratory disease in a herd of pigs. Veterinary Record 151,135-140.

[33] Djordjevic SP, Eamens GJ, Romalis LF, Nicholls PJ, Taylor V, Chin J., 1997. Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing a denatured membrane antigen pool and adjuvant. Australian Veterinary Journal 75, 504-511.

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

PART II.

Interaction between single-dose *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus vaccines on dually infected pigs

SJ Park, HW Seo, C Park, C Chae, 2014.
Research in Veterinary Science, 96, 516-522

Abstract

The objective of this study was to determine the effects of *Mycoplasma hyopneumoniae* and/or Porcine reproductive and respiratory syndrome virus (PRRSV) vaccination on dually infected pigs. In total, 72 pigs were randomly divided into nine groups (eight pigs per group), as follows: five vaccinated and challenged groups, three non-vaccinated and challenged groups, and a negative control group. Single-dose vaccination against *M. hyopneumoniae* alone decreased the levels of PRRSV viremia and PRRSV-induced pulmonary lesions, whereas single-dose vaccination against PRRSV alone did not decrease nasal shedding of *M. hyopneumoniae* and mycoplasma-induced pulmonary lesions in the dually infected pigs. *M. hyopneumoniae* challenge impaired the protective cell-mediated immunity induced by the PRRSV vaccine, whereas PRRSV challenge did not impair the protective cell-mediated immunity induced by the *M. hyopneumoniae* vaccine. The present study provides swine practitioners and producers with efficient vaccination regimes; vaccination against *M. hyopneumoniae* is the first step in protecting pigs against co-infection with *M. hyopneumoniae* and PRRSV.

1. Introduction

Porcine respiratory disease complex (PRDC) is a serious respiratory disease in most pig-raising countries and is caused by multiple pathogens. Among these pathogens, *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus (PRRSV) are two of the pathogens that are commonly isolated from pigs suffering from PRDC and are two of the major contributors to this disease condition [21].

The relationship between *M. hyopneumoniae* and PRRSV is well known. *M. hyopneumoniae* potentiates pneumonia induced by PRRSV, whereas PRRSV does not potentiate pneumonia induced by *M. hyopneumoniae* [22, 18, 19]. In addition, two-dose vaccination against *M. hyopneumoniae* decreases the potentiation of PRRSV-induced pneumonia on dually infected pigs [19]. However, previous studies were limited to determining the microbiological (viremia and nasal shedding) and immunological IFN-γ-SC effects of PRRSV (or *M. hyopneumoniae*) infections in pigs that received only a *M. hyopneumoniae* (or PRRSV) vaccine and were then dually challenged.

Currently, a single-dose *M. hyopneumoniae* bacterin-based vaccine is widely used in pig production worldwide. Nevertheless, no studies have evaluated the effect of single-dose vaccination against *M. hyopneumoniae*, PRRSV, or both on dually infected pigs. Therefore, the objective of the present study was to determine the effects of a single-dose *M. hyopneumoniae* (or PRRSV) vaccine on dually challenged pigs based on microbiological (PRRSV viremia and nasal shedding of *M. hyopneumoniae*), immunological (anti-IgG antibodies and IFN-γ-SC), and pathological outcomes.

2. Materials and methods

2.1. Commercial vaccines

The inactivated *M. hyopneumoniae* vaccine (RespiSure-One, Zoetis, Medison, NJ, USA) and modified live PRRSV vaccine (Fostera PRRS, Zoetis) were used in this study. The *M. hyopneumoniae* vaccine is an inactivated whole cell culture of *M. hyopneumoniae*, coupled with an oil adjuvant. The modified live PRRSV vaccine is based on a virulent US PRRS isolate (P129) attenuated using CD163 expressing cell lines. All of the vaccines that were used in this study were administered according to the manufacturer's instruction (one dose, via intramuscular route).

2.2. Animals

A total of 72 colostrum-fed, cross-bred, conventional piglets were weaned and purchased at six days of age from a PRRSV-free commercial farm. They were all negative for porcine circovirus type 2 (PCV2), PRRSV, swine influenza virus and *M. hyopneumoniae* according to routine serological testing. *M. hyopneumoniae* and PRRSV were not detected in the nasal and serum samples, respectively, by the real-time polymerase chain reaction (PCR) [6, 23]. Individual piglets from seven days of age were uniquely identified by their ear notches.

2.3. Experimental design

In total, 72 pigs were randomly divided into nine groups (eight pigs per group), as follows: five vaccinated challenged (VC) groups, three unvaccinated challenged (UVC)

groups, and one unvaccinated unchallenged group (**Table 1**). At seven days of age (-42 days post challenge, dpc), the pigs in Groups 1, 2, and 5 were injected intramuscularly in the right side of the neck with 2.0 ml of the *M. hyopneumoniae* vaccine. At 21 days of age (-28 dpc), the pigs in Groups 3, 4, and 5 were injected intramuscularly in the left side of the neck with 2.0 ml of the PRRSV vaccine. An equal volume of phosphate-buffered saline (PBS) (2.0 ml) was injected in the same anatomical location in the positive (Groups 6, 7, and 8) and negative (Group 9) control pigs at 7 and 21 days of age.

At 49 days of age (0 dpc), pigs in the some of the VC (Groups 1, 2, 4, and 5) and UVC (Groups 6 and 8) groups were intratracheally administered a 10-ml dose of a frozen lung homogenate of *M. hyopneumoniae* strain SNU98703 (1:100 dilution in Friis medium) at a final concentration of 10^4-10^5 color-changing units (CCU)/ml in the morning, as previously described [9]. In the afternoon of the same day, the pigs in some of the VC (Groups 2, 3, 4, and 5) and UVC (Groups 7 and 8) groups were intranasally administered a 2-ml dose of PRRSV (strain SNUVR090851; 5th passage in MARC-145 cells) containing 1.2 x 10^5 tissue culture infective dose of 50% ($TCID_{50}$)/ml (**Table 1**).

Blood samples and nasal swabs were collected at -42, -28, 0, 7, 14, 21, and 28 dpc. The pigs from each group were sedated by intravenous injection of sodium pentobarbital and then euthanized by electrocution at 14 and 28 dpc. Tissues were collected from each pig at necropsy. All of the methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

2.4. Quantification of M. hyopneumoniae DNA in nasal swabs

DNA was extracted from the nasal swabs using the QIAamp DNA Mini Kit (QIAGEN Ltd, Crawley, UK). The DNA extracts were used to quantify the *M. hyopneumoniae* genomic DNA copy numbers by real-time PCR as previously described (Dubosson et al., 2004). Real-time PCR was performed with primers based on the putative ABC transporter (GenBank no. U02537) [6].

2.5. Quantification of PRRSV RNA

RNA was extracted from serum samples and nasal swabs to quantify North American PRRSV genomic cDNA copy numbers, as previously described [23].

2.6. Serology

The serum samples were tested using the commercially available *M. hyopneumoniae* and PRRSV enzyme-linked immunosorbent assay (ELISA; IDEXX M.hyo Ab TEST and PRRS X3 Ab Test, IDEXX Laboratories Inc., Westbrook, Maine, USA). Serum samples were considered positive for *M. hyopneumoniae* and PRRSV antibody if the S/P ratio was greater than 0.4 according to the manufacturer's instructions.

2.7. Preparation of M. hyopneumoniae antigen

M. hyopneumoniae antigen was prepared as previously described [1]. Briefly, *M. hyopneumoniae* (strain SNU98703) was cultured in Friis' medium. When the pH of the culture reached 6 or lower, the organisms were harvested by continuous-flow

centrifugation at 70,000*g*. The harvested *M. hyopneumoniae* was resuspended in Tris-sodium chloride (TN) buffer (pH 7.2 to 7.4) in 1/100 of the original volume of the culture and washed three times by centrifugation, each with the same proportion of TN buffer. The washed *M. hyopneumoniae* was inactivated by one freeze-thaw cycle and then b sonic disruption *M. hyopneumoniae* was solubilized with 1% NP-40 and the antigen concentration was adjusted to 4 mg/ml.

2.8. Preparation of PRRSV antigens

The same PRRSV strain that was used for the challenge in the pigs was propagated in MARC-145 cells to a titer of 10^4 $TCID_{50}$/ml and treated with two freeze-thaw cycles. The PRRSV antigen was prepared by concentrating the virus that was present in the cell culture by ultracentrifugation at 100,000*g* at 4°C for 3 h. The virus pellet was resuspended with PBS. The concentrated PRRSV was inactivated by exposure to an 8 W germicidal UV lamp at a distance of 15 cm for 1 h. Inactivation was confirmed by the absence of the virus antigen from the MARC-145 cells as determined by an immunoperoxidase assay as previously described [2].

2.9. Enzyme-linked immunospot (ELISPOT) assay

The numbers of *M. hyopneumoniae*- and PRRSV-specific IFN-γ-SCs were determined in peripheral blood mononuclear cells (PBMCs) as previously described [12, 5]. Briefly, 100 µl containing 2×10^6 PBMCs in RPMI 1640 medium that was supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., SelectScience,

Bath, UK) was seeded into plates that were precoated overnight with anti-porcine IFN-γ monoclonal antibody (5 μg/ml, MABTECH, Mariemont, OH, USA) and incubated with 100 μl of *M. hyopneumoniae* antigen (20 μg/ml) and phytohemagglutinin (10 μg/ml, Roche Diagnostics GmbH, Mannheim, Germany) as positive controls or with PBS as a negative control for 40 h at 37°C in a 5% humidified CO_2 atmosphere. For PRRSV, the cells were stimulated with PRRSV in RPMI 1640 medium for 20 h at 37°C in 5% humidified CO_2; the linear response was tested between 0.01 and 0.1 multiplicity of infection. Then, the wells were washed five times with PBS (200 μl per well). Thereafter, the procedure was conducted according to the manufacturer's instructions using the commercial ELISPOT Assay Kit (MABTECH, Mariemont, OH, USA). 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 numbers of IFN-γ-SCs per million PBMCs.

2.10. In situ hybridization

In situ hybridization (ISH) for *M. hyopneumoniae* was performed as previously described [10].

2.11. Immunohistochemistry

Immunohistochemistry (IHC) for PRRSV was performed using SR30 monoclonal antibody (Rural Technologies Inc., Brookings, SD, USA) as previously described [8]. SR30 monoclonal antibody (Rural Technologies Inc.), capable of specifically

recognizing nucleocapsid protein of PRRSV, was diluted 1:10,000 in PBS (0.01M, pH 7.4) containing 0.1% Tween 20.

2.12. Morphometric analysis

Lung tissue sections were blindly examined by two veterinary pathologists (Park and Chae). Mycoplasmal pneumonia lesions were scored (0 to 4) based on the severity of peribronchiolar and perivascular lymphoid tissue hyperplasia as previously described (Thacker et al., 1999). PRRSV pneumonia lesions were score (0 to 6) based on the severity of interstitial pneumonia as previously described [18].

For the morphometric analyses of ISH and IHC, 3 sections were cut from each of three blocks of tissue from lung of each pig. The slides were analyzed using the NIH Image J 1.43m program (http://image.nih.gov/i/j/download.html) to obtain the quantitative data. For the analysis of *M. hyopneumoniae*, 10 fields were randomly selected and slides were scored ranging from 0 (no signal detectable) to 3 (intense labeling on the surface of several airway) as previously described [15]. For the analysis of PRRSV, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm^2) was determined as previously described [7]. The mean values were also calculated.

2.13. Statistical analysis

Summary statistics were calculated for all of the groups to assess the overall quality of the data, including normality. For single comparisons, an ANOVA with a post-hoc Tukey's test was used to compare the primary variables (ISH and IHC scores) among

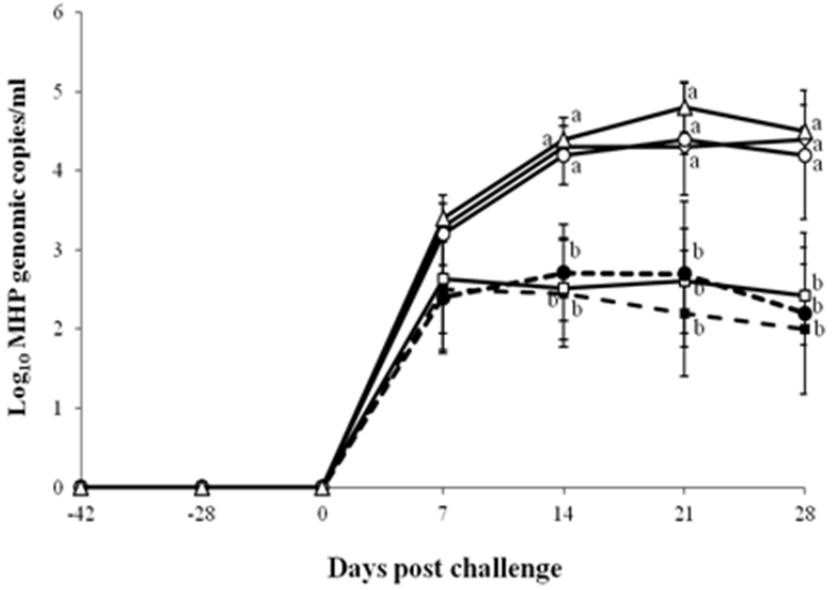
groups. The continuous data for the *M. hyopneumoniae* and PRRSV serology, *M. hyopneumoniae* DNA, and PRRSV RNA were analyzed using an ANOVA for each time point. When a one-way ANOVA revealed a significance of $P < 0.05$, the Tukey's Honestly Significant Difference test was used to determine the significance of individual between group differences. Discrete data (histopathological lung lesion scores) were analyzed by the Chi-square and Fisher's exact tests. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Quantification of M. hyopneumoniae DNA in nasal swabs

At the time of the challenges, no genomic copies of *M. hyopneumoniae* were detected in any of the serum samples from any of the 8 groups. The pigs that received the *M. hyopneumoniae* vaccine followed by only the *M. hyopneumoniae* challenge (Group 1), the pigs that received the *M. hyopneumoniae* vaccine followed by the dual challenge (Group 2), and the pigs that received the *M. hyopneumoniae* and PRRSV vaccines followed by dual challenge (Group 5) had a significantly ($P < 0.05$) lower number of genomic copies of *M. hyopneumoniae* in their nasal swabs than the pigs that received the PRRSV vaccine followed by dual challenge (Group 4), the pigs challenged with *M. hyopneumoniae* alone (Group 6), and dually challenged pigs (Group 8) at 14, 21, and 28 dpc (**Fig. 1**). No genomic copies of *M. hyopneumoniae* were detected in any of the serum samples from non-challenged pigs (Groups 3, 7, and 9) throughout the experimental period.

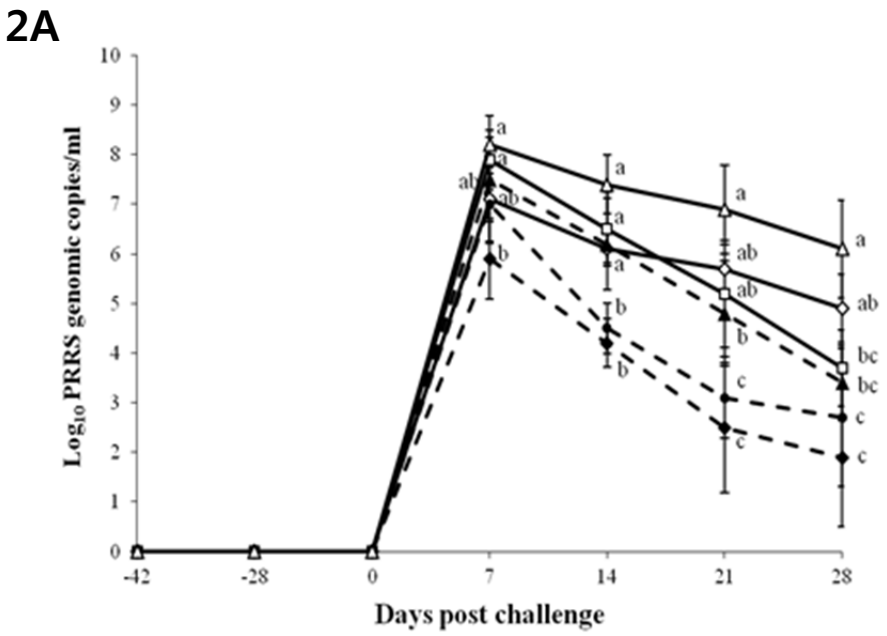
Fig. 1. Quantification of *M. hyopneumoniae* DNA. Mean values of the genomic copy numbers of *M. hyopneumoniae* DNA in nasal swabs in the different groups; pigs which received *M. hyopneumoniae* vaccine followed by *M. hyopneumoniae* challenge only (Group 1, ■), pigs which received *M. hyopneumoniae* vaccine followed by dual challenge (Group 2, □), pigs which received PRRSV vaccine followed by PRRSV challenge only (Group 3, ◆), pigs which received PRRSV vaccine followed by dual challenge (Group 4, ◇), pigs which received *M. hyopneumoniae* and PRRSV vaccine followed by dual challenge (Group 5, ●), pigs which were challenged with *M. hyopneumoniae* (Group 6, ○), pigs which were challenged with PRRSV (Group 7, ▲), and pigs which were challenged with *M. hyopneumoniae* and PRRSV (Group 8, △). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.



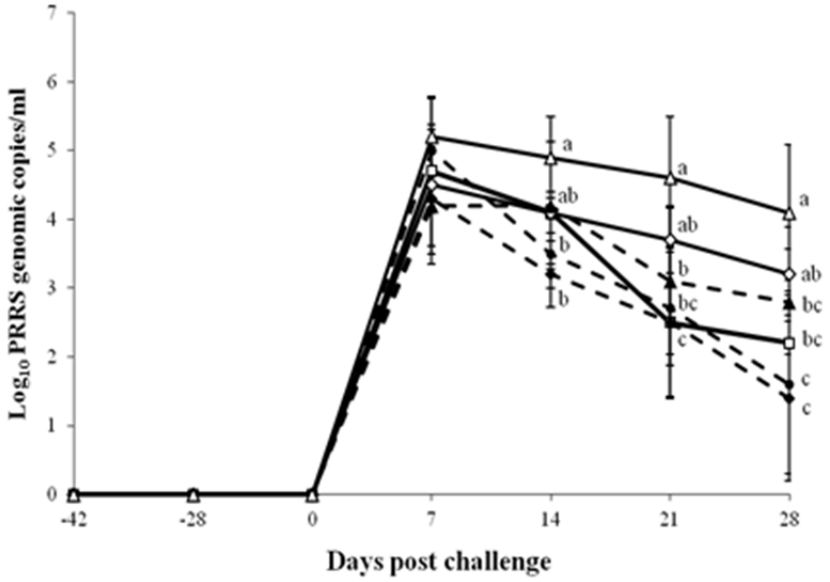
3.2. Quantification of PRRSV RNA in blood and nasal swabs

At the time of the challenges, no genomic copies of PCV2 were detected in any of the serum samples or nasal swabs from any of the 8 groups. Pigs that were given the *M. hyopneumoniae* vaccine followed by the dual challenge (Group 2) and pigs challenged with PRRSV alone (Group 7) had a significantly ($P < 0.05$) lower number of genomic copies of PRRSV in their sera (**Fig. 2A**) and nasal swabs (**Fig. 2B**) than dually challenged pigs (Group 8) at 21 and 28 dpc. Pigs that received a PRRSV vaccine followed by PRRSV challenge only (Group 3) had a significantly ($P < 0.05$) lower number of genomic copies of PRRSV in their sera (**Fig. 2A**) and nasal swabs (**Fig. 2B**) than pigs that received the PRRSV vaccine followed by the dual challenge (Group 4) at 14, 21, and 28 dpc. No genomic copies of PRRSV were detected in any of the serum samples or nasal swabs from non-challenged pigs (Groups 1, 6, and 9) throughout the experimental period.

Fig. 2. Quantification of PRRSV RNA. Mean values of the genomic copy numbers of PRRSV RNA in the serum samples (**2A**) and nasal swabs (**2B**) in the different groups; pigs which received *M. hyopneumoniae* vaccine followed by *M. hyopneumoniae* challenge only (Group 1, ■), pigs which received *M. hyopneumoniae* vaccine followed by dual challenge (Group 2, □), pigs which received PRRSV vaccine followed by PRRSV challenge only (Group 3, ◆), pigs which received PRRSV vaccine followed by dual challenge (Group 4, ◇), pigs which received *M. hyopneumoniae* and PRRSV vaccine followed by dual challenge (Group 5, ●), pigs which were challenged with *M. hyopneumoniae* (Group 6, ○), pigs which were challenged with PRRSV (Group 7, ▲), and pigs which were challenged with *M. hyopneumoniae* and PRRSV (Group 8, △). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.



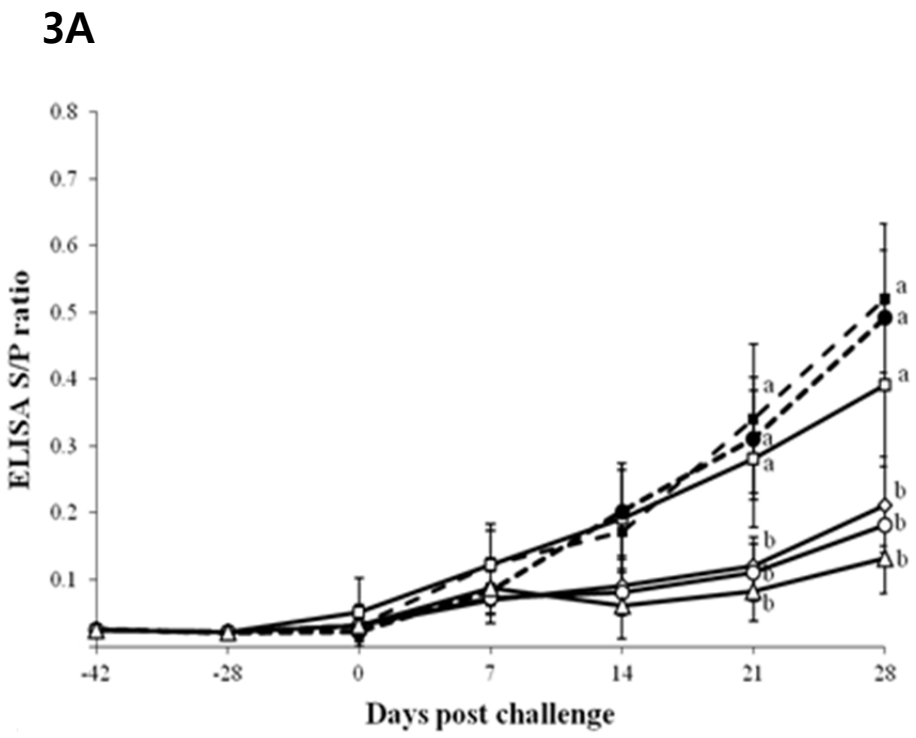
2B


Log10 PRRS genomic copies/ml
Days post challenge
-42
-28
0
7
14
21
28
a
a
a
ab
ab
ab
b
b
b
bc
bc
bc
c
c
c

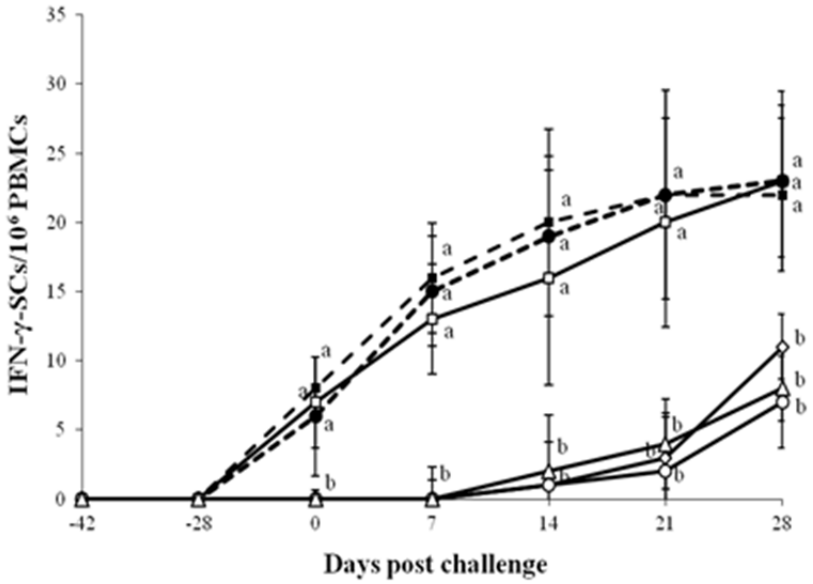
3.3. Immunological responses of M. hyopneumoniae

All pigs in the 9 groups were seronegative by 14 dpc. *M. hyopneumoniae*-specific IFN-γ-SCs were not detected among PBMCs at -28 dpc in any of the pigs but detected among PBMCs at 0 dpc in *M. hyopneumoniae*-vaccinated pigs (Groups 1, 2, and 5). *M. hyopneumoniae*-vaccinated pigs followed by either only the *M. hyopneumoniae* challenge or the dual challenge (Groups 1, 2, and 5) had significantly ($P < 0.05$) higher anti-*M. hyopneumoniae* IgG antibody levels (**Fig. 3A**) and numbers of *M. hyopneumoniae*-specific IFN-γ-SCs (**Fig. 3B**) than non-*M. hyopneumoniae*-vaccinated pigs followed by either only the *M. hyopneumoniae* challenge or the dual challenge (Groups 4, 6, and 8) at various dpc. No anti-*M. hyopneumoniae* IgG antibodies and *M. hyopneumoniae*-specific IFN-γ-SCs were detected in pigs from Groups 3, 7, and 9.

Fig. 3. Immunological responses against *M. hyopneumoniae*. (**3A**) Mean values of the anti-*M. hyopneumoniae* IgG antibody levels. (**3B**) Mean number of *M. hyopneumoniae*-specific IFN-γ-SCs in the different groups; pigs which received *M. hyopneumoniae* vaccine followed by *M. hyopneumoniae* challenge only (Group 1, ■), pigs which received *M. hyopneumoniae* vaccine followed by dual challenge (Group 2, □), pigs which received PRRSV vaccine followed by dual challenge (Group 4, ◇), pigs which received *M. hyopneumoniae* and PRRSV vaccine followed by dual challenge (Group 5, ●), pigs which were challenged with *M. hyopneumoniae* (Group 6, ○), and pigs which were challenged with *M. hyopneumoniae* and PRRSV (Group 8, △). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.



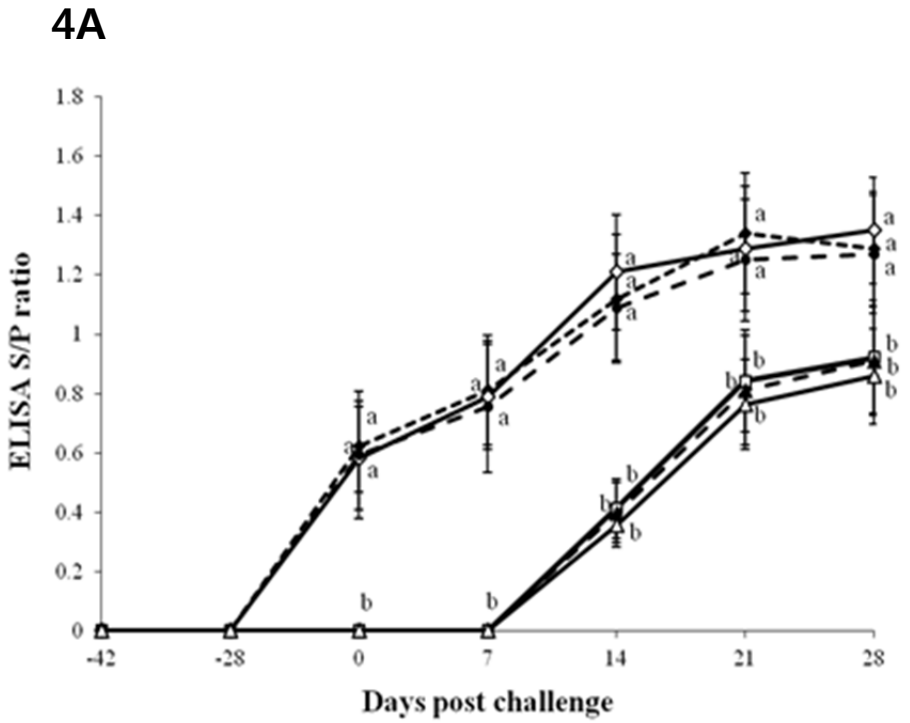
3B


IFN-γ-SCs/10⁶ PBMCs
35
30
25
20
15
10
5
0
-42
-28
0
7
14
21
28
Days post challenge

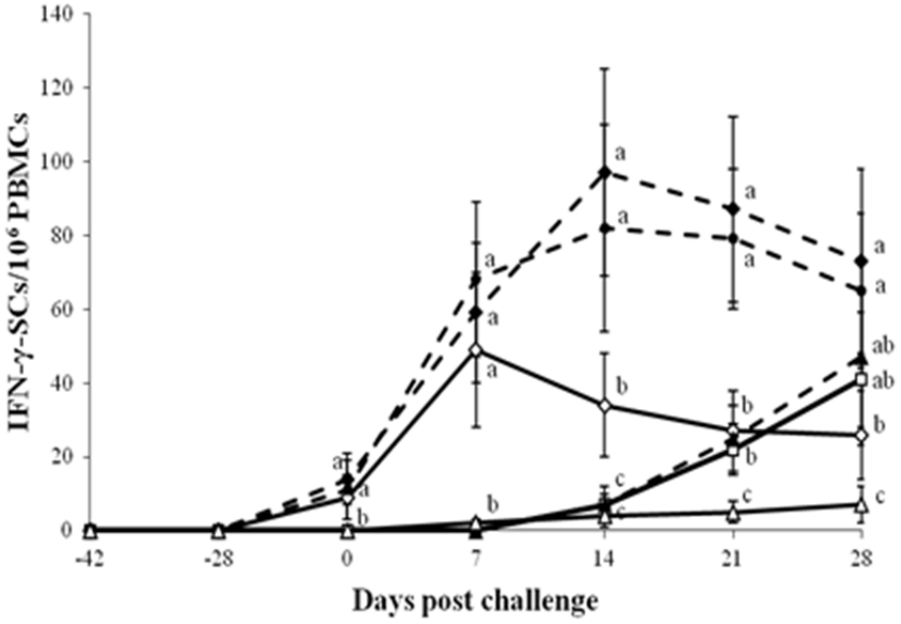
3.4. Immunological responses by PRRSV

At the time of the challenges, all of the pigs that were vaccinated against only PRRSV (Groups 3, 4, and 5) were seropositive by ELISA at 0 dpc. No PRRSV-specific IFN-γ-SCs were detected among PBMCs at -28 dpc in any of the pigs. PRRSV-vaccinated pigs followed by either only the PRRSV challenge or the dual challenge (Groups 3, 4, and 5) had significantly ($P < 0.001$) higher anti-PRRSV IgG antibody levels (**Fig. 4A**) and numbers of PRRSV-specific IFN-γ-SCs (**Fig. 4B**) than non-PRRSV-vaccinated pigs followed by either only the PRRSV challenge or the dual challenge (Groups 2, 7, and 8) at various dpc. No anti-PRRSV IgG antibodies and PRRSV-specific IFN-γ-SCs were detected in pigs from Groups 1, 6, and 9.

Fig. 4. Immunological responses against PRRSV. (**4A**) Mean values of the anti-PRRSV IgG antibody levels. (**4B**) Mean number of PRRSV-specific IFN-γ-SCs in the different groups; pigs which received *M. hyopneumoniae* vaccine followed by dual challenge (Group 2, □), pigs which received PRRSV vaccine followed by PRRSV challenge only (Group 3, ◆), pigs which received PRRSV vaccine followed by dual challenge (Group 4, ◇), pigs which received *M. hyopneumoniae* and PRRSV vaccine followed by dual challenge (Group 5, ●), pigs which were challenged with PRRSV (Group 7, ▲), and pigs which were challenged with *M. hyopneumoniae* and PRRSV (Group 8, △). Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.



4B


IFN-γ-SCs/10⁶ PBMCs
140
120
100
80
60
40
20
0
-42
-28
0
7
14
21
28
Days post challenge

3.5. Histopathological lesion score

M. hyopneumoniae-vaccinated pigs followed by either only the *M. hyopneumoniae* challenge or the dual challenge (Groups 1, 2, and 5) had significantly ($P < 0.05$) lower scores for mycoplasmal pneumonia lesions than non-*M. hyopneumoniae*-vaccinated pigs followed by either only the *M. hyopneumoniae* challenge or the dual challenge (Groups 4, 6, and 8) at 14 and 28 dpc (**Table 1**).

For PRRSV-induced pulmonary lesions, pigs that were vaccinated for *M. hyopneumoniae* followed by the dual challenge (Group 2) and pigs that were vaccinated for both *M. hyopneumoniae* and PRRSV followed by the dual challenge (Group 5) had significantly ($P < 0.05$) lower scores for PRRSV pneumonia lesions than dually challenged pigs (Group 8) at 14 and 28 dpc. The other significant results of scores for interstitial pneumonia lesions are summarized in **Table 1**.

Table 1. Experimental designs and results of lesion score, *Mycoplasma hyopneumoniae* (Mhp) DNA, and porcine reproductive and respiratory syndrome virus (PRRSV) antigen in different groups at 14 and 28 days post challenge (dpc).

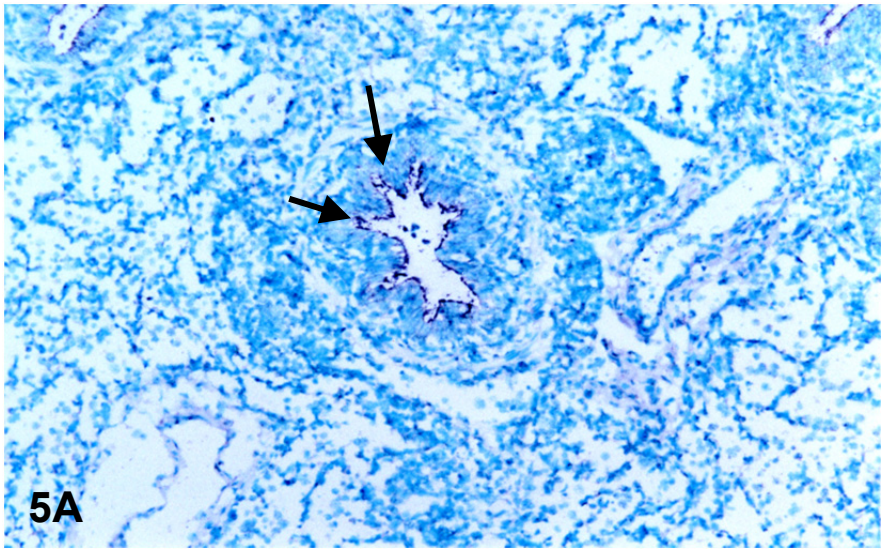
Groups	Vaccination	Challenge	dpc	Lesion score		Mhp DNA	PRRSV antigen
				Mycoplasmal pneumonia*	PRRSV pneumonia†		
1	Mhp	Mhp	14	0.33±0.55^{a}	0.17±0.41^{a}	0.33±0.52^{a}	0
			28	0.50±0.52^{a}	0.33±0.50^{a}	0.33±0.52^{a}	0
2	Mhp	Mhp + PRRSV	14	0.67±0.52^{ab}	2.17±0.41^{c}	0.67±0.52^{ab}	25.67±6.25^{ab}
			28	0.65±0.47^{a}	1.83±0.41^{b}	1.00±0.63^{a}	18.65±6.12^{ab}
3	PRRSV	PRRSV	14	0.17±0.41^{a}	1.33±0.52^{b}	0	18.00±5.02^{a}
			28	0.31±0.48^{a}	0.83±0.43^{a}	0	9.33±5.89^{a}
4	PRRSV	Mhp + PRRSV	14	1.17±0.75^{bc}	2.50±0.55^{c}	1.17±0.75^{b}	27.33±5.75^{ab}
			28	2.17±0.75^{b}	2.33±0.52^{c}	2.00±0.63^{b}	24.00±6.16^{bc}
5	Mhp + PRRSV	Mhp + PRRSV	14	0.65±0.51^{ab}	2.00±0.63^{bc}	0.67±0.48^{ab}	20.50±3.62^{a}
			28	0.67±0.52^{a}	1.83±0.75^{b}	0.83±0.41^{a}	13.00±5.55^{a}
6	-	Mhp	14	1.19±0.42^{bc}	0.50±0.55^{a}	1.00±0.63^{ab}	0
			28	2.00±0.63^{b}	0.67±0.52^{a}	1.83±0.41^{b}	0
7	-	PRRSV	14	0.33±0.52^{a}	2.83±0.41^{c}	0	31.00±5.06^{b}
			28	0.50±0.55^{a}	2.00±0.63^{bc}	0	17.67±4.76^{ab}
8	-	Mhp + PRRSV	14	1.50±0.83^{c}	3.33±0.51^{c}	1.15±0.38^{b}	35.00±4.77^{b}
			28	2.33±0.52^{b}	2.84±0.43^{c}	2.17±0.41^{b}	28.83±4.67^{c}
9	-	-	14	0	0	0	0
			28	0	0	0	0

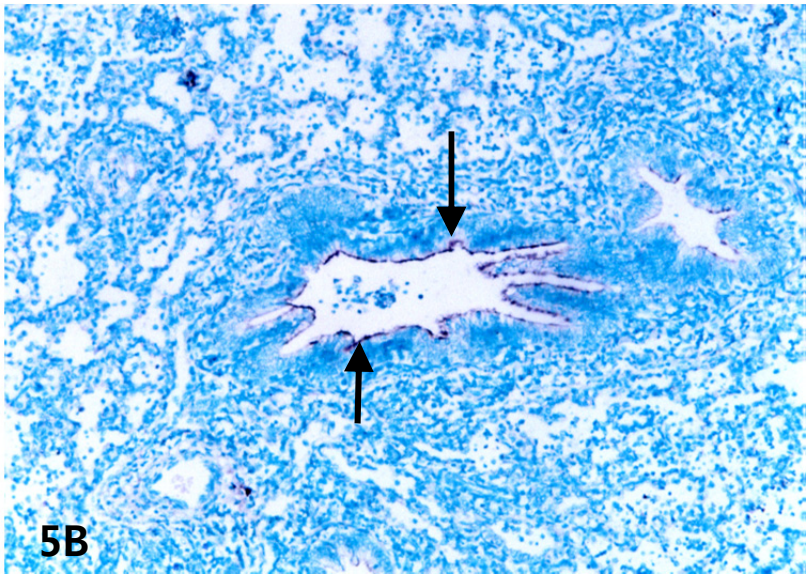
*Mycoplasmal pneumonia = peribronchiolar and perivascular lymphoid tissue hyperplasia.
†PRRSV pneumonia = interstitial pneumonia.
[a,b,c]Different letters (a, b, and c) indicate significant ($P < 0.05$) difference among groups.

3.6. In situ hybridization of M. hyopneumoniae

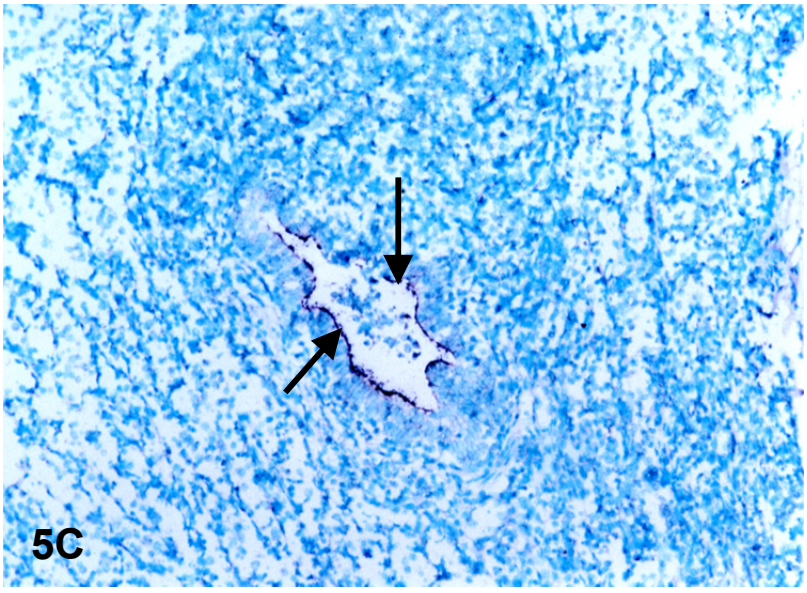
M. hyopneumoniae DNA was associated with the surface of epithelial cells in the bronchi and bronchioli of the lungs from pigs challenged with *M. hyopneumoniae* (Groups 1, 2, 4 [**Fig. 5A**], 5, 6 [**Fig. 5B**], and 8 [**Fig. 5C**]). At 14 dpc, pigs that were vaccinated for *M. hyopneumoniae* followed by *M. hyopneumoniae* challenge (Group 1) had a significantly ($P < 0.05$) lower amount of *M. hyopneumoniae* DNA in their lungs than pigs in Groups 4 and 8. At 28 dpc, pigs in Groups 2 and 5 had a significantly ($P < 0.05$) lower amount of *M. hyopneumoniae* DNA in their lungs than pigs in Groups 4, 6, and 8 (**Table 1**). The other significant results of scores for hybridization signals of *M. hyopneumoniae* are summarized in **Table 1**.

Fig. 5. In situ hybridization of *M. hyopneumoniae*. Positive signals (arrows) were detected in the surface of bronchiolar epithelium in the different groups; pigs which received PRRSV vaccine followed by dual challenge (Group 4, **5A**), pigs which were challenged with *M. hyopneumoniae* (Group 6, **5B**), and pigs which were challenged with *M. hyopneumoniae* and PRRSV (Group 8, **5C**). No difference in mycoplasmal hybridization signals between Group 4 and 6 indicated that single-dose vaccination against PRRSV alone was unable to reduce the enhancement of *M. hyopneumoniae* replication on dually challenged pigs. No difference in mycoplasmal hybridization signals between Group 6 and 8 indicated that PRRSV was unable to potentiate the enhancement of *M. hyopneumoniae* replication on dually challenged pigs.





5B

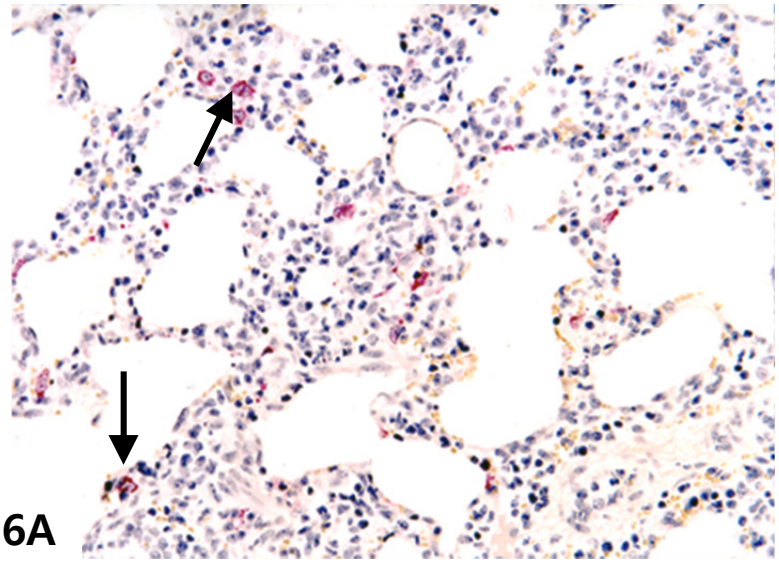


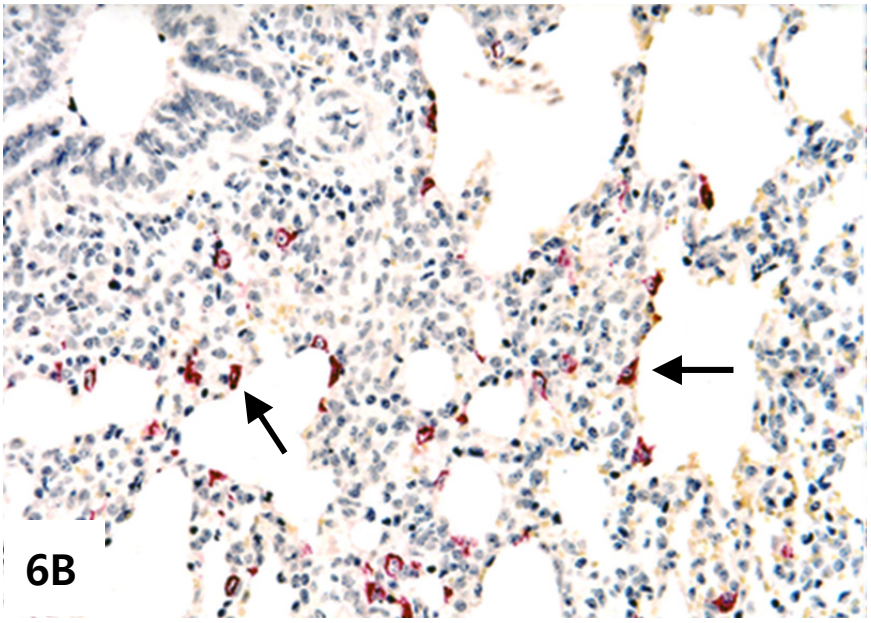
5C

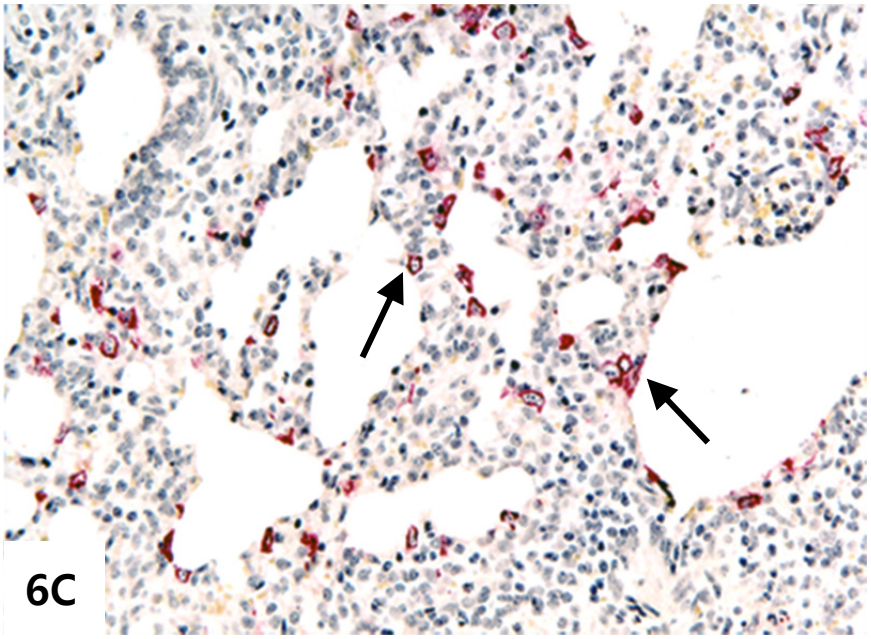
3.7. Immunohistochemistry for PRRSV

The PRRSV antigen was detected in the lungs of pig challenged with PRRSV (Groups 2 [**Fig. 6A**], 3, 4 [**Fig. 6B**], 5, 7, and 8 [**Fig. 6C**]). Pigs which received *M. hyopneumoniae* vaccine followed by dual challenge (Group 2) had a significantly ($P < 0.05$) lower number of PRRSV-positive cells per unit in their lungs than pigs which were challenged with *M. hyopneumoniae* and PRRSV (Group 8) at 28 dpc. There were no significant difference between pigs in Group 4 and Group 8 at 14 and 28 dpc (**Table 1**). The other significant results of scores for immunohistochemical antigen of PRRSV are summarized in **Table 1**.

Fig. 6 Immunohistochemistry of PRRSV. Positive signals (arrows) were detected in the macrophages in the different groups; pigs which received *M. hyopneumoniae* vaccine followed by dual challenge (Group 2, **6A**), pigs which received PRRSV vaccine followed by dual challenge (Group 4, **6B**), and pigs which were challenged with *M. hyopneumoniae* and PRRSV (Group 8, **6C**). Significant difference in immunohistochemical signals of PRRSV between Group 2 and 4 indicated that single-dose vaccination against *M. hyopneumoniae* alone was able to reduce the enhancement of PRRSV replication on dually challenged pigs. No difference in immunohistochemical signals of PRRSV between Group 4 and 8 indicated that single-dose vaccination against PRRSV alone was able to reduce the enhancement of PRRSV replication on dually challenged pigs




6B


6C

4. Discussion

The results of this study demonstrated that single-dose vaccination against *M. hyopneumoniae* alone was able to decrease PRRSV viremia and PRRSV-induced pulmonary lesions on dually infected pigs. In recent years, a single-dose *M. hyopneumoniae* vaccine has been increasingly used because it has several advantages, such as decreased labor costs, reduced stress to animals, and better meat quality because of fewer injection sites. These results agree with previous findings under experimental and field conditions [19, 14]. Based on these results, one way to minimize the effect of the *M. hyopneumoniae*-associated potentiation of PRRSV-induced pneumonia may be *M. hyopneumoniae*-based vaccination of preweaned pigs in *M. hyopneumoniae*/PRRSV-co-infected herds.

Interestingly, pigs vaccinated for PRRSV followed by PRRSV challenge induced a higher level of PRRSV-specific IFN-SCs than pigs vaccinated for PRRSV followed by the dual challenge. PRRSV-specific IFN-γ-SCs play key roles in protective cell-mediated immunity against PRRSV infection [12, 24] and IFN-γ is known to inhibit PRRSV replication [3, 16]. Similarly, *M. hyopneumoniae* modulated the effectiveness of the immune response to PRRSV, particularly levels of IFN-γ on dually infected pigs without vaccination [20, 13]. These results suggest that *M. hyopneumoniae* infection impairs PRRSV vaccine-induced protective cell-mediated immunity and may affect the rate of PRRSV vaccine failure. Therefore, control of *M. hyopneumoniae* infections by vaccination against *M. hyopneumoniae* may positively affect the full induction of

immunity by the PRRSV vaccine in herds suffering from co-infection with *M. hyopneumoniae* and PRRSV.

In contrast with earlier findings that vaccination against both PRRSV and *M. hyopneumoniae* prior to *M. hyopneumoniae* challenge appeared to decrease the efficacy of the *M. hyopneumoniae* vaccine [19, 11], vaccination against *M. hyopneumoniae* prior to a co-challenge did not decrease the efficacy of the mycoplasma vaccine, as determined by the induction of *M. hyopneumoniae*-specific IFN-γ-SCs and the scores for microscopic *M. hyopneumoniae*-induced lung lesions in the present study as well as determined by the percentage of macroscopic mycoplasmal lung lesions in the previous study [4]. Differences in the results suggest that the timing of mycoplasma vaccination in relation to PRRSV vaccination may be an important consideration in establishing successful vaccine programs.

Regardless of their PRRSV infection status, vaccination of pigs against *M. hyopneumoniae* not only induced *M. hyopneumoniae*-specific IFN-γ-SCs, which is an important protective immune parameter for control of *M. hyopneumoniae* infection but also reduced nasal shedding of *M. hyopneumoniae*. Transmission through nasal secretions has been suggested as a potential mode of horizontal spread [17]; therefore, vaccination is expected to decrease the risk of transmission to other pigs and decrease the amount of *M. hyopneumoniae* circulating within the herd. The results of this study may explain why the *M. hyopneumoniae* vaccine is effective under field conditions in which *M. hyopneumoniae* and PRRSV infections are widespread in the pig population. The present study provides swine practitioners and producers with efficient vaccination

regimes; vaccination against *M. hyopneumoniae* is the first step in protecting pigs against co-infection with *M. hyopneumoniae* and PRRSV.

References

[1] Bandrick, M., Pieters, M., Pijoan, C., Molitor, T.W., 2008. Passive transfer of maternal *Mycoplasma hyopneumoniae*-specific cellular immunity to piglet. Clinical Vaccine and Immunology 15, 540-543.

[2] Bautista, E.M., Molitor, T.W., 1997**.** Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral Immunology 10, 83-94.

[3] Bautista, E.M., Molitor, T.W., 1999. IFN-γ inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Archives of Virology 144, 1191-1200.

[4] Boettcher, T.B., Thacker, B.J., Halbur, P.G., Waters, W.R., Nutsch, R., Thacker, E.L., 2002. Vaccine efficacy and immune response to *Mycoplasma hyopneumoniae* challenge in pigs vaccinated against porcine reproductive and respiratory syndrome virus and *M hyopneumoniae*. Journal of Swine Health and Production 10, 259-264.

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

[6] Dubosson, C.R., Conzelmann, C., Miserez, R., Boerlin, P., Frey, J., Zimmermann, W., Hani, H., Kuhnert, P., 2004. Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples. Veterinary Microbiology 102, 55-65.

[7] Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.-J., Andrews, J.J., Lum, M.A., Rathje, J.A., 1996. Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Veterinary Pathology 33, 159-170.

[8] Han, K., Seo, H.W., Oh, Y., Kang, I., Park, C., Kang, S.H., Kim, S.H., Lee, B.H., Kwon, B., Chae, C., 2012. Evaluation of monoclonal antibody-based immunohistochemistry for the detection of European and North American *Porcine reproductive and respiratory syndrome virus* and a comparison with in situ hybridization and reverse transcription polymerase chain reaction. Journal of Veterinary Diagnostic Investigation 24, 719-724.

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

[10] Kwon, D., Chae, C., 1999. Detection and localization of *Mycoplasma hyopneumoniae* DNA in lungs from naturally infected pigs by in situ hybridization using a digoxigenin-labeled probe. Veterinary Pathology 36, 308-313.

[11] LeRoith, T., Hammond, S., Todd, S.M., Ni, Y., Cecere, T., Pelzer, K.D., 2011. A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with *Mycoplasma hyopneumoniae*. Veterinary Immunology and Immunopathology 140, 312-316.

[12] Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2003. Gradual development of the interferon-γ response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309, 18-31.

[13] Messier, S., Ross, R.F., 1991. Interactions of *Mycoplasma hyopneumoniae* membranes with porcine lymphocytes. American Journal of Veterinary Research 52**,** 1497-1502.

[14] Moreau, I.A., Miller, G.Y., Bahnson, P.B., 2004. Effects of *Mycoplasma hyopneumoniae* vaccine on pigs naturally infected with *M. hyopneumoniae* and porcine reproductive and respiratory syndrome virus. Vaccine 22, 2328-2333.

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

[16] Rowland, R.R., Robinson, B., Stefanick, J., Kim, T.S., Guanghua, L., Lawson, S.R., Benfield, D.A., 2001. Inhibition of porcine reproductive and respiratory syndrome virus by interferon-γ and recovery of virus replication with 2-aminopurine. Archives of Virology 146, 539-555.

[17] Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F., Segales, J., 2009. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. Veterinary Journal 181, 221-231.

[18] Thacker, E.L., Halbur, P.G., Ross, R.F., Thanawongnuwech, R., Thacker, B.J., 1999. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. Journal of Clinical Microbiology 37, 620-627.

[19] Thacker, E.L., Thacker, B.J., Young, T.F., Halbur, P.G., 2000. Effect of vaccination on the potentiation of porcine reproductive and respiratory syndrome (PRRSV)-induced pneumonia by *Mycoplasma hyopneumoniae*. Vaccine 18, 1244-1252.

[20] Thanawongnuwech, R., Thacker, E.L., 2003. Interleukin-10, interleukin-12, and interferon-γ levels in the respiratory tract following *Mycoplasma hyopneumoniae* and PRRSV infection in pigs. Viral Immunology 16, 357-367.

[21] Van Alstine, W.G., 2012. Respiratory system. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, 10th edn. Wiley-Blackwell Publishing, Ames, IA, USA, 348-362.

[22] Van Alstine, W.G., Stevenson, G.W., Kanitz, C.L., 1996. Porcine reproductive and respiratory syndrome virus does not exacerabate *Mycoplasma hyopneumoniae* infection in young pigs. Veterinary Microbiology 49, 297-303.

[23] Wasilk, A., Callahan, J.D., Christopher-Hennings, J., Gay, T.A., Fang, Y., Dammen, M., Reos, M.E., Torremorell, M., Polson, D., Mellencamp, M., Nelson, E., Nelson, W.M., 2004. Detection of U.S., Lelystad, and European-like porcine reproductive and respiratory syndrome viruses and relative quantitation in boar semen and serum samples by real-time PCR. Journal of Clinical Microbiology 42, 4453-4461.

[24] Zuckermann, F.A., Garcia, E.A., Luque, I.D., Christopher-Hennings, J., Doster, A., Brito, M., Osorio, F., 2007. Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of γ-IFN-producing cells and virological parameters

of protection upon challenge. Veterinary Microbiology 123, 69-85.

GENERAL CONCLUSION

The objective of this thesis is to determine the relationship of several major PRDC related pathogens and vaccine efficacy throughout experimental model and challenge test. Part I was designed to clarify the interaction between the PCV2 and *M. hyopneumoniae* by dual challenged model. Pigs in dual challenged group had significantly increased PCV2-viremia, more severe PCV2-induced pulmonary and lymphoid lesions, and higher levels of lesion-associated PCV2 antigen than single PCV2 challenged group.

In this study, although *M. hyopneumoniae* potentiates the severity of PCV2-associated lesions in dually challenged pigs, single-dose vaccination with *M. hyopneumoniae* alone did not reduce the PCV2 viremia or the PCV2-associated lymphoid lesions in pigs that were dually challenged. In addition, vaccination against PCV2 did not reduce the nasal shedding of *M. hyopneumoniae*, the *M. hyopneumoniae*-induced pulmonary lesions or the lesion-associated *M. hyopneumoniae* DNA in dually challenged pigs.

But the efficacy of PCV2 vaccines was not affected by a subsequent infection with *M. hyopneumoniae*. That means PCV2 vaccination elicits PCV2-specific NA and IFN-γ-SCs, even in pigs with dual challenge. Likewise, a single vaccination against *M. hyopneumoniae* induced *M. hyopneumoniae*-specific IFN-γ-SCs, even in pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge. Dual challenge with PCV2 and *M. hyopneumoniae* did not interfere with the induction of active

immunity induced by a previous single vaccination for either PCV2 or *M. hyopneumoniae*. These results suggest *M. hyopneumoniae* vaccines may be effective for the *M. hyopneumoniae* control under field conditions where PCV2 is widespread in the swine population.

In Part II, Interestingly, single-dose vaccination against *M. hyopneumoniae* alone could decrease PRRSV viremia and PRRSV-induced pulmonary lesions on dually infected pigs. It means the protective cell-mediated immunity induced by the *M. hyopneumoniae* vaccine was not impaired by PRRSV challenge. The result of this study can provide swine practitioners with the guideline for effective vaccination program; vaccination against *M. hyopneumoniae* is the first step in protecting the farms suffering from PRDC or the pigs with the co-infection with *M. hyopneumoniae* and PRRSV or PCV2.

국문 논문 초록

마이코플라즈마, 써코바이러스, 돼지 생식기 호흡기 증후군 바이러스의 상호작용 연구

(지도 교수: 채찬희, 수의사, 수의학박사)

박 수 진

서울대학교 대학원

수의학과, 수의병인생물학 및 예방수의학전공

돼지 호흡기 복합 질병 Porcine Respiratory Disease Complex (PRDC)은 우리나라와 세계의 양돈 산업에서 심각한 피해를 끼치는 원인 중의 하나이다. 이러한 PRDC의 가장 흔한 바이러스 원인체로는 돼지 호흡기 생식기 증후군 바이러스 Porcine reproductive and respiratory syndrome virus (PRRS), Classical swine fever (CSF), Swine influenza virus (SIV), Pseudorabies (PRV), 그리고 Porcine Circovirus type 2 (PCV2) 등이 있다. 세균원인체로는, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Streptococcus suis* 그리고 *Actinobacillus spp.* 등이 있다. 이와 같은 원인체 상호간의 작용을 이해하는 것은 질병의 정도와 밀접한 관계가

있으므로 매우 중요하다. 이러한 PRDC를 효과적으로 예방, 관리하기 위해서는 우선 적절한 진단이 필요하며, 농장의 상황에 맞춰 항생제나 백신을 사용하는 것이 매우 중요한 관리요소로 지목되고 있다. 따라서 본 연구에서는 실험적인 모델 및 공격접종을 통해 각 주요 병원체와 백신의 효과를 검증해 보고자 하였다.

Part I은 실험적으로 유발시킨 PCV2-*M. hyopneumoniae* 복합감염 모델에서 PCV2와 마이코플라즈마 백신의 상호작용을 검증해 본 것이다. 백신의 효과는 PCV2 viremia, 및 *M. hyopneumoniae*의 비강 배출을 통해 미생물학적으로 평가하였고, 또한 면역학적으로도 중화항체와 인터페론감마분비세포를 측정함으로써 체크하였다. 병리학적으로는 폐와 림프조직의 육안병변, 조직병변과 병변 내 PCV2 항원 및 마이코플라즈마 DNA를 확인함으로써 평가하였다. 실험결과, 마이코플라즈마와 PCV2를 복합 감염시킨 돼지에서, 마이코플라즈마에 의해 PCV2의 병변이 심해지고, PCV2항원이 더욱 많이 관찰되기는 하였으나, 마이코플라즈마 백신의 단독처방만으로는 복합감염 모델에서 PCV2의 바이러스 혈증이나, PCV2에 의한 병변, PCV2 항원의 분비량을 감소시킬 수는 없었다. 또한 PCV2 백신만으로는 복합감염모델에서 마이코플라즈마의 비강 분비, 및 마이코플라즈마에 의한 폐 병변과, 병변내 관찰되는 마이코플라즈마 DNA의 양을 감소시킬 수는 없었다. PCV2와 마이코플라즈마의 복합감염 모델에서, 두개의 백신은 각각 서로의 능동면역 유발에 영향을 미치지 않는 것이 확인 되었다. 이 실험의 결과로써, 마이코플라즈마 및 PCV2의 복합감염 모델에서, (i) 마이코플라즈마 단독백신 만으로는 마이코플라즈마에 의해 심화되는 PCV2와 관련된 병변을 경감시킬수 없고 (ii) PCV2 단독백신으로 마이코플라즈마로 인해 심화되는 PCV2 관련 병변을 경감시킬 수 있다는 결론을 얻게 되었다.

Part II 는 마이코플라즈마 및 PRRS의 복합감염 모델에서 각 백신의 효과를 확인 하였다. 전체 72두의 돼지를 무작위로 9개의 그룹으로 나누어서 5군은 백신 접종 및 공격감염군, 3군은 비백신 접종 및 공격감염군, 그리고 음성 대조군으로 나누었다. 1회 마이코플라즈마 단독백신은 복합감염 모델에서 PRRS 바이러스 혈증을 줄였고, PRRSV에 의해 발생하는 폐 병변을 어느정도 감소시켰다. 이에 비하여 1회의 PRRS 백신은 복합감염 모델에서 마이코플라즈마의 비강 배출이나 마이코플라즈마에 의한 폐 병변을 감소시키지 못하였다. 마이코플라즈마 공격감염은 PRRS 백신으로 발생되는 세포성 면역기능을 무력화시켰으나, PRRSV 공격감염은 마이코플라즈마 백신으로 인해 발생하는 세포성 면역을 무력화 시키지는 못하였다. 본 연구로 인하여 양돈농가 및 양돈관련 수의사들이 더욱 효과적인 백신 프로그램을 고안하고, 복합감염이 만연하는 한국의 농장 상황에서, 마이코플라즈마의 백신이 농장의 효과적인 방어를 위하여서 가장 먼저 처방해야 할 지침임을 알려주는 결론을 도출하게 되었다.

주요어:
마이코플라즈마; 돼지써코바이러스 2 형; 돼지 생식기 호흡기 증후군 바이러스; 돼지 호흡기 복합 질병; 백신

학번: 2014-30548