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Development and validation of an attenuated *Mycoplasma hyopneumoniae* aerosol vaccine



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ABSTRACT

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) causes a chronic respiratory disease with high morbidity and low mortality in swine, and has been presented as a major cause of growth retardation in the swine industry. Aerosol vaccination presents a needle free, high throughput, and efficient platform for vaccine delivery, and has been widely applied in poultry vaccination. However, aerosol vaccines have rarely been used in swine vaccination primarily because the long and curving respiratory track of swine presents a barrier for vaccine particle delivery. To develop an effective *M. hyopneumoniae* aerosol vaccine, three major barriers need to be overcome: to optimize particle size for aerosol delivery, to maintain the viability of mycoplasma cells in the vaccine, and to optimize the environmental conditions for vaccine delivery. In this study, an aerosol mycoplasma vaccine was successfully developed based on a conventional live attenuated *M. hyopneumoniae* vaccine. Specifically, the Pari LCD nebulizer was used to produce an aerosol vaccine particle size less than 5 μm ; and a buffer with 5% glycerol was developed and optimized to prevent inactivation of *M. hyopneumoniae* caused by aerosolization and evaporation. Before nebulization, the room temperature and relative humidity were control to 20–25 °C and 70–75%, respectively, which helped maintain the viability of aerosol vaccine. Animal experiments demonstrated that this newly developed aerosol vaccine was effectively delivered to swine low respiratory track, being confirmed by nested-PCR, in situ hybridization and scanning electron microscope. Moreover, *M. hyopneumoniae* specific *slgA* secretion was detected in the nasal swab samples at 14 days post-immunization. To our knowledge, this is the first report on a live *M. hyopneumoniae* aerosol vaccine.

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1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) causes mycoplasmal pneumoniae in swine (MPS), a chronic respiratory disease with high morbidity and low mortality. MPS induces growth retardation in pigs by reducing gain to feed ratio (Ross, 1999). In addition, *M. hyopneumoniae* facilitates the invasion of the respiratory tract by other pathogens, such as porcine circovirus type 2, porcine

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respiratory and reproductive syndrome virus, and *Pasteurella multocida* (Thacker, 2006).

Vaccination is one of the most important strategies to prevent and control MPS. However, only several inactivated whole-cell vaccines have till now been licensed and used in the commercial farms around the world, and an attenuated vaccine (attenuated *M. hyopneumoniae* 168 strain) was permitted to enter farms in China in 2007. A few new subunit and DNA vaccines are still in development (Chen et al., 2006a,b, 2009; Fagan et al., 1996, 2001; Ma et al., 2011; Ogawa et al., 2009; Okamba et al., 2010; Shimoji et al., 2003). Inactivated vaccines could increase the daily gain and decrease lung lesions but cannot prevent the colonization of *M. hyopneumoniae* (Thacker et al., 1998). The IgG antibody concentrations in serum and respiratory tract washings did not correlate with protection against pneumonia (Djordjevic et al., 1997; Messier et al., 1990; Thacker et al., 1998, 2000). Our previous research indicated that the attenuated vaccine administered by the intrapulmonary route could produce favorable protection by activating both cellular and mucosal immunity of the host, specifically, the level of specific sIgA antibody and IFN γ in the respiratory tract and systemic cellular immunity may be directly responsible for immune protection in MPS (Feng et al., 2010b). However, conventional needle based injection methods, such as intramuscular injection for the inactivated vaccines and intrapulmonic injection for the attenuated vaccine, are labor intensive and time consuming. Practically, these two factors represent a great challenge to the implementation of these vaccines at a large scale in the swine industry. Thus, it is necessary to develop an alternative needle-free method, such as oral or spray administration.

Aerosol vaccine is a popular vaccine being delivered using spray administration. Aerosol vaccine delivery can be used to achieve mass vaccination and can induce rapid immunization of the hosts. Because of its safety, labor-saving, and high efficacy, aerosol vaccine has been widely used in poultry (Chernyshev et al., 1977; Corbanie et al., 2008; Evans and Hafez, 1992; Singer and Malkinson, 1979). However, to date, aerosol vaccination has rarely been applied in swine. The long and curving respiratory tract of pig may present a major barrier for aerosol administration in pigs. Studies have shown that most aerosol vaccine particles (diameter above 10 μm) fail to penetrate the lower respiratory tract (Hatch, 1961).

In this study, our goal was to develop an effective aerosol vaccine for swine. An attenuated *M. hyopneumoniae* vaccine developed in our laboratory (Feng et al., 2010b) was used as a template. A Pari LCD nebulizer was chosen to produce an aerosolized vaccine with the particle diameter below 5 μm . The spray pressure, vaccine additives, and environmental factors, such as temperature and relative humidity, were optimized to improve the viability of swine aerosol vaccine. The swine experiment confirmed the successful delivery of the live vaccine to the lower respiratory tracts (behind the larynx) of pigs. The results implied that the attenuated *M. hyopneumoniae* vaccine could be administrated as aerosol immunization in swine. The immune dose and the protective effect of the *M.*

hyopneumoniae live aerosol vaccine shall be studied in the future.

2. Materials and methods

2.1. Vaccine strain and ingredients

A commercial freeze-dried attenuated *M. hyopneumoniae* vaccine (*M. hyopneumoniae* 168 strain) for intrapulmonic administration was obtained from Nanjing Tianbang Bio-industry Company, China (40-dose vials, 1×10^7 CCU/dose). Four vaccine diluents were optimized, deionized water, 5% glycerol in deionized water, 0.1% polyvinylpyrrolidone (PVP) in deionized water and 0.1% Tween-80 in deionized water.

2.2. Aerosol-generating procedures

The aerosol vaccine was produced by a Pari LCD nebulizer (Pari, Starnberg, Germany) coupled to an air compressor (Xingbao HP2.5; HLX, Xiamen, China). Briefly, one vial of frozen vaccine (40-dose) was dissolved with 4 ml vaccine diluents, and then transferred into the Pari nebulizer reservoir for producing the aerosol vaccine. The room temperature and relative humidity (RH) were controlled to 20–25 °C and 70–75%, respectively. Aerosol-generation was performed in a piglet's incubator (300 l, 1 m \times 0.5 m \times 0.6 m, Hongrun Co., Xinxiang, China).

Four vaccine diluents and two nebulizing air pressures (0.2 and 1 bar) were optimized to produce an aerosol vaccine with high vitality and desired drop size (1–10 μm). The aerosol vaccine was then validated in vivo.

2.3. Collection and titration of aerosol vaccine

The *M. hyopneumoniae* vaccine suspensions in four diluents were added into the Pari nebulizer reservoir separately for spraying. The nebulizing air pressure was set at 0.2 or 1 bar, respectively. For collecting the aerosol vaccine, the nebulizer nozzle was connected to a self-made aerosols gathering unit (Fig. 1). The aerosol vaccine was collected and transformed to liquid vaccine.

The titers of the liquid vaccine were determined by the CCU₅₀ method (Litamoi et al., 1996) and compared with the vaccine suspension in deionized water before nebulization.

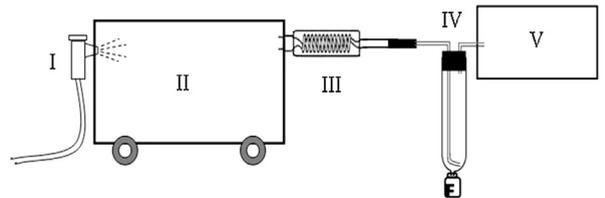


Fig. 1. The aerosol gathering unit including five components: (I) Pari nebulizer; (II) sealed chamber; (III) air-liquid converter; (IV) liquid vaccine collecting tube; and (V) adjustable aspirator pump. Nebulization was performed first to fill the chamber with dissolved vaccine in diluents, then the aspirator pump is turned on to collect aerosols. The same airflow amount was used between the aspirator pump and nebulizer.

2.4. Measurement of aerosol vaccine particle size

The particle sizes of vaccine aerosol produced at the air pressures of 0.2 and 1 bar were measured by both paraffin–vaseline cushion method (Li and Zhang, 1995) and a retrofitted laser particle size analyzer (JL9200, Weina, Ji'nan, China). Before nebulization, the room temperature (RT) and relative humidity (RH) in the exposure chamber were controlled to 20–25 °C and 70–75%, respectively. As for the paraffin–vaseline cushion method, a slide covered with a soft cushion composed by a mixture of liquid paraffin and vaseline 2:1 (v/v) was placed at the bottom of the exposure chamber to collect aerosol particles for 10 s. The particle sizes of vaccine aerosols on the soft cushion were measured immediately by optical microscope when the collection is done. Also, the exposure chamber was opened at the bottom and placed on top of the laser particle size analyzer with the inlet nozzle of the analyzer in the middle of the chamber base. The mass median diameter ($D[v, 0.5]$) was measured as the volume particle size distributions by a retrofitted laser particle size analyzer.

2.5. Swine experiment

Six 5-day-old piglets born from a *M. hyopneumoniae* free sow were chosen. Before the experiments were initiated, sera and nasal swab samples were collected from each pig. Anti-*M. hyopneumoniae* IgG antibody in sera and sIgA antibody in nasal swabs were respectively detected by the *M. hyopneumoniae* antibody test kit (IDEXX Co.) and the *M. hyopneumoniae* sIgA-ELISA method (Feng et al., 2010a) to confirm that all the piglets were free of *M. hyopneumoniae* infection. Three piglets were put into a piglet's incubator described above for *M. hyopneumoniae* aerosol vaccine administration (3 ml, 30 doses) with the optimal vaccine diluents and air pressure. The piglets were kept in the chamber about 5 min until all the vaccine solution in nebulizer reservoir was sprayed. Another 3 piglets were not vaccinated and housed in a separated room as controls. All the piglets were fed with milk not containing antibiotics. On 7 and 14 days post-immunization (dpi), nasal swab samples of each piglet were collected for mucosal immune response detection by *M. hyopneumoniae* sIgA-ELISA method (Feng et al., 2010a). All the piglets were slaughtered on 14 dpi, and lung tissues with bronchi and bronchioles from the apical lobe of each piglet were sampled for vaccine strain detection by scanning electron microscope (SEM) and in situ hybridization (ISH). The condition of ciliated cells was assessed by SEM. Bronchoalveolar lavage fluids (BALFs) were collected for detecting *M. hyopneumoniae* strain by nested-PCR.

M. hyopneumoniae in situ hybridization (ISH) was performed as described by our previous research (Wang et al., 2012b). A 5'-DIG labeled 25-mer probe was designed according to the P36 genome in GenBank (GeneID:49109), 5'-DIG-AATCGCAAAAAGAGCAAAAGTATCG-3'. The probe was diluted to 2 µg/ml for the ISH assay. The ISH assay was conducted according to the manufacturers' instructions (Boster Co., China).

For SEM, the isolated bronchi were gently washed with 0.1 mol/l PBS, and then immediately fixed with 2.5%

glutaraldehyde in 0.1 mol/l PBS at pH 7.4. The fixed tissues were rinsed twice with PBS, subsequently dehydrated in ascending concentrations of ethanol, critical-point dried using carbon dioxide, and coated by vacuum-evaporated carbon and ion-sputtered gold. All samples were observed under scanning electron microscope (S-3000N, HITACHI) at an accelerated voltage of 15 kV.

To perform nested-PCR, BALFs were taken and treated as described before (Feng et al., 2010a). Briefly, 1 ml BALFs samples were concentrated by centrifugation at $12,000 \times g$ for 30 min, and the pellets was resuspended in lysis buffer for DNA extraction described by Blanchard et al. (1996). A 5 µl sample of treated DNA was used as templates for *M. hyopneumoniae* detection with nested-PCR method described by our previous research (Feng et al., 2010a).

2.6. Data analysis

The CCU₅₀ titers of four aerosol vaccines as well as control and the *M. hyopneumoniae* specific sIgA antibody titers of piglets in two groups on 7 and 14 dpi were compared using one-way ANOVA method with the post hoc Tukey's test by using the Graphpad Prism Version 5 Software. A *p* value < 0.01 was considered statistically significant.

3. Results

3.1. Effectiveness of diluent buffer on stability of aerosol vaccines

To minimize the impact of nebulization on cell viability in the aerosol vaccine, we tested four buffer conditions: 5% glycerol, 0.1% PVP, 0.1% Tween-80, or deionized water. The titers of the aerosol vaccine under each of these four conditions were measured and compared with those for the live vaccine before nebulization. Our results showed that, nebulization can dramatically reduce the viability of live vaccine cells: the difference of titers before and after nebulization was extremely significant ($P < 0.0001$) with the value of $7.9 \pm 0.17 \log_{10}$ CCU₅₀/ml (control) and $6.57 \pm 0.12 \log_{10}$ CCU₅₀/ml (deionized water vaccine), respectively (Fig. 2). Among other three vaccines tested with different additives, the glycerol vaccine (dissolved in 5% glycerol) can greatly reduce the effects of nebulization, with a titer of \log_{10} CCU₅₀/ml values of 7.43 ± 0.12 , which was not significantly different when compared with the control group (deionized water vaccine before nebulization). However, titers of PVP vaccine ($7.10 \pm 0.17 \log_{10}$ CCU₅₀/ml, dissolved in 1% PVP) and Tween-80 vaccine ($6.37 \pm 0.17 \log_{10}$ CCU₅₀/ml, dissolved in 0.1% Tween-80) were significantly lower when compared with the control group ($P < 0.01$ and $P < 0.0001$).

3.2. Particle diameter of vaccine aerosol

The diameter of glycerol vaccine particles was detected by both paraffin–vaseline cushion method and a retrofitted laser particle size analyzer. The particle size of aerosol vaccine produced at the pressure of 0.2 bar was $4.80 \mu\text{m}$ $D[v, 0.5]$ (Fig. 3A and C), while that at the pressure of 1 bar was $4.50 \mu\text{m}$ $D[v, 0.5]$ (Fig. 3B and D). Before the aerosol

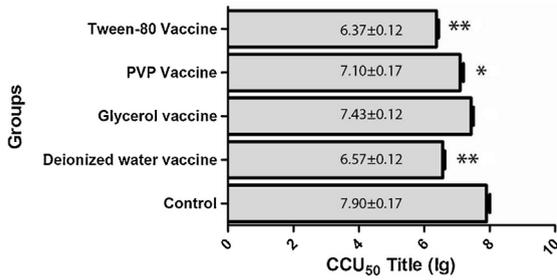


Fig. 2. Titration of aerosol vaccines with the CCU₅₀ method. All the aerosol vaccines were produced with the nebulizing air pressure of 1 bar, the RT and RH were 23 °C and 70%, respectively. Every 100 μl of each vaccine solution from collecting tube of aerosols gathering unit as well as 100 μl control vaccine solution was used for titration. The assay was triplicate. The values within the bars represent CCU₅₀ titer (log₁₀) showed as mean ± SD. Significant differences among the means were performed by one-way ANOVA and Tukey's post hoc test. **P* < 0.01; ***P* < 0.0001.

vaccine was produced, the RT and RH in chamber were 25 °C and 72%, respectively.

3.3. Aerosol vaccine can be delivered to low respiratory track of pigs

ISH demonstrated that DNA of *M. hyopneumoniae* was detected on the luminal surface of bronchiolar lining epithelial cells of all vaccinated piglets (Fig. 5A) whereas no positive hybridization signal was found in the same area of any non-vaccinated piglet as control (Fig. 5B). On the other hand, SEM showed that Mycoplasma-like particles were observed on the surface of the bronchi ciliated cells of all

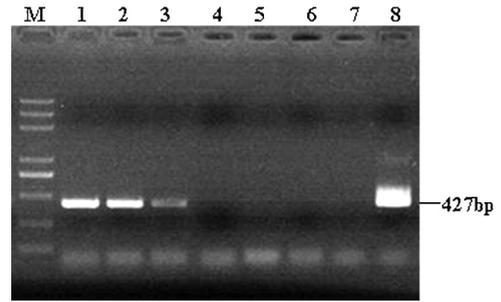


Fig. 4. *M. hyopneumoniae* genome detection in BALF samples by the nested-PCR method. A 427-bp fragment specific for the *M. hyopneumoniae* P36 genome was amplified. M, DL5000 DNA ladder; 1–3, samples from 3 vaccinated piglets; 4–6, samples from 3 control piglets; 7, PCR negative control; 8, PCR positive control.

vaccinated piglets, and that the light aggregation of ciliated cells was also identified (Fig. 6A). However, no Mycoplasma-like particle and aggregation was shown in the ciliated bronchial epithelial cells of non-vaccinated piglets (Fig. 6B). The results of ISH and SEM were confirmed by nested-PCR (Fig. 4), which showed that the *M. hyopneumoniae* DNA could be detected in BALF samples of all three vaccinated piglets but not in unvaccinated controls.

Also, the mucosal immune response was detected in the respiratory tract of all the vaccinated piglets, but not in unvaccinated ones (Fig. 7). Specific sIgA antibody secretion in the nasal cavities of vaccinated pigs were detectable on 14 dpi compared those in control (*P* < 0.01), but the difference was not significant on 7 dpi (*P* > 0.01).

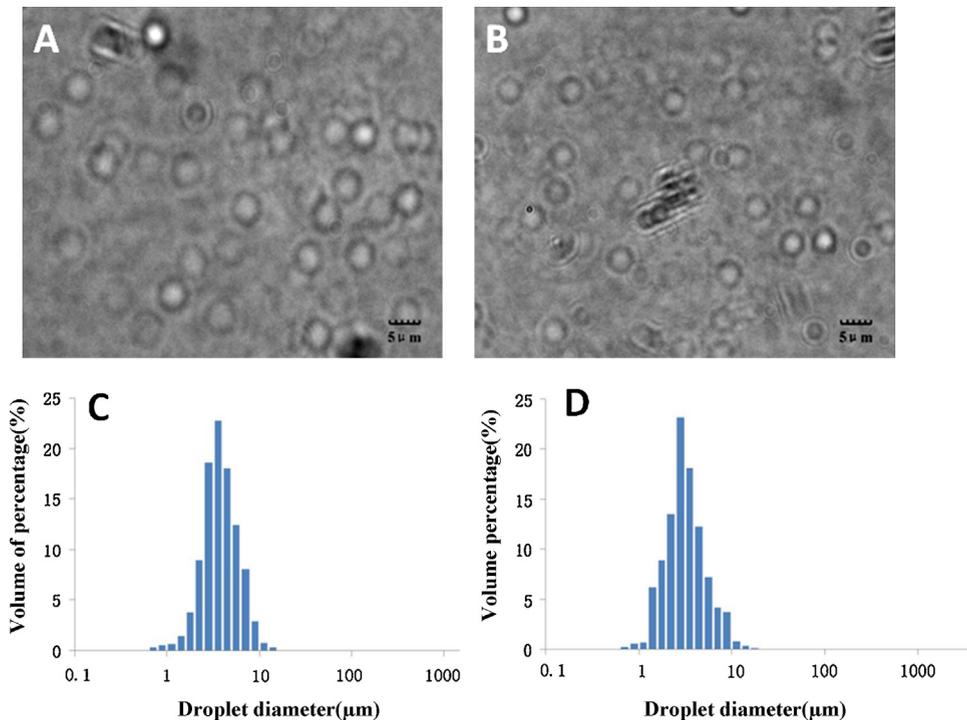


Fig. 3. Particle sizes detection. The attenuated *M. hyopneumoniae* aerosol vaccine was produced at 0.2 bar (A and C) and 1 bar (B and D), respectively. The particle size of the aerosol vaccine was detected by the paraffin-vaseline cushion method (A and B) and a retrofitted laser particle size analyzer (C and D).

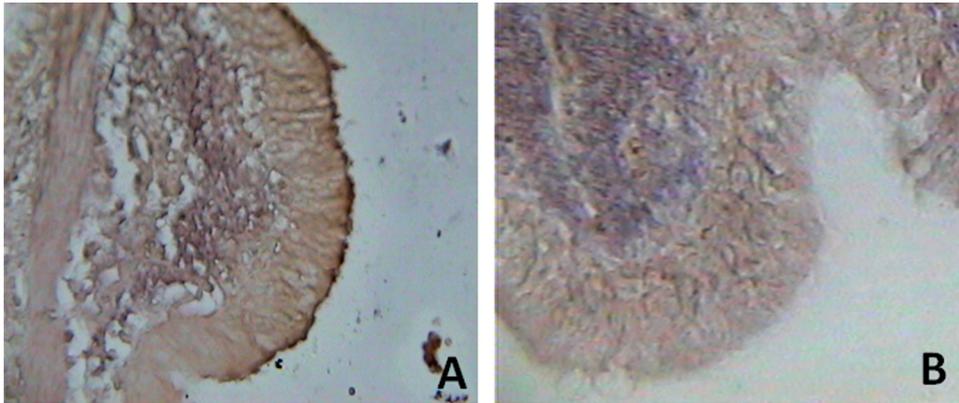


Fig. 5. (A) Lung; pig No. 1, vaccinated with *M. hyopneumoniae* aerosol vaccine, *M. hyopneumoniae* DNA (dark brown reaction) is detected in the luminal surface of bronchiolar epithelial cells. (B) Lung; pig No.4, non-vaccinated as control, no hybridization signal is detected. In situ hybridization; 3,3'-diaminobenzidine/H₂O₂ ($\times 20$).

4. Discussion

MPS is characterized by high morbidity due to the airborne transmission capability of *M. hyopneumoniae*. In many countries, vaccination for controlling *M. hyopneumoniae* infections is applied to more than 70% of the pig herds. To meet the need for time and labor-saving *M. hyopneumoniae* vaccine delivery systems for mass vaccination, a number of new needle free injection techniques have recently been attempted, such as oral administration (Lin et al., 2002, 2003) and aerosol vaccination (Murphy et al., 1993). Both strategies could activate mucosal (pulmonary) immunity which is important in preventing the disease. However the previous inactivated *M. hyopneumoniae* aerosol vaccine failed to protect pigs against MPS. Failure of vaccine deposition or retention in the lung was considered as the possible reason as well as the inadequate vaccine dose and immune response (Murphy et al., 1993). Although another report described that most of pigs induced pathologic lesions after aerosol challenge by the different doses of *M. hyopneumoniae* virulent strain Beaufort (Czaja et al., 2002), the threshold dose for the induction of MPS by a virulent strain is much lower than

that for eliciting enough immune protection by a vaccine strain. To improve the actual attenuated vaccine intake, more attention should be paid on the characteristic and viability of *M. hyopneumoniae* aerosol. Location of aerosol particles in the respiratory tract is dependent on the size of the aerosol droplets, while the viability of the aerosol vaccine was responsible for avoiding rapid clearance by the mucociliary apparatus. In this study, the particle size and viability of an attenuated *M. hyopneumoniae* aerosol vaccine were the critical parameters considered for the improvement of vaccine deposition and retention in the lung.

The ciliated bronchial epithelial cells are the target cells for *M. hyopneumoniae* infection. As a result, the particle sizes of *M. hyopneumoniae* aerosol vaccine should be controlled to make vaccine particles accessible to the lower respiratory tract of piglets. According to previous reports (Hatch, 1961; Pritchard, 2001), deposition of aerosols in trachea and bronchioles was maximal when aerosol droplet size was 2–5 μm . The particle size spectra of aerosols mainly depend on the construction of the nebulizer and spray-head. The Pari LCD nebulizer was selected to produce the *M. hyopneumoniae* aerosol vaccine

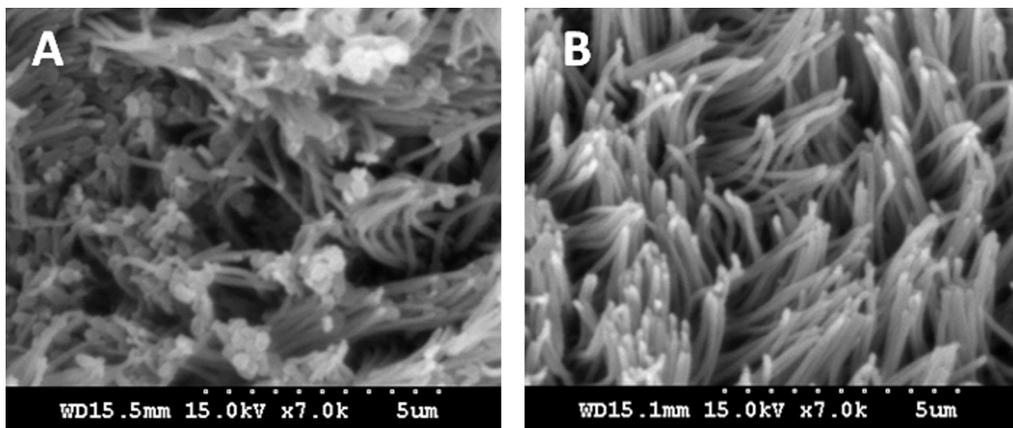


Fig. 6. Scanning electron micrograph of a section from the apical lobe bronchi showing many mycoplasma-like particles adhering to the ciliated epithelial cells with light aggregation in vaccinated piglets (A) compared normal area in non-vaccinated piglet (B). $\times 7000$.

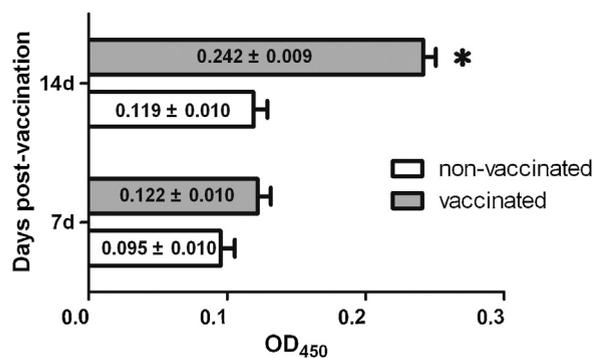


Fig. 7. *M. hyopneumoniae* specific sIgA antibody response in nasal cavities of vaccinated and non-vaccinated piglets on 7 and 14 dpi, respectively. The values within the bars represent OD₄₅₀ showed as mean ± SD. Significant differences among the means were performed by one-way ANOVA and Tukey's post hoc test. * $P < 0.01$.

in this study. According to the product instructions, when 0.9% NaCl solution was used for the nebulization test, the Pari nebulizer could produce an aerosol with a $D[v, 0.5]$ of 2.90 μm , which is the appropriate size for aerosol penetration into the lower respiratory tract of piglets. When the attenuated *M. hyopneumoniae* vaccine was tested, the particle size of aerosolized vaccine increased to 4.50 and 4.80 μm $D[v, 0.5]$ at the two different air pressures, which may due to the impact of glycerol in diluents (Ganderton et al., 2002). Moreover, after the piglets were immunized with the *M. hyopneumoniae* aerosol vaccine, the vaccine strains could be detected in the bronchi and bronchioles of the apical lobe by SEM and ISH, respectively. The results confirmed that the particle size of the *M. hyopneumoniae* aerosol vaccine produced in this study was sufficient for deposition in lower respiratory of piglets. The aerosol vaccine showed little damage to ciliated epithelial cells.

Nebulizing air pressure is another factor determining the particle size of aerosols. Generally, the median droplet sizes of aerosol particles can be decreased by increasing the nebulizing air pressure. This is explained by the droplet forming mechanism. More energy is added to stretch liquid into a thin layer, at which point the liquid splits into droplets. It is possible to increase the nebulizing air pressure to extend this air-contact surface and to form smaller droplets (Swift, 1980). However, no significant differences were found when two different pressures (0.2 and 1.0 bar) were used to produce an aerosol vaccine with the Pari nebulizer. This may have occurred because of the nebulizer's construction in which the baffles in the reservoir removed large particles from the aerosol (Atkins et al., 1992). Because of the structure of the nebulizer, we were unable to produce an aerosol vaccine with a droplet size above 10 μm as control in this study; however, some previous reports had confirmed that particle diameters above 10 μm seldom penetrated to the lobules (Hatch, 1961; Pritchard, 2001).

The physical and cellular barriers composed of the nasal airways, mucociliary apparatus and pulmonary macrophage cells represent the major protective mechanisms in the pig's respiratory system to prevent entry of dust and

infectious agents or to eliminate the agents that have come in contact with the tissues. The adhesion ability and immune evasion mechanisms of *M. hyopneumoniae* can delay the clearance from respiratory tract. On the contrary, the inactivated microbe is easily and quickly cleared by the barrier defenses. Maybe this would be one of the reasons that the previous inactivated *M. hyopneumoniae* aerosol vaccine failed to induce immune protection (Murphy et al., 1993). A number of factors are responsible for the viability of microbial aerosol during the nebulization, such as RT, RH, evaporation efficiency, impact forces, and the shear forces (Gough and Allan, 1973; Landman and van Eck, 2001; Swift, 1980; Yadin and Orthel, 1978). Higher temperature and lower humidity leads to faster evaporation, which is helpful in generating smaller particles that can enter the lower airway tract. However, the viability of the aerosol vaccine can be reduced because of the rapid increasing of ion concentrations in vaccine droplets (Gough and Allan, 1973; Landman and van Eck, 2001; Yadin and Orthel, 1978). The inactivation of *M. hyopneumoniae* due to the high ion concentration was also proved by our previous research which showed that the titer of *M. hyopneumoniae* cells were treated with a hypertonic solution for 2 min (Wang et al., 2012a), whereas no inactivation was detected in hypotonic solution treated for the same time (not published). As a result, a moderated RT (20–25 °C) and a relatively high RH (70–75%) were respectively set in this study to avoid fast evaporation. Also, deionized water was used as basic reagent instead of PBS or 0.9% NaCl solution to remove the deleterious effects of ions concentrated by evaporation.

Besides the factors of RT and RH, the impact forces and the shear forces generated during the nebulization were considered as major factors responsible for inactivation of the aerosol vaccine (Swift, 1980). This situation was also shown in our study. When the deionized water was simply used as vaccine diluents, the titers of the aerosol vaccine decreased sharply from $7.90 \pm 0.17 \log_{10} \text{CCU}_{50}/\text{ml}$ before nebulization to $6.57 \pm 0.12 \log_{10} \text{CCU}_{50}/\text{ml}$. Some reagents with surface activity and permeability, such as surfactants, Gelatin, and carbohydrates, are usually added into the vaccine solution as an excipient to reduce inactivation of the aerosol vaccine by nebulization and evaporation. Gelatin and sucrose are generally added to maintain viability during the freeze-drying process for the *M. hyopneumoniae* attenuated vaccine. However, no significant protective effect was shown during nebulization according to the titer reduction of water vaccine (dissolved in deionized water). Another three diluents, 5% glycerol, 0.1% PVP and 0.1% Tween-80, were optimized as well for keeping the stability of aerosol vaccine. In this study, glycerol showed the highest protection. Glycerol was usually added as a non-volatile component to delay the speed of evaporation. However, our results implied that glycerol may also reduce the shear forces and impact forces of aerosolization, because a high RH in this study was controlled to prevent rapid evaporation. Tween-80, a nonionic surfactant, could be preferentially concentrated at the air–water interface during droplet production, excluding the protein, and thereby protecting the protein from denaturation at that interface (Albasarah et al., 2010).

Contrary to our expectations, the lowest titer was observed with the Tween-80 vaccine. The damaging effect may be due to the severe foaming phenomenon of Tween-80 vaccine during aerosolization, which may due to the backflow of vaccine from the baffles in the nebulizer reservoir, increasing the surface tension, and thereby reducing the stability of the vaccine. Considering the viability of the aerosol vaccine, only the gelatin vaccine was used in this study for particle size measuring in vitro and deposition testing in vivo. The viability of the *M. hyopneumoniae* aerosol vaccine was also proved in vivo. The vaccine strains could be detected in BALF (by nested-PCR method) and on the luminal surface of lower airway tract (by IHS and SEM method) on 14 days post-immunization implied that the *M. hyopneumoniae* aerosol vaccine could adhere to the ciliated cells of the bronchi and propagate on them.

In this study, we used 3 ml (about 3×10^8 CCU) *M. hyopneumoniae* attenuated vaccine to vaccinate 3 piglets by aerosolization. An increase of specific *M. hyopneumoniae* sIgA antibody in nasal cavities was detected on 14 dpi, which could imply that the aerosol vaccine developed in this study has potential for immune protection. As further study, we will perform challenge study and optimize the dose to produce desirable efficacy.

Although the observation of *M. hyopneumoniae*-like particles in the bronchi by SEM was similar to the studies reported by Underdahl et al. (1980), it was presumed according to Hatch's report (Hatch, 1961) that only a small part of the aerosol vaccine particles below $3 \mu\text{m}$ could be easily inhaled into the lobules when the vaccine was sprayed. The *M. hyopneumoniae* particles detected in the bronchioles at slaughter may result from the replication and spread of the inhaled vaccine strains. This may also explain why specific sIgA antibody was undetectable until to 14 dpi. Our future studies will focus on generating more inhalable droplets by the change of nebulizers or vaccine forms, such as dry power vaccine. In addition, the Pari nebulizer designed for individual people has a low output, which is not competent for mass spray and aerosol vaccination. The electric nebulizer with a large-capacity should be used in the clinic.

In this study, the first attenuated *M. hyopneumoniae* aerosol vaccine was prepared and demonstrated to penetrate and adhere to the lower respiratory tract of piglets successfully.

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