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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 15 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

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