The Veterinary Journal 199 (2014) 268–274

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Protective efficacy of a live attenuated *Mycoplasma hyopneumoniae* vaccine with an ISCOM-matrix adjuvant in pigs

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ARTICLE INFO

Article history:
Accepted 1 November 2013

Keywords:
Pig
Adjuvant
Intramuscular vaccine
Mycoplasma hyopneumoniae

ABSTRACT

An attenuated *Mycoplasma hyopneumoniae* vaccine that requires intrathoracic administration is commercially available for use against mycoplasmal pneumonia in China. Given the limitations of such a route of administration, this study was undertaken to assess the capacity of an ISCOM-matrix adjuvant to enhance immunogenicity following intramuscular use. Immune responses in pigs following vaccination and subsequent intra-tracheal bacterial inoculation were examined using lymphocyte proliferation, serology and mucosal IgA in both nasal and saliva swabs.

Vaccination induced clear lymphocyte proliferation, but only slight serum antibody responses although these were significantly increased following experimental infection. Mucosal IgA was not detected in either nasal or salivary secretions. Following bacterial challenge, animals vaccinated with the adjuvant-containing live vaccine exhibited less severe pulmonary lesions (median score 3.67) than unvaccinated pigs (median score 13.58). The degree of ciliary loss on the respiratory tract surface was reduced in vaccinated pigs compared with experimentally infected controls. The findings indicated that the adjuvant vaccine administered IM provided protection against experimentally induced mycoplasmal pneumonia and could have commercial potential.

Introduction

Mycoplasma hyopneumoniae plays an important role in the development of porcine respiratory disease complex (PRDC) (Maes et al., 2008; Straw et al., 2008; Sibila et al., 2009), causing considerable economic losses through reduced growth rates and feed conversion efficiency, and in increasing the susceptibility of pigs to infection by other organisms (Straw et al., 2008). Infection with *M. hyopneumoniae* strain 168 results in mycoplasmal pneumonia of swine (MPS) (Liu et al., 2011), but an attenuated strain of this pathogen is available in China as a commercially available vaccine (Feng et al., 2010b). However, the vaccine must be given by the intrathoracic route in order to achieve optimal protection and this greatly limits its current use (Feng et al., 2010b).

The development of an intramuscular (IM) preparation is a desirable objective, although previous work has indicated that the immune response induced following delivery via this route gave insufficient protection against subsequent experimental infection. We therefore investigated the use of the ISCOM-matrix, a particulate adjuvant consisting of Quil A, phospholipid and cholesterol, to assess whether this might help to enhance protection.

Unlike an immune stimulatory complex (ISCOM), the ISCOM-matrix contains no antigen (Sun et al., 2009; Lovgren et al., 2011), and combines the advantages of a particulate carrier system with the additional adjuvant effect of Quil A (Heldens et al., 2010; Schiott et al., 2011; Paillot and Prowse, 2012). This formulation avoids the use of the detergent Mega-10 in the subsequent preparation of the vaccine. The objectives of the present study were to assess the potential toxicity of such an adjuvant formulation for *M. hyopneumoniae*, and to examine whether it was efficacious when used as part of an attenuated live vaccine to protect pigs against infection with the organism.

Materials and methods

Preparation of ISCOM-matrix

A lipid solution was generated by dissolving 5 mg of phosphatidylcholine (Amresco) and 5 mg of cholesterol (Sigma) in 0.5 mL 20% Mega-10 (Sigma) by gentle heating at 50 °C for 1 h, and then at 37 °C overnight. A 2% Quil A (Accurate) solution was prepared by dissolving 10 mg of Quil A in 0.5 mL of phosphate buffered saline (PBS). A 0.2 mL lipid solution was then diluted with 1.3 mL PBS and mixed with 0.5 mL of 2% Quil A. The mixture was treated by ultra-sonification in an ice bath and then incubated at room temperature for 2 h. After dialysis with five changes of PBS (for 48–60 h in total), the solution was filtered through a 0.22 μm sterile filter and scanned using transmission electron microscopy (EM) (H-7650, Hitachi).

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http://dx.doi.org/10.1016/j.tvjl.2013.11.001

Assessment of toxicity of ISCOM-matrix for Mycoplasma hyopneumoniae

A series of ISCOM-matrix solutions were incubated with *M. hyopneumoniae* cultures (1:1, v/v) at room temperature, with final concentrations of 0, 0.025, 0.25, or 2.5 mg/mL of ISCOM-matrix (denoted by the concentration of Quil A), respectively. An identical volume of PBS was used as control for the ISCOM-matrix. The samples (100 μL) were removed after 5 min, 0.5 h or 2 h, respectively, to detect the colour changing units (CCU). The sample solution was added into 900 μL of medium, and serial ten-fold dilutions were made until a dilution of 10^{-10} was achieved. The cultures were incubated at 37 °C for 14 days. The highest dilution at which colour change was detected was recorded in CCU/mL. The experiment was repeated three times.

Lymphocyte proliferation response

Peripheral blood mononuclear cells (PBMC) from pigs were isolated and dispensed into a flat-bottomed 96-well microtitration plate (3×10^5 cells/well). For one assay, 5 μL of different concentrations of ISCOM-matrix solution (diluted in PBS) were added into each well, with five repeats for each concentration. Concanavalin A (Con A, 1.25 μg/mL, Sigma) was used as a positive control. In a second assay, 1.25 μg/mL of Con A was used as the basic stimulant, and 5 μL of different concentrations of the ISCOM-matrix solutions were added into each well. After 72 h incubation, lymphocyte proliferation was detected using a methyl thiazolyl tetrazolium (MTT) assay. The magnitude of the proliferative response was expressed as the stimulation index (SI), which is the mean absorption of cells stimulated by the ISCOM-matrix (with or without Con A) divided by the mean absorption of cells stimulated by PBS or Con A alone, respectively.

Preparation of adjuvanted live vaccine

The prepared ISCOM-matrix was diluted 10 times with sterile PBS to a final concentration of 0.05% Quil A, 0.01% phosphatidylcholine and 0.01% cholesterol. During the first immunisation, the adjuvant was mixed with the live vaccine solution (5×10^6 CCU/mL) at a ratio of 1:1 (v/v), and each pig received 2 mL of this vaccine (5×10^6 CCU). The adjuvant volume was doubled for the second vaccination, in line with the weight increase of the pigs in the intervening period. Each animal received 3 mL of adjuvant-containing vaccine solution consisting of 1 mL of vaccine (5×10^6 CCU) and 2 mL of diluted ISCOM-matrix adjuvant. An identical volume of distilled PBS was used to prepare the live vaccine formulation where no adjuvant was used.

Animal selection and experimental procedure

All experimental procedures were approved by the Ethical and Animal Welfare Committee of the Jiangsu Academy of Agricultural Sciences (Reference 136).

Forty-five Suzhong pigs (Duroc × Taihu, 23 males, 22 females) were obtained from a *M. hyopneumoniae*-free herd: animals from this herd did not exhibit clinical signs or lesions of pneumonia, and sows were negative for *M. hyopneumoniae* in both repeated serological tests and in the testing of nasal swabs by PCR. Seronegative pigs aged 5–10 days, were randomly divided into five groups: Group A (not vaccinated or infected); Group B (given vaccine without adjuvant and infected); Group C (vaccinated with ISCOM-matrix adjuvant and infected); Group D (vaccinated with commercially-available inactivated vaccine [RespiSure, Pfizer, A058703] and infected), and Group E (infected).

The pigs were vaccinated IM twice at 2 week intervals. Eight weeks after the first vaccination, bacterial infection was carried out by inoculating 5 mL of a stock of frozen lung homogenate from pigs infected with a virulent strain (JS), and supplemented with 10^6 of CCU of live *M. hyopneumoniae* (strain JS) by the intratracheal route. The infected and non-infected groups were housed separately.

Evaluation of vaccine safety

Rectal temperatures were recorded on the day of vaccination and for three consecutive days. Any side-effects were recorded over the experimental period. Following euthanasia, muscle from the injection sites was collected for histopathological examination.

Serology, mucosal antibody and lymphocyte proliferation responses

Serum samples were tested for antibodies to *M. hyopneumoniae* using a commercially available ELISA (HerdChek *Mycoplasma hyopneumoniae*, IDEXX Laboratories). On days 0, 3, 7, 14, 28, 42 and 56, nasal and saliva swabs were placed in 0.5 mL of PBS and stored overnight at 4 °C. These samples were then centrifuged at 10,000 *g* for 5 min. Secretory IgA was measured as previously described (Feng et al., 2010a).

Suspensions of PBMC were added to microtitration plate wells and stimulated with *M. hyopneumoniae* antigen at a final concentration of 10 μg/mL for 72 h (five repeated wells/sample). Cell proliferation was detected using an MTT assay, with Con A (at 1.25 μg/mL) as a positive control. The proliferative response was expressed as the stimulation index (SI), in this case the mean absorption of cells cultured with antigen divided by the mean absorption of cells cultured with medium alone.

Necropsy

All pigs were euthanased 28 days after experimental infection, and the proportion of the lungs of each animal that was pneumonic was estimated without prior knowledge of treatment group (Madec and Kobisch, 1982); these pneumonia scores ranged from 0 (no lesions) to 28 (entire lung infected). Vaccine efficacy was calculated as the extent of pneumonia score reduction, using group median lung scores as follows:

$$\text{Vaccine efficacy} = [(\text{challenged control group median score} - \text{vaccinated group median score})/(\text{challenged control group median score} - \text{negative control group median score})] \times 100.$$

Effective protection was defined as ⩾60% decrease in lesion score. Samples containing bronchi were taken from the apical lobe of each lung, and the ultrastructure of the bronchial surfaces was examined by scanning EM (S-3000N, Hitachi). To facilitate objective evaluation, micrographs were randomly taken from the bronchial lining in all groups of pigs. Samples of lung lesions from all pigs were collected for bacteriological and histopathological examination. Where no lesions were present, samples were taken routinely from the apical lobes of the right lung. A nested PCR specific for the P36 (L-lactate dehydrogenase) gene was used to detect *M. hyopneumoniae* in lung tissue (Lu et al., 2010).

Statistical analysis

Data were expressed as means ± SD, and repeated measures of ANOVA with time as the repeating factor, was used to determine if vaccinated animals exhibited significant lymphocyte proliferation and antibody responses. All other statistical analyses including difference in lung lesion scores between the groups were carried out using one-way ANOVA, and a *P*-value <0.05 was considered statistically significant.

Results

Potential toxicity of ISCOM-matrix for live vaccine

On examination by transmission EM, the ISCOM-matrix had a typical 'cage-like' structure, each measuring approximately 20–30 nm in diameter (Fig. 1A and B). A small proportion of helical structures was also observed (Fig. 1B). The matrix maintained good stability after storage for 3 months at 4 °C (data not shown). After 5 min, 0.5 h or 2 h incubation with 0, 0.025, 0.25, or 2.5 mg/mL ISCOM-matrix, respectively, there was no reduction in the CCU of the live vaccine, regardless of concentration (Fig. 1C), indicating no toxic effect.

In vitro stimulation of PBMCs

Prior to the animal experiment, the ability of the ISCOM-matrix to activate lymphoid cells in vitro was determined. Different concentrations of the ISCOM-matrix were added to a 96-well microtitration plate containing isolated swine PBMCs. After 72 h incubation, proliferation was measured using the MTT assay. The results indicated that the ISCOM-matrix could stimulate PBMC, both on its own and together with con A (Table 1). This finding indicated the capacity of the adjuvant to activate cellular immunity, which is considered to be an important prerequisite in protecting pigs against *M. hyopneumoniae* infection.

Evaluation of vaccine safety

No differences were observed in body temperature or other clinical parameters following treatment with the ISCOM-matrix and/or vaccine, and no significant changes were detected at the injection sites on histopathological examination.

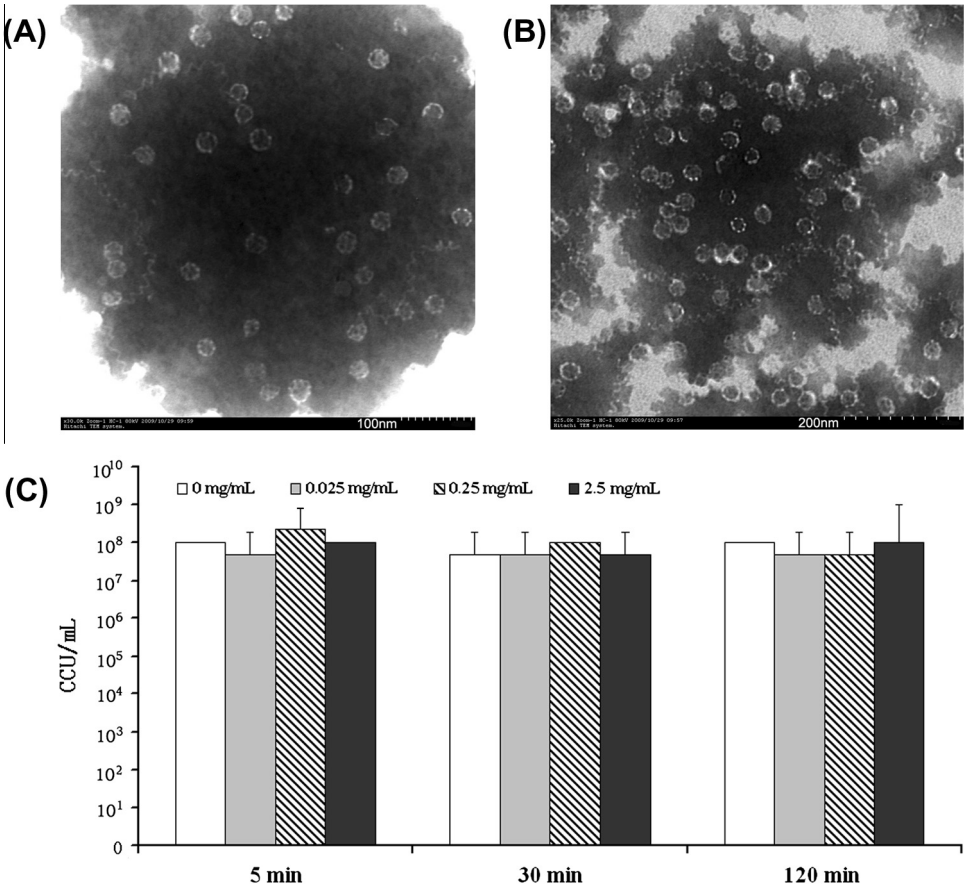

Fig. 1. Preparation of the ISCOM-matrix and assessment of its potential toxicity for *Mycoplasma hyopneumoniae*. Particles of the negatively-stained ISCOM-matrix imaged using transmission electron microscopy illustrated (A and B). The prepared ISCOM-matrix exhibited no obvious toxicity at concentrations of 0, 0.025, 0.25 and 2.5 mg/mL (denoted by concentration of Quil A in the matrix) (C). CCU, colour changing units.

Table 1
Capacity of the ISCOM-matrix to activate lymphoid cells in vitro determined using different concentrations of adjuvant in a 96-well microtitration plate containing porcine peripheral blood mononuclear cells (PBMCs). After 72 h incubation, proliferation of the cells by the ISCOM-matrix adjuvant both with and without Concanavalin (Con) A was measured using a methyl thiazolyl tetrazolium assay.

Concentration (ng/mL)	0	0.01	0.1	1	10	100	1000
Without Con A							
SI	1.00 ± 0.17	1.61 ± 0.12**	1.72 ± 0.25**	2.54 ± 0.21**	2.46 ± 0.47**	2.64 ± 0.47**	2.21 ± 0.32**
With Con A							
SI	1.00 ± 0.24	1.27 ± 0.16	1.82 ± 0.21**	2.54 ± 0.34**	2.74 ± 0.39**	2.62 ± 0.75**	1.78 ± 0.24**

** $P < 0.01$ compared with negative control.

Serum IgG and mucosal IgA responses

Large amounts of specific serum antibody were induced following treatment with the commercial inactivated vaccines ($P < 0.01$, compared with negative controls; Fig. 2). However, the live vaccine without adjuvant did not significantly stimulate humoral immunity ($P > 0.05$, compared with negative controls). When used in conjunction with the ISCOM-matrix adjuvant, administration of the live vaccine resulted in a slight increase in serum antibody prior to challenge, and after experimental challenge, titres were significantly increased ($P < 0.01$, compared with negative controls). No responses were detected in nasal or saliva samples from any group prior to experimental infection indicating that IM vaccination with both live and inactivated formulations failed to induce local mucosal immunity.

Cellular immune response

The animals that were not vaccinated (i.e. the negative controls and 'infected' control groups) did not demonstrate lymphocyte proliferation responses following 72 h of stimulation (Fig. 3). Significant increases in the SI were detected for PBMCs from the adjuvant-containing live vaccine group ($P < 0.01$, compared with negative controls), and for PBMCs from pigs given the inactivated vaccine ($P < 0.01$, compared with negative controls). Administration of the live vaccine without adjuvant resulted in increased cell proliferation on day 21, but not on day 42 ($P < 0.05$, compared with negative controls). Differences in this response between animals receiving the live vaccine with and without accompanying adjuvant were statistically significant ($P < 0.01$).

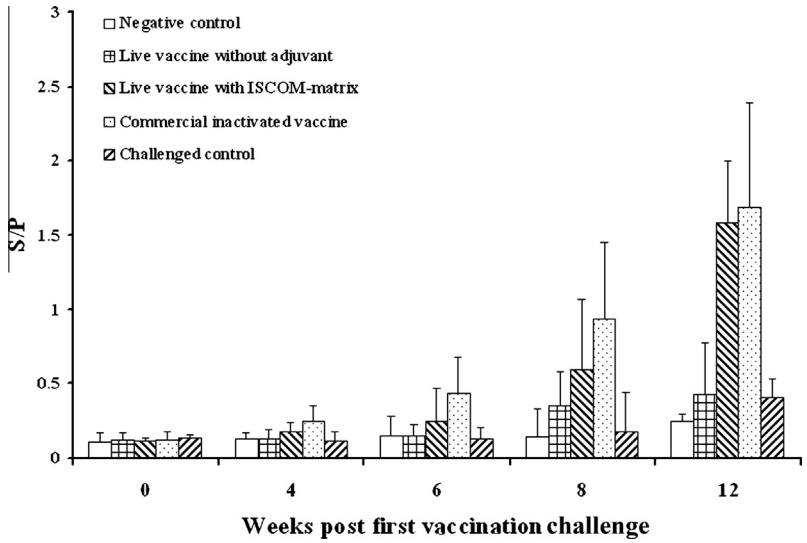

Fig. 2. Serum IgG concentrations against *Mycoplasma hyopneumoniae* in pigs following vaccination as measured using a commercial ELISA. S/P = (sample OD – negative control OD)/(positive control OD – negative control OD). Sample: positive control ratios ⩾0.4 considered positive.

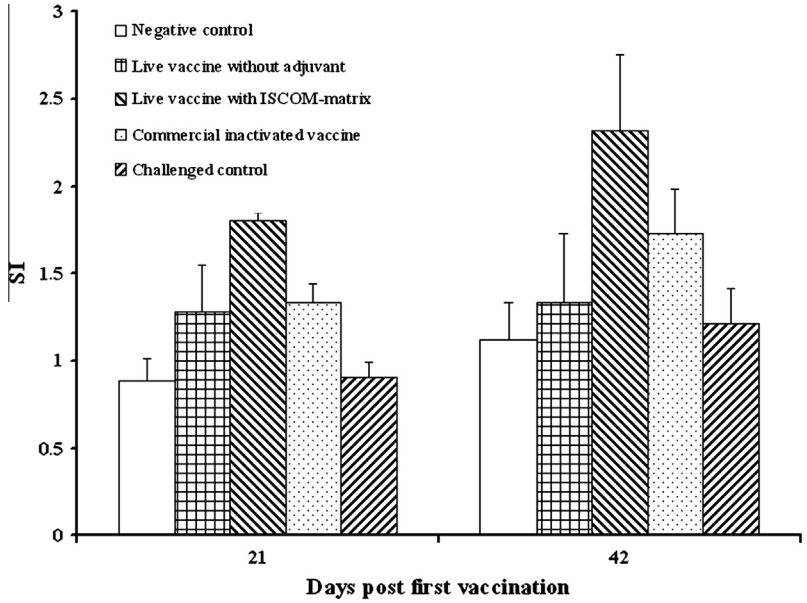

Fig. 3. Lymphocyte proliferation responses to *Mycoplasma hyopneumoniae* antigen. Purified lymphocytes from five groups of animals were stimulated in vitro with 10 μg/mL of *M. hyopneumoniae* antigen for 72 h. The stimulation index (SI) was detected using a methyl thiazolyl tetrazolium assay.

Protective efficacy of vaccine

Post-mortem examination was carried out on all pigs 28 days following experimental infection, and histopathological examination was carried out on lung samples. Lesions typical of mycoplasmal infection were observed, namely, hyperplasia of bronchus-associated lymphoid tissue along with infiltration of lymphocytes and macrophages. The majority of experimentally inoculated pigs were confirmed as infected with *M. hyopneumoniae* on PCR except for one animal from the 'adjuvant-free' live vaccine group, two from the 'adjuvant-containing' live vaccine group, and two given the commercial inactivated vaccine (Table 2). Whereas pigs from the challenged control group exhibited severe pneumonic lesions (median score 13.58), those from the live vaccine/ISCOM-matrix adjuvant group had significantly less pulmonary pathology (median score 3.67; $P < 0.01$).

The pigs given the commercial inactivated vaccine also had less severe pneumonia (median score 4.83; $P < 0.01$). Although the pigs given the live vaccine without adjuvant also had a significantly reduced mean lesion score (median score 7.39; $P < 0.05$), the protection afforded was not considered sufficient to justify its use in the field. The lesions scores in these animals were not significantly different from those in the other vaccinated groups ($P > 0.05$ compared with the adjuvant-containing live and inactivated vaccine groups). However, this score was still significantly greater than that of the negative control group ($P < 0.05$), whereas the scores of pigs immunised with the adjuvant-containing live vaccine was not significantly different from that of negative control group ($P > 0.05$).

Table 2
Protective efficacy of a live attenuated *Mycoplasma hyopneumoniae* vaccine administered with an ISCOM-matrix adjuvant assessed by measuring lung lesion scores and PCR detection in experimentally-challenged pigs.

Treatment group	Negative control ($n = 9$)	Live vaccine without adjuvant ($n = 9$)	Live vaccine with ISCOM-matrix ($n = 9$)	Commercial inactivated vaccine ($n = 9$)	Challenged control ($n = 9$)
Lung lesion score	0.02 ± 0.04**	7.39 ± 7.57*	3.67 ± 4.97**	4.83 ± 6.88**	13.58 ± 6.45
Number of pigs positive by PCR	1	8	7	7	9
Vaccine efficacy	–	45.72%	73.08%	64.53%	–
Number of pigs protected	–	4	7	6	–

* $P < 0.05$ compared with challenged control group.
** $P < 0.01$ compared with challenged control group.

The efficacies of the three vaccines were 45.72% (live vaccine without adjuvant), 73.08% (live vaccine with ISCOM-matrix adjuvant), and 64.53% (commercial inactivated vaccine). The number of pigs we considered to be protected were four in the live vaccine without adjuvant group ($n = 9$), seven in the live vaccine with ISCOM-matrix adjuvant group ($n = 9$), and six in the commercial inactivated vaccine group ($n = 9$). One pig from the negative control group was positive for *M. hyopneumoniae* by PCR, but no histopathological evidence of disease was observed in any of the pigs from this group (Table 2).

Impairment of ciliary function

Scanning EM of the bronchial cilia revealed that pigs given only the experimental infection exhibited the greatest impairment of ciliary function. Cilia were decreased in number and had sloughed (Fig. 4E). Notably, only a slight degree of ciliary loss was observed after challenge in the groups given the adjuvant-containing live and commercial inactivated, vaccines, respectively (Fig. 4C and D). Some of the pigs immunised with the live vaccine without the adjuvant exhibited obvious ciliary impairment (Fig. 4B). No abnormalities were observed in the negative control group (Fig. 4A), and no differences were observed ultrastructurally between pigs from the adjuvant-containing live (Fig. 4C), and commercial inactivated (Fig. 4D) vaccine groups.

Discussion

Given the potential health and welfare implications of administering an attenuated *Mycoplasma hyopneumoniae* vaccine by the

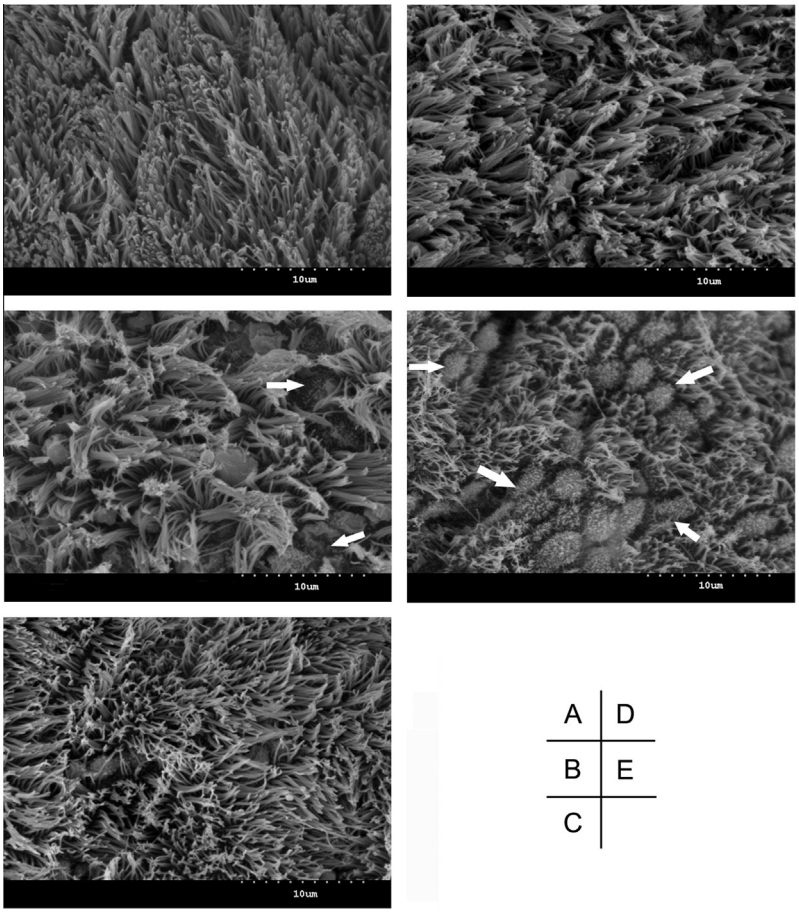

Fig. 4. Scanning electron micrographs illustrating bronchial surfaces 28 days after experimental challenge of pigs with *Mycoplasma hyopneumoniae*: (A) negative control; (B) live vaccine without adjuvant; (C) live vaccine with ISCOM-matrix adjuvant; (D) commercial inactivated vaccine; and (E) challenged control. Loss of cilia indicated by arrows.

intrathoracic route, the focus of this study was to determine if a more appropriate (but equally efficacious) route of administration could be identified. Following previous assessment of various routes (including intranasal, aerosol and intramuscular), IM vaccination was selected as a potential alternative given the advantages of less risk of injury to the animals and greater ease of application. However, it was anticipated that the vaccine given by this route would require an accompanying adjuvant in order to generate a sufficiently protective immune response.

As a systemic cellular immune response is considered critical in preventing *M. hyopneumoniae* infection (Thacker et al., 2000; Maes et al., 2008), we selected adjuvant components accordingly. The present study demonstrates that when given IM, an ISCOM-matrix adjuvant-containing live vaccine can induce a cellular, and to a lesser extent, humoral immune response. Previous reports support the findings of our study that serum antibody concentrations do not correlate well with protection against *M. hyopneumoniae* (Djordjevic et al., 1997; Thacker et al., 1998; Shimoji et al., 2003; Maes et al., 2008). In a previous unpublished experiment where a live vaccine using a chitosan adjuvant was assessed, high levels of serum IgG along with a poor cellular immune response was induced, and minimal protection of pigs against pathogen challenge was achieved.

ISCOM and ISCOM-matrix systems are flexible systems comprising different phospholipid and saponin components (Sanders et al., 2005). In forming ISCOM or ISCOM-matrix nanoparticles, the adjuvant activity of Quil A is maintained, while its potential haemolytic activity/toxicity is reduced. The pre-formed ISCOM-matrix, which can be used directly as an adjuvant, is just as effective at stimulating immune responses as ISCOM (Pearse and Drane, 2005). Its ease of application has led to the use of ISCOM-matrix as a commercial adjuvant (Sun et al., 2009; Ahlberg et al., 2012; Paillot and Prowse, 2012), and it can be prepared by dialysis (Garcia et al., 2008; Schiott et al., 2011), centrifugation (Homhuan et al., 2004), lipid-film hydration (Liang et al., 2008; Pandey et al., 2010), or by using ethanol (Lendemans et al., 2005) or ether (Pham et al., 2006) injection. Of these, the dialysis method is most commonly used due to its ability to produce more homogenous formulations with a narrow particle size distribution (in our study a diameter of approximately 25 nm).

Evidence of mucosal IgA production was previously only detected in bronchoalveolar lavage fluid (BALF) after challenge (Djordjevic et al., 1997; Thacker et al., 2000). In our experiment significant IgA was not induced in the nasal mucus or saliva of any of the vaccine formulations assessed. Lin et al. (2003) found that an oral microencapsulated *M. hyopneumoniae* vaccine induced IgA in serum, faeces, nasal mucus and saliva. The ability to stimulate local mucosal immunity appears to be influenced by inoculation route. The IM route was less effective in inducing mucosal immunity against *M. hyopneumoniae* relative to direct mucosal immunisation.

The mechanisms underlying the protection induced by live *M. hyopneumoniae* vaccine remain to be elucidated. Calus et al. (2009) reported that seven *M. hyopneumoniae* field isolates of varying virulence did not differ in their ability to adhere to cilia, suggesting that adherence is not the only factor responsible for virulence. We speculate that low virulence strains of *M. hyopneumoniae* adhere to cilia without causing damage but induce a protective immune response.

During the present experiment, fluorescent dye-labelled live *M. hyopneumoniae* vaccine was found to persist in the bloodstream for more than 1 week, and when given at a high dose (8×10^6 CCU), could be detected in BALF (unpublished data). It is possible that attenuated strains such as 168 adhere to bronchial cilia thus blocking the attachment of organisms inoculated subsequently. However, further experiments will be required to elucidate the mechanisms underlying the protective effect of this vaccine.

Conclusions

When administered IM with an ISCOM-matrix adjuvant, an attenuated *Mycoplasma hyopneumoniae* vaccine reduced pulmonary lesion scores and ciliary loss in experimentally infected pigs. Our findings suggest that such a vaccine formulation and route of administration is efficacious and could have potential application in the field.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

This work was supported by grants from the Special Fund for Independent Innovation of Agricultural Science and Technology in Jiangsu Province of China (Grant No. cx [12] 5047) and State Key Laboratory of Veterinary Biotechnology (Grant Number SKLVBF201305).

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