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Effect of different adjuvant formulations on the immunogenicity and protective effect of a live *Mycoplasma hyopneumoniae* vaccine after intramuscular inoculation

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ABSTRACT

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) vaccine strain 168 is an intrapulmonically injected attenuated live vaccine that is available in the Chinese market. The aim of this study was to develop suitable adjuvants for this live vaccine to provide effective protection after intramuscular inoculation. Several adjuvant components were screened to assess their toxicity for the live vaccine, and various adjuvant formulations were then designed and prepared. Vaccines supplemented with these adjuvants were used to immunize mice intramuscularly to assess the capacity of the adjuvants to induce a specific immune response. The screened formulations were then evaluated in pigs. Seven of the eight adjuvant components did not affect the viability of the live vaccine, and seven different adjuvant formulations were then designed. In mice, the ISCOM-matrix adjuvant and the levamisole–chitosan mixture adjuvant significantly enhanced serum IgG responses against *M. hyopneumoniae*, while lymphocyte proliferation was enhanced by the ISCOM-matrix adjuvant, the carbomer–astragalus polysaccharide mixture adjuvant and an oil-in-water emulsion adjuvant. These four adjuvants were evaluated in pigs. Enhancement of specific lymphocyte proliferation responses was observed in the groups vaccinated with the ISCOM-matrix adjuvant and the carbomer–astragalus polysaccharide mixture adjuvant. Significant enhancement of serum IgG antibody production was observed before challenge in pigs vaccinated with the carbomer–astragalus polysaccharide mixture adjuvant and the levamisole–chitosan mixture adjuvant, while after challenge, all of the animals that received vaccines containing adjuvants had higher antibody concentrations against *M. hyopneumoniae* than unvaccinated animals. Animals inoculated with a vaccine containing the ISCOM-matrix adjuvant (median score 3.57) or the carbomer–astragalus polysaccharide mixture adjuvant (median score 5.28) had reduced lesion scores compared to unvaccinated animals (median score 14.81). These studies will help in the development of appropriate adjuvants for intramuscular administration of this live *M. hyopneumoniae* vaccine.

1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) causes enzootic pneumonia in pigs and plays an important role in the porcine respiratory disease complex (PRDC) [1–3]. The organism adheres to and damages the ciliated epithelium of the respiratory tract. It causes considerable economic losses through retarded growth, poor feed conversion efficiency, and increased susceptibility to infection with other organisms [1]. *M. hyopneumoniae* strain 168 was isolated in 1974 from an Er-hua-lian pig (a local Chinese breed) that had typical mycoplasmal pneumonia of swine (MPS) [4]. The field strain was attenuated by continual passage through KM2 cell-free liquid medium (a modified Friis medium) *in vitro* [5]. After 300 passages, a stable attenuated strain, named 168, with the patent certificate (ZL 99 1 14276.4, the People's Republic of China) was obtained. The strain induced good immunological protection against MPS (approximately 80–85%) and had a good safety profile. The attenuated strain 168 was subsequently developed into a live

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vaccine (Freeze-dried, KM2 medium, Tianbang Bio-Industry Co., Ltd, Nanjing, China) against MPS and made commercially available in the Chinese market [6].

However, the current live vaccine requires intrapulmonic inoculation to obtain optimal protection [6], which greatly limits its wide application. The development of a more convenient route of administration is thus an important aim for further studies. Intramuscular (i.m.) injection is the most common route of administration of vaccines in pigs. However, our previous studies indicated that the immune response induced by the strain 168 vaccine after i.m. inoculation was not sufficient to provide satisfactory protection against challenge. Therefore, in the present study, different adjuvants were assessed for their capacity to enhance the immunogenicity and protective efficacy of the strain 168 vaccine after i.m. inoculation.

Adjuvants were initially evaluated for their toxicity for the live vaccine and then as suitable adjuvants for it, initially in mice. Thereafter, the capacity of the optimized adjuvant formulations to enhance the immune response to the vaccine and to enhance immunological protection was assessed in experimentally challenged pigs.

2. Materials and methods

2.1. Adjuvant components

Levamisole (purity >99%), saponin (sapogenin content, 20–35%), chitosan (purity >93%), cholesterol (purity >99%) and squalene (purity >98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Carbomer 934P was obtained from Lubrizol (Wickliffee, OH, USA). Quil A was obtained from Accurate Chemical & Scientific (Westbury, NY, USA). Phosphatidylcholine (purity >98%) was obtained from Amresco (Solon, OH, USA). Astragalus polysaccharide (purity >70%) was obtained from Shanxi Jintai Biological Engineering (Shanxi, China). Two commercial adjuvants, Montanide ISA 15A and Montanide GEL 01, were kindly provided by Seppic (Shanghai) Chemical Specialities Co., Ltd (Shanghai, China). Polysorbate 80 (Tween 80) and sorbitan trioleate (Span 85) were purchased from Sinopharm Chemical Reagents (Shanghai, China).

2.2. Adjuvant preparation and examination of toxicity for M. hyopneumoniae

Different adjuvant components were examined for their toxicity for the live vaccine.

Levamisole, saponin and astragalus polysaccharide were dissolved in sterile PBS at a concentration of 100 mg/ml, 5 mg/ml and 300 mg/ml, respectively, and filter-sterilized by 0.22 μm filter (Millipore AS, Oslo, Norway). ISCOM-matrix, containing 0.5% Quil A, 0.1% cholesterol and 0.1% phosphatidylcholine, was prepared by dialysis using MEGA 10 as a surfactant as described previously [7–10]. Briefly, a solution of 5 mg of cholesterol and 5 mg of phosphatidylcholine dissolved in 0.5 ml of 20% MEGA 10 was added to 1.25 ml of 20 mg Quil A/ml. The volume was adjusted to 5 ml with PBS. The mixture was incubated with slow agitation for 1 h and then dialyzed against PBS. The solution was then passed through a 0.22 μm filter. Chitosan was dissolved in sterile 1% acetic acid solution (20 mg/ml), and neutralized with sterile 0.2 M NaOH to pH 7.2. The concentration was adjusted to 12 mg/ml with sterile PBS. Then the solution was filter-sterilized by 0.22 μm filter. Carbomer was added to sterile deionized water at a concentration of 15 mg/ml. After complete swelling, sterile 1 M NaOH was used to neutralize the solution to pH 7.2. The concentration was adjusted to 7 mg/ml with sterile PBS. Then the solution was sterilized by autoclave.

A fresh culture of attenuated strain 168 was prepared in KM2 medium. Serial dilutions of the adjuvant solutions in sterile PBS were made and then mixed with 1 ml of bacterial culture at a ratio of 1:1 (v/v), expect for chitosan, which was added at a ratio of 5:1 (chitosan: bacterial culture). Final concentrations of adjuvants in the mixtures are shown in Table 1. The mixtures of the two commercial adjuvants and bacterial culture were prepared aseptically according to the manufacturer's instructions (GEL 01 2.5%, v/v; ISA 15A 15%, v/v). An identical volume of PBS was used as the control for the adjuvants. After 10 or 30 min of incubation at room temperature, samples (100 μl) were removed for titration. Briefly, each sample was added to 900 μl of KM2 medium, and serial 10-fold dilutions were made with medium until a 10^{-10} dilution was obtained. The cultures were incubated at 37 °C for 14 days, and the highest dilution at which a color change detected was regarded as the endpoint and the titre determined in color changing units (CCU)/ml. The experiment was repeated three times.

Different formulations were then designed based on this toxicity trial, and the toxicity of these formulations was further evaluated. Aqueous adjuvant A was prepared by diluting the previously prepared ISCOM-matrix solution (0.5% Quil A, 0.1% cholesterol and 0.1% phosphatidylcholine) in sterile PBS. Aqueous adjuvants B–D were prepared aseptically by mixing the previously prepared solutions of the specific adjuvant components (100 mg/ml levamisole, 300 mg/ml astragalus polysaccharide, 12 mg/ml chitosan and 7 mg/ml carbomer) together at room temperature, without any particular order. Sterile PBS was added to adjust the concentration, and the solutions were thoroughly mixed with a shaker. Oil-in-water adjuvants E and F were prepared aseptically using the process and composition published for MF59 [11,12] with or without an addition of 7.5 mg levamisole/ml. The commercial adjuvant GEL 01 (2.5%, v/v) was chosen as adjuvant G. Details about the final concentrations of components in every adjuvant are listed in Table 2. All of the adjutants were stored at 4 °C for less than two months before use. Toxicity was assessed by directly dissolving the freeze-dried live vaccine (40-dose vials, 1×10^7 CCU/dose, Tianbang Bio-Industry Co., Ltd, Nanjing, China) in 4 ml of adjuvant. An identical volume of PBS was used as the control for the adjuvants.

2.3. Immunization in mice

Male BALB/c mice obtained from the Comparative Medicine Center of Yangzhou University (Yangzhou, China) were divided into 9 groups of 11 mice each. The animals in the negative control group received no immunization, while the animals in the other groups were immunized with the live vaccine with or without adjuvant. The vaccine was prepared by dissolving the freeze-dried vaccine with adjuvant or PBS at room temperature; the final formulation contained 10^7 CCU/ml of live strain 168 and was administered within 10 min. All of the mice received 2 i.m. immunizations with 100 μl of vaccine using sterile 26 gauge needles 2 weeks apart. Three of the 11 mice were killed at 7 days after the second immunization, and their lymphocyte proliferative responses were evaluated. The remaining eight mice were kept for determination of serum IgG responses.

2.4. Detection of IgG antibodies in mouse serum

Cultured *M. hyopneumoniae* strain 168 cells were collected by centrifugation at 10,000 × *g* for 15 min, washed three times and resuspended in saline. After lysis by ultrasonication in saline for 20 min (3 s for each time with 5 s interval) at 20 kHz using an JY92-IIDN ultrasonic apparatus (NINGBO SCIENTZ Biotechnology Co., Ltd, Ningbo, China), the supernatant was collected and diluted to 10 μg/ml of total protein in coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6) to be used as a coating antigen. Flat bottomed 96-well ELISA plates (Costar 9018, Corning, NY, USA) were coated with antigen (100 μl/well) overnight

Table 1
Toxicities of adjuvant components for the *M. hyopneumoniae* live vaccine.

Adjuvant	Final concentration	Titer (CCU/ml)		Adjuvant	Final concentration	Titer (CCU/ml)	
		10 min	30 min			10 min	30 min
PBS		$10^{8.33\pm0.58}$	$10^{8.00\pm0.00}$	PBS		$10^{7.00\pm0.00}$	$10^{6.67\pm0.58}$
Levamisole	0.25 mg/ml	$10^{8.00\pm1.00}$	$10^{8.33\pm0.58}$	Chitosan	0.5 mg/ml	$10^{7.33\pm0.58}$	$10^{7.33\pm0.58}$
	2.5 mg/ml	$10^{8.00\pm0.00}$	$10^{7.67\pm0.58}$		5 mg/ml	$10^{6.67\pm0.58}$	$10^{6.67\pm0.58}$
	25 mg/ml	$10^{7.67\pm0.58}$	$10^{8.00\pm0.00}$		10 mg/ml	$10^{7.00\pm0.00}$	$10^{7.33\pm0.58}$
PBS		$10^{7.67\pm0.58}$	$10^{7.33\pm0.58}$	PBS		$10^{7.67\pm0.58}$	$10^{8.00\pm0.00}$
Saponin	5 μg/ml	$10^{7.33\pm0.58}$	$10^{6.67\pm0.58}$	Astragalus	5 mg/ml	$10^{8.00\pm0.00}$	$10^{7.67\pm0.58}$
	50 μg/ml	$10^{5.67\pm0.58}$ **	$10^{4.33\pm0.58}$ ***	polysac-	50 mg/ml	$10^{7.67\pm0.58}$	$10^{8.00\pm0.00}$
	500 μg/ml	0 ± 0.00 ***	0 ± 0.00 ***	charide	100 mg/ml	$10^{7.67\pm0.58}$	$10^{7.67\pm0.58}$
PBS		$10^{8.33\pm0.58}$	$10^{8.33\pm0.58}$	PBS		$10^{7.33\pm0.58}$	$10^{7.67\pm0.58}$
ISCOM-	0.025 mg/ml	$10^{8.67\pm0.58}$	$10^{8.67\pm0.58}$	Carbomer	1 mg/ml	$10^{7.67\pm0.58}$	$10^{7.33\pm0.58}$
matrix[a]	0.25 mg/ml	$10^{8.33\pm0.58}$	$10^{8.00\pm0.00}$		2.5 mg/ml	$10^{7.67\pm0.58}$	$10^{7.67\pm0.58}$
	2.5 mg/ml	$10^{8.33\pm0.58}$	$10^{8.67\pm0.58}$		3.5 mg/ml	$10^{8.00\pm0.00}$	$10^{7.67\pm0.58}$
PBS		$10^{9.00\pm0.00}$	$10^{8.67\pm0.58}$	PBS		$10^{8.00\pm0.00}$	$10^{8.33\pm0.58}$
Montanide ISA 15A, 15%		$10^{8.67\pm0.58}$	$10^{7.67\pm0.58}$	Montanide GEL 01, 2.5%		$10^{8.00\pm0.00}$	$10^{8.33\pm0.58}$

[a] The concentration of ISCOM-matrix is indicated by the concentration of Quil A.
** $P<0.01$.
*** $P<0.001$, compared with PBS control.

at 4 °C. After blocking with 3% bovine serum albumin, each well was incubated for 1 h at 37 °C with 100 μl of serum diluted in PBS containing 1% bovine serum albumin at 1:1600 (optimized previously for detecting the differences between groups of different adjuvants), followed by 100 μl horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Boster, Wuhan, China) at a dilution of 1:10,000. After washing, the substrate containing 3,3′,5,5′-tetramethylbenzidine and H_2O_2-urea was added and the plates were incubated at 37 °C for 15 min, then H_2SO_4 was added to stop the reaction, and the absorbance was measured at 450 nm.

2.5. Lymphocyte proliferation assay

Lymphocytes were prepared from the spleen of three mice or the peripheral blood of three pigs from each group. Briefly, spleen was removed from each mouse immediately after killing; erythrocytes were then lysed, and spleen-derived lymphocytes from each mouse were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Lymphocytes were isolated from peripheral blood of pigs with a commercial lymphocytes separation medium according to the manufacturer's instructions (Huajing Biological Hi-tech Co., Ltd, Shanghai, China). Then, 100 μl of lymphocyte culture (3×10^6 cells/ml) was dispensed into each well of a flat-bottomed 96-well microtitration plate (Costar 3599, Corning, NY, USA). The cells were then stimulated with 11.1 μl of *M. hyopneumoniae* antigen (prepared as described above, sterilized by filtration and diluted with sterile PBS to 0.1 mg/ml) at a final concentration of 10 μg/ml [13] for 72 h (37 °C, 5% CO_2, 5 wells per animal). An identical volume of PBS was used as the control for the antigen solution. Lymphocyte responses were determined by adding 20 μl of 5 mg methyl thiazolyl tetrazolium (MTT)/ml to each well. Four hours later the supernatant was discarded. The formazan generated was dissolved in DMSO (100 μl/well), and the OD at 490 nm was measured. The magnitude of the proliferative response was expressed as the stimulation index (SI): the mean absorption of cells cultured with antigen divided by the mean absorption of cells cultured with medium alone.

2.6. Immunization and challenge in pigs

Forty-nine Suzhong pigs (Duroc × Taihu crossbreeds) were obtained from a *M. hyopneumoniae*-free herd. Animals of this herd had no clinical signs of respiratory infection or pneumonic lesions. The sows were negative for *M. hyopneumoniae* infection based on both repeated serological tests and PCR tests of nasal swab samples. Briefly, the serological tests were conducted using a commercial ELISA kit, the HerdChek *M. hyopneumoniae* (IDEXX Laboratories, Westbrook, ME, USA). Nasal swabs were obtained by swabbing the nasal mucosa of both nostrils reaching deeply into the turbinates. Swab specimens were resuspended in 500 μl of sterile saline. DNA

Table 2
Adjuvant formulations used in this study.

Adjuvant	Type	Formulation			
Adjuvant A	Aqueous	Quil A 0.25 mg/ml	Cholesterol 0.05 mg/ml	Phosphatidylcholine 0.05 mg/ml	
Adjuvant B	Aqueous	Levamisole 7.5 mg/ml	Carbomer 5 mg/ml		
Adjuvant C	Aqueous	Carbomer 5 mg/ml	Astragalus polysaccharide 50 mg/ml		
Adjuvant D	Aqueous	Levamisole 7.5 mg/ml	Chitosan 10 mg/ml		
Adjuvant E	Oil-in-water	Squalene 4.3% (v/v)	Tween 80 0.5% (w/v)	Span 85 0.5% (w/v)	
Adjuvant F	Oil-in-water	Squalene 4.3% (v/v)	Tween 80 0.5% (w/v)	Span 85 0.5% (w/v)	Levamisole 7.5 mg/ml
Adjuvant G	Aqueous	Montanide GEL 01 2.5% (v/v)			

Table 3
Toxicity of the formulated adjuvants for the live vaccine.

Incubation time (min)	Titer (CCU/ml)							
	PBS	Adjuvant A	Adjuvant B	Adjuvant C	Adjuvant D	Adjuvant E	Adjuvant F	Adjuvant G
10	$10^{8.00\pm0.00}$	$10^{8.00\pm0.00}$	$10^{8.00\pm0.00}$	$10^{7.67\pm0.58}$	$10^{8.00\pm0.00}$	$10^{7.67\pm0.58}$	$10^{7.67\pm0.58}$	$10^{8.00\pm0.00}$
30	$10^{7.67\pm0.58}$	$10^{8.00\pm0.00}$	$10^{7.67\pm0.58}$	$10^{7.33\pm0.58}$	$10^{8.00\pm0.00}$	$10^{7.33\pm0.58}$	$10^{4.67\pm0.58}$ ***	$10^{7.67\pm0.58}$

*** $P<0.001$, compared with PBS control.

was extracted, and 5 μl DNA samples were used as template for *M. hyopneumoniae* detection by nested PCR, as described previously [14]. Pigs aged 5–10 days that were confirmed to be seronegative were selected. Animals were randomly separated into seven groups (Table 6). Pigs received i.m. immunizations twice into the lateral cervical musculature using sterile 18 gauge needles at 2 week intervals with 2 ml of vaccine solution containing 10^7 CCU/ml of live strain 168 with or without adjuvant. Freeze-dried live vaccine was dissolved in adjuvant or PBS at room temperature and inoculated within 10 min. The antibody response and the lymphocyte proliferation were subsequently evaluated. Eight weeks after the first immunization, the challenge was performed by intratracheal inoculation. Briefly, prior to the challenge, the pigs were sedated with ketamine hydrochloride (15 mg/kg i.m.) The animals were placed in dorsal position and fixed. A needle was inserted through the wall of the trachea. Once the tip of the needle was in the lumen of the trachea, aspiration of air was used to confirm that the needle was in the right position. Then, 5 ml of challenge inoculum was injected into the trachea. The challenge inoculum contained 4 ml of sterile PBS, dissolved frozen lung homogenate from pigs exposed to the virulent strain JS, and 1 ml of live *M. hyopneumoniae* strain JS (10^6 CCU/ml) culture. Pigs from the challenged groups and the unchallenged group were then housed in different rooms until the end of the experiment.

All animal study procedures were approved by the Ethical and Animal Welfare Committee of the Jiangsu Academy of Agricultural Sciences (No. 92).

2.7. Detection of IgG antibodies in swine serum

All serum samples were assayed for antibodies using a commercial ELISA kit: the HerdChek *M. hyopneumoniae* (IDEXX Laboratories, Westbrook, ME, USA).

2.8. Necropsy

All pigs were slaughtered 28 days after challenge. The lungs were removed from the thorax, and the proportion of each lobe affected by pneumonic lesions was estimated visually in a blinded manner using previously reported methods [15]. The values could theoretically range from 0 (no lesions) to 28 (entire lung affected). Vaccine efficacy at reducing lung scores was calculated as follows: vaccine efficacy = [(challenged control group median score – vaccinated group median score)/(challenged control group median score – negative control group median score)] × 100%.

2.9. Statistical analysis

Data are expressed as the mean ± SD. The statistical analysis of adjuvant toxicity was performed using a one-way ANOVA. For the experiments in mice, one-way ANOVA and repeated measures ANOVA were used to analyze lymphocyte proliferation assay data and serum antibody data, respectively. For the experiments in pigs, the data, which were unlikely to be normally distributed, were assessed using the Kruskal–Wallis test with a Dunn's multiple comparison test for selected pairs. A *P*-value <0.05 was considered significant.

3. Results

3.1. Toxicity of adjuvants for the live vaccine

Specific adjuvant components were initially assessed for their toxicities for the live vaccine. Different concentrations were chosen for each adjuvant component based on their typical usage concentrations. The results (Table 1) showed that saponin inactivated the live vaccine at concentrations of 50 μg/ml and above. The other adjuvant components did not have detectable toxicity for *M. hyopneumoniae*.

Six adjuvant formulations (adjuvants A–F) were designed based on the nontoxic adjuvant components. The commercial adjuvant Montanide GEL 01 was chosen as adjuvant G (Table 2). The toxicity of all of the adjuvants for the freeze-dried live vaccine was subsequently assessed (Table 3). None of these seven formulated adjuvants had any detectable toxicity, except for adjuvant F, which caused a decrease in titer after 30 min incubation ($P<0.001$).

3.2. Enhancement of the specific immune response in mice

The ability of adjuvants A–G to enhance the immunogenicity of the live vaccine was evaluated in mice. As shown in Table 4, the adjuvants A, C and E significantly enhanced the antigen-specific cellular immune response ($P<0.05$, compared with the group inoculated with the vaccine without an adjuvant). There were clear differences between adjuvants A and D, adjuvants A and G, adjuvants C and D, and adjuvants D and E ($P<0.05$). Based on the humoral immune responses, all of the animals immunized with adjuvanted vaccines had a distinctly higher level of serum antibody against *M. hyopneumoniae* than the animals immunized with the vaccine alone or with PBS ($P<0.001$). Adjuvants A and D induced greater responses than the others ($P<0.001$, compared with adjuvants B, C, E–G, Table 5). Adjuvant A was more effective at inducing serum antibodies at a very early stage. Both the level and the duration of the response in this group were much greater than those of mice inoculated with vaccines containing the other adjuvants. Adjuvant D was also effective in enhancing

Table 4
Lymphocyte proliferative response against *M. hyopneumoniae* antigen in mice after immunization.

Group	Stimulation index
Negative control	1.26 ± 0.27
Without adjuvant	1.40 ± 0.24
Adjuvant A	2.15 ± 0.69** #
Adjuvant B	1.61 ± 0.22
Adjuvant C	2.02 ± 0.25* #
Adjuvant D	1.42 ± 0.18
Adjuvant E	2.01 ± 0.58* #
Adjuvant F	1.84 ± 0.37
Adjuvant G	1.46 ± 0.22

* $P<0.05$.
** $P<0.01$, compared with the negative control group.
\# $P<0.05$, compared with the group inoculated with live vaccine without adjuvant.

Table 5
Specific serum IgG antibody in mice after immunization.

Group	OD450 nm						
	Week[a] 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 9
Negative control	0.08 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.06 ± 0.01	0.08 ± 0.00	0.07 ± 0.01	0.06 ± 0.01
Without adjuvant	0.25 ± 0.20	0.51 ± 0.28	0.46 ± 0.16	0.46 ± 0.15	0.30 ± 0.10	0.21 ± 0.02	0.14 ± 0.01
Adjuvant A	0.72 ± 0.26	1.15 ± 0.04	1.26 ± 0.11	1.18 ± 0.08	1.39 ± 0.10	1.50 ± 0.15	1.43 ± 0.11
Adjuvant B	0.21 ± 0.08	0.63 ± 0.27	0.71 ± 0.20	0.67 ± 0.20	0.62 ± 0.30	0.78 ± 0.16	0.59 ± 0.26
Adjuvant C	0.29 ± 0.16	0.75 ± 0.29	0.77 ± 0.20	0.72 ± 0.19	0.74 ± 0.21	0.57 ± 0.15	0.45 ± 0.14
Adjuvant D	0.29 ± 0.16	0.95 ± 0.18	1.10 ± 0.17	1.10 ± 0.12	1.21 ± 0.21	1.13 ± 0.16	1.09 ± 0.11
Adjuvant E	0.35 ± 0.10	0.88 ± 0.38	0.93 ± 0.19	0.98 ± 0.16	0.82 ± 0.26	0.65 ± 0.16	0.86 ± 0.20
Adjuvant F	0.38 ± 0.18	0.91 ± 0.14	1.00 ± 0.19	0.99 ± 0.16	0.88 ± 0.25	0.80 ± 0.08	0.75 ± 0.01
Adjuvant G	0.33 ± 0.17	0.90 ± 0.29	0.92 ± 0.37	0.85 ± 0.28	0.76 ± 0.21	0.44 ± 0.06	0.37 ± 0.01

[a] Weeks after the first immunization.

Table 6
Design of vaccine efficacy experiment in pigs.

Group		Vaccine	Adjuvant	Adjuvant type	Adjuvant formulation	Challenge
G1	Negative control	–	–	–	–	–
G2	Live vaccine without adjuvant	Live vaccine	–	–	–	+
G3	Live vaccine with adjuvant A	Live vaccine	Adjuvant A	Aqueous	ISCOM-matrix	+
G4	Live vaccine with adjuvant C	Live vaccine	Adjuvant C	Aqueous	Carbomer + astragalus polysaccharide	+
G5	Live vaccine with adjuvant D	Live vaccine	Adjuvant D	Aqueous	Levamisole + chitosan	+
G6	Live vaccine with adjuvant E	Live vaccine	Adjuvant E	Oil-in-water	Squalene + Tween 80 + Span 85	+
G7	Challenged control	–	–	–	–	+

antibody production, although this was not apparent over the first two weeks. The antibody levels of the animals inoculated with vaccines containing adjuvants E, F or G began to decrease at around 5 to 6 weeks, indicating a relative short duration of the response.

3.3. Immune response detected in pigs

Four adjuvants (adjuvants A, C–E) were selected based on the results of the experiment in mice and further evaluated in pigs. The detailed experimental design is shown in Table 6. Pigs were immunized with the live vaccine resuspended in these adjuvants and their specific immune responses were assessed.

Blood samples were collected to test IgG antibody levels every two weeks after the second immunization (week 2) until challenge (Table 7). The animals immunized with live vaccine without adjuvant had relatively low antibody levels ($P > 0.05$). Animals immunized with vaccine containing adjuvants C or D had significantly higher antibody levels (adjuvant C on week 4 and 6; adjuvant D on week 4, 6 and 8). Animals from the groups administered vaccines containing adjuvants A or E had higher antibody levels at only one time point (adjuvant A: week 8, $P < 0.05$ *vs.* negative control; adjuvant E: week 6, $P < 0.05$ *vs.* negative control). Challenge was performed on week 8 and all animals were tested again just before necropsy on week 12. All of the animals immunized with adjuvanted vaccines had higher antibody levels than the unvaccinated animals or the animals that were immunized with vaccine without adjuvant, except for the animals inoculated with vaccine containing adjuvant E ($P < 0.01$ *vs.* negative control; $P > 0.05$ *vs.* live vaccine without adjuvant).

The lymphocyte proliferative response was used to evaluate the specific cellular immune response (Table 8). Animals inoculated with vaccine containing adjuvants A or C exhibited significant levels of proliferation, while the animals inoculated with vaccine without adjuvant or with vaccine containing adjuvant D or E did not have strong lymphocyte proliferative responses.

Table 7
Specific serum IgG antibody in pigs after immunization.

Group	Sample-to-positive ratio[a]			
	Week[b] 4	Week 6	Week 8[c]	Week 12
Negative control	0.19 ± 0.08	0.13 ± 0.13	0.19 ± 0.16	0.23 ± 0.07
Without adjuvant	0.15 ± 0.06	0.16 ± 0.07	0.36 ± 0.17	0.74 ± 0.60
Adjuvant A	0.18 ± 0.06	0.25 ± 0.22	0.69 ± 0.49*	1.63 ± 0.42*** $^{\#}$
Adjuvant C	0.33 ± 0.17$^{\#}$	0.49 ± 0.41*** $^{\#}$	0.39 ± 0.24	1.73 ± 0.49*** $^{\#}$
Adjuvant D	0.49 ± 0.25* $^{\#\#}$	0.53 ± 0.48** $^{\#}$	1.01 ± 0.59**	2.14 ± 0.57*** $^{\#\#\#}$
Adjuvant E	0.28 ± 0.20	0.46 ± 0.46*	0.43 ± 0.32	1.42 ± 0.40**
Challenged control	0.14 ± 0.08	0.11 ± 0.08	0.21 ± 0.20	0.55 ± 0.39

[a] The sample-to-positive ratio (S/P ratio) is defined as (sample OD − negative control OD)/(positive control OD − negative control OD). S/P ratios of ≥0.4 were considered positive, S/P ratios of <0.4 but ≥0.3 were classified as suspect, and S/P ratios of <0.3 were classified as negative. All samples were performed in duplicate, and the average of the two wells was used to calculate the S/P ratio.
[b] Weeks after the first immunization.
[c] The sera samples of week 8 were taken before challenge.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$, compared with the negative control group.
$^{\#}$ $P < 0.05$.
$^{\#\#}$ $P < 0.01$.
$^{\#\#\#}$ $P < 0.001$, compared with the group inoculated with live vaccine without adjuvant.

Table 8
Lymphocyte proliferative response against *M. hyopneumoniae* antigen in pigs after immunization.

Group	Stimulation index	
	Week[a] 3	Week 6
Negative control	1.07 ± 0.20	0.88 ± 0.05
Without adjuvant	1.48 ± 0.25	1.11 ± 0.33
Adjuvant A	2.06 ± 0.42[*]	2.30 ± 0.39[**] [#]
Adjuvant C	2.50 ± 0.05[**]	1.88 ± 0.26[**] [#]
Adjuvant D	1.63 ± 0.45	1.25 ± 0.28
Adjuvant E	1.53 ± 0.38	1.41 ± 0.17
Challenged control	1.05 ± 0.16	1.08 ± 0.23

[a] Weeks after the first immunization.
[*] $P < 0.05$.
[**] $P < 0.01$, compared with the negative control group.
[#] $P < 0.05$, compared with the group inoculated with vaccine without adjuvant.

3.4. Protection against challenge

Twenty-eight days after challenge, all groups of animals were killed. No macroscopic changes were observed in the muscles of the injection sites of all the animals. Pigs from the challenged control group had typical and severe pneumonic lesions, namely, hyperplasia of bronchus-associated lymphoid tissue along with infiltration of lymphocytes and macrophages. The median lesion score was 14.81. Pigs that received the live vaccine without an adjuvant did not acquire significant protection, with a median lesion score of 9.28 ($P > 0.05$). When delivered with adjuvant A or C, the live vaccine induced significant protection (adjuvant A, median score of 3.57, $P < 0.01$; adjuvant C, median score of 5.28, $P < 0.05$). The vaccine efficacies in these two groups were 80.62 ± 36.30% (adjuvant A) and 68.27 ± 48.10% (adjuvant C), respectively. The decreases in lesion score in the groups inoculated with vaccines containing adjuvants D or E were not significant (Fig. 1).

4. Discussion

The current intrapulmonic route of administration for the strain 168 live vaccine requires greater training, and exposes animals to a higher risk of infection. Therefore, considerable effort has been taken to improve its route of delivery. Different approaches, including intranasal, aerosol and intramuscular immunization, have been tested. Intranasal immunization with live strain 168 with a CpG adjuvant evokes a local cellular and humoral immune response in the respiratory tract and a systemic immune response [16], but the protective effect of this has not been reported. Intramuscular immunization is the most common route used for vaccine inoculation, with the advantages of ease of administration and a reduced risk of injury to the animal. However, previous research has indicated the need for an appropriate adjuvant to generate sufficiently effective protection from this vaccine when it is delivered *via* the i.m. route. In the study described here, different adjuvants were designed to determined the most suitable formulation for i.m. administration of the live vaccine.

Previous reports have suggested that cellular immunity is important in conferring protection against *M. hyopneumoniae* [1,17]. The role of systemic humoral immunity remains unclear, but is generally considered to play no role in protection [1,13,18,19]. Therefore, adjuvants that are capable of stimulating cellular immunity, such as levamisole [20–22], astragalus polysaccharide [23,24] and ISCOM-matrix [7–10], might be anticipated to enhance the protective efficacy of the *M. hyopneumoniae* vaccine. Adjuvants A and C were shown to be effective in up-regulating the lymphocyte proliferative response in mice and pigs. Pigs immunized with vaccine containing adjuvants A or C were also significantly protected from challenge. These results suggest a relationship between the cellular immune response and protection. Of the four adjuvants assessed in our challenge experiment, adjuvant D significantly enhanced the serum antibody response but had no effect on protection. Although the animals from the groups inoculated with vaccines containing adjuvants A or C had some increase in antibody levels (adjuvant A on week 8, adjuvant C on week 4 and 6), the antibody titer

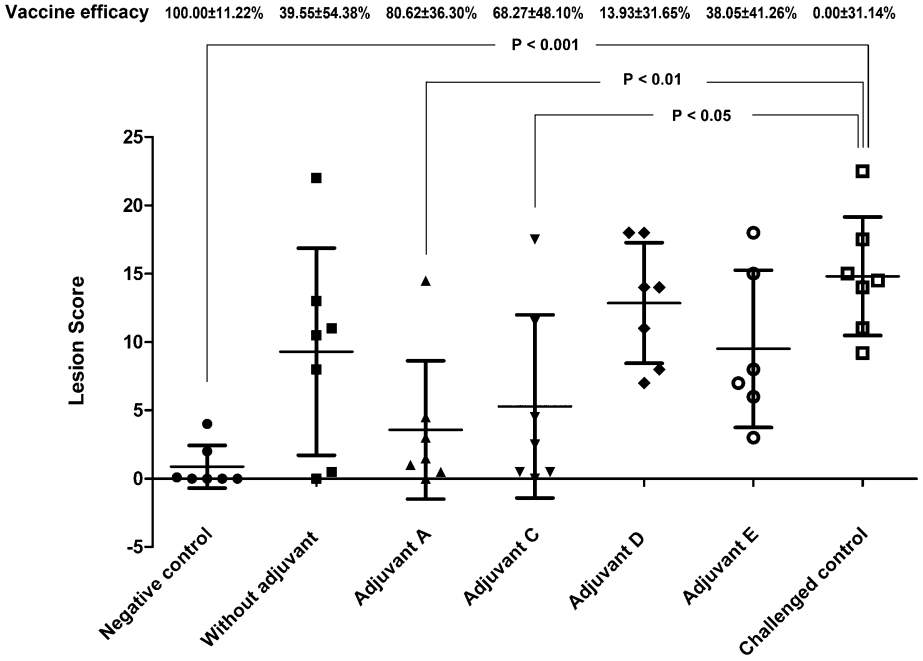

Fig. 1. Lung lesion scores after challenge. The lung lesion scores were determined using the method described by Madec et al., with the score potentially ranging from 0 to 28. The efficacy of the vaccine was calculated as follows: vaccine efficacy = [(challenged control group median score – vaccinated group median score)/(challenged control group median score – negative control group median score)] × 100%.

remained at a relatively low level until challenge. Moreover, comparison of serum antibody levels and the lesion scores of individual animals in these groups revealed no obvious relationship. This further suggested that cellular immune responses rather than humoral immunity plays the major role in protecting animals from disease caused by *M. hyopneumoniae*.

Although a systemic humoral immune response is probably not necessary for conferring protection, a high level of serum IgG antibody could help provide an easier and more practical method for confirming effective vaccination during clinical use. In the current study, adjuvant C induced an increase in IgG levels until week 6, but this response was not sufficiently high nor of sufficient duration for effective use in the field. The effect of adjuvant A could not be detected until week 8. In further research, other components that could strongly stimulate humoral immunity could be added into these two formulations to generate adjuvants more suited to routine clinical use.

There were differences between the mice and the pigs both in their cellular and humoral immune responses. Adjuvant A enhanced the serum antibody response in mice but failed to do so in pigs. Adjuvant E significantly enhanced the specific lymphocyte proliferative response in mice but not in pigs. In spite of these variations between species, the cost of performing experiments in pigs suggests that mice are still suitable for the primary screening of adjuvants.

Over the entire course of the experiment there were no detectable SIgA antibodies in the nasal swab samples (data not shown). In previous studies SIgA could only be detected in bronchioalveolar lavage fluid after challenge [17,18]. However, study by Lin et al. found that a microencapsulated *M. hyopneumoniae* vaccine delivered orally could induce IgA production in serum, feces, nasal discharges and saliva [25]. The stimulation of local mucosal immunity appears to be greatly influenced by the route of inoculation. Intramuscular immunization with vaccines against *M. hyopneumoniae* is less effective in inducing a strong local mucosal immunity than delivery by mucosal routes.

5. Conclusions

In this study, seven adjuvant formulations were designed and assessed for their ability to enhance the specific immune response induced by a live *M. hyopneumoniae* vaccine delivered i.m. to mice. Subsequently, four of these adjuvants were further evaluated in pigs to assess their capacity to enhance protective immunity. Two of the adjuvants were effective in augmenting the protective efficacy of the vaccine. These studies on adjuvants will assist in the development of an intramuscularly injected live vaccine against *M. hyopneumoniae*.

Conflict of interest

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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.03.071.

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