



Immune Responses in Mice Immunized with Mastitis Multiple Vaccines Using Different Adjuvants

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ABSTRACT

Background: Bovine mastitis, a serious disease associated with both high incidence and significant economic losses, posing a major challenge to the global dairy industry. The development of vaccines for protection from new infections by mastitis pathogens is of considerable interest to the milk production industry. Vaccination is a common and easy strategy for the control of infectious diseases, and the adjuvants used in the formulation is a critical factor for vaccine efficacy improvement. The main objective of the present study was to evaluate three different adjuvants for their ability to enhance immune responses of mice that vaccinated with Bovine Mastitis Multiple Vaccine.

Materials, Methods & Results: The thymus and spleen index, the phagocytic ability of macrophage and the serum antibody levels of mice were detected after vaccination, respectively. The results showed that the thymus index, spleen index, and the phagocytic ability of macrophage of mice in Aluminum group exhibited a significant higher level ($P < 0.05$) compared with those in the control groups. The difference of the serum antibody levels was significant ($P < 0.05$) between experimental groups and control group after vaccination. The serum antibody concentration of mice in FIA group was higher compared with other groups and had a longer duration. The antibody concentration of mice in France 206 oil group can not increase as fast as the antibody concentration of Aluminum group, but it could last a longer time at a high level. In conclusion, multiple vaccines mixed with three different adjuvants (Aluminum, France 206 oil and Freund's incomplete adjuvant) for bovine mastitis could enhance the immunity of mice and could decrease mortality of mice against challenge. Take all results from this work into consideration, Freund's incomplete adjuvant (FIA) would be the best candidate as the adjuvant for mastitis multiple vaccines.

Discussion: The goal of vaccination is to generate strong immune response providing protection against infection for a time. Unlike attenuated live vaccines, killed whole organism or sub-unit vaccines generally require the addition of an adjuvant to be effective. What's more, different protective effects will usually obtained by different adjuvants even use same antigen. Adjuvants play an important role in increasing the efficacy of a number of different vaccines. In this study, three kinds of adjuvants (Aluminum hydroxide, France 206 oil and FIA) were evaluated for their adjuvant effects for multiple vaccine of bovine mastitis in mice and aluminum hydroxide did best as the vaccine adjuvant from the results. Aluminum hydroxide is a universally accepted adjuvant for both human and veterinary vaccines. The goal of vaccination is to generate strong immune response providing protection against infection for a time. Different protective effects will usually obtained by different adjuvants even use same antigen. In this work, FIA, Alum and 206 oil were chosen as adjuvants for inactivated antigens of *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Staphylococcus aureus*. The results showed that there was a significantly higher antibody levels in vaccinated mice compared with those in control group. In addition, the mice in France 206 oil and FIA group performed a higher antibody levels and stronger immunity than mice in Aluminum hydroxide groups. These findings suggest that Freund's incomplete adjuvant (FIA) would be the best candidate as the adjuvant for mastitis multiple vaccines investigated in this study.

Keywords: bovine mastitis, vaccine, adjuvant, immune response.

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INTRODUCTION

Bovine mastitis, a serious disease associated with both high incidence and significant economic losses [11,30], posing a major challenge to the global dairy industry [16]. When pathogenic microorganisms invade the mammary gland via the teat orifice and cause an inflammation of the secretory tissue, manifesting itself in either clinical or sub-clinical mastitis [29]. Over 200 agents have been recorded to cause bovine mastitis [2], but the vast majority of cases were caused by *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Escherichia coli* and *Streptococcus uberis* [15,17,23].

Current practices to prevent and control mastitis based on diagnosis, segregation and the use of improved hygiene can contribute to a decrease in the occurrence of the disease [34]. However, such measures are often expensive and are not always practical or efficacious. Hence, the treatment for bovine mastitis still relies heavily on the use of antibiotics [25]. Antibiotics are used extensively in food-producing animals to combat disease and to improve animal performance. In approximately 80% of dairy farms in America, antibiotics are administered routinely to entire herds to prevent mastitis during the dry-period. Benefits of antibiotic use include lower incidence, reduced mortality, and more productive cows [22]. However, presence of antibiotic residues in milk is the greatest disadvantage of use of antibiotics for the treatment of mastitis, which is not only a public health issue but a severe economic loss for dairy farmers. In addition, use of antibiotics is largely responsible for the emergence of antimicrobial-resistant bacteria [6].

Due to these limitations of antibiotics, the development of vaccines for protection from new infections by mastitis pathogens and reduce the need for the use of antibiotic is of considerable interest to the milk production industry. Vaccination is a common and easy strategy for the control of infectious diseases, and the adjuvants used in the formulation is a critical factor for vaccine efficacy improvement. The present study was designed to evaluate the immune responses of mice after immunisation with multiple vaccine composed of field isolates and a combination of three different adjuvants.

MATERIALS AND METHODS

Formulation of vaccine against bovine mastitis

Staphylococcus aureus (RFY84184), *Streptococcus agalactiae* (RFY271) and *S. dysgalactiae*

(RFY2531) used in this study were clinical bovine mastitis isolates which previously characterized as virulent strains and conserved in our laboratory. The bacteria were cultured by shaking at 37°C for 12 h in optimized Todd-Hewitt Broth (THB). The cells were harvested by centrifugation at 5000 g at 4°C for 10 min [18], and washed extensively in PBS before inactivation in 0.3% formalin for 18 h at 37°C [21]. Complete inactivation of the bacteria was confirmed by plating on blood agar plate for 48 h. Killed whole bacteria cells of *S. aureus*, *S. agalactiae* and *S. dysgalactiae* were mixed at 1:1:1 and then was mixed with Freund's incomplete adjuvant (FIA)¹, Montanide ISA-206 (ISA-206)² or Aluminium hydroxide (alum)³, respectively. Vaccines formulated with different adjuvants were stored at 4°C and can be used within 1 year.

Mice immunization

Three to four-week old Kunming mice (n = 264), purchased from Lanzhou University (Lanzhou, China), were used in the experiment. All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee of Lanzhou University. Mice were randomly distributed into four groups with half male and half female, and subcutaneously vaccinated (day 0) and revaccinated (day 21) with 0.5 mL of vaccine with FIA (n = 64), ISA-206 (n = 64), or alum (n = 64). The rest of 64 mice were injected with 0.5 mL PBS by subcutaneous injection twice as control.

Assessment of phagocytic function of macrophages

Phagocytic function of macrophages of mice was evaluated by a previously reported method with some minor modification [33]. Eight mice were selected randomly from each group at 35th, 50th, and 65th day after first immunization, and 0.5 mL of 5% chicken-red blood cells (CRBC) were injected intraperitoneally into each mouse. After 12 h, the abdominal liquid of each mouse was collected and dropped on a slide, and fixed with a mixture of acetone/methanol (1:1, v/v) for 5 min, then stained with giemsa for 3 min, rinsed with running water and dried. After drying, peritoneal macrophages were counted under microscope. The effect of vaccines on phagocytosis of macrophage was evaluated by the chicken-red cell phagocytic index, which were calculated using the following formulas: phagocytic index = the number of phagocytized CRBC / the number of macrophages which phagocytizing CRBC.

Samples collection and detection of thymus and spleen indexes

Eight mice were selected randomly from each group at 21st, 35th, 50th, and 65th day after first immunization. Blood of each mouse was collected and the serum samples obtained by centrifugation were kept frozen at -20°C. The entire spleen and thymus of mice were promptly removed after killed and weighed. The spleen and thymus indexes were calculated according to the formulas: (spleen or thymus weight / body weight) × 100% [23].

Determination of antibody levels by indirect EISA

Purified antigens were obtained by ultrasonication of *S. aureus*, *S. agalactiae* and *S. dysgalactiae*, and antigen concentrations were determined by Lowry method [10]. Ninety six-well plates were coated with 100 µL of purified antigen in carbonate coating buffer at 37°C for 2 h, and then was transferred to 4°C, overnight. The coated plates were washed three times with 0.05% PBS-Tween 20, and the non specific binding was blocked by adding 120 µL per well of BSA-PBS and incubated at room temperature for 2 h. Then the coated plates were washed again, and 100 µL of each serum in PBS was added and incubated for 1 h at 37°C. Following washed with PBST, and incubated in goat anti-mouse Ig G HRP (Abcam, UK) diluted with PBS for 1 h at 37°C. Then the plates were washed three times with PBS-Tween 20 and 100 µL of TMB per well was added and incubated for 10 min at RT. Finally, the enzymatic reaction was stopped by adding 50 µL 2M H₂SO₄ per well [5]. The optical densities were measured at 450 nm using a micro plate reader within 15 min [4,9].

Evaluation of protective effect of vaccines

Forty-five days after first vaccination, 10 mice from each group were challenged intravenously with 0.1 mL zymotic fluid containing 108 CFU *S. aureus*. This strain was grown in Nutrient Broth (Oxoid, UK) for 12 h at 37°C and the bacterial concentration was determined by serial dilution on blood agar plates [24]. The mice were monitored daily for 14 days and the changes of the spirit, appetite and temperature were observed, and the death case was recorded.

Statistical analysis

Significant differences between groups were evaluated by using One-way ANOVA analysis in SPSS, and the difference was considered statistically significant when $P < 0.05$.

RESULTS

Effect of vaccines on phagocytic function of macrophages

To assess the influence of vaccines on phagocytic function of macrophages, eight mice of each group were injected intraperitoneally with CRBC at 35th, 50th, and 65th day after first immunization, and the phagocytic index of macrophages were calculated. As shown in Figure 1, compared with mice in control group, there is a higher level of phagocytic index of macrophages in vaccinated groups. One-way ANOVA analysis showed that there is a significant difference between Alum group and control group ($P < 0.05$), whereas the difference between ISA-206 group, FIA group and control group was not significant ($P > 0.05$) except for FIA group on 65th day after first vaccination.

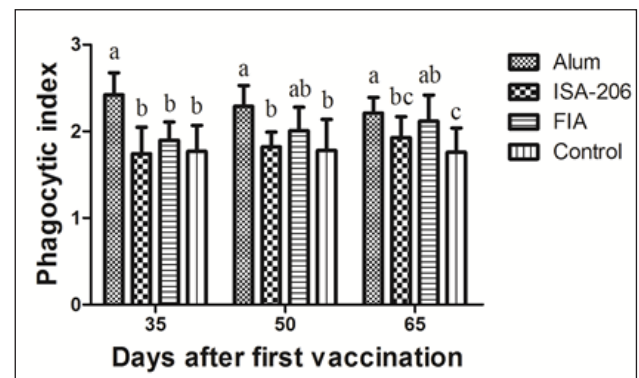


Figure 1. Phagocytic index of mice in different groups over time. *^{a-c}Different letters indicate statistically significant differences ($P < 0.05$).

Effect of vaccines on thymus and spleen index

As shown in Figure 2, thymus and spleen index of mice in alum groups is higher than those mice in control groups ($P < 0.05$) on the 35th day after first vaccination. However there is no significant difference between ISA-206, FIA and control group ($P > 0.05$).

Antibody response in mice

ELISA results showed that as compared with control groups, higher levels of IgG in vaccinated groups were observed in 21st day after the first immunization (Figure 3). The highest levels of antibody in FIA and Alum groups were observed in 35th day after the first immunization, but antibody level of ISA 206 group was increased slowly. As shown in Figure 3, antibody titer decreased slowly with time after reached the highest point, which conform to change regularity of antibody.

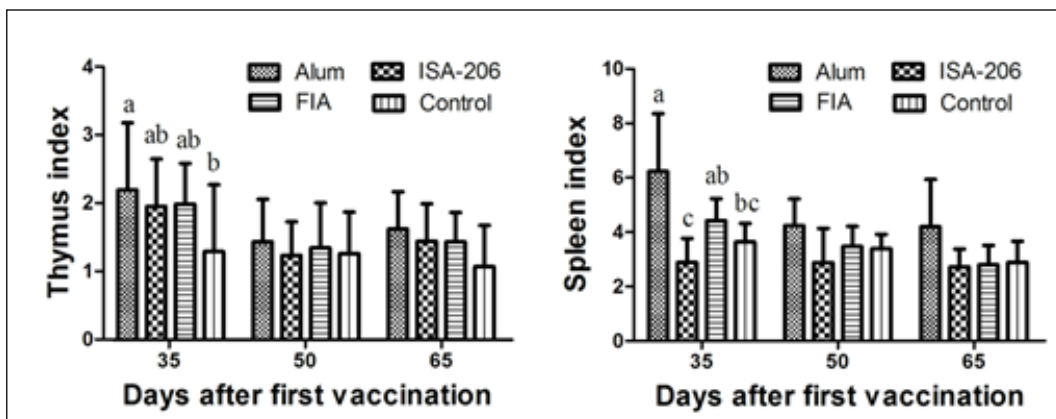


Figure 2. Thymus index (A) and spleen index (B) of mice in different groups over time. ^{a-c}Different letters indicate statistically significant differences ($P < 0.05$).

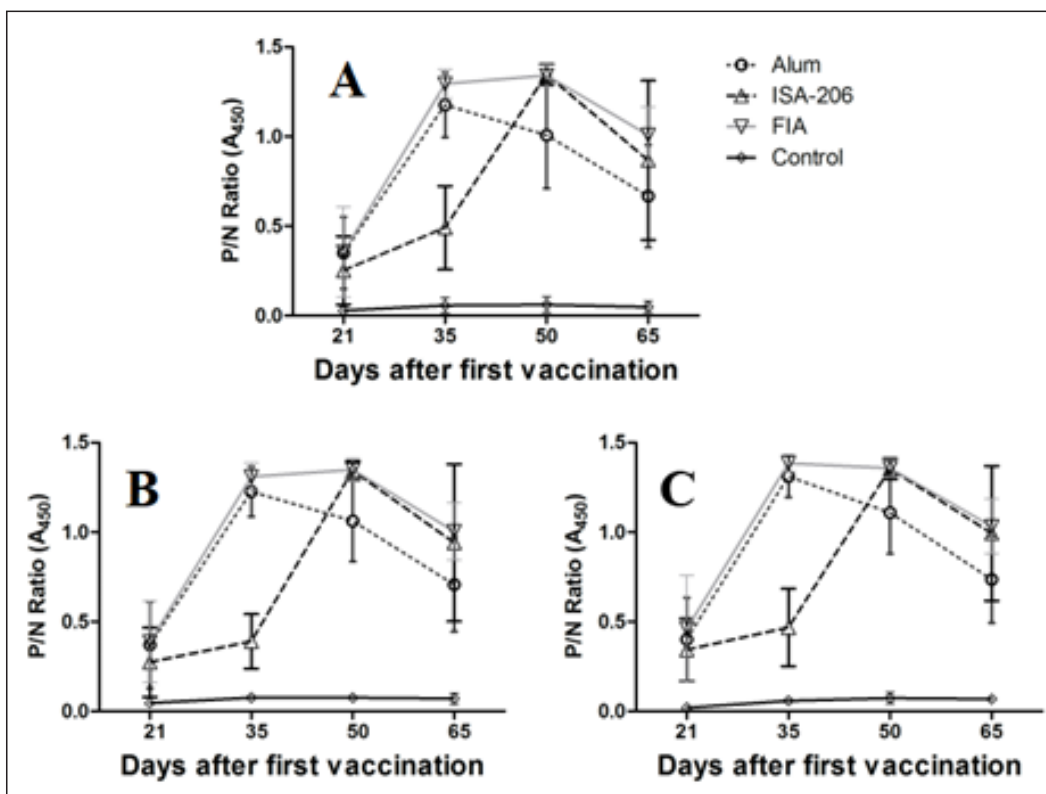


Figure 3. Specific serum antibody titer of *Staphylococcus aureus* (A), *Streptococcus agalactiae* (B) and *S. dysgalactiae* (C) in different groups over time.

Protection effect of vaccines against challenge

In order to compare the protective efficacy of vaccines with different adjuvants, 10 mice from each group were challenged via caudal vein with about 1×10^8 CFU *S. aureus* per mouse on day 45 after primary immunization. The mortality of mice was observed

and recorded during the first week post-challenge (Figure 4). These results indicate that vaccination conferred significantly higher ($P < 0.05$) protection as compared to control group. However, there was no distinctly difference between the three of vaccinated groups ($P > 0.05$).

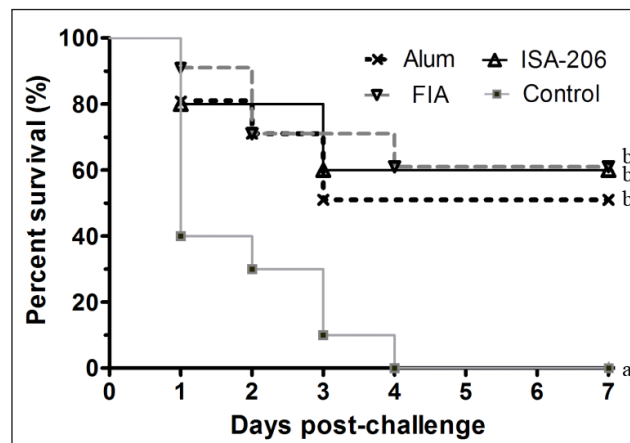


Figure 4. Survival curve of mice in different groups. ^{a-c}Different letters indicate statistically significant differences ($P < 0.05$).

DISCUSSION

Immune system is the major contributor to host defense against infection and maintaining health, which consists of immune organs, immunocytes and immune molecules [19,32]. The thymus, one of the most important immune organ, is the location of T-cell development where bone marrow-derived progenitors differentiate to become mature thymocytes before emigration into the periphery as new T cells [14]. The spleen, the largest peripheral immune organ, hosts major types of mononuclear phagocytes, including macrophages, DCs, and monocytes, which are key protectors of the organism because they identify pathogens and cellular stress, remove dying cells and foreign material, regulate tissue homeostasis and inflammatory responses, and shape adaptive immunity [3]. Thus, thymus, spleen and phagocytic index are important index reflecting the immune status.

In the present study, the results showed that macrophages collected from mice immunized with vaccine that aluminum hydroxide was used as adjuvant showed a higher activity, which represents an encouraging result compared to other groups, especially to control group (Figure 1). However, the promotion effect of vaccines on macrophages activity reduced gradually as time goes on. Vaccination enhanced the thymus and spleen index of mice, but the difference between vaccinated and control groups were not significant except for mice in aluminum hydroxide group on 35th day after the first immunization (Figure 2).

Adjuvants play an important role in increasing the efficacy of a number of different vaccines [8]. In this study, three kinds of adjuvants (Aluminum hydroxide, France 206 oil and FIA) were evaluated for

their adjuvant effects for multiple vaccine of bovine mastitis in mice and aluminum hydroxide did best as the vaccine adjuvant from the results. Aluminum hydroxide is a universally accepted adjuvant for both human and veterinary vaccines [13]. Although it has been investigated since 1926, the exact mechanism by which aluminium hydroxide adjuvates vaccine antigens is unknown [31]. The proposed mechanisms of action of aluminum hydroxide include (1) formation of a depot allowing slow release of antigen, (2) adsorption of antigen onto micron-sized mineral particles that are avidly taken up by APCs, (3) stimulation of immunoreactive cells and (4) direct effects on macrophages [27]. Rimaniol *et al.* [28] had investigated the effect of aluminum hydroxide adjuvant on isolated macrophages in vitro, the results showed that aluminum hydroxide vaccine stimulated macrophages, and these mature antigen-presenting macrophages were particular importance in the establishment of memory responses and in vaccination mechanisms leading to long-lasting protection. In another study, Lögdberg *et al.* [20] reported that vaccination with the adhesin of candidal with aluminum hydroxide adjuvant reduced the tissue infectious burden of mice, and they also showed that the mechanism of protection was a Th1/Th17 response, resulting in recruitment and activation of phagocytes at sites of infection and more effective clearance of *S. aureus* and *Candida albicans* from tissues.

The goal of vaccination is to generate strong immune response providing protection against infection for a time [1]. Unlike attenuated live vaccines, killed whole organism or sub-unit vaccines generally require the addition of an adjuvant to be effective [26]. What's more, different

protective effects will usually be obtained by different adjuvants even use same antigen. In a previous study reported by Hu *et al.* [12], 18 heifers were randomly divided into three groups and were immunised twice intramuscularly with the *S. aureus* bacterin (control), or with the bacterin in combination with different adjuvants (crude ginseng extract and purified ginsenoside). The specific antibody response to *S. aureus* antigen was evaluated in blood samples taken before and after immunisations, and the results showed that crude ginseng extract had stronger adjuvant effects, when used for immunisation against *S. aureus* in dairy cattle. In this work, FIA, Alum and 206 oil were chosen as adjuvants for inactivated antigens of *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Staphylococcus aureus*. The results showed that there was a significantly higher antibody levels in vaccinated mice compared with those in control group. In addition, the mice in France 206 oil and FIA group performed a higher antibody levels and stronger immunity than mice in Aluminum hydroxide groups.

CONCLUSION

In conclusion, results from this investigation showed that: multiple vaccines mixed with three

different adjuvants (Aluminum, France 206 oil and Freund's incomplete adjuvant) for bovine mastitis could enhance the immunity of mice and could decrease mortality of mice against challenge; Take all results from this work into consideration, Freund's incomplete adjuvant (FIA) would be the best candidate as the adjuvant for mastitis multiple vaccines investigated in this study.

MANUFACTURERS

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Ethical approval. This experiment was approved under the guidelines of Ethics Committee for Animal Use of Lanzhou University of China, under Registration No. 2017041606.

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Declaration of interest. The authors report no conflict interest. The authors alone are responsible for the content and writing of paper.

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