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Establishment of a model of *Mycoplasma hyopneumoniae* infection using Bama miniature pigs



Yuan Gan[†], Xing Xie[†], Lei Zhang, Qiyan Xiong, Guoqing Shao and Zhixin Feng^{*} 

Abstract

Mycoplasma hyopneumoniae (*M. hyopneumoniae*), is the primary aetiological agent of enzootic pneumonia leading to chronic respiratory disease prevalent worldwide. Conventional pigs are the only animals used for pathogenicity studies and vaccine evaluations of *M. hyopneumoniae*. Considering that the challenge animals have better genetic stability and a smaller body size to operate with, an alternative experimental animal model of *M. hyopneumoniae* infection with Bama miniature pigs was established. Nine seven-week-old snatch-farrowed, porcine colostrum-deprived (SF-pCD) Bama miniature pigs and nine conventional pigs were randomly divided into two infected groups (Bama miniature-infected (BI) and conventional-infected groups (CI), BI and CI, $n = 6$) and two control groups (Bama miniature control (BC) and conventional control (CC) groups, BC and CC, $n = 3$). Every piglet was tracheally inoculated with 5×10^8 CCU/mL containing 10% suspension of a stock of frozen lung homogenate from SF-pCD pigs infected with virulent strain JS or sterilized KM2 medium. Typical lung lesions appeared in all infected pigs after necropsy, and the mean gross lung lesions was 17.3 and 13.7 in groups of BI and CI. Serum IgG and nasal sIgA antibody titres were increased significantly. Cilia shedding and mucus staining increased greatly in JS-infected bronchi. Obvious reddish gross lesions and *M. hyopneumoniae* antigen were detected, especially apparently observed in group of BI. Moreover, DNA copies of *M. hyopneumoniae* from bronchoalveolar lavage fluid (BALF) of each JS-infected piglet reached more than 10^8 , and *M. hyopneumoniae* could be re-isolated from each infected BALF. These results indicate that Bama miniature pigs could be used as an alternative and more maneuverable experimental infection model for *M. hyopneumoniae* and display typical clinical and pathological features consistent with those in conventional pigs.

Keywords: *Mycoplasma hyopneumoniae*, Bama miniature pigs, Conventional pigs, Infection model

Highlights

- A more sensitive infection model of *M. hyopneumoniae* using SF-pCD Bama miniature pigs infected with a highly virulent strain JS was established for the first time.
- The mean gross lung lesions scoring with the 28 points assessment method was 17.3 and 13.7 in infection groups of Bama miniature pigs (BI) and conventional pigs (CI).
- Serum IgG and nasal sIgA antibody titres tested for *M. hyopneumoniae* from infected pigs were increased significantly compared with pigs in each control group, and IgG antibody titers of pigs in BI was higher than those in CI.
- Cilia shedding and mucus staining increased significantly in JS-infected bronchi.
- Obvious reddish gross lesions and *M. hyopneumoniae* P97 antigen were detected in infected pigs. DNA copies from bronchoalveolar lavage fluid (BALF) of each JS-infected piglet

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reached more than 10^8 , and *M. hyopneumoniae* could be re-isolated from BALF of infected pigs.

Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the aetiologic agent of enzootic pneumonia in swine (EPS), a prevalent chronic respiratory disease worldwide that causes great economic losses to the pig industry (Maes et al. 2018). The main clinical features of pigs infected with *M. hyopneumoniae* usually include symptoms such as dry non-productive cough (Sibila et al. 2009), laboured breathing, anorexia and lethargy (Maes et al. 1996). Lung lesions in infected animals are mainly “EP-like” lesions, which are composed of purple-to-grey consolidated areas in the apical, cardiac, intermediate and the anterior portions of the diaphragmatic lobes and are the typical pathological features of the disease (Maes et al. 2008). In addition, affected pigs are much more likely to suffer from mixed or secondary infections of other respiratory pathogens, including viruses such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine pseudorabies virus (PRV), and porcine circovirus type 2 (PCV2); bacteria such as *Haemophilus parasuis* (HPS) and *Streptococcus suis* (*S. suis*); and parasites, which can increase the severity of the illness (Maes et al. 2008; Sibila et al. 2009). Moreover, *M. hyopneumoniae* is also considered to be one of the primary agents involved in the porcine respiratory disease complex (PRDC) (Maes et al. 2008; Sibila et al. 2009). Due to pigs being the natural host of *M. hyopneumoniae*, establishment of an infection model with a single pathogen *M. hyopneumoniae* is necessary for research on its pathogenesis mechanisms and host-pathogen immune interactions. Conventional pigs have been the only animals widely used in pathogenicity and vaccine evaluation studies of *M. hyopneumoniae* for a long time. For example, in our previous study, the model of *M. hyopneumoniae* infection in Suzhong pigs (Duroc × Taihu crossbreeds) was established, which was used to test the effects of different adjuvants on the immunogenicity and protective effect of a *M. hyopneumoniae* live vaccine after intramuscular inoculation with *M. hyopneumoniae* strain JS (Xiong et al. 2014a). Existing studies have found that Chinese local breed pigs such as the Erhualian pig (Huang et al. 2017) and the Jinhua pig (Xu et al. 2020) are more sensitive to swine respiratory diseases, including swine enzootic pneumonia, compared with conventional pigs such as Large White, Duroc and Landrace breeds or crossbreeds. Major challenges and disadvantages, however, including poor test repeatability and the difficulty of handling conventional pigs, thus prompting us to find an alternative, more convenient animal model with characteristics of good genetic stability, small size

and susceptibility to infection for the study of *M. hyopneumoniae*.

Bama miniature pigs have been inbred for more than thirty years in southern China and have the characteristics of all miniature pigs (Xia et al. 2015). Bama miniature pigs are generally selected for basic breeding or inbreeding with half-siblings, which guarantees that they are genetically stable. The small size of the animals makes them an ideal infection model and an attractive alternative compared to conventional pigs, especially for long-term trials, which is suitable for *M. hyopneumoniae* infections because this pathogen has a morbidity of at least 28 days in pigs (Liu et al. 2015). Analysis of the homologous sequences of important cytokines and major innate immune-related molecules in Bama miniature pigs has indicated that it is feasible to use Bama miniature pigs instead of conventional pigs as ideal experimental animals at the molecular level (Gao et al. 2014). In addition, previous studies have reported that miniature pigs have been used as experimental infection models for pathogens such as *E. coli* (Bronner et al. 2002), *S. suis* (Madsen et al. 2001), and dengue virus (Cassetti et al. 2010). All of these advantages make Bama miniature pigs suitable for wide use as an important experimental animal model in medical research (Ju et al. 2011; Sun et al. 2011).

Here, for the first time, snatch-farrowed, porcine colostrum-deprived (SF-pCD) Bama miniature pigs and conventional pigs (three-way crossbreeding of the Landrace, Large White and Duroc breeds) were experimentally infected with a highly virulent strain JS of *M. hyopneumoniae*, the virulence of which was assessed in our previous report (Xiong et al. 2014). Clinical symptoms, serology, aetiology, lung gross lesions and histopathology were recorded for EPS evaluation and side-by-side comparison.

Materials and methods

Animals, housing and experimental design

Nine seven-week-old snatch-farrowed, porcine colostrum-deprived (SF-pCD) Bama miniature pigs and nine SF-pCD weaner conventional piglets (predominantly three-way crossbreeding of the Landrace, Large White and Duroc breeds) were all purchased from Zhubang Biological Technology Company of Nanjing, China. At the time of clinical examination, the piglets did not show any signs of cardiopulmonary disorders and respiratory symptoms. None of the piglets had received any drug treatment or vaccination prior. Before inoculation, serological tests of the piglets were conducted for anti-*M. hyopneumoniae* antibody detection. Nasal swabs collected from all piglets were tested by nested PCR (Lu et al. 2010). In addition, except for *M. hyopneumoniae*, pigs were free of the following pathogens based on serology detection test,

detection pathogens included: classical swine fever virus (CSFV), porcine respiratory and reproductive syndrome virus (PRRSV), porcine circovirus type 2 (PCV2) and porcine pseudorabies virus (PRV).

Both Bama miniature pigs and conventional pigs were separately housed in the Experimental Animal Center of Jiangsu Academy of Agricultural Sciences under standard conditions determined by institutional guidelines. In total, 18 seven-week-old SF-pCD piglets were randomly divided into four groups according to the breed. Group 1 ($n = 6$), Bama miniature pigs were inoculated with a virulent strain JS (totally 5×10^8 CCU/mL, cultured with KM2 medium, containing 10% suspension of a stock of frozen lung homogenate from JS-infected SF-pCD pigs, named as BI); group 2 ($n = 3$), Bama miniature pigs inoculated with sterilized KM2 medium (prepared in our lab) which served as a negative control (named as BC); group 3 ($n = 6$), conventional pigs infected with the same amount of 5×10^8 CCU/mL strain JS containing 10% suspension of a stock of frozen lung homogenate from JS-infected SF-pCD pigs (named as CI); group 4 ($n = 3$), conventional pigs inoculated with sterilized KM2 medium which served as another negative control (named as CC). Pigs were kept under the same feeding and management conditions but were housed in four separate rooms until the end of the experiment.

Ethics statement

All animal experiments were performed according to animal welfare standards, which were approved by the Ethics Committee for Animal Experiments of Jiangsu Academy of Agricultural Sciences, China, with the approval number SYXK (Su) 2015–0019.

Bacterial strains and culture conditions

M. hyopneumoniae strain JS is a virulent strain that can induce typical characteristics of enzootic pneumonia in conventional pigs, as described previously (Xiong et al. 2014). It was confirmed that there were no other respiratory tract bacteria causing lung diseases except *M. hyopneumoniae* in the suspension of a stock of frozen lung homogenate from SF-pCD pigs infected with a virulent strain JS. The titer of *M. hyopneumoniae* strain JS was quantified using a colour-changing unit (CCU) assay (Furr & Taylor-Robinson 1993) in KM2 cell free liquid medium (a modified Friis medium) containing 20% (v/v) swine serum at 37 °C (Xiong et al. 2014), and confirmed by TaqMan quantitative PCR described below (Wu et al. 2019).

Animal inoculation, clinical observation and necropsy

Briefly, prior to inoculation, pigs were stabilized and anaesthetized by intramuscular injection of Zoletil (60

mg/kg, Virbac, Carros, France). Every piglet in two infected groups (BI and CI, each group contains six Bama miniature piglets and six conventional piglets) was tracheally inoculated once with 5 mL a dose of 5×10^8 CCU/mL containing 10% suspension of a stock of frozen lung homogenate from SF-pCD pigs infected with a *M. hyopneumoniae* virulent strain JS. Five millilitres of sterile KM2 medium per piglet was inoculated in the same manner in a total of six pigs in the two control groups. Pigs were observed daily during the same time period for 15 min to determine the occurrence of respiratory signs. In addition, behavioural changes in the infected pigs, including appetite, breathing and cough were also assessed (Maes et al. 1996). All pigs were euthanized 28 days after inoculation (dpi), and every lung was evaluated to determine whether it showed typical lesions, such as “EP-like” consolidation located in the apical, intermediate, accessory and cranial portions of the diaphragmatic lobes (Garcia-Morante et al. 2016).

Sample collection and scanning electron microscopy observation

Serum and nasal swab samples from all pigs at 0 dpi (before the experiment) and 28 dpi (at the time of necropsy) were aseptically collected. In addition, the BALF of each pig was collected after necropsy as well, centrifuged and stored at -70 °C until use. Lungs from the two infected groups (samples were taken from lesion margins) and from the two control groups (samples were taken routinely from the intermediate lobes of the right lung) were harvested from the pigs after death (after euthanasia at the end of the trial) and were collected for further pathological observation and immunohistochemical assessment. Bronchus samples were taken from the apical lobe of each lung for frozen sectioning, and the ultrastructure of the bronchial surfaces was examined by scanning electron microscopy (EVO-LS10, Zeiss, Germany).

Serological and mucosal antibody tests

Serum samples were collected from all piglets at 0 and 28 dpi and tested for *M. hyopneumoniae*-specific IgG antibodies using a commercially available ELISA kit (IDEXX, Liebefeld-bern, Switzerland) according to the manufacturer's instructions (Bai et al. 2018). In addition, nasal swabs were collected where swabs were inserted deeply in both nostrils to reach the turbinates (Feng et al. 2010). Swab specimens were resuspended in 1 mL of PBS and stored for 2 h at 4 °C before centrifugation at $10,000 \times g$ for 5 min. Then *M. hyopneumoniae*-specific secretory IgA (sIgA) antibodies were measured as previously described (Bai et al. 2018).

Indirect immunofluorescence assay (IFA) for detection of mucus secretion in the bronchus

Bronchi were taken from the apical lobe of each lung and fixed in 4% paraformaldehyde for 4 h. The bronchi were then soaked in a 30% sucrose solution for 6 h before being embedded in optimal cutting temperature compound for 20 min. The embedded frozen tissue pieces (longitudinal section) were then sectioned at 8 μm (CM1950, Leica, Germany), placed at room temperature (RT) for more than 30 min to prevent stripping before IFA was conducted as described previously (Wang et al. 2018; Xie et al. 2018a). Briefly, to detect the secretion of mucus in JS-infected bronchi, frozen tissue pieces were blocked in 2% bovine serum albumin (BSA) for 1 h before incubation with primary and secondary antibodies, respectively.

Primary antibody against mucin 5B (MUC5B) (1:100 dilution; Cat. No ab 87,376, Abcam, Cambridge, UK) for testing mucus secretion was incubated with slices overnight at 4 °C in 1% BSA. After washing three times with PBS, the slices were incubated with Texas Red-conjugated goat anti-rabbit IgG (H + L) (1:1000 dilution, Cat. No ab6719, Abcam, Cambridge, UK) for 1 h at 37 °C. Cell nuclei were stained with DAPI for 5 min at RT before the cells were visualized using an LSM 710 confocal laser scanning microscope (Zeiss, Germany).

Histopathology and immunohistochemistry analysis

Lung samples obtained from pigs in two *M. hyopneumoniae* strain JS-infected groups and two control groups were collected for pathological examination as previously described (Xie et al. 2018). In brief, four micrometre-thick sections were prepared from the paraffin-embedded tissues by immersing the lung tissues in 10% neutral buffered formalin, and the sections were stained with haematoxylin and eosin (H&E). Histopathological pneumonic lesions of each lung tissue sample were evaluated according to the criteria lymphocyte infiltration: it was graded as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe) depending on the lesion severity (Sun et al. 2011; Xie et al. 2018).

M. hyopneumoniae infection in the airway was detected using an immunohistochemistry assay. After bronchial sections were blocked with 1% bovine serum albumin, sections were stained with a monoclonal antibody against the P97R1 protein produced in our laboratory at a dilution of 1:1000 for 1 h at 37 °C. Then sections were probed by biotin-conjugated goat anti-mouse immunoglobulin (Cat.No BA1001, Boster, Wuhan, China) and HRP-conjugated streptavidin (Cat.No 22393, Boster, Wuhan, China) at a dilution of 1:200 for 30 min at 37 °C before being developed with an HRP-DAB chromogenic substrate kit (Tiangen, Beijing, China) for 10 min and finally counterstained with haematoxylin.

Quantitative real-time PCR assay of *M. hyopneumoniae*

The whole lung was removed through the throat, and the throat was cut with sterilized scissors. After slowly injecting 50 mL of sterile PBS into each trachea using a syringe, a haemostat was clamped on the tracheal incision, and a total of eighteen lungs were gently massaged for no less than one minute to ensure sufficient contact between the PBS and alveoli. Bronchoalveolar lavage fluid (BALF) of each piglet was collected and recorded before being stored at -70 °C until use.

DNA of *M. hyopneumoniae* was extracted from 0.2 mL of BALF of each piglet using a DNA Extraction Kit (Axygen, Cat.No: AP-MN-BF-VNA-250, New York, USA). Extracted DNA was quantified by quantitative real-time PCR using the TaqMan system, and primers and TaqMan-TAMRA probes were designed based on the conserved sequence of the P97 gene of *M. hyopneumoniae* (Wu et al. 2019). In addition, a standard curve was generated using a 10-fold diluted standard plasmid, PMD-T-P97, as a template before using a QuantStudio R5 Real-Time PCR System to determine the DNA copies as described previously (Wu et al. 2019). The samples were run in triplicate biological replicates.

Re-isolation, Wright's staining, PCR detection of *M. hyopneumoniae*

M. hyopneumoniae strains were re-isolated from BALF in two infected groups and identified using Wright's staining and nested PCR assay (Lu et al. 2010). First, 0.4 mL of BALF per pig were filtered through a 0.45 μm filter (Millipore), mixed with 1.6 mL of KM2 medium and incubated in an incubator with a 5% CO₂ environment at 37 °C. A positive control of culture of strain JS and a negative control of KM2 medium alone were simultaneously prepared. The cultures were observed every half day until the colour of the medium changed completely from red to yellow, in addition, pH value was detected as well after colour changing. The discoloured culture was the first passage of the re-isolation, and a 1:10 inoculation ratio was subcultured for two additional passages. Cultures of passage 2 were subjected to Wright's staining and electron microscopic observation. Each culture passage was subjected to nested PCR specific for the P36 (L-lactate dehydrogenase) gene of *M. hyopneumoniae*. Two pairs of specific primer sequence were as follows: Outer forward primer, 5'-TTAGTGTCTCCGTTATG-3'; Outer reverse primer, 5'-GAAATCCGTATTCTCCTC-3'; Inner forward primer, 5'-TTACAGCGGGAAGACC-3'; Inner reverse primer, 5'-CGGCGA GAAACTGGATA-3', and the annealing temperature was set as 42 °C and 51 °C, respectively. Tissue samples from all pigs were also collected for routine detection of the other porcine pathogens mentioned above (Xiong et al. 2014).

Statistical analysis

Data were collected and analysed using SPSS Statistics v20.0 software. Differences in serum IgG titres and sIgA antibody titres of nasal swabs between pigs in two infected groups (BI and CI) and two control groups (BC and CC) were evaluated using a multiple *t* test analysis, with $P < 0.05$ considered a significant difference, while $P < 0.01$ considered an extremely significant difference. Variations in number of DNA copies per piglet against P97 between two infected groups (BI and CI) and two control groups (BC and CC) were analysed using an unpaired *t* test.

Results

Evaluation of clinical symptoms and lung gross lesions after pigs were infected with *M. hyopneumoniae*

Bama miniature pigs and conventional pigs were divided into two control groups ($n = 3$) and two infection groups ($n = 6$) according to their breeds, respectively, which were housed in separate animal rooms. Porcine serums were collected for antibody detection against *M. hyopneumoniae*, PRRSV, PCV2, PRV and CSFV. The results (Table S1) demonstrated that the pigs were antibody negative to these listed respiratory pathogens, with the

exception of one Bama miniature pig (numbering 491) which was a suspected antibody positive to PCV2. During the experimental process, no coughing symptoms were observed for any pigs in the two control groups. However, dry, non-productive coughing typical of *M. hyopneumoniae* infection was observed in three inoculated Bama miniature pigs (numbering 274, 273, 759) and two conventional pigs (numbering 143, 157) from 14 to 20 dpi. Interestingly, all inoculated piglets in BI and CI groups exhibited symptoms similar to labored breathing, but this was not observed in the control group (BC and CC) two weeks after infection.

At 28 dpi, gross lung lesions were observed in all pigs and evaluated after necropsy. For Bama miniature piglets, lungs from BI group demonstrated obvious boundaries, and typical “EP-like” lesions with dark red-to-purple areas of consolidation were observed, which were mainly located in the apical and intermediate lobes of the left and right lungs and accessory lobes (Fig. 1 a and b). In contrast, there were no visible lesions in the lung tissue of the BC group (Fig. 1 c and d). Similarly, for conventional piglets, lesions were also found in all piglets from CI group, where EP-like lesions appeared in the ventro-cranial aspect of the left and right

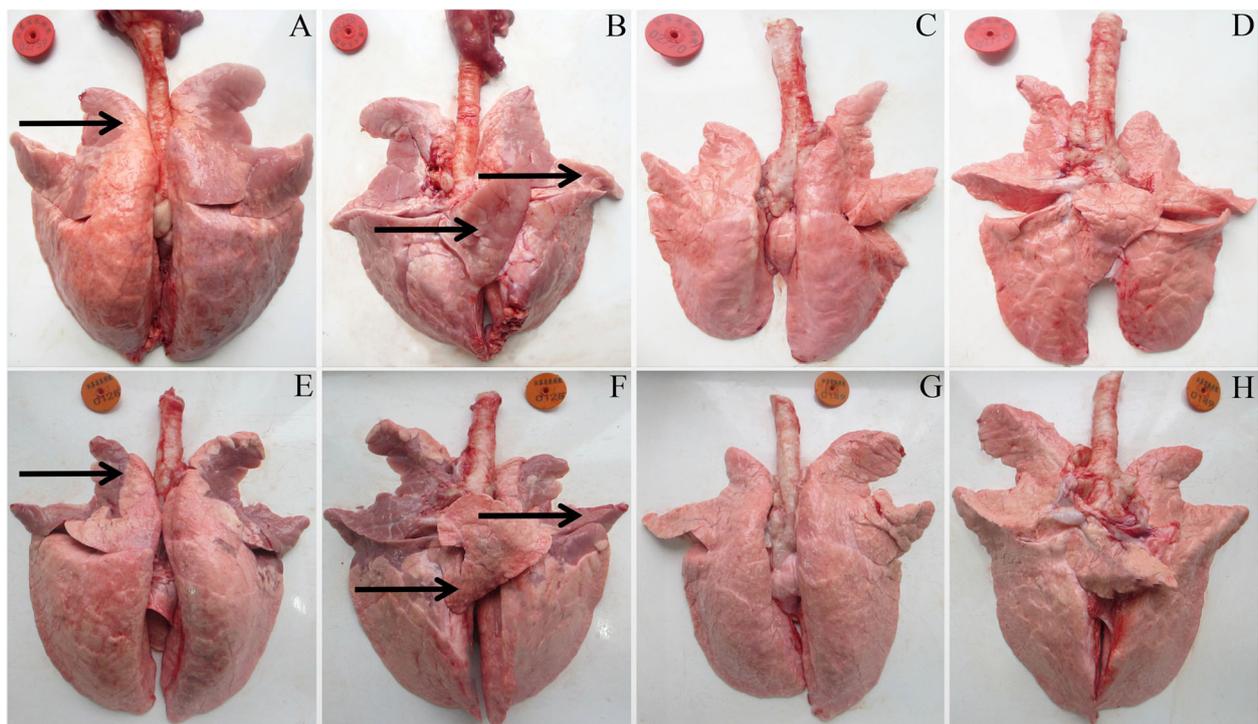


Fig. 1 Gross lesions evaluation of lung samples at 28 dpi after necropsy. Whole lung samples connected to the trachea of each piglet were imaged after the animals were sacrificed. **a** and **b** Dorsal and ventral views of representative morphology images of lung gross lesions in the *M. hyopneumoniae* strain JS-infected group of the Bama miniature pigs; **(c)** and **(d)**: Dorsal and ventral views of representative morphology images of lung samples in the control group of the Bama miniature pigs; **(e)** and **(f)**: Dorsal and ventral views of representative morphology images of lung gross lesions in the *M. hyopneumoniae* strain JS-infected group of the conventional pigs; **(g)** and **(h)**: Dorsal and ventral views of representative morphology images of lung samples in the control group of the conventional pigs

diaphragmatic lobes (Fig. 1e and f), while no lesions were observed in piglets from group CC (Fig. 1g and h). Gross lung lesions (dark red-to-purple areas of consolidation) scoring of piglets in four groups using the 28 point evaluation method is summarized in Table S2. The mean lung lesions scoring with the 28 points assessment method was 17.3 and 13.7 in BI and CI group, respectively, although no statistically significant differences of lung lesion scoring were found between BI and CI groups.

Serum IgG and mucosal nasal sIgA antibody responses

As shown in Fig. 2a, serum IgG antibody test results showed that all the piglets in both the two infected groups and two control groups were all negative for *M. hyopneumoniae* at 0 dpi (D0). At 28 dpi (D28), all infected piglets in BI and CI groups showed seroconversion to a mean S/P value of 1.22 and 0.84, respectively, while S/P values of serum IgG titers of those in the two control groups remained negative. Comparison of S/P values of serum IgG titers between D0 and D28 of two infected groups revealed a significant difference in BI group ($P < 0.01$) and CI group ($P < 0.01$), respectively. In addition, at D28, serum IgG titers in BI and CI groups were also significantly higher than those in BC group ($P < 0.01$) and CC group ($P < 0.05$). Moreover, serum IgG titers in BI at D28 was higher than those in CI, although no statistically significant differences were found between the two groups.

Similarly, as depicted in Fig. 2b, the sIgA antibody levels from nasal swab samples of infected piglets in BI

($P < 0.01$) and CI ($P < 0.01$) groups both increased extremely significantly at D28 with those at D0. No sIgA antibody conversion was detected in nasal swab samples of the two control groups throughout the whole sampling period. At D28, significant differences were also found of sIgA antibody titers in both BI and CI groups compared with those in BC group ($P < 0.05$) and CC group ($P < 0.05$).

Bronchial cilia distribution and mucus secretion after piglets inoculated with *M. hyopneumoniae*

Scanning EM of the bronchial tissues revealed that both Bama miniature pigs and conventional pigs infected with the *M. hyopneumoniae* strain JS exhibited the severe destruction of ciliary function. At 2000 \times magnification, a large number of cilia was decreased and had shed in the BI group (Fig. 3a) whilst no obvious abnormality on the bronchial cilia was observed (Fig. 3b). When observed at 10,000 \times magnification, it was observed that a large number of elliptical forms of *M. hyopneumoniae* had adhered to the tip of cilia in group BI (Fig. 3c), in contrast, cilia were neatly arranged in clusters in the negative control group BC (Fig. 3d). Similar results were observed in bronchial from conventional pigs (data not shown).

To further test the susceptibility of the bronchi to *M. hyopneumoniae* infection, mucus secretion in bronchi were analysed by IFA from frozen sections. Results from BI and CI showed similar trends. For the immunostaining of mucus from the bronchi of Bama miniature piglets and conventional pigs (data not shown) infected with *M. hyopneumoniae* strain JS, it was found that there

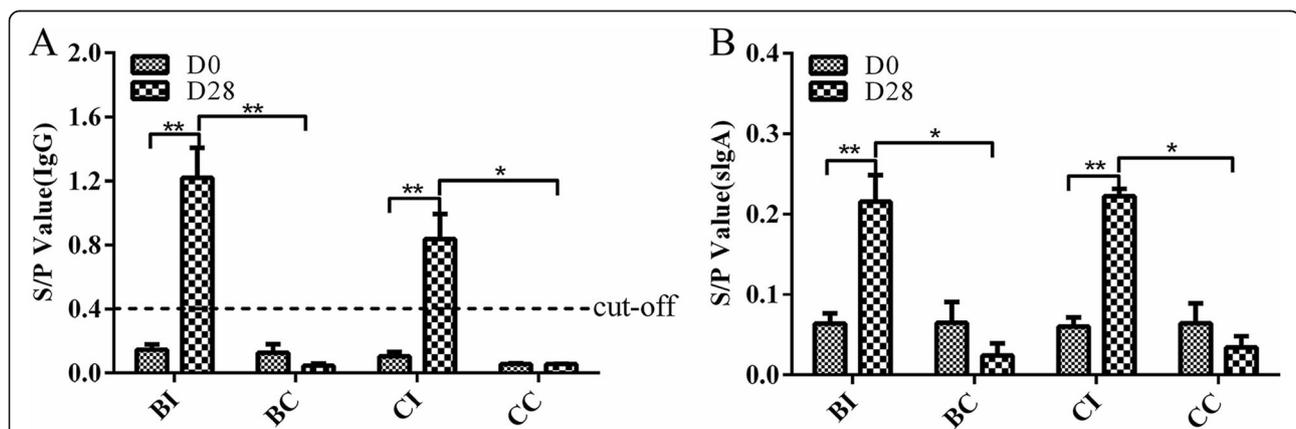


Fig. 2 Serum IgG antibody and nasal sIgA antibody titres detection of all pigs from both Bama miniature pigs and conventional pigs. Serum and nasal swabs from all pigs were collected at 0 dpi and 28 dpi. D0: 0 dpi; D28: 28 dpi. **a** Serum IgG titres of *M. hyopneumoniae* from all pigs were tested by a commercialized ELISA kit. S/P value = (sample OD₆₅₀ - negative control OD₆₅₀)/(positive control OD₆₅₀ - negative control OD₆₅₀). S/P values higher than the cutoff value of 0.4 were considered positive. *, $P < 0.05$, indicates serum IgG S/P values between the infected and control groups of conventional pigs were significantly different. **, $P < 0.01$, indicates serum IgG S/P values at 0 dpi and 28 dpi were extremely significantly different. **b** sIgA titres of *M. hyopneumoniae* collected from nasal swabs of pigs measured using a lab-prepared ELISA kit. S/P value = (sample OD₄₅₀ - negative control OD₄₅₀)/(positive control OD₄₅₀ - negative control OD₄₅₀). *, $P < 0.05$, indicates nasal swab sIgA S/P values between the infected and control groups of both Bama miniature and conventional pigs were significantly different. **, $P < 0.01$, indicates nasal swab sIgA S/P values at 0 dpi and 28 dpi of infected pigs in both Bama miniature and conventional pigs were extremely significantly different

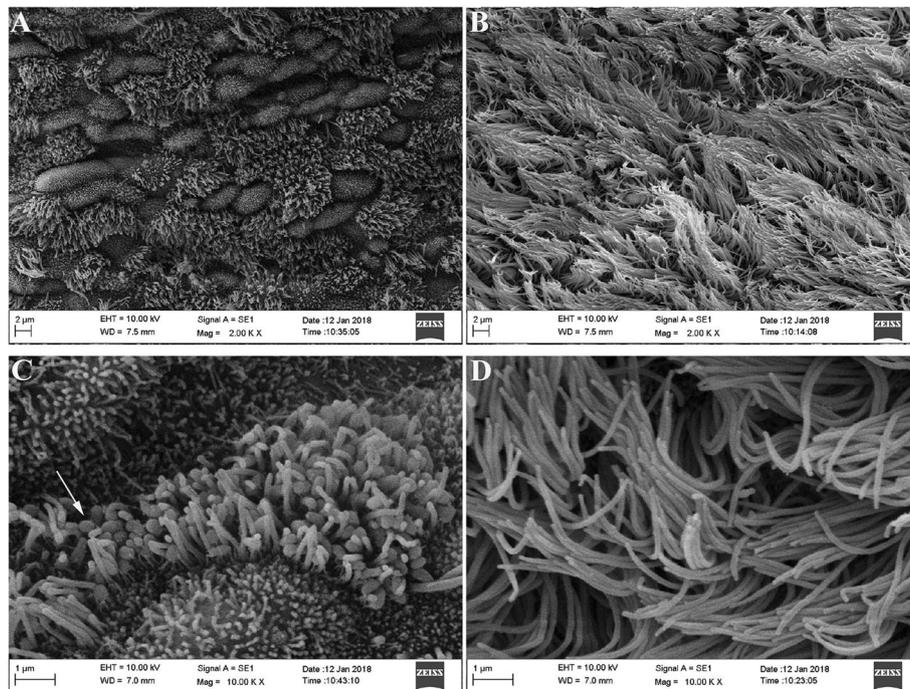


Fig. 3 Scanning electron microscope observation of bronchi ciliary structure. Lungs containing bronchi of all pigs were removed for gross lesion observation and sample collection. **a** and **c** represent cilia observations of bronchi samples taken from Bama miniature pigs infected with the *M. hyopneumoniae* strain JS at 2000 and 10,000 \times magnifications, respectively. Elliptical erythrocyte-like forms of *M. hyopneumoniae* are marked by white arrows on the truncated cilia (**c**). **b** and **d** represent cilia observations of bronchi samples taken from Bama miniature pigs in the control group at 2000 and 10,000 \times magnifications, respectively

exists some part of red mucus color distribution around the bronchial cavity, and a large amount of mucus distribution in the vacuole-like mucous gland as demonstrated in bronchi from BI group (Fig. 4a and b), indicating that mucus secretion increased in *M. hyopneumoniae* strain JS-infected bronchi. However, only sporadic mucus distribution was observed in the bronchial tissue of the BC group (Fig. 4c and d) and CC group (data not shown).

Histopathological and immunohistochemical analysis of lung tissues infected with *M. hyopneumoniae*

After lung gross lesions in four groups were scored, histopathological and immunohistochemical analyses were performed. Histopathological observation of lungs from *M. hyopneumoniae* strain JS-infected groups BI (Fig. 5a) and CI (Fig. 5c) showed widened alveolar space, resulting in nearly no visible alveolar structures. In addition, the bronchial lumen became narrowed, and there was a large number of infiltrating inflammatory cells in the alveolar space and bronchial cavity, together with some evidence of congestion or haemorrhage. In the lungs of control piglets from control groups BC (Fig. 5b) and CC (Fig. 5d) showed normal lung tissue morphology with normal alveolar septa. To compare the histopathological lesions more directly, the histopathological

lesions were evaluated based on lymphocyte infiltration and were graded as 0 (normal), 1 (mild), 2 (moderate) and 3 (severe). Based on this analysis, the lesion scores of the four groups were as follows: BI: CI: BC: CC was 3, 3, 0 and 0, respectively.

Based on the immunohistochemical analyses, obvious brown P97 antigen staining was observed in the luminal surface of bronchial lining epithelial cells, as indicated by the black arrows from both BI group (Fig. 5e) and CI group (Fig. 5g), and the area indicated by the arrow was enlarged and displayed in the upper right corner. While in two control groups BC (Fig. 5f) and CC (Fig. 5h), no brown antigen staining of *M. hyopneumoniae* was detected.

Quantitative PCR assay for the detection of *M. hyopneumoniae* in BALF

BALF samples from all piglets were collected, and DNA copies of *M. hyopneumoniae* in each piglet were quantified using quantitative real-time PCR. As shown in Fig. 6, the DNA copies/piglet of *M. hyopneumoniae* in the two infected groups were both more than 10^8 , while no DNA of *M. hyopneumoniae* was detected in the BC and CC groups. In addition, comparison between infected and control Bama miniature and conventional piglets revealed that DNA copies/piglet in both BI and CI groups

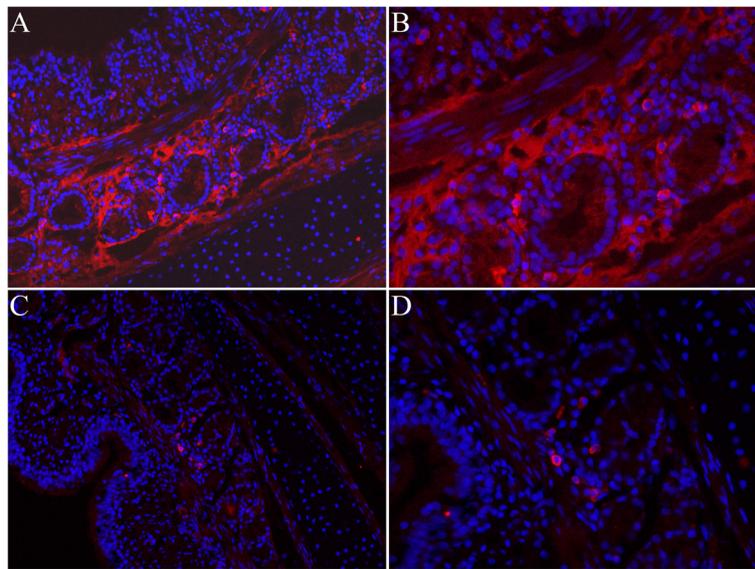


Fig. 4 IFA for the detection of mucus secretion in bronchi (longitudinal section) infected with *M. hyopneumoniae*. **a** and **b** represent immunostaining with MUC5B in bronchi of Bama miniature pigs infected with *M. hyopneumoniae* strain JS at 200× magnification and 400× magnification, respectively. **c** and **d** represent immunostaining with MUC5B in bronchi of control group of Bama miniature piglets at 200× magnification and 400× magnification, respectively

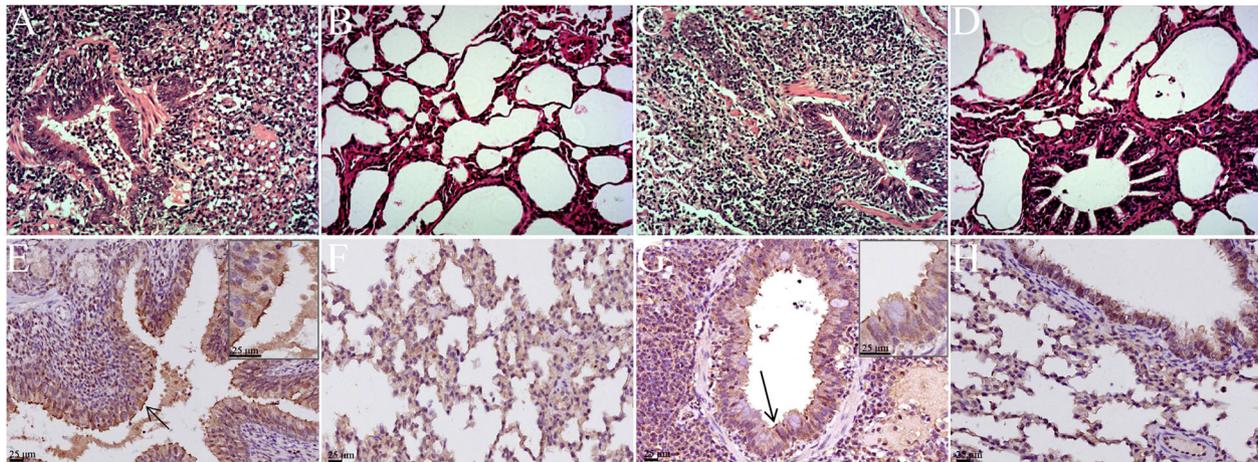
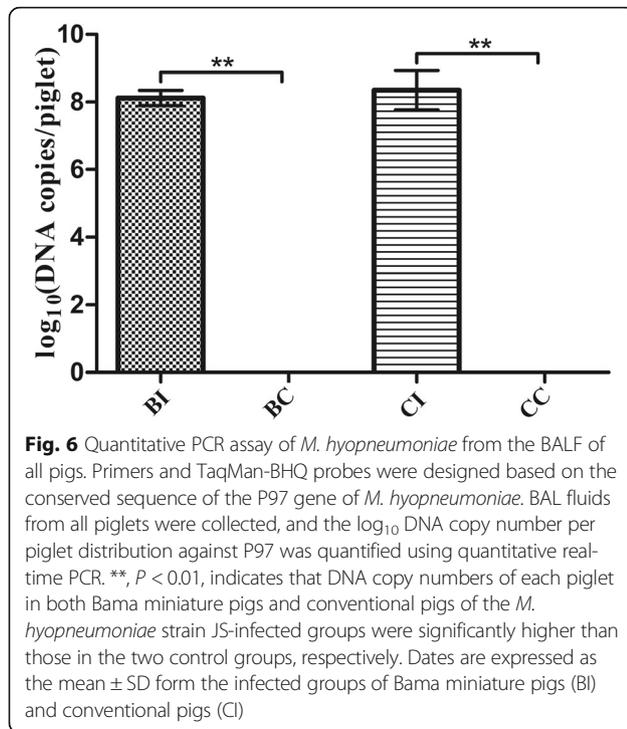


Fig. 5 Histopathological and immunohistochemical analysis of lung samples. **a** and **b** represent histopathological findings of lungs collected from Bama miniature pigs in the *M. hyopneumoniae* strain JS-infected group (BI) and control group (BC) at 200× magnification, respectively. **c** and **d** are histopathological findings of lungs collected from the conventional pigs in *M. hyopneumoniae* strain JS-infected group (CI) and control group (CC) at 200× magnification, respectively. All pictures were captured with the microscope of Nikon (TI-S-EJOY). **e** and **f** represent immunohistochemical observation of lungs against *M. hyopneumoniae* antigen P97 collected from Bama miniature pigs in the *M. hyopneumoniae* strain JS-infected group (BI) and control group (BC), respectively. Brown staining appeared around inner edge of bronchial lumen in the *M. hyopneumoniae* strain JS-infected group of Bama miniature pigs against P97R1 and was marked by short black arrows (**e**). **g** and **h** are immunohistochemical observation of lung against *M. hyopneumoniae* antigen P97 collected from conventional pigs in the *M. hyopneumoniae* strain JS-infected group (CI) and control group (CC), respectively. Similarly, brown staining appeared in group CI against P97R1 and is marked by long black arrows (**g**), and the enlarged image of the *M. hyopneumoniae* antigen P97-stained area pointed by the arrow (captured at 200× magnification) is shown in the upper right corner (**e** and **g**). The black bar represents the scale: 25 μm



were extremely significantly higher than those in BC group ($P < 0.01$) as well as CC group ($P < 0.01$).

Re-isolation and detection of *M. hyopneumoniae* from BALF samples

The BALF of each piglet was filtered before inoculation at a ratio of 1:5 for static culture in an incubator. After 120 h, the colour of the cultures from all pigs in two *M. hyopneumoniae* strain JS-infected groups BI and CI turned yellow (pH value 6.5–6.8), while re-isolated cultures from two control groups BC and CC remained red in colour, as did the KM2 medium negative control (pH value 7.2–7.4) (Fig. 7a). Cultures after colour change from BALF of Passage 2 in both infected groups BI and CI (data not shown) were subjected to Wright's staining and electron microscopy observation, as shown in Fig. 7b, Wright's staining results from culture of piglet in BI group preliminary indicated that the culture was *M. hyopneumoniae*, with ring-shaped as well as spherical, bipolar, crescent, filamentous, and other mycoplasma-like cells. In addition, cultures from all piglets were identified using the P36 gene specific for *M. hyopneumoniae* by nested PCR, from which outer and inner results were shown in Fig. 7c and Fig. 7d, respectively, indicating that *M. hyopneumoniae* was re-isolated from the BALF of all infected Bama miniature pigs and conventional pigs, while piglets from control group were all negative. Similarly, as expected, PCR detection for cultures from BALF in all pigs were all negative to other respiratory pathogens listed in Table S1 mentioned above (data not shown).

Discussion

Pigs infected with *M. hyopneumoniae*, the aetiologic agent of enzootic pneumonia in swine, demonstrate mainly poor growth and a reduced feed conversion rate. As experimental pigs from different backgrounds are not well controlled during the actual feeding process, pigs often exhibit mixed infections with other respiratory pathogens, including PRRSV, PRV, PCV2, CSFV, etc., causing porcine respiratory disease syndrome, which contributes to the severity of lung lesions demonstrated in infected pigs (Maes et al. 2018). Once pigs are infected with other respiratory pathogens, the accuracy and reliability of *M. hyopneumoniae* experimental infection is greatly reduced. In addition, the pathological changes and inflammatory reactions in pigs caused by *M. hyopneumoniae* are usually not typical and specific with unclear general background conditions, which seriously affects the aetiology, pathogenic mechanism and evaluation of vaccine immune responses (Xiong et al. 2014). Thus, in this study, snatch-farrowed, porcine colostrum-deprived (SF-pCD) pigs were housed according to the method described previously (Huang et al. 2013), and pigs raised with weaning directly after birth at day 0 were successfully used in our previous study to establish immortalized porcine bronchiolar epithelial cells (Xie et al. 2018), indicating that weaning directly after birth in order to produce highly healthy SF-pCD pigs is crucial for establishing a pathogenesis model of *M. hyopneumoniae* infection.

The advantages of Bama miniature pigs, including stable heritability, low feeding cost, easily operated, ease of exclusion of secondary bacterial infections as well as antibody positive cases, have raised the interest of researchers. It has been confirmed that Bama miniature, highly inbred pigs have normal reproductive characteristics consistent with conventional pigs (Liu et al. 2010), and studies using Bama miniature pigs were mainly focused on body effects, such as the effects of heat stress (Ju et al. 2011) or a long-term high-energy diet (Xia et al. 2015). In addition, Bama miniature pigs, applied as specific pathogen infection models, have also been confirmed. Shen et al. (2008) successfully constructed a Bama miniature pig infection model for *S. suis* type 2, and *S. suis* that can artificially infect Bama miniature pigs with good reproducibility, besides, obvious clinical symptoms and pathological changes caused by *S. suis* are consistent with natural infections. Moreover, Bama miniature pigs support productive CSFV infection and display clinical signs and pathological changes consistent with CSFV infections observed in conventional pigs, as reported previously (Sun et al. 2011). The aim of this study was to establish an alternative infection model for *M. hyopneumoniae* in Bama miniature pigs with more sensitivity and to apply it to the pathogenesis studies of

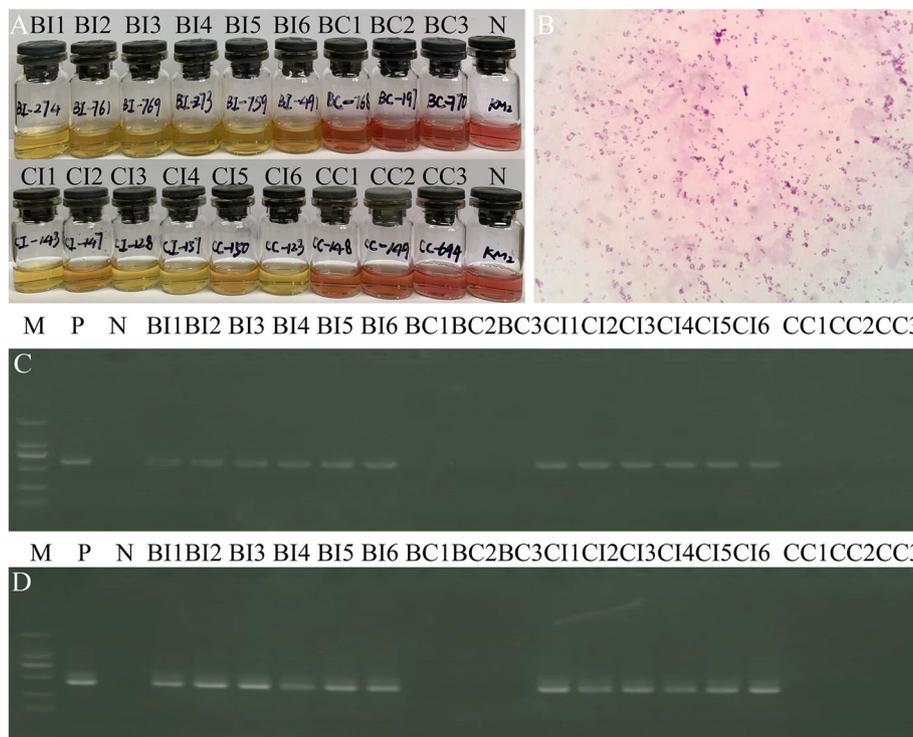


Fig. 7 Re-isolation and identification of *M. hyopneumoniae* from BALF of all pigs. **a** Growth condition and colour observation of samples re-isolated from the BALF of all pigs after 120 h of culture at 37 °C. Infected group of Bama miniature pigs (BI): BI1, BI2, BI3, BI4, BI5, BI6; Control group of Bama miniature pigs (CI): BC1, BC2, BC3; Infected group of conventional pigs (CI): CI1, CI2, CI3, CI4, CI5, CI6; Control group of conventional pigs (CC): CC1, CC2, CC3; N: KM2 culture medium of *M. hyopneumoniae*. **b** BALF cultures after colour change and pH value between 6.5–6.8 after tested were collected, and cultures among infected groups of Bama miniature pigs (BI) and infected groups of conventional pigs (CI, data not shown) were subjected to Wright’s staining and electron microscopic observation at 1000 × magnification. *M. hyopneumoniae* mainly displayed ring-shaped, spherical, bipolar, crescent, filamentous, and other mycoplasma-like cells. **c** and **d** Nested PCR test of P36 for re-isolated *M. hyopneumoniae* of passage 2 using specific outer and inner primers, respectively. M, DNA 2000 marker; P, positive control; N, negative control; Infected group of Bama miniature pigs (BI): BI1, BI2, BI3, BI4, BI5, BI6; Control group of Bama miniature pigs (BC): BC1, BC2, BC3; Infected group of conventional pigs (CI): CI1, CI2, CI3, CI4, CI5, CI6; Control group of conventional pigs (CC): CC1, CC2, CC3; the amplified product sizes of outer and inner product were 621 bp and 427 bp, respectively

M. hyopneumoniae. Here, genetically stable, highly inbred, small-sized (average of 6 kg of 50-day-old animals) pigs and conventional pigs (average of 12 kg of 50-day-old animals) were selected, together with weaning technology at day 0 directly after birth, to ensure that all pigs were negative for common swine respiratory pathogens, as listed in Table S1 by serum antibody detection.

After two weeks of infection, some infected piglets in BI group (3/6) and CI group (2/6) started to show clinical symptoms of cough, which demonstrated similar symptoms as those in natural infections and were consistent with a previous report (Procajlo et al. 2010). Lung lesions of piglets in the two infected groups revealed obvious boundaries through gross lesion observation, which are mainly located in the accessory lobe and part of the apical, intermediate and diaphragmatic lobes in both the left and right sides, indicating that pigs depicted obvious lung lesions and were highly infected with *M. hyopneumoniae* as previously reported (Garcia-Morante

et al. 2017). In addition, comparison between lung lesion scoring assessed by 28 points method between infected lungs from Bama miniature pigs and conventional pigs, it was found that the mean lung lesions scoring of piglets in BI group was higher than that of conventional pigs, but with no significant difference. The ELISA method is usually thought to be the main test standard for detecting antibodies expression levels against *M. hyopneumoniae* (Petersen et al. 2019), thus, here, the serum antibody level of pigs at 28 dpi increased significantly after infection, indicating that Bama miniature pigs and conventional pigs infected with *M. hyopneumoniae* strain JS can stimulate humoral immunity to produce increased serum IgG antibody levels, as previously reported that experimental infection could induce IgG antibody secretion against *M. hyopneumoniae* and produce certain level of immunity (Villarreal et al. 2009). In addition, the result of serum IgG antibody levels of pigs in BI was higher than those in CI, and was consistent

with the serological performance of pigs after natural infection as well (Leon et al. 2001).

Similarly, the detection of expression levels of sIgA antibodies against *M. hyopneumoniae* from collected nasal swabs also showed that this infection could stimulate all infected piglets to produce specific sIgA antibodies, indicating that *M. hyopneumoniae* infection in both Bama miniature and conventional pigs could induce a local mucosal immune response and the production of increased sIgA antibody levels, which is consistent with results reported previously (Bai et al. 2018; Feng et al. 2014). It was found that increased secretion of specific antibodies is most likely associated with lung lesion severity during infection, here compared between sIgA antibody level in BI and BC, CI and CC, which is consistent with previous findings (Garcia-Morante et al. 2017). However, here, although the mean lung lesion scores of pigs in BI group were higher than those of infected conventional pigs, the sIgA antibody level of infected Bama miniature pigs was little lower compared with conventional pigs, the reason of which may partially because the peak value has fallen back at 28 dpi, as demonstrated in our previous research showed that the peak of sIgA antibody level was at 14 dpi and decreased in some extent at 28 dpi (Bai et al. 2018). In addition, here, pigs inoculated with 10% suspension of a stock of frozen lung homogenate of *M. hyopneumoniae* strain JS and strain JS culture alone may also induce some effect on sIgA antibody level, which remains to reveal in the future work.

M. hyopneumoniae is found mainly on the mucosal surface of swine trachea, bronchi and bronchioles, inducing ciliostasis and loss of cilia. The first step of *M. hyopneumoniae* infection is the successful adhesion of *M. hyopneumoniae* to the cilia of the epithelium along swine respiratory tract (Blanchard et al. 1992). Here, a bronchus ciliary structure was observed through scanning electron microscopy, and many cilia were detached and cut off in both the *M. hyopneumoniae* strain JS-infected Bama miniature group similar to that of the conventional group which was performed in our previous study (Xiong et al. 2014), and was also consistent with previous findings that pigs infected with *M. hyopneumoniae* induced serious lesions in swine tracheal epithelial cells, cilia accumulation, injury and shedding (Maes et al. 2008). To further test the susceptibility of the bronchi to *M. hyopneumoniae* infection, and to confirm whether Bama miniature pigs could be used as an alternative and more sensitive host animal model instead of conventional pigs, mucus secretion was evaluated by IFA of frozen sections. Here, we took bronchi from BI group as an example, and the bronchi of the Bama miniature pigs infected with *M. hyopneumoniae* strain JS showed red mucus staining distribution in the vacuole-like mucous glands mostly, but a few showed a

small distribution in the bronchial cavity as well. As infection with *M. hyopneumoniae* can cause mucinous gland hypersecretion, thus leading to mucus secretion increase, which may partially explain the reason that mucus and inflammatory exudate retention in the mucous glands and bronchi, which can further trigger secondary infection in infected pigs, finally resulting in the opposite relationship that cilia decreased while mucus increased in *M. hyopneumoniae* strain JS-infected bronchi.

Histopathological changes of lung samples in the two *M. hyopneumoniae* strain JS-infected groups BI and CI were manifested mainly by increased alveolar septum, lymphoid aggregation around the bronchi and bronchioles, vascular and bronchial-associated lymphoid tissue hyperplasia, and inflammatory cell infiltration, leading to bronchial lumen and peripheral alveolar occlusion. This is consistent with results described previously (Lorenzo et al. 2006). Moreover, it was found that there were some hyperemia and hemorrhage lesions in the lung samples of two infected groups BI and CI. As the P97/P102 paralogue families are recognized as the primary antigens of *M. hyopneumoniae*, and served as an important adhesin, repeat region 1 is the most important domain of P97 (P97R1) for its adhesion functions (Adams et al. 2005). Thus, here, P97R1, as a main antigen of *M. hyopneumoniae*, was selected to observe the immunohistochemical staining of lung tissues. As expected, obvious brown staining around the luminal surface of bronchial epithelial lining cells appeared in both *M. hyopneumoniae* strain JS-infected groups, in addition, brown antigen of P97R1 staining also appeared around some extruded alveolar spaces, although some non-specific coloration appeared on infiltrating inflammatory cells. In comparison, real-time PCR directed to the detection of bacterial DNA is considered the most sensitive test for samples obtained at necropsy (Maes et al. 2008). Previous reports demonstrated that the BALF was the best site to sample for detection of *M. hyopneumoniae* (Kurth et al. 2002; Vranckx et al. 2012), which was also confirmed by the DNA copies calculated by quantitative PCR in the alveolar lavage of each pig both in BI and CI group in this experiment, with DNA copies in the two JS-infected groups reaching more than 10^8 . In addition, *M. hyopneumoniae* could be re-isolated from BALF of infected pigs in both the BI and CI group to confirm the replication of *M. hyopneumoniae* in the respiratory tract of Bama miniature pigs as well as conventional pigs.

Conclusions

Compared with conventional pigs, for the first time, we established an alternative infection model of *M. hyopneumoniae* using SF-pCD Bama miniature pigs. Through clinical symptom observation, serology, aetiology, lung gross lesion, histopathology, and quantitative PCR evaluation

for *M. hyopneumoniae*, together with assessment of cilia shedding and mucus secretion in bronchi, the results of which demonstrated that Bama miniature pigs with better genetic stability and smaller body size could be used as an alternative and a more maneuverable experimental challenge model of *M. hyopneumoniae*, which displayed typical clinical and pathological features consistent with those of *M. hyopneumoniae* infections observed in conventional pigs.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s43014-020-00034-w>.

Additional file 1: Table S1. Serum antibody test information of porcine respiratory pathogens from piglets used in this study.

Additional file 2: Table S2. Gross lung lesions scoring of four group piglets using 28 point evaluation method.

Abbreviations

dpi: Day(s) post infection; BI: Bama miniature pigs of the infected group; CI: Conventional pigs of the infected group; BC: Bama miniature pigs of the control group; CC: Conventional pigs of the control group

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Authors' contributions

YG, XX carried out most of the experiments described in the manuscript and wrote the article; LZ, QYX and GQS participated in the animal management, lung gross lesion evaluation and histopathological analysis; ZXF conceived the study and contributed in its design and coordination. The authors read and approved the final manuscript.

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Availability of data and materials

All the data we used and analyzed during the current study are demonstrated in the manuscript and all datasets on which the conclusions of the manuscript rely are included in the supplementary tables of the manuscript.

Ethics approval and consent to participate

This study did not involve human participants. Veterinarians took the samples for analysis purposes and/or to check the health or infected status from Bama miniature pig and conventional pig populations. Before conducting the study, approval for conducting the animal experiments was obtained from the Ethics Committee for Animal Experiments of Jiangsu Academy of Agricultural Sciences, China, with the approval number SYXK (Su) 2015–0019.

Competing interests

The authors declare that they have no competing interests.

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