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Evaluation of *Mycoplasma hyopneumoniae* Bacterin based on Microbiological, Immunological and Pathological Analysis

마이코플라즈마 하이오뉴모니애 박테린 백신 효능의 미생물학적, 면역학적, 병리학적 평가

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Abstract

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Mycoplasma hyopneumoniae is the primary pathogenic agent of enzootic pneumonia in pigs, and it becomes very important in the

etiology of the porcine respiratory disease complex (PRDC) as it gives damage to the ciliated epithelium of the trachea, bronchi, and bronchioles so makes pig susceptible to secondary bacterial and viral invaders. In the pig industry, *M. hyopneumoniae* is a high prevalent worldwide, causes significant economic losses to farms resulting from growth retardation and poor feed efficiency.

Although there have been many efforts to control and prevent *M. hyopneumoniae*, it is very difficult to eradicate and maintain *M. hyopneumoniae* free due to the nature of airborne pathogens can spread several kilometers and the dense breeding practices of pig farms. Therefore, vaccination is considered the most efficient tool of controlling *M. hyopneumoniae*.

The purpose of this study is to analyze the efficacy and safety of the new single-dose inactivated *M. hyopneumoniae* bacterin in Korean field and laboratory condition using a clinical, microbiological, immunological, and pathological technique. The efficacy of the vaccine was evaluated microbiologically through the nasal shedding of *M. hyopneumoniae* and was assessed humoral and cell-mediated immunity through enzyme-linked immunosorbent assay (ELISA) and measuring the number of interferon gamma secreting cells (IFN-γ -SC) in peripheral blood mononuclear cells (PBMC). Pathological evaluation was performed by the observation of gross and microscopic in lung lesion, and the isolation of *M. hyopneumoniae* antigen in the lesion. The safety of the vaccine was evaluated as an index for clinical evaluation of respiratory diseases and average of

daily weight gain (ADWG).

Chapter I is with the efficacy evaluation of the vaccine under laboratory condition using challenge model of Korean pathogenic *M. hyopneumoniae* isolate. As a result of the experiment, the vaccinated group significantly induced more *M. hyopneumoniae*-specific ELISA antibodies and IFN-ɣ-SC in PBMC compared to the unvaccinated. The nasal shedding and lung lesion analysis of *M. hyopneumoniae* also showed significantly lower levels in the vaccinated group than the unvaccinated. Therefore, the vaccine is considered to be effective in controlling infection of Korean pathogenic *M. hyopneumoniae* isolates.

Chapter II is with the evaluation of efficacy and safety of the vaccine at three commercial pig farms with a history of swine enzootic pneumonia in Korea. As a result of the experiment, the vaccinated groups in all three farms had significantly lower respiratory clinical symptom, and the higher average of daily weight gain than the unvaccinated groups. Furthermore, in all three farms, the vaccinated group significantly induced more *M. hyopneumoniae*-specific IFN-ɣ-SC than the unvaccinated group. The severity of lung lesions of the vaccinated groups was significantly lower than that of the unvaccinated groups. Therefore, it was confirmed that the vaccine effectively induced cell-mediated immunity in the environment in which the *M. hyopneumoniae* pathogen is present, and effectively improved the daily weight gain by alleviating the severity of lung lesions and respiratory clinical symptoms.

Above two series studies, the new single-dose inactivated *M. hyopneumoniae* bacterin was assessed for clinical, microbiological, immunological, and pathological parameters. The vaccine demonstrated the protection ability against challenging domestic pathogenic isolates, and the improved daily weight gain was confirmed when applied in field condition. The new single-dose inactivated *M. hyopneumoniae* whole-cell bacterin can provide another option to control *M. hyopneumoniae* and considering that it is the primary agent for the Porcine Respiratory Disease Complex and causing a severe economic impact to swine farmers, this can suggest an important implication to sustainable growth in animal welfare and the swine production industry.

Keywords: *Mycoplasma hyopneumoniae*; *Mycoplasma hyopneumoniae* bacterin; Enzootic pneumonia; Secondary infection; Porcine Respiratory Disease Complex; Cell-mediated immunity; Average of daily weight gain

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LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
AIAO	All-in/all-out
CMI	Cell mediated immunity
dpc	Days of post-challenge
dpv	Days of post-vaccination
EP	Enzootic pneumonia
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Ennzyme-linked immunospot assay
IFN-ɣ-SC	Interferon-gamma secreting cells
IL	Interleukin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCV2	Porcine circovirus type 2
PCR	Polymerase chain reaction
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
SEP	Swine enzootic pneumonia

GENERAL INTRODUCTION

Swine mycoplasmal pneumonia (SMP), or swine enzootic pneumonia (SEP) is caused by *Mycoplasma hyopneumoniae* and it is one of critical contributors that can bring out economic loss in the industry of swine production. After Mare and Switzer reported first in 1965, *M. hyopneumoniae* is still a high prevalent worldwide despite several efforts for control continuously.

M. hyopneumoniae infection alone causes relatively mild disease in condition of the absence of environmental stress factors. The clinical signs are characterized by dry cough, growth retardation and poor feed efficiency in growing pigs. Moreover, the importance of *M. hyopneumoniae* has been emphasized as well in perspective of interaction with secondary infection pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), and *Pasteurella multocida*, etc. Hence, control of *M. hyopneumoniae* infections in pig herds is major concern of pig practitioners.

Improvement of housing technique and biosecurity practice for pig herds are highly recommended to control *M. hyopneumoniae* infection. However, *M. hyopneumoniae* can be transmitted by vertical, horizontal, and airborne. It suggests that preventing transmission in physical way is very difficult and needs to take a lot of effort to accomplish a desired consequence. Antimicrobial medication is another option for the control of *M. hyopneumoniae* infection because it helps

the severity of disease relieved, and the infection load decreased. However, this access does not prevent *M. hyopneumoniae* infection and is against current trend of reducing antimicrobial medication for resistance concern. Therefore, vaccination against *M. hyopneumoniae* is commonly considered as a most effective tool for a control of *M. hyopneumoniae* infection.

Several inactivated and whole-cell bacterins are commercially available and vaccination is frequently practiced worldwide. Several studies have shown that vaccination relieves clinical signs and lung lesions due to *M. hyopneumoniae* infections, and helps economical profits increased through better average daily weight gain (ADWG) and lower medication cost. Despite these positive effects, overall performance of vaccination is various per each farm. This indicates that the vaccination induces only a partial protection as well as the efficacy of *M. hyopneumoniae* vaccination may vary depending on the character of different field isolates of *M. hyopneumoniae*. Therefore, it is important for the evaluation of vaccine that the performance may be demonstrated in laboratory as well as field condition with proper parameter and technique.

This dissertation was designed to investigate the efficacy of new single-dose inactivated *M. hyopneumoniae* whole-cell bacterin against challenge of *M. hyopneumoniae* Korean field isolate in laboratory condition (Chapter I), and the performance in field condition at 3 Korean farms (Chapter II) based on clinical, microbiological, immunological and pathological analysis.

LITERATURE REVIEW

Mycoplasma hyopneumoniae

1. Introduction

Mycoplasmas are belonged to the phylum *Tenericutes,* class *Mollicutes,* order *Mycoplasmatales*, and family *Mycoplasmataceae* [1]. Mycoplasmas are the smallest and simplest organism which can self-replicate and have phylogenetical relation with gram-positive bacteria in spite of fundamental differences between ones and other bacteria [2]. *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae* and *Mycoplasma suis* are identified to have relation with diseases of swine.

M. hyopneumoniae is one of important pathogens in porcine respiratory disease complex (PRDC) [3] as well as the primary pathogen of swine enzootic pneumonia (SEP), a chronic respiratory disease in growing pigs resulting from combined infections of *M. hyopneumoniae* and one or more secondary bacterial pathogens [2]. SEP is characterized by a persistent non-productive cough with a reduced growth rate, a poor feed conversion ratio, high morbidity, and low mortality [1, 4]. The economic impact of *M. hyopneumoniae* infections in swine farms is considered as significant worldwide.

M. hyopneumoniae colonizes the ciliated epithelial cells of the respiratory tract of the infected pigs, damaging the cells so that predisposes the pig to secondary bacterial and/or viral invaders [5].

All ages of pig are susceptible, especially the animals in the growing to finishing period are most affected [6]. However, in herds without immunity, the disease can affect pigs from all age groups, including suckling and breeding animals [7].

Several strategies may be applied to prevent and control *M. hyopneumoniae* successfully including optimized management practices and vaccination [8]. While all-in/all-out (AIAO) production and multi-site operations are well established as great management tools for the control of pathogen, vaccination remains an important and a cost-effective method for reducing the impact of *M. hyopneumoniae* infections. It is estimated that 70% of pig herds are being applied vaccination against *M. hyopneumoniae* worldwide [9]. Korea has a similar tendency as well, approximately 70% of total piglets farrowed were vaccinated against *M. hyopneumoniae* according to the survey in 2018 (http://www.kahpa.or.kr). The major benefit of vaccination comes from an improvement of animal welfare and a decrease of the performance losses due to *M. hyopneumoniae* infections [10]. Although several different types of vaccine are marketed and being practiced in pig production industry, their effects sometimes seem various per farm and herd, and the detrimental impacts from *M. hyopneumoniae* infections still go on worldwide. In addition, even if a farm would achieve *M. hyopneumoniae*-free status of herd through several hard practices, its maintenance is very difficult particularly for in pig-dense areas because the airborne spread of this pathogen may occur across over several kilometers [11]. Controlling of *M.*

hyopneumoniae infection has always been challenged since the first isolates from field had been reported in 1960s. Many monumental findings have been reported so far, but much of *M. hyopneumoniae* are still unknown and need further research.

2. Etiology

M. hyopneumoniae is a small size (0.2-0.4 μm) and lacks a cell wall, so inevitably pleomorphous [3]. The genomes of *M. hyopneumoniae* strains, the pathogenic strains 232 and 7448, and the nonpathogenic strain J, were first sequenced in 2004 [12, 13], and total 23 genomes of *M. hyopneumoniae* have been entirely sequenced and available now [10]. Generally, the genomes are small size of 0.86-0.96 Mb, and there are 528 to 691 protein-encoding genes [14]. Although maximum 30% of the *M. hyopneumoniae* genes are known to encode surface proteins, the function of many of them is still unknown [15]. Comparing to other bacteria, *M. hyopneumoniae* has low (28.54%) guanine and cytosine (GC) content genome for which influence genome organization and gene expression. It suggests that *M. hyopneumoniae* needs to obtain amino acids and membrane components from their growth environment, particularly for host cells [6]. This character of low GC content also makes *M. hyopneumoniae* have a complex transcriptional organization, unique intrinsic terminator stem-loop formation and individual ribonuclease P (RNase P) structure [16]. *M. hyopneumoniae* can produce adhesins, modulins, aggresins and impedins which can help adhesion and modulation to

the host immune system [17]. These surface proteins of *M. hyopneumoniae* proteolytically cleave upon translocation across the membrane [18, 19], which can alter the bacterial surface structure. This complexity contributes that *M. hyopneumoniae* can avoid a detection and an attack by the host immune response.

Several research in genomic level of *M. hyopneumoniae* also indicate that it has a high diversity at genomic, antigenic and proteomic level among strains [20]. These diversities in various levels may trigger virulence differentiation of individual *M. hyopneumoniae* strain [21]. Infection of low virulent *M. hyopneumoniae* strain was reported not to protect pigs against following challenge of high virulent *M. hyopneumoniae* [22]. It provides an important implication to understand the possible reasons for various effects of vaccines, and to develop more efficacious one.

M. hyopneumoniae is difficult to cultivate *in vitro*. It requires some strict conditions such as a specific nutritional medium like Friis supplemented with serum and relatively long time for incubation [1]. Furthermore, an identification success rate is low due to overgrowth or sample contamination with another *Mycoplasma* spp., such as *M. hyorhinis* or *M. flocculare* [2]. This fastidious character in isolation and identification of *M. hyopneumoniae* is one of principal factors which may hinder to understand *M. hyopneumoniae*.

3. Pathogenesis

Pathogenesis of *M. hyopneumoniae* is still not fully understood. However, putting together the results of pertinent research, *M. hyopneumoniae* pathogenesis may be elucidated by its own factors and the interaction with host immune response.

Once *M. hyopneumoniae* is introduced in respiratory tract of pigs, it attaches to the ciliated epithelial cells of the trachea, bronchi and bronchioles underneath the mucous layer. This mechanism is attributed by interaction between adhesins, synthesized by *M. hyopneumoniae,* and host ligands, displayed on cilia surface or in the extracellular matrix [14]. Adhesion makes *M. hyopneumoniae* can overcome mucociliary clearance of host, the primary barrier against respiratory pathogen, and is followed by the induction of ciliostasis, loss of cilia, and death of epithelial cell [23]. This event is considered as critical in pathogenesis of *M. hyopneumoniae*, several studies has tried to elucidate adhesins in proteomic level. P97 and its paralogues is known as the primary adhesin [23], which is the first identified one of *M. hyopneumoniae*. The other family of adhesins, related with P97, is formed by P102 and its paralogues [24]. Another adhesin is identified as P159, is not related with P97 and P102 [25]. The receptor of adhesin on eukaryotic cell are mainly glycosaminoglycans (GAGs) on cilia surface, and fibronectin and plasminogen in extracellular matrix. Most of the proteins from the P97/P102 paralogues and P159 are post-transcripted and cleaved, a system observed with several other surface-associated proteins [26]. To date,

at least 35 *M. hyopneumoniae* proteins which involved in cell adhesion have been identified [27, 28]. They are endoproteolytically cleaved in *M. hyopneumoniae.* These include adhesins as well as lipoproteins and multifunctional cytosolic proteins "moonlight" at the cell surface. The cleaved fractions of P97/P102 paralogues and P159 remain on the cell surface and work as receptors of heparin, plasminogen and fibronectin, which can become a trigger for colonization of *M. hyopneumoniae* into host tissue [6, 29]. For this reason, these bacterial proteins are considered as one of important pathogenic factors of the organism and have been evaluated as one of antigen candidates in many experimental studies of vaccine engineering against *M. hyopneumoniae* [30]. Adhesion is a starting point of the infection and may be exaggerated by other virulence factors. In general, *M. hyopneumoniae* can produce toxic metabolites like H_2O_2 because it deprives glycerol as a carbon source from the host cell. This mechanism is strain-dependent, because the attenuated type of strain J didn't produce detectable amounts of H_2O_2 [31]. *M. hyopneumoniae* can take up myo-inositol and use it as energy source in condition of absence of glycerol. Since myo-inositol is plenty of in pig's serum, it might be an optimal energy source for *M. hyopneumoniae* infiltrating in the highly vascularized lungs [31].

Cell-surface lipoproteins, called lipid associated membrane proteins (LAMPs), have also been considered as one of pathogenic factors. They mediate inflammation process through interaction with the host immune system related to Toll-like receptors (TLRs) [32]. In addition,

LAMPs have been found to induce apoptosis in various cell types, including porcine peripheral blood mononuclear cells (PBMC) [33], and to activate production of nitric oxide (NO) and reactive oxygen species in the host cell [34].

M. hyopneumoniae can release extracellular DNA that allows the organism to make biofilms on host surface. It makes the pathogen more resistant to antimicrobial and the host immune response and affect to host immunity persistently [35].

The interaction with host immune system may aggravate pathogenesis of *M. hyopneumoniae* infection. The organism elicits acute inflammatory response in pig lungs. This is represented by an excessive infiltration and accumulation of neutrophil, macrophage and lymphocyte. High level of secretion of pro-inflammatory cytokines from those lymphocytes are accompanied. This induces microbicidal response to host respiratory tissue as well as invading organism [14]. Moreover, because *M. hyopneumoniae* can prolong in respiratory tract, chronic immune response is also occurred subsequently. It modulates the secretion of anti-inflammatory cytokines from dendritic cells and macrophages, and apoptosis on immune cells is induced by LAMPs [14]. As a result, the number of immune cells decreases, infected pigs lead to an immunosuppressive state. The detail of interaction between host immune mechanism is stated in section 7.

4. Swine enzootic pneumonia

The major clinical sign of SEP is a dry, non-productive cough. This typical sign shows gradual onset and prolonged weeks to even months inconsistently [3]. Intensity of disease is various from subclinical course with little or no coughing and tends the greatest in pigs at growing-finishing period of the production cycle [36]. Coughing of the infected pigs helps the microorganisms of *M. hyopneumoniae* spread easily to other animals in their herd. Therefore, SEP is well characterized high morbidity and low mortality. Whereas, if other pathogens associated with secondary infection and poor housing environment are involved, clinical signs become more severe including dyspnea, fever, anorexia, lethargy and even death [4].

Basically, coughing is caused by the lung lesions observed in the infected pigs. Gross lesions of lungs affected *M. hyopneumoniae* is commonly recognized with dark red to purple areas of consolidation [37]. In the lungs of pig with SEP, the lesions are usually located in the ventral part of the cranial and middle lobes, and the cranial part of the caudal lobes [36]. The overall appearance of the affected lung looks like atelectatic [36]. Once cutting into the affected lung, the solidity is not firm. However, if secondary infection is involved, the lesion is appeared aggravated and firmer and its range is more diffused [27].

Microscopic changes in the lungs with SEP is well defined as broncho-interstitial pneumonia. In the early phase of pneumonia, the

number of lymphocytes is getting increased in perivascular, peribronchiolar tissue and lamina propria of the airway lumen. In addition, pneumocyte type II hyperplasia and edema fluid in the alveolar space are observed [38]. As disease progresses, these lesions worsen with developing follicles from peribronchial and perivascular lymphocytic hyperplasia [7], with an expanded goblet cells and hyperplasia of submucosal glands [5].

Growth retardation is another principal consequence of SEP. Most pigs suffering from SEP may appear as normal but low vitality, with rough hair coat even though appetites are usually normal [36]. Taking account into the major characters of SEP, low mortality, and high morbidity, this makes SEP becoming as a major economical disease in pig production industry. Although there are few studies to assess economic impact on a growth from SEP, 6-16% of reduction in the growth rate at the age of slaughter pigs has been reported [39]. Another study investigated ADWG of seronegative and seropositive herds against *M. hyopneumoniae* under subclinical status. The seronegative herds were reported more than 38 g/day of ADWG when compared with the seropositive ones [40].

The intensity of impact from SEP looks various case by case. Some factors that affects this variance are recognized including environmental distress, practitioner's capability for herd management and *M. hyopneumoniae* strain involved in the disease, etc. Although the influence of diversity in *M. hyopneumoniae* strains to the severity of clinical signs and lung lesions is not fully understood yet, it has

been reported that most pigs are co-infected with more than one strains of *M. hyopneumoniae* [41, 42], and those showed the increased severity and prevalence of the typical lung lesions of *M. hyopneumoniae* infection from abattoir monitoring [43].

5. Porcine respiratory disease complex

M. hyopneumoniae is closely associated in the pathogenesis of PRDC [3]. The concept of PRDC was introduced to describe the complicated characters of respiratory symptoms and poor growth performance in grow-finishing ages of pigs [44]. This disease has been called as '18 weeks wall' given its high prevalence in this ages [45]. PRDC typically affects finishing pigs of between 16 and 22 weeks of age and is characterized by growth retardation, poor feed efficiency, lethargy, anorexia, pyrexia, cough, and labored respiration [46]. PRDC is now defined as a multi-factorial problem intervened by several viral and/or bacterial pathogens, environmental distress, production systems. Pathogens involved in PRDC are porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), swine influenza virus (SIV), Aujeszky's disease virus, porcine respiratory coronavirus (PRCV), *Actinobacillus plueropneumoniae*, *Pasteurella multocida*, *Streptococcus suis*, *Glaesserella parasuis* and *M. hyopneumoniae* [27, 47]. The most affecting pathogens to PRDC are different depending on herd and outbroken region. PRRSV and *M. hyopneumoniae* are considered

principal pathogens among PRDC related ones mentioned above, it's because those are most isolated commonly from pigs with PRDC [49]. In addition to these infectious agents, PCV2 has been emphasized its criticality to contribute PRDC recently [49]. PRRSV, PCV2 and *M. hyopneumoniae* have something in common influencing host immune system. PRRSV and PCV2 infect macrophages and lymphocytes respectively. *M. hyopneumoniae* influences non-specifically macrophages and lymphocytes both, makes them hyperplasia and infiltrate into pulmonary tissue. Hence, infections of these organisms together with another respiratory pathogen can aggravate the severity of respiratory disease and make the treatment difficult.

The role of *M. hyopneumoniae* in PRDC has been researched focusing on the interaction with PRRSV and PCV2. According to the study investigating the interaction between PCV2 and *M. hyopneumoniae* using experimental dual infection model, pigs infected with both pathogens had significantly more severe lung and lymphoid lesions, and PCV2 antigens were identified more frequent and longer period in these lesions compared to controls [50]. Another similar study investigated the interaction between PRRSV and *M. hyopneumoniae* has shown that PRRSV-associated clinical respiratory disease and lung lesions were observed more severe and longer period in pigs infected with *M. hyopneumoniae* compared to other groups infected with single pathogen [51]. These results indicate that *M. hyopneumoniae* infection can exacerbate the negative impacts of other two major viral organisms involved in PRDC. It also implies

that a control of *M. hyopneumoniae* may be the first priority in perspective of swine respiratory disease control particularly for viral diseases. In case of PRRSV infected pigs in natural condition, *M. hyopneumoniae* vaccination contributed to compensate loss of ADWG following PRRSV-induced disease [52].

6. Epidemiology

Pig is the only host of *M. hyopneumoniae.* There is no clear evidence of susceptibility depends on an age, although clinical signs of disease are typically observed in grow-finishing to slaughter age and rarely observed before 6 weeks age of piglets [3].

M. hyopneumoniae can be mainly transmitted by close contact between infected and susceptible pigs [27]. *M. hyopneumoniae* may be introduced into a herd by direct transmission following the introduction of purchased, subclinically infected replacement gilts or other pigs and by airborne transmission. *M. hyopneumoniae* may infect animals via the inhalation of muco-respiratory droplets emitted during coughing from the infected animals. This pathogen may spread horizontally to susceptible pigs or vertically from sows to offspring. In epidemiology of transmission, persistently infected pigs with subclinical disease are critical, it because they are remained as a carrier able to transmit the pathogen continuously to another susceptible animals [53]. It tends that low parity sows or gilts have low levels of antibodies and excrete *M. hyopneumoniae* organisms

more than older sows [54]. The transmission of *M. hyopneumoniae* between pen mate is reported slow [55]. In recent, the transmission from wild boar has also been reported [56]. The spreading of *M. hyopneumoniae* through the air is principal challenging to control the pathogen. Airborne particles containing the microorganism are generated during sneezing and coughing, and also exhaled by infected pigs [57]. Especially airborne transmission between farms may occur, and the risk of a herd becoming infected with *M. hyopneumoniae* is inversely proportional with a distance from other pig farms [3]. It suggests airborne transmission can be a major risk of *M. hyopneumoniae* infection to mycoplasma-free SPF pig herds if the breakout of *M. hyopneumoniae* is occurred in neighbor.

In general swine production system, sows and their offspring are considered the reservoir of *M. hyopneumoniae* infection. Circulation of *M. hyopneumoniae* is occurred among existing sows and be spread to incoming gilts, which are probably enabling to maintain the pathogen within the production system and are main route of shedding to newborn piglets [58, 59]. The continuous inflow of gilts and birth of piglets provide important susceptible populations needed to maintain *M. hyopneumoniae* circulation in this condition [27]. Piglets are considered free from *M. hyopneumoniae* at birth, and they are firstly exposed to the pathogen during lactation period by contact with dams shedding it [58]. This indicates that the length of the lactation period can play a role of risk factor which piglet becomes colonization with *M. hyopneumoniae* prior to weaning [60]. Piglet colonized by *M.*

hyopneumoniae at weaning age is an important factor in epidemiology in multi-site production system, in which pigs are transferred to clean facilities for the growing and finishing phases under the circumstances that AIAO is able to be practiced.

Effective diagnostic tool to assess *M. hyopneumoniae* infection is also critical for mitigating the risk of transmission. Clinical signs and lung lesions are used for a tentative diagnosis but needs a confirmation test in laboratory. Bacterial isolation can be a confirmatory method to diagnose *M. hyopneumoniae* infection. However, it is usually not used routinely because the procedure is laborious, time-consuming and interference with another *Mycoplasma* spp., such as *M. hyorhinis* or *M. flocculare* [3, 4]. Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are the most utilized commonly to monitor swine respiratory diseases of which are associated with *M. hyopneumoniae* infection. ELISA can accomplish detection of antibodies to *M. hyopneumoniae*, so be utilized to monitor serological status of pig herds. ELISA is a rapid, cost-efficient, and easy to process. This method is useful to determine the presence of maternally derived and acquired antibodies, as well as on the time required for animals to be seroconverted [3]. The antibody profiling by ELISA cannot differentiate natural infection from vaccination. In addition, serological antibody titers don't guarantee the extent of protection against the infection [4]. Studies for the time of seroconversion using ELISA method indicates that seroconversion under natural infection with *M. hyopneumoniae* in field

conditions is occurred slowly, mainly in grower-finishing phase (8-24 weeks of age), between 6-9 weeks post-infection (PI) [61, 62]. This delay in seroconversion after infection with *M. hyopneumoniae* is possibly attributed to that *M. hyopneumoniae* adheres to the ciliated epithelium and does not infiltrate into the lung tissue to the same extent as other pathogens, this may cause slower antigen presenting to the host immune system [3].

The application of PCR is allowed for a significant increase in the detection of *M. hyopneumoniae* in multiple types of sample, and real-time PCR is one of the most common methods for *M. hyopneumoniae* detection [63, 64]. This is more rapid, specific, and sensitive than bacteriological culture. Since *M. hyopneumoniae* attaches to the ciliated epithelial cell in respiratory tract, the sample for PCR is usually tracheo-bronchial swabs or bronchoalveolar lavage fluid (BALF). In condition of natural infection, the use of PCR to diagnose from nasal swab sample was reported reliable and a correlation was observed between the detection of *M. hyopneumoniae* in the nasal cavities and bronchi with lesions of enzootic pneumonia [65]. Nowadays, nasal swabs for nested PCR testing for *M. hyopneumoniae* in live pigs has been known as the most sensitive tool to detect the pathogen with extremely low levels of nucleic acids [66, 67]. These findings, the worth of nasal swab as an appropriate sample for PCR and the benefit of the nested PCR requires small volume of sample, can support that the PCR is more precise and convenient method of determining when animals become infected.

7. Immunity

The interaction of *M. hyopneumoniae* with host immune system is not fully understood yet. However, some factors of the immune system may enhance as well as hamper the progress of SEP [27, 68]. The infection generates multiple pro-inflammatory (Interleukin (IL)-1ß, IL-6, Tumor necrosis factor (TNF)-α) and immunoregulatory (IL-10) cytokines by macrophage, neutrophils and lymphocytes in the lung. This immoderate inflammatory response brings out lymphoid hyperplasia, it is thought to be a major enhancer of lung lesions [69]. Toll-like receptor 2 (TLR2) and TLR6 have been reported important for porcine alveolar macrophages to recognize *M. hyopneumoniae* [70]. Macrophages reduce the production of TNF-α in accordance with the blocking of TLR2 and TLR6. This indicates that alveolar macrophages are related in inflammatory and innate immune responses during *M. hyopneumoniae* infection [71]. It can modulate gene expression of swine epithelial cells. Multiple genes related to immune response and inflammation were found, such as C3 complement, SAA3, chemokines (CXCL2 and CCL20) and galectins [72]. These chemokines may attract myeloid cells. It suggests that ciliostasis caused by *M. hyopneumoniae* might partially be supported by the down-regulation of ciliary genes.

The infection of *M. hyopneumoniae* usually induces slow seroconversion and generates local specific antibodies prior to serum specific antibodies. but reduced faster [73]. *M. hyopneumoniae*-specific serum IgG antibodies are detected 3-4 weeks post-infection (PI), peak

after 11-12 weeks and then decrease very gradually in experimental condition [74]. In another study, the virulence of strain also affected to the time of seroconversion, pigs infected with a highly virulent strain had earlier seroconversion compared with pigs infected with low virulent strain [75]. *M. hyopneumoniae*-specific IgM in serum can be detected as early as 9 days PI, and the proportion of positive pigs peaked at 14 days PI and decreased rapidly in experimental condition [76]. In Regard to IgA to specific *M. hyopneumoniae*, it can be detected in nasal swabs approximately 6 days PI and peaked 12-16 days PI and decreased steadily afterwards to return pre-immune levels by 84 days PI [73]. *M. hyopneumoniae*-specific IgG levels in serum don't have correlation with the severity of lung lesions in the infected pigs. This indicates the systemic antibodies induced by vaccination may play a minor role in protective immunity [77]. Mycoplasma-specific IgA can prevent adhesion of *M. hyopneumoniae* to the ciliated cells of the respiratory tract [78, 79]. Mycoplasma-specific IgG spreading from the blood into the lung tissue or produced locally in the bronchus-associated lymphoid tissue (BALT) could opsonize *M. hyopneumoniae* and initiating phagocytosis by macrophages and neutrophils [80].

T-cell mediated immune responses are known as important for protection against *M. hyopneumoniae*. T cells mediate immune responses and have enormous impact on the progress of SEP [68]. In the study of *M. hyopneumoniae* vaccine and challenge, the vaccinated group was observed the secretion of specific antibodies, as well as

interferon-ɣ secreting cells (IFN-ɣ-SC) in blood before and after challenge and had significant low lung lesions compared with the unvaccinated group [78]. In another study using *M. hyopneumoniae*-resistant pig line, higher serum levels of IFN-ɣ-SC and IL-17A but lower levels of IL-4 and $CD4^+$ T cells were detected in the resistant line compared to non-resistant one after vaccination [81]. In addition, pigs vaccinated with *M. hyopneumoniae* bacterin had a lower $CD4^+/CD8^+$ ratio, and thus a higher relative number of $CD8^+$ cells, it suggests that $CD8^+$ T cells have a role of protector against *M. hyopneumoniae* infection and contribute to positive effects observed after vaccination [80]. These results also indicate that Th1, Th17 and $CD8^+$ T cell response play an important role in protection against mycoplasmal disease. Th1 response induces the activation of macrophage killing by IFN-ɣ. The immune response of Th17, which produces IL-17A, is critical to protect a mucosal surface, to promote epithelial cell regeneration, mucosal and antimicrobial protein production, and the release of neutrophil recruitment [68]. Th17 cells attract other immune cells which can sweep out the pathogen, and elevate secretory IgA level, consequently, protect the lung mucosa [82, 83]. $CD8^+$ T cells is well defined its character as killing infected cells [84]. Studies performed in *Mycoplasma pulmonis* mouse model suggested that $CD8^+$ T cell may reduce the pro-inflammatory Th cell responses that leads to lung damage and clinical disease [68].

8. Vaccine

Currently, the best way for controlling *M. hyopneumoniae* infection in swine farm has been accepted as well-combined application of the improvement in management and housing such as AIAO and enhancement of ventilation, treatment with antibiotics, and vaccination. Among them, vaccination is considered as the most effective tool to accomplish the desired purpose. It has been estimated that 70% of pig herds are being applied vaccination against *M. hyopneumoniae* worldwide [9]. To date, 11 monovalent vaccines against for *M. hyopneumoniae* are approved in Korea (**Table 1**). Almost of marketed vaccines worldwide are based on whole-cell bacterin preparation with inactivated *M. hyopneumoniae* strain, which is formulated with adjuvant, and are administered by intramuscularly [8]. Korea is also consistent with this tendency of vaccine. The vaccination timing is mainly around 3 weeks of age, but early administration has become a major concern in swine practitioners to manage transmission of *M. hyopneumoniae* from sow to offspring.

Studies with vaccines have demonstrated that vaccination can reduce clinical signs and lung lesions, help economic performance improved. In addition, vaccines decreased the number of organisms in the respiratory tract and reduced the infection level in the applied herd [7, 75, 85, 86]. These effects seem like various per pig herd. It may be caused by several factors like infection level, the age of infection, and the diversity between different field isolates of *M. hyopneumoniae* [22, 87]. The benefits of vaccination are mainly focused

Table 1. Summary of characters for *M. hyopneumoniae* monovalent vaccines approved in Korea

Commercial vaccine name	Manufacturer	Strain	Vaccine type	Usage	Route
MYPRAVAC	HIPRA (Spain)	Strain J	Inactivated	≥7-10 days of age, 2 mL, double injection apart from 2 wks	IM
Porcillis M hyo IN Once	MSD Animal Health (Netherland)	Strain 11	Inactivated	≥2 wks of age, 0.2 mL, single injection	ID
Ingelvac MycoFLEX	Boehringer Ingelheim Animal Health (USA)	Strain J	Inactivated	≥3-4 wks of age, 1 mL, single injection	IM
MH Guard Inj.	GCVP (Korea)	-	Inactivated	≥3 wks, 1 mL, double injection apart from 2 wks	IM
M+PAC	Schering-Plough Animal Health (USA)	Strain J	Inactivated	≥6 wks, 2 mL, single injection/ ≥7-10 days of age, 1 mL, double injection apart from 2 wks	IM IM or SC
SuiShot MycoGuard	CAVAC (Korea)	NSM	Inactivated	≥4 wks, 2 mL, double injection apart from 2 wks	IM
Bayovac MH-PRIT-5 One	Tafoong vaccine & Biotech (Taiwan)	PRIT-5	Inactivated	≥3 wks, 2 mL, single injection	IM
Myco Shield	Pharmgate (USA)	VMRI-11	Inactivated	≥2 wks, 1 mL, double injection apart from 2 wks	IM
MycoGard-1 TIME	Pharmgate (USA)	ATCC strain #25095	Inactivated	≥2 wks, 1 mL, single injection	IM
RespiSure One	Zoetis (USA)	P5722-3	Inactivated	≥1 wks, 2 mL, single injection	IM
Hyogen	CEVA (Hungary)	Strain 2940	Inactivated	≥3 wks, 2 mL, single injection/ ≥2 wks, 1 mL, double injection apart from 2-3 wks	IM

-: No records were found/wks: weeks/IM: Intramuscular/ID: Intradermal/SC: Subcutaneous

to economic aspects including the improvement of the ADWG (2-8%) and the feed conversion ratio (2-5%), saving the cost of treatment due to reduction of clinical symptoms of respiratory disease [88]. However, vaccines cannot prevent *M. hyopneumoniae* colonizing to ciliated epithelial cells in respiratory tract, and have no significant reduction in transmission [78, 85]. It indicates that vaccination can provide partial protection against *M. hyopneumoniae* infection, so the vaccination alone may be insufficient to achieve for controlling the pathogen, thus other practices such as optimizing management and biosecurity measure should be executed together [89].

The mechanism of protection that commercial vaccines confer to the host is still not fully understood. Cell-mediated immune (CMI) responses are regarded as a principal key for the protective capability of vaccines [80]. The pathogenicity of *M. hyopneumoniae* infection is mainly from its distortion to the innate immune system of host, such as massive infiltration of macrophages and lymphocytes to the lung tissue, it leads to pneumonia lesion [80, 90, 91]. This hyperplasia of host immune cells is mediated by several chain reactions of pro-inflammatory cytokines [92]. Vaccinations against *M. hyopneumoniae* have demonstrated the effect of adjustment to the immune response, such as a lower infiltration of macrophages in the lung tissue in vaccinated pigs upon *M. hyopneumoniae* infection [93, 94]. Several studies have reported that inactivated whole-cell bacterins significantly elevate the level of IFN-ɣ-SC in the blood and lung tissue of vaccinated pigs compared to the unvaccinated before

and after challenge with virulent strain [9, 78, 80, 95]. Moreover, vaccinated pigs had lower levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 that may potentially induce the hyperplasia of immune cells [80, 94], and had higher level of cells producing anti-inflammatory cytokines like IL-10 in their bronchial lymph node comparing with the unvaccinated ones [80].

Antigen-specific antibodies in serum is usually considered as one of assessment points for the efficacy of vaccine. Commercial vaccines against *M. hyopneumoniae* can induce pathogen-specific antibodies in host's blood, although the proportion of seroconversion is various (30-100%) per an individual vaccine [96, 97]. In condition that natural infection is not involved, and booster vaccination is applied, antibody titers decreased below detection limits within 1-3 months after vaccination [88]. However, no correlation between the level of antibodies in serum and protection against *M. hyopneumoniae* was observed [77, 97]. In other words, measuring serum *M. hyopneumoniae*-specific antibodies after vaccination may not be proper to investigate the capability of vaccine for protection. This perspective also can be supported by that current serological analysis cannot differentiate the source of identified *M. hyopneumoniae*-specific antibodies in serum, either vaccination or natural infection [98]. This parameter may be only available to determine the time of onset immunity of vaccine under experimental condition using specific pathogen-free (SPF) pigs.

Besides the capability of commercial vaccines inducing CMI and

specific antibodies in serum against *M. hyopneumoniae* infection, what strain of *M. hyopneumoniae* is used for vaccine formulation also should be considered for foreseeing the effect of vaccines. Mostly non-pathogenic strain J, isolated UK in middle of 1950s, is commonly used as an antigen of vaccine [99]. Since *M. hyopneumoniae* is the most prevalent pathogen in pig farm worldwide, it can be doubt whether strain J shows similar efficacy and safety against circulating strain in another continent and region [42, 100]. Recently, several pharmaceutical makers are utilizing to formulate alternative strain such as strain 2940 (Ceva Sante Animal) isolated in Unites States and Prit-5 (Bayer) isolated in Taiwan. This indicates that the interested parties in swine production are aware of that vaccine strain is one of factors may induce variable results of the performance after vaccine application. Therefore, the efficacy of vaccine needs to be evaluated prior to introducing to new region under experimental condition with a local pathogenic isolates challenge.

A commercial vaccine subjected to be evaluated in Chapter I and II is formulated inactivated *M. hyopneumoniae* whole-cell bacterin based on strain 2940, oil adjuvant and preservative. This is a general composition for inactivated *M. hyopneumoniae* vaccines. The vaccine had been evaluated its efficacy against European pathogenic strains of *M. hyopneumoniae* [101]. Since the vaccine strain was never applied into Korea, the confirmation study to demonstrate efficacy and safety was essential under experimental and field condition in Korea. There are several parameters commonly observed in studies for the

evaluation of vaccines against *M. hyopneumoniae* [95, 102-106]. Regarding the efficacy, ADWG, lung lesion scoring and inducing immune responses particularly for CMI. Clinical observations, injection site reaction (ISR) and rectal temperature were commonly monitored for safety evaluation. These parameters can be considered as a guideline for further candidates of vaccine to measure and compared in development studies before commercialized.

Although several commercial vaccines against *M. hyopneumoniae* infection are available, a whole mechanism of vaccine conferring protection ability is not yet understood. A tremendous loss in performance coming from the infection of that pathogen is still going on in pig production industry worldwide. Developing new vaccines through continuous efforts for the improvement will provide better and more various options for protection against *M. hyopneumoniae.*

9. References

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CHAPTER I.

Experimental evaluation of *Mycoplasma hyopneumoniae* bacterin against a Korean *M. hyopneumoniae* challenge

1. Abstract

The objective of this study was to evaluate the efficacy of a new *Mycoplasma hyopneumoniae* bacterin against a Korean *M. hyopneumoniae* challenge under experimental conditions. 15 pigs were allocated randomly into 3 groups (5 pigs per group) that were designated in 1 of 3 ways: vaccinated-challenged, unvaccinated-challenged, or unvaccinated-unchallenged. The pigs in the vaccinated-challenged group were immunized with an *M. hyopneumoniae* whole-cell bacterin at a 2.0 mL dose-level at 21 days of age. At 42 days of age (0 days post challenge (dpc)), the pigs in the vaccinated-challenged and unvaccinated-challenged groups were inoculated intranasally with a strain of Korean *M. hyopneumoniae.* Vaccinated-challenged pigs elicited a strong cell-mediated immunity as measured by *M. hyopneumoniae*-specific interferon-gamma secreting cells (IFN-ɣ-SC) when compared with unvaccinated-challenged pigs. Vaccination of pigs with this new *M. hyopneumoniae* bacterin reduced nasal shedding and lung lesions. The evaluated vaccine was therefore considered effective in controlling of *M. hyopneumoniae* infection.

Keywords Bacterin, Enzootic pneumonia, *Mycoplasma hyopneumoniae*

2. Introduction

M. hyopneumoniae infection alone causes relatively mild disease in the absence of environmental stressors, but when complicated by secondary bacterial invaders, may result in obvious clinical disease and severe production losses in intensively reared pigs [1]. This respiratory disease is referred to as enzootic pneumonia. *M. hyopneumoniae* is probably the most frequent bacterial respiratory infection in pig production and continues to be economically significant worldwide [1].

Vaccination is the most effective strategy for reducing economic losses and the clinical effects of *M. hyopneumoniae* infection on the Asian pork industry. A new single-dose *M. hyopneumoniae* whole-cell bacterin (Hyogen®, CEVA Santé Animale) was recently introduced into the Asian market to protect pigs against *M. hyopneumoniae* infection. In Europe, the same single-dose *M. hyopneumoniae* whole-cell bacterin provided protection against Belgian *M. hyopneumoniae* field isolates [2]. *M. hyopneumoniae* field isolates are known to be highly genetic, antigenic, and pathogenically variable between herds and geographical locations [3-5]. Moreover, the genetic diversity of *M. hyopneumoniae* field isolates may be one of the factors that affects the efficacy of *M. hyopneumoniae* vaccines [6].

These results strongly suggest that protection of this bacterin against Belgian *M. hyopneumoniae* field isolates does not guarantee the same effective protection against Korean *M. hyopneumoniae* field isolates. The objective of this study was to evaluate the efficacy of

the new single-dose *M. hyopneumoniae* whole-cell bacterin based on strain BA 2940-99, oil adjuvanted with paraffin and *Escherichia coli* J5 LPS with thimerosal as excipient, in pigs experimentally infected with *M. hyopneumoniae* for registration as recommended by the Republic of Korea's Animal and Plant Quarantine Agency (APQA, http://qia.go.kr).

3. Materials and methods

3.1. Animals

Unnecessary animal usage was eliminated in accordance with APQA guidelines by selecting and assigning the recommended 5 piglets for treatment group. A total of 15 colostrum-fed, crossbred, conventional piglets were weaned and purchased at 18 days of age from a commercial farm that was free of porcine reproductive and respiratory syndrome virus (PRRSV) and *M. hyopneumoniae* based on serological testing of the breeding herd and long-term clinical and slaughter history. At 21 days of age, serum samples from pigs were found seronegative for porcine circovirus type 2 (PCV2), PRRSV, and *M. hyopneumoniae* according to routine serological testing. Serum samples were negative for PCV2 and PRRSV and nasal swabs were negative for *M. hyopneumoniae* when tested by real-time polymerase chain reaction (PCR) [7].

3.2. Experimental design

For the study, 15 pigs were allocated into 3 groups (5 pigs per group) using the Excel random number generator function (Microsoft Corporation). At -21 days post challenge (dpc, 21 days of age), the pigs in the vaccinated-challenged (Vac/Ch) group were administered a single, 2.0 mL dose of *M. hyopneumoniae* whole-cell bacterin (Hyogen®, Lot No.1405582B) intramuscularly based on the manufacturer's instructions. The pigs in unvaccinated-challenged (UnVac/Ch) and unvaccinated-unchallenged (UnVac/UnCh) groups were administered an equal volume of phosphate buffered saline (PBS, 0.01M, pH 7.4, 2.0 mL) at 21 days of age. At 0 dpc (42 days of age), the pigs in the Vac/Ch and UnVac/Ch groups were inoculated with *M. hyopneumoniae* (strain SNU98703). Infection of pigs with *M. hyopneumoniae* strain SNU98703 caused severe mycoplasmal pneumonia [8].

Pigs in the Vac/Ch and UnVac/Ch groups were anesthetized with a mixture of 2.2 mg/kg body weight (BW) xylazine hydrochloride (Rompun®, Bayer), 2.2 mg/kg tiletamine hydrochloride, and 2.2 mg/kg BW zolazepam hydrochloride (Zoletil 50®, Virbac) by intramuscular injection. Post-anesthetization, pigs were inoculated intratracheally with 7.0 mL of *M. hyopneumoniae* (strain SNU98703) culture medium containing 10^7 color-changing units (CCUs)/mL. Pigs in the UnVac/UnCh group were inoculated with 7.0 mL of PBS in the same manner. After challenge, the pigs in the Vac/Ch and UnVac/Ch groups were randomly assigned to 1 room. The rooms each contained

2 pens with 5 pigs housed per pen. Pigs in the UnVac/UnCh group were randomly placed into 1 pen in the remaining room.

Blood and nasal swabs were collected at -21, 0, 7, 14 and 21 dpc. All 15 pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 21 dpc as described in a previous study [9]. Tissues were collected from each pig at necropsy. Post-collection, the tissues were fixed for 24 hours in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee (approval number SNU-181018-4).

3.3. Clinical observation

After *M. hyopneumoniae* inoculation, the pigs were monitored daily for physical condition and scored weekly for severity of clinical respiratory disease severity using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) [10].

3.4. Growth performance

The live weight of each pig was measured at 2 time points throughout the study as follows: -21 (21 days of age) and 21 dpc (63 days of age). On conclusion of the study, the average daily weight gain (ADWG; g/pig/day) was calculated over production stage from 21 to 63 days of age. Data for dead or removed pigs were included

in the calculation.

3.5. Quantification of M. hyopneumoniae DNA in nasal swabs

Genomic DNA copies of *M. hyopneumoniae* were quantified by real-time quantitative PCR after DNA was extracted from nasal swabs using a commercial kit (QNAamp DNA Mini Kit, QIAGEN) as described in previous study [7].

3.6. Enzyme-linked immunosorbent assay

Serum samples were tested for antibodies against *M. hyopneumoniae* (*M. hyo* Ab test, IDEXX Laboratories Inc.). Serum samples were considered positive for *M. hyopneumoniae* antibodies if the sample-to-positive (S:P) ratio was 0.4.

3.7. Enzyme-linked immunospot assay

An enzyme-linked immunospot (ELISpot) assay was conducted to measure the number of *M. hyopneumoniae*-specific IFN-ɣ-SC. *M. hyopneumoniae* (strain SNU98703) antigens were prepared as described in previous studies [11, 12]. The numbers of *M. hyopneumoniae*-specific IFN-ɣ-SC stimulated by the aforementioned challenge *M. hyopneumoniae* antigen were determined in peripheral blood mononuclear cells (PBMC) [11, 12]. The IFN-ɣ positive spots on the membranes were imaged, analyzed, and counted using an automated ELISPOT Reader (AID ELISPOT Reader; AID GmbH).

The results were expressed as the numbers of IFN-γ-SC per million PBMC. The ELISpot assay was done in duplicate.

3.8. Pathology

Morphometric analysis of the macroscopic pulmonary lesion was scored on a total scale of 100 points as follows: 10 points each to the right cranial lobe, right middle lobe, left cranial lobe, and left middle lobe; 27.5 points each to the right caudal lobe and left caudal lobe; and 5 points to the accessory lobe [10]. Microscopic mycoplasmal pulmonary lesions were scored (0 to 6) based on the severity of peribronchiolar and perivascular lymphoid tissue hyperplasia [13]. All lung section scoring was evaluated blindly by 2 pathologists.

3.9. Statistical analysis

Prior to statistical analysis, RT-PCR data were transformed to $\log_{10}$ values. Data were tested for normal distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to examine whether there were statistically significant differences at each time point within the 3 groups. A 1-way ANOVA test result with such a statistical significance was further evaluated by conducting a *post-hoc* test for a pairwise comparison with Tukey's adjustment. If the normality assumption was not met, the Kruskal-Wallis test was conducted. A result from the Kruskal-Wallis test that showed statistical significance was further evaluated with

the Mann-Whitney test to include Tukey's adjustment to compare the differences among the groups. Results were reported in P value in which a value of $P < 0.05$ was considered to be significant.

4. Results

4.1. Clinical observations

The mean scores for respiratory disease were significantly lower ($P < 0.05$) in pigs from the Vac/Ch group when compared with the UnVac/Ch group at 14 and 21 dpc. The pigs from the UnVac/UnCh group remained normal throughout the experiment. There was no significant difference in ADWG among 3 groups from 21 and 63 days of age (**Table I**).

Table I. ADWG from 21 to 63 days of age and pathological data (mean ± standard deviation) of 5 pigs in each of 3 groups at 21 dpc. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 3 groups.

Groups	Vaccinated -challenged	Unvaccinated -challenged	Unvaccinated -unchallenged
ADWG	295.71 ± 22.30	291.90 ± 26.76	301.90 ± 16.62
Macroscopic lung lesion scores	7.3 ± 6.53[a]	22.7 ± 11.42[b]	0 ± 0[a]
Microscopic lung lesion scores	1.68 ± 0.39[a]	3.64 ± 0.57[b]	0 ± 0[c]

4.2. Quantification of M. hyopneumoniae DNA in nasal swabs

Pigs in the Vac/Ch group had significantly less ($P < 0.05$) *M. hyopneumoniae* genomic copies in their nasal swabs compared to the UnVac/Ch group at 14 and 21 dpc (**Figure 1**). No *M. hyopneumoniae* was detected in the pigs from the UnVac/UnCh group.

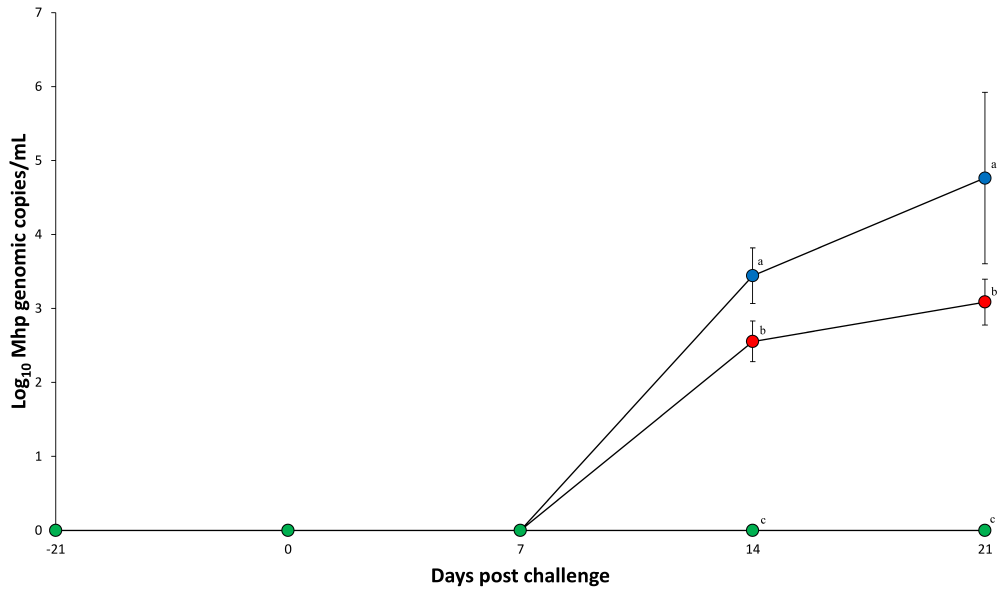

Figure 1. Mean values of *M. hyopneumoniae* DNA genomic copy number in nasal swabs from vaccinated-challenged (Vac/Ch, ●), unvaccinated-challenged (UnVac/Ch, ●), and unvaccinated-unchallenged (UnVac/UnCh, ●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, c) indicate significant ($P < 0.05$) difference among the 3 groups.

4.3. Immune responses against M. hyopneumoniae

Pigs in the Vac/Ch group had a significantly higher ($P < 0.05$) *M. hyopneumoniae* enzyme-linked immunosorbent (ELISA) assay S:P ratio in their serum samples when compared with the UnVac/Ch group from 0 to 7 dpc (**Figure 2**), as well as a significantly higher number of *M. hyopneumoniae*-specific IFN-ɣ-SC in their PBMC (**Figure 3**) when compared with the UnVac/Ch group from 0 to 21 dpc. No *M. hyopneumoniae* - specific antibodies and IFN-ɣ-SC were detected in pigs from the UnVac/UnCh group.

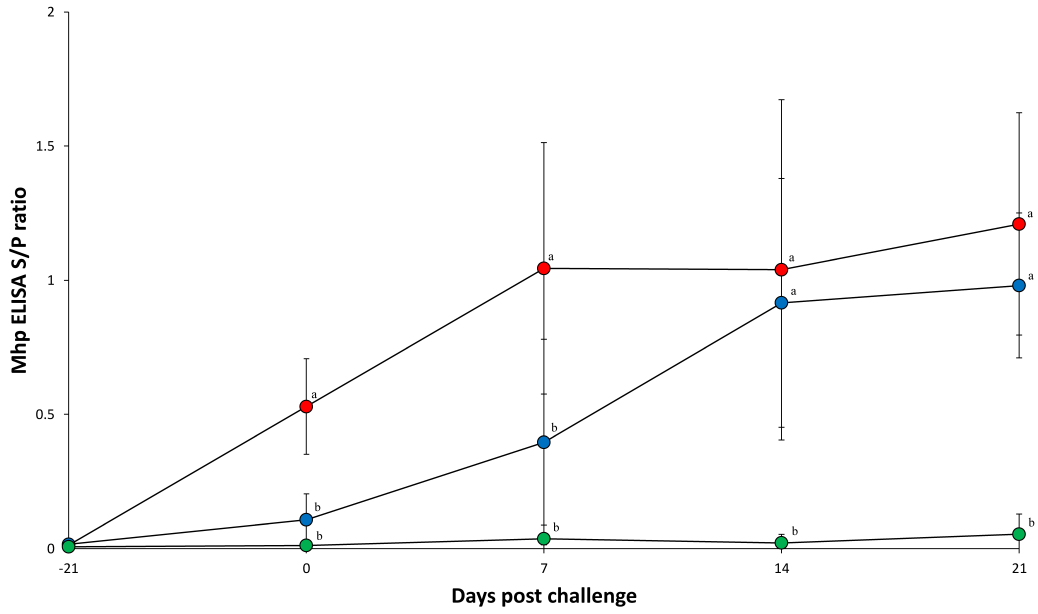

Figure 2. *M. hyopneumoniae*-specific ELISA antibody levels in serum from vaccinated-challenged (Vac/Ch, ●), unvaccinated-challenged (UnVac/Ch, ●), and unvaccinated-unchallenged (UnVac/UnCh, ●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among the 3 groups.

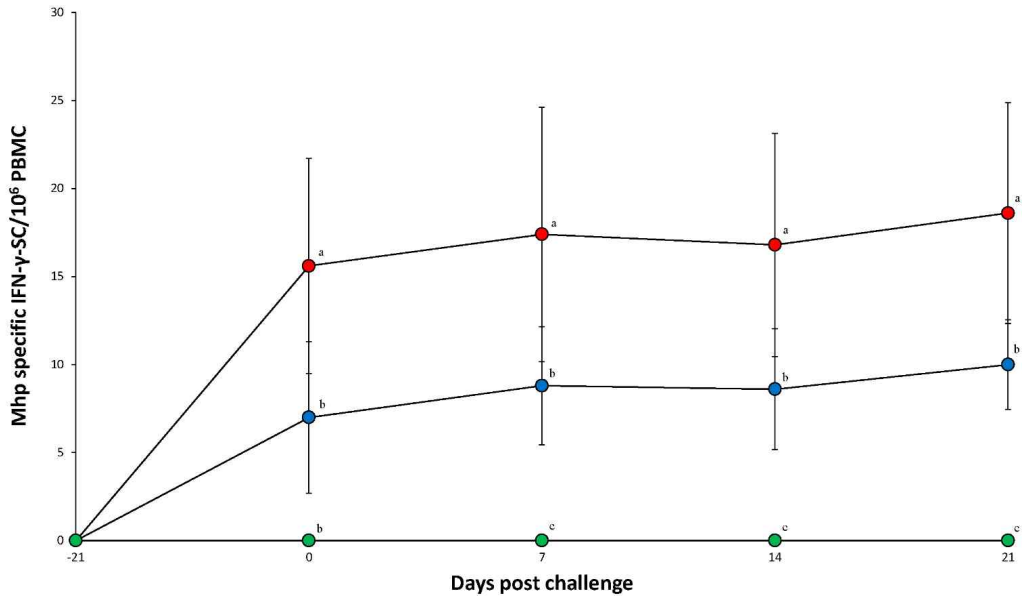

Figure 3. Frequency of *M. hyopneumoniae*-specific IFN-ɣ-SC in PBMC from vaccinated-challenged (Vac/Ch, ●), unvaccinated-challenged (UnVac/Ch, ●), and unvaccinated-unchallenged (UnVac/UnCh, ●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among the 3 groups.

4.4. Pathology

Pigs in the Vac/Ch group had significantly lower ($P < 0.05$) macroscopic and microscopic lung lesion scores when compared with the UnVac/Ch group at 21 dpc. No macroscopic and microscopic lung lesions were detected in pigs from the UnVac/UnCh group (**Table I**).

5. Discussion

The results of the present study demonstrate that vaccinated-challenged pigs develop fewer lung lesions and nasal route excretion than unvaccinated-challenged pigs. This variance between the 2 groups is probably due to differences in protective immunity. Protective immunity against *M. hyopneumoniae* is not fully understood. The fact that the pathogen is non-invasive, but can still induce pneumonia, implies that cellular immune response plays a significant role [14, 15]. Vaccinated-challenged pigs elicited a strong cell-mediated immunity as measured by *M. hyopneumoniae*-specific IFN-ɣ-SC when compared with unvaccinated-challenged pigs. Induction of cell-mediated immunity by *M. hyopneumoniae* vaccine plays a significant role in protecting pigs against *M. hyopneumoniae* infection, as implied by previous studies [12].

There are 2 ways to assess the efficacy of vaccines: field clinical and experimental challenge trials. Field clinical trials are suitable for evaluating pig productivity. Vaccination against *M. hyopneumoniae*

improved pig productivity and was reported as increased growth performance and decreased mortality under field conditions [16-20]. Despite vaccination efforts, *M. hyopneumoniae* continues to circulate within pig herds, leading to the possibility of exposure and re-exposure to the organisms by horizontal transmission under field conditions. Meanwhile, experimental challenge trials are suitable for microbiological, immunological, and pathological evaluation.

Growth performance was also evaluated in the present experimental challenge study. There was no significant difference in ADWG between vaccinated-challenged and unvaccinated-challenged groups because of the small number of pigs in each group and the short duration observed after challenge with *M. hyopneumoniae*. These results agree with a previous study in which the same vaccine showed no significant difference in growth performance under experimental conditions [3]. Nevertheless, vaccination of pigs with this newly evaluated *M. hyopneumoniae* bacterin benefits the pig by eliciting cell-mediated immunity and reducing nasal shedding and lung lesions. The newly evaluated vaccine may therefore be an effective tool in controlling *M. hyopneumoniae* infection.

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CHAPTER II.

Field evaluation of a new single-dose *Mycoplasma hyopneumoniae* bacterin effects on growth performance

1. Abstract

This study was to evaluate the efficacy of a new single-dose bacterin against *Mycoplasma hyopneumoniae* under field conditions. Three separate farms were selected based on their history of enzootic pneumonia. On each farm, vaccinated pigs (n = 20; 10 male and 10 female) were administered a single dose of the *M. hyopneumoniae* bacterin at 21 days of age while unvaccinated pigs (n = 20; 10 male and 10 female) were administered a single dose of phosphate buffered saline (PBS) at the same age.

Vaccination against *M. hyopneumoniae* reduced the severity of lung lesions and clinical signs such as coughing, which leaded to improved growth performance of the pig. Vaccinated pigs had a significantly higher ($P < 0.05$) average daily weight gain (ADWG) between 21 to 175 days of age (0 to 154 days post vaccination) and elicited cell-mediated immunity, as measured by *M. hyopneumoniae*-specific interferon-ɣ secreting cells (IFN-ɣ-SC), when compared with unvaccinated pigs located at all 3 farms. The data presented in this field study demonstrated that the *M. hyopneumoniae* bacterin improved growth performance effectively in 3 farms suffering from enzootic pneumonia.

Kewords: swine, enzootic pneumonia, *Mycoplasma hyopneumoniae,* vaccine

2. Introduction

M. hyopneumoniae can be an important pathogen in porcine respiratory disease complex (PRDC) as well as the primary pathogen of enzootic pneumonia, a chronic respiratory disease in growing pigs resulting from combined infections of *M. hyopneumoniae* and one or more secondary bacterial pathogens [1, 2]. Enzootic pneumonia is characterized by a persistent non-productive cough with a reduced growth rate, a poor feed conversion ratio, high morbidity, and low mortality [3, 4]. The economic impact of *M. hyopneumoniae* infections in swine farms worldwide can be considered significant.

Several strategies may be implemented to successfully prevent and control *M. hyopneumoniae* including optimized management practices and vaccination [5]. While all-in/all-out (AIAO) production and multisite operations are great management tools, vaccination remains an important and cost-effective method for reducing the impact of *M. hyopneumoniae* infection. The *M. hyopneumoniae*-free status of herds is difficult to maintain especially in pig-dense areas, since the airborne spread of this pathogen may occur over several kilometers [6].

In Korea, approximately 70% of total piglets farrowed in 2018 were vaccinated with *M. hyopneumoniae* (http://www.kahpa.or.kr). Therefore, vaccination is one of the tools used to control *M. hyopneumoniae.* The objective of this study was to evaluate the efficacy of a new single-dose *M. hyopneumoniae* whole-cell bacterin (Hyogen®, CEVA Santé Animale) based on strain BA 2940-99, oil

adjuvanted with paraffin and *Escherichia coli* J5 LPS with thimerosal as excipient under field conditions in accordance with the registration guidelines of the Republic of Korea's Animal and Plant Quarantine Agency (APQA, http://www.qia.go.kr).

3. Methods

The protocol for this field study was approved by the Seoul National University Institutional Animal Care and Use Committee (approval number SNU-180621-13).

3.1. Farm history

The Clinical field trial was conducted on 3 Korean farms (denoted as Farms A, B, and C) between August 2018 and February 2019. Status of porcine reproductive and respiratory syndrome (PRRS) was stable with no active PRRS virus circulation (high-parity sows were the only seropositive animals in the herd). Porcine circovirus type 2 (PCV2) was circulating in the postweaning and growing period without overt clinical signs of porcine circovirus-associated disease (PCVAD) on the 3 farms.

Farm A was a conventional 400-sow farrowed-to finish swine farm where the owner complained about a dry recurrent cough beginning at 40 days of age accompanied by growth retardation. Real-time polymerase chain reaction (PCR) testing [7] of pneumonic and atelectatic lung samples from pigs at 49 days of age was

conducted for *M. hyopneumoniae* at the Veterinary Diagnostic Center, College of Veterinary Medicine, Seoul National University in May 2018. The testing returned positive results for 5 of the 7 lung samples submitted for *M. hyopneumoniae*. The combined occurrence of clinical signs, detection of *M. hyopneumoniae* by PCR, and histopathological lesions (peribronchiolar and perivascular lymphoid tissue hyperplasia) were indicative of an ongoing infection with *M. hyopneumoniae.*

Farm B consisted of a conventional 150-sow farrow-to-finish swine farm managed in a 2-week batch system and included a history of enzootic pneumonia. Infection with *M. hyopneumoniae* was evident by severe dry coughing, histopathological peribronchiolar lymphoid tissue hyperplasia, and detection of *M. hyopneumoniae* in lung samples by real-time PCR [7] in all three of the 38-day-old pigs tested.

Farm C, a conventional 450-sow-farrow-to-finish swine farm, was suggested to our clinical study team by its practitioner to participate in this field trial on *M. hyopneumoniae* vaccine efficacy. A pilot survey was implemented to assess the circulation of *M. hyopneumoniae* within the herd, as the producers had complained of severe dry coughing and retardation of growth between 10 and 50 days of age. Lung samples from 74-day-old pigs were submitted to the Veterinary Diagnostic Center, College of Veterinary Medicine, Seoul National University in June 2018. Three of the 5 lung samples submissions were positive for *M. hyopneumoniae* using real-time

PCR testing [7]. The histological lesions were characterized by peribronchiolar lymphoid tissues hyperplasia and bronchopneumonia. *Pasteurella multocida* was isolated in four of the 5 lung samples. These results were indicative of enzootic pneumonia by *M. hyopneumoniae* with secondary *P. multocida* infection.

3.2. Study design

The experimental design of the field study strictly adhered to the registration guidelines set by the Republic of Korea's Animal and Plant Quarantine Agency (APQA). Guidelines require that 20 piglets (10 male and 10 female) be selected and assigned to each group of vaccinated and unvaccinated animals. To minimize sow variation, four to six 7-day-old piglets were randomly selected from each sow and assigned to either the vaccinated or unvaccinated group using the random generator function in Excel (Microsoft Corporation). The pigs in the vaccinated groups (VacA, VacB and VacC) were injected intramuscularly in the right side of the neck with 2.0 mL of the *M. hyopneumoniae* bacterin (Hyogen®, CEVA Santé Animale, Lot No. 1405582B) at 21 days of age, while an equal volume of PBS (0.01M, pH 7.4) was injected in the same anatomical location for pigs of the unvaccinated groups (UnVacA, UnVacB, and UnVacC). At 24 days of age, all vaccinated and unvaccinated pigs were transferred to the nursery facility and kept in co-mingled groups until the end of the trial. In the nursery, pigs were then randomly distributed into 4 total pens to include 10 pigs/pen, all within one room. A similar proportion

of each treatment was included in each pen. All pens were identical in design and equipment which included free access to a feed and water trough in accordance with standard farm procedures. The 3 farms did not use feed or water medication effective against *M. hyopneumoniae.* Antibiotics (i.e., penicillin) were given to vaccinated and unvaccinated pigs to help control respiratory diseases during the study. Blood and nasal swabs were collected at study days 0 (21 days of age), 21 (42 days of age), 49 (70 days of age), 77 (98 days of age), and 105 (126 days of age).

3.3. Mortality

Pigs that died were subjected to gross pathological examination within 24 hours at a local veterinary practitioner's clinic. All major organs such as brain, lung, subinguinal lymph node, small and large intestine, liver, kidney, and tonsils were collected from each pig. In case of lung lesions, samples were collected from the edge of these lesions. PCR assays were used to detect specific nucleic acids for PCV2, PRRS virus, swine influenza virus (SIV), and *M. hyopneumoniae* [8–11]. All other bacterial isolation and identifications were carried out by using routine methods.

3.4. Clinical observations

Physical conditions of pigs were monitored daily, and pigs were scored weekly for clinical respiratory disease from study days 0 to 105. Scores ranged from 0 to 6: 0 = normal; 1 = mild dyspnea,

tachypnea, or both when stressed; 2 = mild dyspnea, tachypnea, or both when at rest; 3 = moderate dyspnea, tachypnea, or both when stressed; 4 = moderate dyspnea, tachypnea, or both when at rest; 5 = severe dyspnea, tachypnea, or both when stressed; 6 = severe dyspnea, tachypnea, or both when at rest. Observers were blinded to vaccination status.

3.5. Growth performance

Pigs were weighed at study days 0 (21 days of age), 49 (70 days of age), 91 (112 days of age), and 154 (175 days of age). ADWG was determined for study days 0 to 49, study days 50 to 91, and study days 92 to 154. The ADWG during these various stages was calculated as the difference between the starting and final weight divided by the duration of the stage. Data for dead or removed pigs were included in the calculation.

3.6. Quantification of M. hyopneumoniae DNA in nasal swabs

Sterile polyester swabs (Fisher Scientific Inc.) were used to swab the nasal mucosa of both nostrils, reaching deeply into the turbinate. Swabs were stored in 5.0 mL plastic tubes (Fisher Scientific Inc.) containing 1.0 mL of sterile saline solution. A commercial kit (QIAamp DNA Mini Kit, QIAGEN) was used to extract DNA from nasal swabs to quantify the *M. hyopneumoniae* genomic DNA copy numbers by real--time PCR as previously described [7]. To construct

a standard curve, real-time PCR was performed in quadruplicate in 10-fold serial dilution of chromosomal DNA from *M. hyopneumoniae* strain SNU98703, with concentrations ranging from 10 ng/μL to 1 fg/μL. One femtogram of chromosomal DNA from *M. hyopneumoniae* is considered to be approximately one genomic equivalent [12]. A negative control was included in each run using double distilled water as the template.

3.7. Enzyme-linked immunosorbent assay

Blood samples were collected from each pig by jugular venipuncture. Serum samples were tested for *M. hyopneumoniae* antibodies using a commercial enzyme-linked immunosorbent assay (ELISA; IDEXX Laboratories Inc.). Serum samples were considered positive for *M. hyopneumoniae* antibodies if the sample-to-positive (S:P) ratio was ≥ 0.4 in accordance with the manufacturer's instructions.

3.8. Enzyme-linked immunospot assay

Blood samples were collected from each pig by jugular venipuncture. The enzyme-linked immunospot (ELISpot) assay was conducted to measure the numbers of *M. hyopneumoniae*-specific IFN-ɣ-SC in peripheral blood mononuclear cells (PBMC) [13]. *M. hyopneumoniae* antigens were prepared as previously described [14]. The IFN-ɣ positive spots on the membranes (MABTECH) were

imaged, analyzed, and counted using an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH). The results were expressed as the numbers of IFN-γ-SC per million PBMC. The ELISpot assay was completed in duplicate.

3.9. Pathological evaluation

Lung samples were collected in pigs from each group at study day 154 (175 days of age). Lung pathology was evaluated blindly by two pathologists. Macroscopic lesion scores were estimated, and a score was given to reflect the amount of pneumonia in each lobe. For the entire lung, up to 100 points were assigned as follows: 10 points each to the right cranial lobe, right middle lobe, left cranial lobe, and left middle lobe; 27.5 points each to the right caudal lobe and left caudal lobe; and 5 points to the accessory lobe [15]. Eight pieces of lung tissues (two pieces from the right cranial lobe, two from the right middle lobe, one from the ventromedial part of the right caudal lobe, one from the dorsomedial part of the right caudal lobe, one from the midlateral part of the right caudal lobe, and one from the accessory lobe) were collected from each pig. Three tissue sections of the eight lung pieces were examined blindly by two veterinary pathologists. Lung sections were scored for presence and severity of type II pneumocyte hypertrophy and hyperplasia, alveolar septal infiltration with inflammatory cells, peribronchial lymphoid hyperplasia, amount of alveolar exudate, and amount of inflammation in the lamina propria of bronchi and bronchioles ranging from 0 to 6: 0 = normal; 1 = mild

multifocal; 2 = mild diffuse; 3 = moderate multifocal; 4 = moderate diffuse; 5 = severe multifocal; 6 = severe diffuse [16].

3.10 Statistical analysis

Prior to statistical analysis, real-time PCR data were transformed to $\log_{10}$ values to reduce variance and positive skewness. The normality of the distribution of the examined variables was evaluated by the Shapiro-Wilk test. Continuous data (ADWG, real-time PCR, ELISA, and ELISpot) were analyzed with a Student's *t*-test to determine the significance of group differences at each time point. Discrete data (clinical signs and pathology lesions) were analyzed by Mann-Whitney test to determine the significance of group differences at each time point. A P value < 0.05 was considered significant.

4. Results

4.1. Mortality

One vaccinated pig from farm A died of bronchopneumonia resulting from a combination of PCV2 that was detected with PCR and *Glaesserella parasuis* that was isolated from the lung at study day 51 (72 days of age). Three unvaccinated pigs from farm A died of pleuropneumonia caused by a combination of *Actinobacillus pleuropneumoniae* and other bacteria. *A. pleuropneumoniae* and *Pasteurella multocida* were isolated from lung tissue at study days

74 (95 days of age) and 77 (98 days of age), and *A. pleuropneumoniae* and *Streptococcus suis* were isolated from lung tissue at study day 93 (114 days of age). Farm C had 1 vaccinated pig die of salmonellosis at study day 42 (63 days of age), and 2 unvaccinated pigs died of bronchopneumonia caused by a combination of PCV2 that was detected with PCR and *P. multocida* that was isolated from lung tissue at study days 72 (93 days of age) and 92 (113 days of age), respectively. But PCV2-associated lesions were not observed in lymph nodes from these 2 pigs.

4.2. Clinical signs

Vaccinated pigs from farm A had significantly lower ($P < 0.05$) clinical respiratory scores when compared with unvaccinated pigs at study days 21 to 56. Farm B vaccinates also had significantly lower ($P < 0.05$) clinical respiratory scores when compared with unvaccinated pigs, but at study days 28 to 56. On farm C, vaccinated pigs had significantly lower ($P < 0.05$) clinical respiratory scores when compared with unvaccinated pigs at study days 21 to 63 (**Figure 1**).

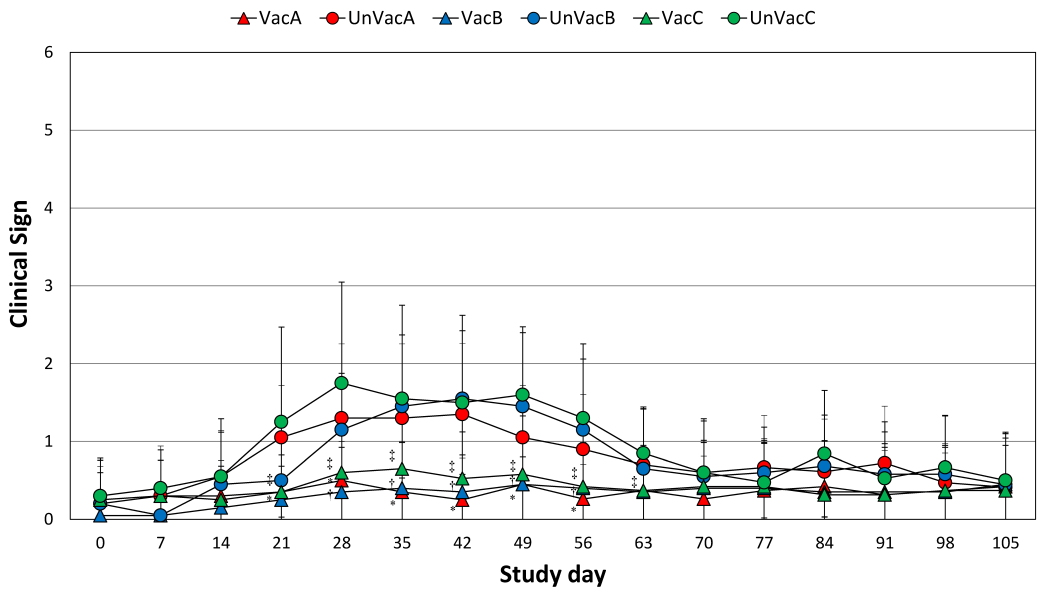

Figure 1. Mean (SD) clinical respiratory disease scores of *M. hyopneumoniae* vaccinated (Vac) or unvaccinated (UnVac) pigs on 3 Korean swine farms (A, B, and C). Mean respiratory scores were scored on a scale from 0 to 6; 0 = normal; 1 = mild dyspnea or tachypnea or both when stressed; 2 = mild dyspnea or tachypnea or both when at rest; 3 = moderate dyspnea or tachypnea or both when stressed; 4 = mild dyspnea or tachypnea or both when at rest; 5 = severe dyspnea or tachypnea or both when stressed; and 6 = severe dyspnea or tachypnea or both when at rest. Significant difference (P value < 0.05; Mann-Whitney test) is indicated between vaccinated and unvaccinated groups within each farm (*Farm A, † Farm B, and ‡ Farm C).

4.3. Growth performance

The body weight of pigs at study day 0 (21 days of age, time of vaccination) did not differ significantly between the vaccinated and unvaccinated groups on all 3 farms (**Table 1**). Vaccinated pigs from all farms (A–C) had significantly higher ($P < 0.05$) ADWG at study days 0 to 49 (21–70 days of age) when compared with unvaccinated pigs from the same farm. Additionally, farm C vaccinated pigs had a significantly higher ($P < 0.05$) ADWG at study days 50 to 91 (70–112 days of age) when compared with the unvaccinated pigs. Overall (study days 0 to 154), the difference between vaccinated and unvaccinated groups was significant ($P < 0.05$) on all 3 farms (**Table 1**).

Table 1 Means (SD) ADWG in pigs vaccinated for *M. hyopneumoniae* (Vac) or unvaccinated pigs (UnVac) on 3 Korean swine farms (A, B, and C)

		ADWG (SD) (g/day) in period between study days			
Farm	Group (n)	0 to 49	50 to 91	92 to 154	0 to 154
A	VacA (20)	402 (19)[a]	745 (30)	763 (21)	643 (10)[a]
	UnVacA (20)	382 (22)[b]	739 (39)	743 (61)	627 (25)[b]
B	VacB (20)	390 (27)[a]	755 (44)	764 (40)	643 (13)[a]
	UnVacB (20)	367 (24)[b]	739 (53)	755 (40)	627 (22)[b]
C	VacC (20)	387 (28)[a]	727 (56)[a]	765 (28)	634 (11)[a]
	UnVacC(20)	366 (26)[b]	704 (34)[b]	760 (44)	620 (22)[b]

[ab] Within a column, values with different superscript letters are significantly different within each farm. ADWG was compared between the two groups within each farm using a Student *t*-test.

4.4. Quantification of M. hyopneumoniae in nasal swabs

On farm A, vaccinated pigs had a significantly lower ($P < 0.05$) number of genomic copies of *M. hyopneumoniae* in their nasal swabs when compared with unvaccinated pigs at study day 21. On farm B, there was a numerical, but not statistically significant ($P < 0.05$), differences in the number of *M. hyopneumoniae* genomic copies on the nasal swabs of vaccinated and unvaccinated pigs. Farm C vaccinated pigs had a significantly lower ($P < 0.05$) number of *M. hyopneumoniae* genomic copies in their nasal swabs when compared with unvaccinated pigs at study days 21, 49, and 77 (**Figure 2**).

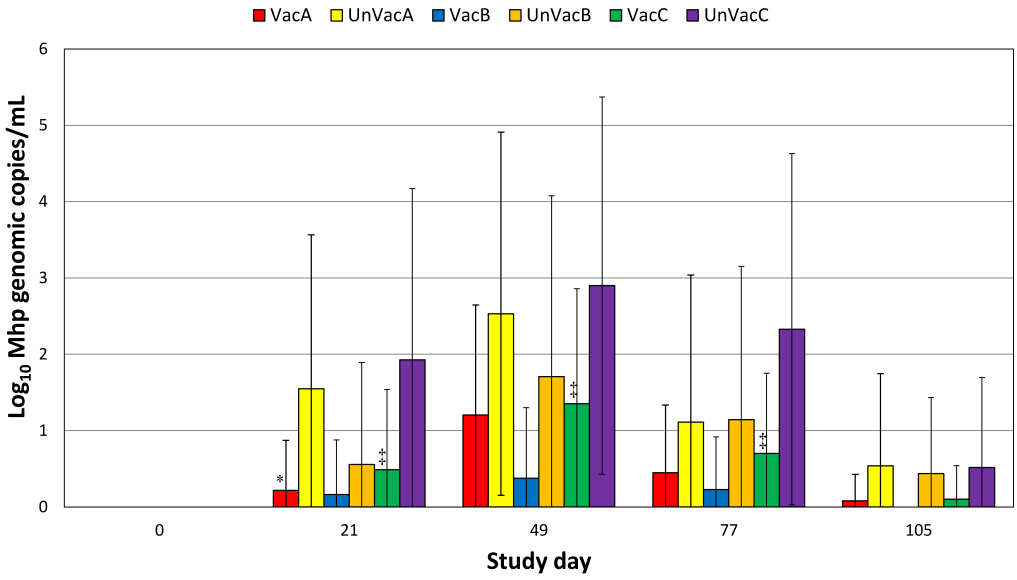

Figure 2. Mean (SD) number of *M. hyopneumoniae* genomic copies in nasal swabs from vaccinated (Vac) and unvaccinated (UnVac) pigs on 3 Korean swine farms (A, B, and C). Significant difference (P value $<$ 0.05; Student's t-test) is indicated between vaccinated and unvaccinated groups within each farm (*Farm A, † Farm B, and ‡ Farm C).

4.5. Serology

On farm A, vaccinated pigs had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S:P ratio at study days 49 and 77 when compared with unvaccinated pigs. On farm B, vaccinated pigs had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S:P ratio at study days 21, 49 and 77 when compared with unvaccinated pigs. On farm C, vaccinated pigs had a significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S:P ratio at study days 49 and 77 when compared with unvaccinated pigs (**Figure 3**).

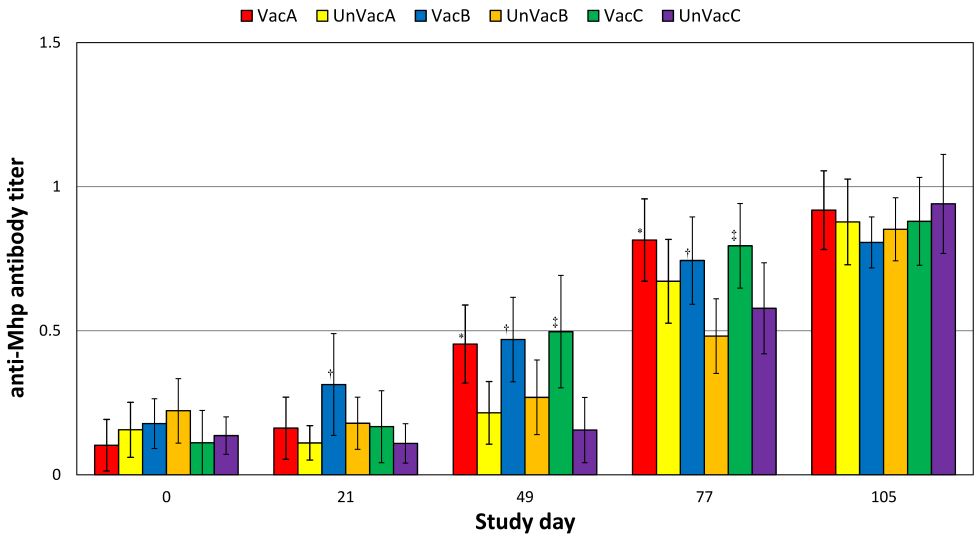

Figure 3. Mean (SD) sample-to-positive (S:P) ratio in serum samples from *M. hyopneumoniae* vaccinated (Vac) and unvaccinated (UnVac) pigs on 3 Korean swine farms (A, B, and C). Significant difference (P value < 0.05; Student's t-test) is indicated between vaccinated and unvaccinated groups within each farm (*Farm A, † Farm B, and ‡ Farm C).

4.6. Enzyme-linked immnospot assay

On farm A, vaccinated pigs had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*-specific IFN-ɣ-SC at study day 49 in their PBMC when compared with unvaccinated pigs. On farm B, vaccinated pigs had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*-specific IFN-ɣ-SC in their PBMC at study days 21 and 49 when compared with unvaccinated pigs. On farm C, vaccinated pigs had a significantly higher ($P < 0.05$) number of *M. hyopneumoniae*-specific IFN-ɣ-SC at study days 49 and 77 in their PBMC when compared with unvaccinated pigs (**Figure 4**).

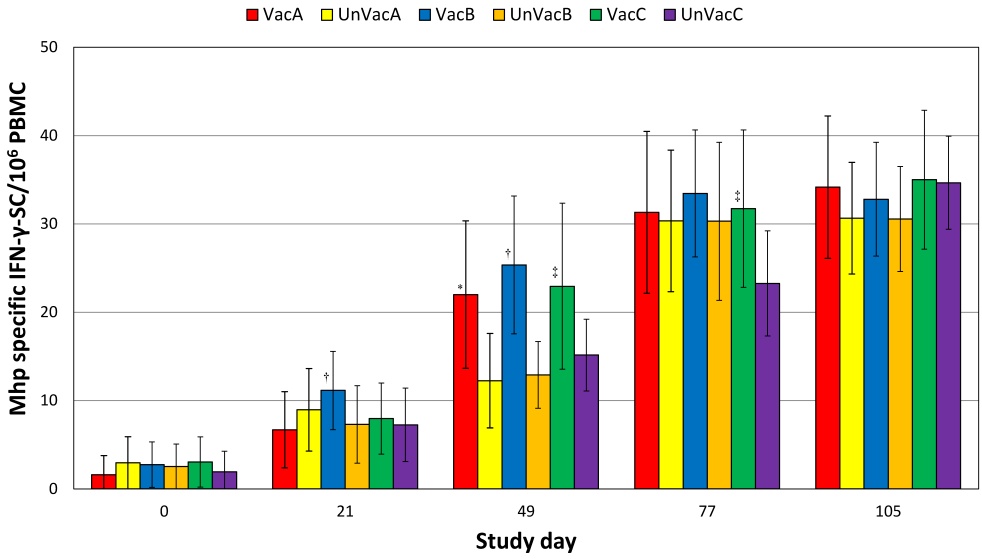

Figure 4. Mean (SD) *M. hyopneumoniae*-specific IFN-γ-SC in PBMC in vaccinated (Vac) and unvaccinated (UnVac) pigs on 3 Korean swine farms (A, B, and C). Significant difference (P value $<$ 0.05; Student's t-test) is indicated between vaccinated and unvaccinated groups within each farm(*Farm A, † Farm B, and ‡ Farm C).

4.7. Pathology

Vaccinated pigs had significantly lower ($P < 0.05$) macroscopic and microscopic lung lesion scores when compared with unvaccinated pigs on the 3 farms at study day 154 (**Table 2**).

Table 2. Means (SD) lung lesion scores of pigs vaccinated for M. hyopneumoniae (Vac) or unvaccinated pigs (UnVac) on 3 Korean swine farms (A, B, and C)

		Lung	
Farm	Group (n)	Macroscopic lesion scores	Microscopic lesion scores
A	VacA (20)	12 (13.57)[a]	0.7 (0.32)[a]
	UnVacA (20)	50 (15.05)[b]	1.7 (0.36)[b]
B	VacB (20)	14 (11.48)[a]	0.9 (0.32)[a]
	UnVacB (20)	46 (21.10)[b]	1.7 (0.38)[b]
C	VacC (20)	14 (12.51)[a]	0.6 (0.27)[a]
	UnVacC (20)	46 (25.51)[b]	1.9 (0.41)[b]

[ab] Within a column, values with different superscript letters are significantly different within each farm.

5. Discussion

In the present field trials, vaccination against *M. hyopneumoniae* reduced the severity of lung lesions and clinical signs including coughing which resulted in improved growth performance. Controlling *M. hyopneumoniae* and its associated diseases in the field can be challenging. Vaccination against *M. hyopneumoniae* using commercial vaccines is the most common strategy within Asian swine production systems. The major advantages of vaccination include reduction of clinical signs and pneumonic lung lesions and improvement of daily weight gain in field trials [17-20]. No statistically significant difference was observed in the growth performance (ADWG) over the nursery period between groups. This confirmed that vaccine did not have a detectable negative impact on growth performance shortly after injection. Overall (study days 0 to 154), the difference in growth performance between vaccinated and unvaccinated pigs was significant on all 3 farms where *M. hyopneumoniae* was circulating.

The mycoplasma organism is a small bacterium without a cell wall. It is a unique pathogen in that it does not invade the body, but instead colonizes the mucosal surface of the respiratory tract damaging the cilia. [21, 22]. Therefore, the serum antibody response to the bacteria may be variable and not a great measurement of protective immunity. No correlation between vaccine-induced serum antibody levels and protection from colonization and disease has been determined [13, 23]. Although protective immunity against *M. hyopneumoniae* is not fully understood, cell-mediated immunity is

likely to play an important role in the protection against *M. hyopneumoniae* infection as described in previous studies [13, 23]. In this study, *M. hyopneumoniae*-specific IFN-ɣ-SC gradually increased from day 21 and reached a peak at day 49. During this period, vaccinated groups improved ADWG and reduced respiratory signs significantly compared with unvaccinated groups on the 3 farms. These results indicate that *M. hyopneumoniae*-specific IFN-ɣ-SC may provide protective immunity. However, since increased levels of IFN-ɣ-SC coincide with the increased amount of mycoplasmal loads in nasal shedding, further studies are needed to determine the functional role of cell-mediated immunity as a protective immunity.

The clinical impact on reducing nasal mycoplasmal shedding by vaccine may be controversial. The vaccine used in this study reduced the genomic copies of *M. hyopneumoniae* on the nasal swabs from vaccinated pigs. Similarly, some studies indicate that other commercial vaccines may also reduce the number of organisms in the respiratory tract and may decrease the infection level in a herd [24]. Contradictory to these findings, additional field studies have shown that vaccination does not significantly reduce the transmission of this respiratory pathogen [25]. In addition, vaccines do not prevent colonization [17-19, 26]. Consequently, vaccination alone will not be sufficient to eliminate *M. hyopneumoniae* from infected pig herds. The producer must still pay attention to stocking density, ventilation, biosecurity and the control of other diseases to be successful in the long-term control of mycoplasma.

Different sampling sites were used to detect *M. hyopneumoniae* infection by PCR on experimentally and naturally infected pigs. Laryngeal swabs were a reliable sample for early detection of *M. hyopneumoniae*, followed by broncho-alveolar lavage fluid (BALF) and nasal swabs in live experimentally infected pigs, especially during the acute period [27]. In contrast, the most sensitive sampling sites in live naturally infected pigs were tracheo-bronchial swabbing and tracheo-bronchial washing, as compared to oral-pharyngeal brushing and nasal swabbing [28]. This may partly explain the relative inaccuracy of the nasal swabbing method [28]. In the present study, sterile swabs were inserted into nasal turbinate deeply and rotated hard enough on the inside of the nose to collect the samples properly for the detection of *M. hyopneumoniae.* In addition, nasal swabs are practical samples for the detection of *M. hyopneumoniae* under field conditions.

M. hyopneumoniae is a slow-growing bacterial organism with a long period between infection and clinical impact [29]. Early infection during the life of a pig is important for the organism to grow and develop clinical disease in pigs. *M. hyopneumoniae* prevalence at weaning can be an important indicator of disease severity in growing pigs [30]. Thus, control measures directed at lowering *M. hyopneumoniae* prevalence at weaning could have a significant impact in disease presentation in grow-finishing pigs. This enhances the criticality that early control of *M. hyopneumoniae* infection by vaccination is essential to control mycoplasma pneumonia. Early

vaccination of piglets (<3 weeks of age) is more common in single-site herds in Korea. Early vaccination has the advantage that immunity can be induced before the pigs become infected, and that fewer pathogens are present to possibly interfere with an immune response. In this field trial, commercial *M. hyopneumoniae* vaccine was also administered to piglets at 3 weeks of age as recommended by company claims.

Single-dose *M. hyopneumoniae* vaccination at 3 weeks of age significantly improved growth performance in pig farms suffering from *M. hyopneumoniae* infection. This field trial was conducted on 3 farms and included housing conditions and a health status reflecting those of conventional facilities in Korea. The results of this study demonstrate that the newly introduced *M. hyopneumoniae* vaccine provided good protection against *M. hyopneumoniae* on farms.

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GENERAL CONCLUSION

M. hyopneumoniae is the most prevalent pathogen in pig production industry worldwide. It is the primary pathogen of swine enzootic pneumonia (SEP), a chronic respiratory disease in pigs of all ages. Being infected with *M. hyopneumoniae* does not result in high mortality but reduces feed conversion rate, results in growth retardation. This disease is often accompanied by secondary infection such as PRRSV, PCV2, *P. multocida* etc., which often to lead to significant economic loss. Vaccination is considered as the most effective tool to control *M. hyopneumoniae* infection. Different types of inactivated whole-cell bacterin are commercially available, they have shown various level of performance in each farm.

The purpose of this experimental study was to investigate the performance of new single-dose *M. hyopneumoniae* bacterin in laboratory and field condition with clinical, microbiological, immunological, and pathological analysis. Chapter I study assessed the efficacy of vaccine against challenge of Korean field isolate of *M. hyopneumoniae* in laboratory. The results indicate that vaccination against *M. hyopneumoniae* is efficacious in all evaluated parameters. For clinical parameter, the mean scores for respiratory disease of pigs in the Vac/Ch group were significantly lower than those in the UnVac/Ch group. For microbiological parameter, the *M. hyopneumoniae*the genomic copies in nasal swab were significantly lower for pigs in the Vac/Ch group than those in the UnVac/Ch

group. For immunological examination, pigs in the Vac/Ch group had a significantly higher *M. hyopneumoniae* ELISA S:P ratio in their serum samples when compared with the UnVac/Ch group, as well as a significantly higher number of *M. hyopneumoniae*-specific IFN-ɣ -SC in their PBMC. Lastly, pigs in the Vac/Ch group had significantly lower macroscopic and microscopic lung lesion scores compared with those in the UnVac/Ch group. An important implication of Chapter I experiment is that the efficacy of vaccine against Korean pathogenic isolate has been demonstrated in laboratory condition where external variables is not intervened.

Chapter II study was to measure overall performance of vaccine in field condition at 3 Korean farms. The farms were diagnosed endemic for *M. hyopneumoniae* based on disease history, so considered optimal environment to suppose a real situation of using a vaccine. The application of vaccine following manufacturer's recommendation showed significantly lower mean score for respiratory disease and higher ADWG in vaccinated groups compared with unvaccinated groups on all 3 farms. In analysis for number of *M. hyopneumoniae* genomic copies in nasal swab, vaccinated groups had significantly lower copies in 2 of 3 trial farms, and numerically lower copies in rest of farm than unvaccinated groups. Furthermore, vaccinated groups significantly induced a higher number of *M. hyopneumoniae*-specific IFN-ɣ-SC than unvaccinated groups on all 3 farms. Regarding the severity of lung lesions, vaccinated groups was significantly lower than that of the unvaccinated groups. Therefore, it

was demonstrated that the investigated vaccine effectively elicited cell-mediated immunity the environment in which the *M. hyopneumoniae* pathogen is present, and effectively improved ADWG by alleviating the severity of lung lesions and respiratory clinical symptoms.

In those two experiments, the new single-dose *M. hyopneumoniae* bacterin was assessed for clinical, microbiological, immunological, and pathological parameters. *M. hyopneumoniae* continues to circulate within pig herds, leading to the possibility of exposure and re-exposure by horizontal transmission in field conditions. It may lead a difficulty to interpret parameters particularly for immunological and pathological analysis. Therefore, field trials are suitable for evaluating pig productivity. Meanwhile, an experimental challenge trials in laboratory conditions are suitable for microbiological, immunological and pathological evaluation. The investigated vaccine demonstrated to provide sufficient protection against challenge of strain of Korean *M. hyopneumoniae* in laboratory conditions, and the improved ADWG compared with unvaccinated pigs in field conditions. Therefore, the newly evaluated vaccine may be an effective tool to control *M. hyopneumoniae* infection and can provide a pig production industry another option of vaccination.

국문 논문 초록

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*Mycoplasma hyopneumoniae*는 돼지에서 일차적으로는 유행성 폐렴(enzootic pneumonia)을 일으키는 병원체이며, 감염 시 호흡기도 상피세포와 섬모를 파괴하여 2차적인 세균이나 바이러스 감염을 유발하므로 돼지호흡기복합감염증(PRDC, Porcine Respiratory Disease Complex)의 병인학에서 매우 중요한 위치를 차지한다. 양돈 산업에 있어서 *M. hyopneumoniae*는 전 세계적으로 유병률이 높고, 성장 정체 및 사료 효

율 저하를 유발하여 농가에 심각한 경제적 손실을 주고 있다.

지금까지 *M. hyopneumoniae*를 통제하고 예방하기 위한 여러 노력이 있었으나 수 킬로미터에 걸친 공기 전파가 가능한 병원체의 특성과 양돈 농가의 밀집 사육 관행으로 인하여 *M. hyopneumoniae*의 박멸 및 청정 상태의 유지는 매우 어렵다. 따라서 백신 접종이 *M. hyopneumoniae* 감염을 통제할 수 있는 가장 효율적인 수단으로 간주된다.

본 연구의 목적은 새로운 돼지 *M. hyopneumoniae* bacterin 백신의 한국 도입을 위해 실험적으로 유도한 *M. hyopneumoniae* 감염 모델과 국내 야외 환경에서 백신의 효능과 안전성을 임상학적, 미생물학적, 면역학적, 병리학적 기법으로 분석하는 것이다. 백신의 효능은 *M. hyopneumoniae*의 비강 배출을 통해 미생물학적으로 평가하였으며, 또한 면역학적으로는 ELISA와 인터페론 감마 분비세포(IFN-ɣ-SC) 수의 측정을 통해 체액성 면역 및 세포 매개성 면역 효능을 평가하였다. 폐의 육안병변과 조직병변을 점수화하여 병리학적 평가를 하였다. 백신의 안전성은 호흡기 질환에 대한 임상적인 평가와 일당 증체율을 지표로 평가하였다.

첫 번째 실험에서는 실험실 조건에서 국내 병원성 *M. hyopneumoniae* 분리주를 사용한 감염 모델에서 백신의 효능을 평가하였다. 실험 결과, 백신을 접종한 그룹은 접종하지 않은 그룹에 비해 *M. hyopneumoniae* 특이적인 ELISA 항체와 IFN-ɣ-SC를 유의적으로 더 많이 유도하였다. *M. hyopneumoniae*의 비강 배출과 폐 병변 분석에서도 백신을 접종한 그룹이 그렇지 않은 그룹에 비해 유의적으로 낮은 수준을 나타내었다. 따라서 평가된 백신은 국내 병원성 *M. hyopneumoniae* 분리주의 감염 컨트롤에도 효과적인 것으로 판단되었다.

두 번째 실험에서는 돼지 유행성 폐렴 진단 이력이 있는 국내 양돈 농장 3개소에서 백신의 효능과 안전성을 평가하였다. 실험 결과, 3개 농장 모두 백신을 접종한 그룹은 접종하지 않은 그룹에 비해 호흡기 임상 증상이 유의하게 낮았으며, 증체율은 유의하게 높았다. 또한 3개 농장 모두 백신을 접종한 그룹에서 백신을 접종하지 않은 그룹에 비해 *M. hyopneumoniae* 특이적인 IFN-ɣ-SC가 유의적으로 더 많이 유도되었으며, 폐 병변의 중증도는 유의하게 낮았다. 따라서 평가된 백신이 *M. hyopneumoniae* 병원체가 상재한 환경에서 세포 매개 면역을 효과적으로 유도하고, 폐 병변의 중증도와 호흡기 임상 증상을 완화시켜 증체율을 효과적으로 향상시켰음을 확인할 수 있었다.

두 가지 실험에서 새로운 돼지 *M. hyopneumoniae* bacterin 백신을 임상학적, 미생물학적, 면역학적, 병리학적 기법으로 평가하였으며, 그 결과 국내 병원성 분리주에 대해서도 효과적인 방어능을 확인하였고, 야외 환경에서 적용하였을 때 개선된 증체율을 확인할 수 있었다. 새로운 돼지 *M. hyopneumoniae* bacterin 백신은 *M. hyopneumoniae* 감염을 통제할 수 있는 또 하나의 선택지를 제공할 수 있으며, *M. hyopneumoniae*가 돼지호흡기복합감염증에서 1차 병원체의 역할을 하고, 양돈 농가에 심각한 경제적 타격을 주는 점을 고려할 때 이는 동물복지와 양돈 산업의 지속적인 성장에 매우 중요한 시사점을 준다.

주요어: *Mycoplasma hyopneumoniae*; *Mycoplasma hyopneumoniae* bacterin 백신; 유행성 폐렴; 2차 감염; 돼지호흡기복합감염증; 세포 매개 면역; 증체율

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