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Reduction of mycoplasmal lesions and clinical signs by vaccination against *Mycoplasma hyorhinis*

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ABSTRACT

Porcine mycoplasmal pneumonia is a significant disease problem in the swine industry. The causative agents include *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. *M. hyopneumoniae* is the major pathogen contributing to the porcine respiratory disease complex, but is difficult to isolate from the respiratory tract and tonsils, whereas *M. hyorhinis* is not. Although *M. hyorhinis* is commonly detected in the lungs, the role of *M. hyorhinis* as a cause of pneumonia remains unclear. Current vaccines for porcine mycoplasmal pneumonia only include *M. hyopneumoniae*, not *M. hyorhinis*. *M. hyopneumoniae* vaccines are widely used, but disease still occurs because of poor vaccine efficacy and possibly the presence of *M. hyorhinis*. In this study, an inactivated vaccine containing a mixture of *M. hyorhinis* and *M. hyopneumoniae* was generated and evaluated for safety, immunogenicity, and protective efficacy against challenge with *M. hyorhinis* in pigs. The inactivated vaccine induced an antibody response and reduced pneumonic lesions in the lungs and tracheas compared with the non-vaccinated group.

1. Introduction

Porcine respiratory disease complex (PRDC), which is caused by a combination of both viral and bacterial agents, is a worldwide problem in the swine industry. Porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* are two of the most common pathogens detected in pigs showing PRDC (Thacker, 2001; Thacker et al., 1999). Pigs infected with viruses that induce immunosuppression, such as PRRSV and porcine circovirus type 2 (PCV2), can be predisposed to secondary bacterial infections, including mycoplasma infections (Drew, 2000; Kim et al., 2003).

Mycoplasmas are small, ubiquitous bacteria without a rigid cell wall that are commensals and pathogens of animals, and can cause serious diseases in humans and animals (Razin et al., 1998). Two mycoplasmas that cause pneumonia in pigs are *M. hyopneumoniae* and *M. hyorhinis* (Friis and Feenstra, 1994; Mare and Switzer, 1965). *M. hyopneumoniae* causes economic losses in the swine industry by inducing chronic pneumonia (Frey et al., 1994). The immune system of the respiratory tract of pigs is modulated by *M. hyopneumoniae*, as the epithelial cell damage it causes disrupts cilial activity (Thacker et al., 2006). Previous studies have shown that *M. hyopneumoniae* also plays a synergistic role in the initiation of diverse bacterial and viral infections under field conditions (Kobisch et al., 1993).

M. hyorhinis is recognized as a causative agent of polyserositis in swine (Friis, 1971; Kawashima et al., 1996). However, *M. hyorhinis* is also frequently found in pneumonic lesions of pigs showing signs of respiratory disease and the pathogenicity may vary with the strain (Friis, 1971; Gois and Kuksa, 1974; Lin et al., 2006). Several strains of *M. hyorhinis* induced slight bronchopneumonia or interstitial pneumonia in gnotobiotic pigs exposed via the respiratory tracts (Friis, 1971; Gois et al., 1971; Kobayashi et al., 1996). *M. hyorhinis* has been reported to cause similar lesions to *M. hyopneumoniae* in Taiwan (Lin et al., 2006), while in Japan, it has been isolated from PRRSV-infected pigs and found to cause severe, chronic pneumonia (Kawashima et al., 1996; Kobayashi et al., 1996). *M. hyorhinis* was detected and isolated in the pneumonic lesions from pigs with respiratory disorders caused by diverse pathogens such as PRRSV, PCV2, swine influenza virus, *M. hyopneumoniae*, *Pasteurella multocida*, and *Haemophilus parasuis* (Falk et al., 1991; Hansen et al., 2010; Kawashima et al., 1996; Makhanon et al., 2012; Shimizu et al., 1994). Particularly, an association with

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PCV2, PRRSV, and *M. hyorhinis* was found in a recent epidemiological study (Kixmoller et al., 2008). In a previous study, we showed that an *M. hyorhinis* strain isolated from the lungs of a pig showing clinical signs of severe pneumonia could potentiate PRRSV-induced pneumonia as *M. hyopneumoniae* (Lee et al., 2016). These results demonstrate that the frequency of co-infections with *M. hyorhinis* and diverse pathogens associated with pneumonia in PRDC-affected animals was significantly higher that than in PRDC-negative animals. Although it is not clear how *M. hyorhinis* enhances viral pneumonia, it does appear to be an important pathogen contributing to porcine respiratory diseases.

Previous studies have reported that only *M. hyorhinis* was isolated from severe lung lesions that were believed to be caused by *M. hyopneumoniae* (Armstrong et al., 1983; Gois and Kuksa, 1974; Lin et al., 2006). In pigs with mycoplasmal pneumonia, no differences were seen between the macroscopic lung lesions caused by *M. hyopneumoniae* and *M. hyorhinis*. Some strains of *M. hyorhinis* can induce mycoplasmal pneumonia in pigs that cannot be distinguished from the pneumonia caused by *M. hyopneumoniae* (Friis, 1971; Poland et al., 1971). Cross-protection between *M. hyorhinis* and *M. hyopneumoniae* is unlikely to occur because the antigenic and serological cross-reactivity between them is low (Lee et al., 2016; Petersen et al., 2016). In previous studies, the efficacy of an *M. hyopneumoniae* bacterin vaccine has been found to vary between herds (Maes et al., 2008). Different levels of infection with *M. hyorhinis* in different herds may be a factor leading to the failure of current vaccines against porcine mycoplasmosis. While vaccines against *M. hyopneumoniae* are widely used, no vaccines are currently used to control disease caused by *M. hyorhinis* in the field (Wu et al., 2000). The objectives of this study were to evaluate the effects of a bivalent vaccine containing *M. hyorhinis* and *M. hyopneumoniae* against challenge with *M. hyorhinis*.

2. Materials and methods

2.1. Animals and experimental design

Sixteen weaned pigs (aged 3 weeks) were purchased from a commercial farm (PIC Korea Inc., Daejeon, Korea) on which there was no serological evidence of exposure to either *M. hyorhinis* or *M. hyopneumoniae*. Weaned pigs had an average weight of approximately 6.0 kg, healthy skin, and no behavioral or spinal disorders, signs of pneumonia, arthritis, or enteritis. The pigs were randomly allocated into three groups (A to C) and housed in individually isolated rooms and fed a commercial feed containing no antimicrobial drugs.

2.2. Vaccination and infection

Pigs in group A were vaccinated intramuscularly once with 2 mL of a formalin-inactivated vaccine consisting of the *M. hyorhinis* strain EH5 ($2 \times 10^{9.5}$ color change units(CCU)/mL), *M. hyopneumoniae* strain NSM ($2 \times 10^{9.5}$ CCU/mL), and Montanide™ ISA201VG (Seppic Inc., Puteaux Cedex, France) as an oil adjuvant. Groups B and C were inoculated with 2 mL of sterile phosphate-buffered saline(PBS). After 4 weeks, *M. hyorhinis* strain EH5 (10^8 CCU/mL) was administered intratracheally to pigs in groups A and B. Clinical signs were observed for 4 weeks and serum samples were collected every week.

2.3. Serology test

All serum samples were tested for antibodies against *M. hyorhinis*, *M. hyopneumoniae*, and PRRSV by using enzyme-linked immunofluorescent assays (ELISA). The *M. hyorhinis* antibody response was determined as previously described (Lee et al., 2016). The *M. hyorhinis* antigen for the ELISA was obtained from strain EH5, which was used for vaccination and infection. *M. hyopneumoniae* and PRRSV antibody titers were determined using commercially available ELISAs (HerdCheck: IDEXX Laboratories, Inc., Westbrook, ME, USA) according to the manufacturer's instructions. Samples were considered positive if the calculated sample-to-positive control ratio was 0.4 or greater.

2.4. Clinical evaluation and necropsy

Pigs were evaluated daily for clinical signs, including appetite, cough, fever, dyspnea, respiration rate, and behavioral changes for 8 weeks. Rectal temperatures and the weights of the pigs were measured until necropsy. Pigs were humanely euthanized at 4 weeks post-infection (WPI) according to the guidelines of the Institutional Animal Care and Use Committee of Konkuk University. Bend the right rib cage of the pigs and the tissues were evaluated for macroscopic and microscopic lesions.

2.5. Pathologic examination

Macroscopic lesions in the lungs of pigs infected with *M. hyorhinis* (dark red to purple consolidated areas) were scored using a previously developed weighting system based on the approximate volume that each lobe contributed to the entire lung (Halbur et al., 1995). Lung lesions were blindly examined by a scorer blinded to the groups and given a score based on the severity and the proportion of each lobe with lesions: 0 = no macroscopic lesions (0%), 1 = mild (< 10% of lobe affected), 2 = moderate (10% to 40% of lobe affected), 3 = severe (41%–70% of lobe affected), 4 = very severe (> 70% of lobe affected).

The microscopic lesions were evaluated by a veterinary pathologist blinded to vaccination and challenge groups. Tissue sections were taken from the cranial lobes, fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin for hematoxylin-eosin staining. The scoring parameters of microscopic lesions were referred as slightly modified version of previous study (Gauger et al., 2012). Lung and tracheal sections were examined and given a score based on the severity and lesions of the microcilial loss, bronchopneumonia, interstitial pneumonia, and peribronchiolar lymphoid cuffing as follows: 0 = no microscopic lesions (0%), 1 = mild (< 10%), 2 = moderate (10% to 40%), 3 = severe (41% to 70%), 4 = very severe (> 70%).

2.6. Statistical analysis

The antibody responses, macroscopic lung lesions, and microscopic lung and trachea lesions were compared between groups using Paired *t*-test for lesion scores and Fisher's exact test for prevalences of antibody responses and microscopic lesions using the SAS 9.2 software package (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Clinical disease

The pigs in group B showed significantly greater signs of respiratory disease, including a rough hair coat, behavioral changes, fever, dyspnea, and coughing, than the pigs in group A (supplementary Table 1). None of the pigs in group C had clinical signs. One of the pigs in group B had severe pneumonia and died 3 weeks after infection. None of the pigs in group A developed any granuloma tissue at the site of vaccination.

3.2. Serology

The rates of deletion of antibody against *M. hyorhinis* antibody by ELISA are summarized in Table 1. Five pigs in group B did not develop anti-*M. hyorhinis* antibodies until the end of the study, and one pig had a detectable immune response at 2 WPI, but this response was not detectable at 3 WPI. The pigs in group A had a detectable antibody response against *M. hyorhinis* at 2 weeks post-vaccination. Their antibody titers increased until 6 weeks post-vaccination (2 WPI) and then