Effects of Selected Adjuvants on Immunogenicity and Protectivity of Pasteurella multocida Bacterin Vaccine in Chickens

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ABSTRACT
Avian pasteurellosis (fowl cholera) is an important disease affecting domestic and wild birds all over the world. While the capsular type A of Pasteurella multocida is mostly involved, other capsular types are occasionally incriminated. Present study aimed at investigating the effect of some adjuvants on immunogenicity and protectivity of P. multocida bacterin in chickens compared with an Iranian commercial vaccine. Eight-weeks-old chicken pullets were double vaccinated with an interval of three weeks. Vaccine immunogenicity testing was conducted using an in-house indirect ELISA and assessing serum antibody titers at 7, 14 and 21 days post-primary and 14 days post-secondary immunization. The possible adverse effects were recorded by a poultry-disease expert. For evaluating the vaccine protection rate, chickens were subjected to 2 × Lethal Dose 50% of a virulent P. multocida strain two weeks post-secondary immunization. The rate of live and normal animals was regarded as protection rate seven days after the exposure. The findings showed that oil adjuvants Montanide ISA 70 and Montanide ISA 71 contained vaccines (with or without saponin) caused a powerful immune reaction than the aluminum adjuvanted vaccine and commercial vaccine (P < 0.05). A significant protection against challenge was merely induced by the oil adjuvanted vaccines (P < 0.05). Majority of the studied chickens showed inflammation at the injection site (yellow) through the trial. Vaccines
made by Montanide ISA 70 and Montanide ISA 71 are novel and effective inactivated vaccines that are able to cause significant protection to fowl cholera disease.

**Key words:** Adjuvants, vaccine, *Pasteurella multocida*, chicken

**INTRODUCTION**

Fowl cholera is an important disease affecting domestic and wild bird species, however turkeys are highly vulnerable. It can be occurred by strains of capsular type A of *Pasteurella multocida*, though other capsular types are occasionally incriminated (Sthitmatee et al., 2008). Fowl cholera is typically occurred as a fulminating disease with tremendous bacteremia and high morbidity and mortality. Among the bacterial diseases of domestic birds, fowl cholera accounts for major economic losses to the poultry industry through death, weight loss, and condemnations (Wilkie et al., 2000). Chemotherapy is lengthy, costly, and ineffectual because of the increased antibiotics resistance of the bacterium and its toxicity for humans. Therefore, vaccination is the most powerful preventive method for the control of the disease (Ahmad et al., 2014).

The aim of vaccination is to procreate immune responses to the administered antigen which should provide long-time protection against infection. In spite of advances in the field of vaccinology, fowl cholera inactivated whole-cell vaccines are still universally used to combat the disease. One of the most important factors that enhances the immunogenicity of these vaccines is the nature of the adjuvant (Petrovsky and Aguilar, 2004). Adjuvants are substances that stimulate the immune system and increase the host reaction against an antigen with no particular antigenic impact. As there are not any universal adjuvants and their function is not yet completely obvious, they must be adapted according to multiple criteria, like the antigens, the route of application, the target species, and the kind of immune reaction or the immunity period (Aucoiturier et al., 2001). Some adjuvants are employed in veterinary vaccines. However, aluminum salts (alum) as well as oil-based emulsions with or without saponin are most frequently used for inactivated fowl cholera vaccines (Bowersock and Martin, 1999; Park et al., 2014).

Aluminium adjuvants are potent immunomodulators and strong Th2 stimulants, a property favorable for a good vaccine against extracellular pathogens such as *P. multocida*. Therefore, alum precipitated vaccines against fowl cholera are extensively used in the field conditions (Cox and Coulter, 1997). However, these vaccines have disparate defects such as the induction of
short-term immune responses which require relatively repetitious revaccinations (Aucouturier et al., 2001).

Oil-based adjuvants such as water-in-oil (W/O) emulsions have widely employed to formulate inactivated avian vaccines. Such emulsions are powerful adjuvants inducing strong and long-term immune responses, leading to more effective protection (Ben Arous et al., 2013). Mineral oils-oriented emulsions have shown effective, however, in some cases, they cause local inflammatory responses with reactive antigens at the inoculation site (Aucouturier et al., 2001). The immunostimulatory effect of adjuvants may be synergised by the application of co-adjuvants. Saponin and its derivatives are widely used as vaccine adjuvants to stimulate antibody and cell-mediated immune responses. Though work with oil-based adjuvants and saponin, as co-adjuvant, is limited, experimental trials found synergistic immune response (Kumar et al., 2012). The Montanide™ ISA series of W/O emulsion adjuvants including Montanide™ ISA 70 VG (ISA 70) and Montanide™ ISA 71 VG (ISA 71) are secure and effective in models for poultry disease (Aucouturier et al., 2001; Jang et al., 2012). Two formulations were solutions made by mineral oil including a highly refined emulsifier achieved by mannitol as well as purified oleic acid collected from vegetables (Aucouturier et al., 2006; Jang et al., 2010). ISA 70 and ISA 71 are similar; however, the former includes an enriched light mineral oil that can stimulate Th1-type cell-mediated immunity (Dupuis et al., 2006). Compared to traditional oil emulsions, these adjuvants are very stable, easy to inject, have high immunopotentiation capacity, and show lesser side-effects (Aucouturier et al., 2002; Mutiso et al., 2010). They have been used for enhancing the immune reaction to diseases affecting poultry, cattle, and small ruminants (Dupuis et al., 2006).

In Iran, fowl cholera is endemic in the northern part of the country, and an inactivated aluminum hydroxide vaccine, containing local *P. multocida* serotype A:1 prepared by Razi Vaccine and Serum Research Institute (RVSRI, Karaj, Iran), is applied to protect the poultries against the disease. However, despite immunization, outbreaks of fowl cholera have been reported from different regions of the country, especially in the endemic areas (Jabbari and Moazeni Jula, 2005; Sotoodehnia et al., 2005; Fereidouni et al., 2006).

In the current study, we have compared the immune responses in chickens immunized with fowl cholera vaccines made by oil-based adjuvants (Montanide ISA 70 and Montanide ISA 71 with or
without saponin) and the alum-based adjuvant (aluminum hydroxide) with a conventional vaccine, and the probable best adjuvant that can stimulate protection to experimental fowl cholera is identified.

**MATERIALS AND METHODS**

**Animals.** Two hundred and eighty-eight lohmann selected leghorn (LSL) laying chickens, eight weeks old at the baseline, were obtained from a commercial laying farm (Qom, Iran). These birds were kept in a breeding enclosed environment with free access to food and water at the poultry department of Veterinary Faculty at Shahid Chamran University of Ahvaz, Iran.

**Antigens.** The used antigens were obtained by inactivation of a local serotype A of *P. multocida* (strain PM CH-4) isolated from a commercial duck with respiratory signs (Shahid Chamran University, Ahvaz, Iran). The isolate was propagated in tryptic soy broth (TSB) (Merck, Germany) via incubation of the bacterial strain (24 h / 37°C) with shaking. After incubation, cell suspension was inactivated with 0.3% formaldehyde at 37°C for 24 h. The cells were separated through centrifuging (5,000 g / 20 min) and the pellet was resuspended in phosphate buffered saline (PBS, pH 7.2). We set the suspension’s optical density (OD) at 1.5 (Equivalent to 8 × 10⁹ organisms per mL) spectrophotometrically (Ultraspec 2000, Pharmacia Biotech, USA) at 540 nm.

**Adjuvants and vaccines.** To prepare the oil adjuvant vaccines (OAVs), standard W/O emulsions including Montanide™ ISA 70 VG and Montanide™ ISA 71 VG were agitated gently on the mixer at room temperature (RT) and the aqueous phase was added at a 70:30 ratio (w/w, adjuvant:antigen or PBS), as recommended by the adjuvant manufacturer (Seppic, France) (Table 1). The oil-based vaccines with saponin as co-adjuvant were prepared in a similar way as of OAV except that their aqueous phase contained 50 µg saponin/mL vaccine. In the alum-precipitated vaccine (APV), the aluminum hydroxide gel was added to the aqueous phase at a 50:50 ratio (w/w), up to 1% final concentration and mixed for 1 h in a low speed agitating mixer at RT. The adjuvants and vaccines were stored at 4°C.

**Immunization of chickens.** The subjects were randomly assigned to 12 groups (n=24 per group). At the age of eight weeks, they were immunized intramuscularly by 1 mL of the adjuvants or vaccine preparation, and immunization was repeated three weeks later (Table 1).
One of the groups was immunized by a commercially P. multocida inactivated alum-based vaccine (Fowl Cholera Vac®, RVSRI, Iran) and others with various experimental preparations according to the various adjuvants studied. Twenty-four chickens were subjected to 1 mL of sterile PBS injection alone.

**Safety assessment.** After immunization, the adjuvants or vaccines were evaluated via daily monitoring of the animals’ general behavior and any abnormality at the areas of injections.

**Challenge procedure.** Median lethal dose (LD$_{50}$) of P. multocida, strain PM3927 obtained from RVSRI (Karaj, Iran), was calculated according to Reed and Muench method (1938) and half of the chickens of each group were challenged intramuscularly with $2 \times$ LD$_{50}$ (equivalent to $1 \times 10^2$ colony forming units per bird (CFU/bird) of virulent PM3927 strain of organism, two weeks post-secondary immunization. Simultaneously, 12 chickens of the control group were injected with 1 mL of sterile PBS alone. They were monitored within seven days to record possible clinical signs. The rate of live and normal animals was regarded as protection rate seven days after the exposure. The liver, spleen and heart blood of these chickens were used to isolate viable organisms (Table 2).

**Serum titer of anti-P. multocida antibody.** Blood samples were randomly collected from the wing web brachial vein of 90 chickens prior to first immunization then from eight chickens/group at days 7, 14 and 21 after primary and day 14 after secondary immunization. Sera were harvested after incubating the clotted blood samples at 4-8°C for 4-5 h and then centrifuged (2,500 g / 5 min at RT) followed by storing at -20°C until analysis of the antibody responses against P. multocida using an in-house indirect enzyme-linked immunosorbent assay (ELISA) (Kedrak et al., 2000). The 96-well microtiter plates (SPL, South Korea) were covered by 100 µL of sonicated P. multocida antigen (20 µg/mL) and incubated at 4°C for a night. PBS including 0.05% Tween 20 (PBST) was used to wash the plates twice and free sites were blocked via 2% skim milk powder at 25°C for 2 h. Test sera were added in duplicate at 1:500 dilution followed by addition of goat anti-chicken IgY horse reddish peroxidase conjugate (Abcam, USA) in each well at 1:10000 dilutions and kept at 25°C for 45 min. The chromogen substrate (tetramethyl benzidine mixed with hydrogen peroxide) (Cytomatingen, Iran) was added and the response was paused 10 min later by adding 0.1 M HCl. The OD values at 450 nm were determined using an
automated ELISA reader (Accu Reader, Taiwan) and their SP% was counted based on the following formula:

\[ \text{SP}\% = \left( \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}} \right) \times 100 \]

**Statistical analysis.** The data from immunized chickens with those from control groups was analyzed by ANOVA (version 19.0, SPSS Inc., USA). Values were compared through the Dunnett’s C test. P < 0.05 was regarded significant.

**RESULTS**

**Humoral immune response to vaccines.** Results of measuring the antibody titer by ELISA in immunized laying chickens are shown in Table 1. According to the outcomes, the chickens have relatively showed low antibody titer prior to the first immunization. Anti- *P. multocida* antibody of the control groups that had been inoculated with different adjuvants or PBS (groups 1-6) has not significantly changed at different days post-immunization. Antibody level in chicks which received tested vaccines (groups 7-12) increased quickly at days 7, 14, 21 and 35 post-immunization, and there was a considerable difference from groups receiving adjuvant and/or PBS (P < 0.05). Based on the results, at days 14, 21 and 35 post-immunization, the highest titers of anti- *P. multocida* antibody among the tested vaccines was related to oil-based adjuvant vaccines that showed a significant difference compared with other groups (P < 0.05).

**Protection against challenge.** The protection level of different adjuvants and vaccines in immunized chickens (groups 2-12) and the PBS control group, with 2 × LD₅₀ of a virulent strain of *P. multocida*, is presented in Table 2. All of the chickens that had been inoculated with adjuvants and/or PBS (groups 1-6) have shown symptoms of the disease and died within 48 h post-challenge. Among the groups which were immunized with different vaccines (7-12), the lowest level of protection (33.30%) was related to the alum-precipitated vaccine. In contrast, ISA 70- and ISA 71-containing vaccines (without saponin) induced the highest amount of protection (100%), while conservation rate of these OAVs plus saponin was 83.30% and 66.60%, respectively. According to the results, only the oil adjuvanted vaccines could induce a significant protection against challenge (P < 0.05). Viable organisms were isolated from liver, spleen and heart blood of dead challenged chickens. Surviving chickens which had been euthanized at day seven post-challenge did not show any signs of disease or infection.
Safety of vaccines. Daily monitoring of the chickens that had been inoculated with tested adjuvants and vaccines showed that in most under study birds, especially in groups which were inoculated with saponified adjuvants or vaccines showed inflammation at the injection site (yellow) through the trial. However, no modification was observed in animals’ behavior.

DISCUSSION
Several adjuvants have been shown with vaccine property; however, the majority of the commercially veterinary vaccines are supplemented with a classical adjuvant, such as aluminum salts as well as oil emulsions (Hilgers et al., 1998). The Montanide ISA series of adjuvants including ISA 70 VG and ISA 71 VG are mineral adjuvants obtained from oil useful to produce W/O emulsions (Aucouturier et al., 2006). Safe W/O emulsion adjuvants can be used to formulate effective inactivated poultry vaccines. Since we could find only a few reports of ISA 70 and ISA 71 adjuvanted fowl cholera vaccines, we hypothesized that these new oil-based adjuvants, with or without saponin, might induce a better immune response and protection in chicken as compared to a conventional alum based adjuvant vaccine.

The results of the measurement of serum anti- *P. multocida* antibody titer in under study chickens, which was performed by ELISA, showed that the immune response in the groups only receiving adjuvant has been a slight increase on the post-immunization weeks. Also, similar to some other studies (Kumar et al., 2012; Ben Arous et al., 2013), the antibody level of the control group has not considerably increased at days 7, 14, 21 and 35 post-immunization.

Generally, adjuvants’ action relies on various mechanisms: (I) the depot impact and the antigen slower secretion from the inoculation area, (II) protecting the antigen to be degraded via enzymes, (III) inflammation and stimulating the use of antigen presenting cells (APC), like macrophages and lymphocytes, (IV) stimulating the lymphocytes accumulation in draining lymph nodes and altering recirculation hence facilitating cell association; and (V) the induction of specific cytokines according to the type of emulsion (Aucouturier et al., 2001). In the present research, among the groups which were immunized with under study vaccines, the highest level of antibody titers was related to oil-based ISA 70- and ISA 71-containing vaccines. In agreement with our findings, Belloc et al. (2008) examined various adjuvants impacts on immunogenicity of *P. multocida* killed vaccines among 16-week-old laying chickens and
reported that the vaccines made by oil adjuvant Montanide ISA 70, ISA 774 and W/O emulsion prepared with Tween/span caused a powerful immune reaction to *P. multocida*, on four and eight weeks post-immunization. Similarly, Mudassar et al. (2014) tested the immunogenicity of Montanide ISA 206, paraffin oil-based and alum-precipitated hemorrhagic septicemia vaccines in rabbits and showed that ISA 206 adjuvanted vaccine induced higher antibody titers as compared with two other vaccines, during consecutive weeks after vaccination. Oil adjuvants used in these experiments (ISA 70, ISA 774 and ISA 206), such as oil-based adjuvants used in the current study, were more robust immune response in the hosts, and antibody titer induced by them were significantly higher than those by others. Also, trials of researchers on other organisms has confirmed strong antibody response obtained from oil-based adjuvants used in the present study (Jang et al., 2010; Ben Arous et al., 2013; Jang et al., 2013). On the basis of our findings, among the oil-based vaccines, saponified oil adjuvant vaccines (S-OAVs) induced the highest level of anti- *P. multocida* antibody titers. In agreement to this result, Kumar et al. (2012) reported that the OAV made by saponin caused potent humoral and cellular immune reaction toward hemorrhagic septicemia among mice as well as calves. Therefore, adding saponin to oil-based adjuvants can increase the antibody titer of the vaccines. According to the results, antibody response of almost all chickens to second immunization was much greater than that of the first, which is consistent with those reported by Hilgers et al. (1998). It seems that a considerable immunological stimulus had been evoked by the second exposure.

In present study, all of the birds that had been injected with different adjuvants died within 48 h post-challenge. However, among the groups which were immunized with various vaccines, the lowest and the highest level of protection were related to the alum-precipitated vaccine (33.30%) and non-saponified oil-based vaccines (100%), respectively. In consistence with the present results, Ben Arous et al. (2013) announced Montanide ISA 71 VG as a beneficial adjuvant to formulate poultry vaccine, which can induce an effective immune reaction in a Newcastle disease (ND) model. Similarly, Sotoodehnia et al. (2005) reported that immunity induced by inactivated oil-based haemorrhagic septicaemia vaccine adjuvanted with Montanide ISA 70 could protect 100% of under study calves up to 150 days post-immunization. Moreover, Rajagopal et al. (2012) compared immunopotency of inactivated oil-based fowl cholera vaccines
formulated with *P. multocida* biofilm, capsule enhanced organisms, and common broth grown organisms in one-month-old ducklings and showed that the serum titers obtained for the biofilm vaccine group were more elevated from other two groups. They can provide 10% more protection after exposure with 200 × LD₅₀ and 100 × LD₅₀ of the virulent strain of organism. Also, Jang et al. (2010, 2013) in two separate studies stated that immunization with the *Eimeria* profilin protein subunit vaccine and an *Eimeria* recombinant profilin protein, combined with Montanide adjuvants, especially ISA 71 VG, enhanced protective immunity against avian coccidiosis. Jabbari and Moazeni Jula (2005) showed that the prepared inactivated trivalent fowl cholera vaccine (serotypes 1, 3 and 4 of the organism) could induce an immune response, and provided protection against challenge with homologous strains from 70% (serotype 3) to 100% (serotypes 1 and 4). In the present experiment, the protection amount of the majority of immunized groups was 66.60-100% against challenge.

In contrast with our results, Kumar et al. (2012) reported that after challenge of several groups of mice with 1000 × LD₅₀ of live *P. multocida*, serotype B, the saponified oil adjuvant vaccine (S-OAV) group had 80% protection as compared to 60% protection by OAV. However, in the current research, the protection level of non-saponified ISA 70- and ISA 71- containing vaccines was 100% as compared to 83.30% and 66.60% by the OAVs plus saponin, respectively.

In the present study, in most tested chickens, especially in groups which were immunized with saponified oil adjuvant vaccines, showed inflammation at the injection site (yellow) through the trial. Similarly, fowl cholera emulsified bacterin using a mineral-oil adjuvant causes intense local tissue reactions characterized by wide caseous necrosis in chickens and turkeys. Fowl cholera or *Mycoplasma gallisepticum* bacterin cause more severe inflammatory reactions at inoculation site than do viral oil-adjuvanted vaccines (Okay et al., 2012). Belloc et al. (2008) also stated that Montanide ISA 774 is an adjuvant including both mineral and non-mineral oils, compared with other tested adjuvants (Montanide ISA 70, Montanide IMS 1112 and W/O emulsion prepared with Tween 80), caused fewer local responses in laying chickens. Also, Belgian researchers’ studies have shown that formalin-killed oil-adjuvanted *Pasteurella* vaccine (OAV) led to local tissue irritation as well as lesion in mice (Plotkin, 2009). The severity of local side effects of OAVs based on the vaccine formulation was associated with oil amount, hydrophilic-lipophilic balance (HLB), and the surfactant quality (Belloc et al., 2008).
Overall, the results of this study showed that oil-based adjuvant vaccines (ISA 70- and ISA 71-containing vaccines) induced high levels of immune response as compared to alum-based adjuvant vaccines (aluminum hydroxide adjuvanted vaccine and commercial vaccine). As well as, only the oil adjuvanted vaccines could induce a significant protection against challenge. In general, it seems that ISA 70- and ISA 71-containing vaccines, which stimulated the immune system at the highest level and resulted in the greatest amount of protection, can be a good alternative to alum-based adjuvant vaccines.

**Ethics**
We hereby declare all ethical standards have been respected in preparation of the submitted article.

**Conflict of Interest**
The authors declare that they have no conflict of interest.

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**References**


Montanide™ ISA 71 VG adjuvant increases protection against experimental necrotic enteritis in commercial broiler chickens. Vaccine 30, 5401-5406.


Table 1. Mean and standard deviation of antibody titers against *P. multocida* (SP%) obtained in LSL laying chickens at different days post-immunization with various adjuvant or vaccine formulations

<table>
<thead>
<tr>
<th>Group</th>
<th>Adjuvant or vaccine formulation</th>
<th>Anti- <em>P. multocida</em> antibody titer (SP%) at days post immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS (control)</td>
<td>27.50±14.50 32.00±4.00 31.70±9.50 118.00±19.30 73.70±17.00</td>
</tr>
<tr>
<td>2</td>
<td>Aluminum hydroxide/PBS</td>
<td>33.70±5.30 15.20±1.70 48.00±3.90 55.80±2.50</td>
</tr>
<tr>
<td>3</td>
<td>Montanide ISA 70/PBS</td>
<td>60.80±9.20 20.40±2.90 65.80±7.90 66.40±9.70</td>
</tr>
<tr>
<td>4</td>
<td>Montanide ISA 71/PBS</td>
<td>56.00±12.50 26.30±5.20 60.20±5.40 72.40±6.60</td>
</tr>
<tr>
<td>5</td>
<td>Montanide ISA 70 /PBS/Saponin</td>
<td>36.70±6.40 36.00±11.40 123.50±6.10 102.50±9.70</td>
</tr>
<tr>
<td>6</td>
<td>Montanide ISA 71 /PBS/Saponin</td>
<td>62.30±9.60 28.00±12.60 94.80±11.50 60.30±12.00</td>
</tr>
<tr>
<td>7</td>
<td>Commercial vaccine</td>
<td>69.70±10.10 66.20±9.80 141.80±16.90 171.00±16.00</td>
</tr>
<tr>
<td>8</td>
<td>Aluminum hydroxide/ Bacterin</td>
<td>121.50±7.20 130.80±4.10 156.60±4.50 207.50±6.40</td>
</tr>
<tr>
<td>9</td>
<td>Montanide ISA 70/ Bacterin</td>
<td>109.50±10.60 196.30±3.20 223.70±2.30 250.50±2.90</td>
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<tr>
<td>10</td>
<td>Montanide ISA 71/ Bacterin</td>
<td>152.90±12.40 198.20±2.50 224.10±4.90 253.00±2.50</td>
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<td>11</td>
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<td>12</td>
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<td>107.40±10.10 202.10±2.20 226.60±3.90 254.70±3.90</td>
</tr>
</tbody>
</table>

abcd Different superscripts in the same column indicate significant differences between groups (P < 0.05).
Table 2. Protection of various adjuvant or vaccine formulations in different treatment groups of LSL laying chickens after intramuscular challenge with \(2 \times \text{LD}_{50}\) (equivalent to \(1 \times 10^2\) CFU/bird) of virulent PM3927 strain of *P. multocida*, 2 weeks post-secondary immunization.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adjuvant or vaccine formulation</th>
<th>Total number of challenged chickens</th>
<th>Number of died chickens</th>
<th>Number of infectious samples</th>
<th>Survived/ challenged</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS (control)</td>
<td>12</td>
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<td>12</td>
<td>0/12</td>
<td>00.00</td>
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<td>2</td>
<td>Aluminum hydroxide/PBS</td>
<td>12</td>
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<td>12</td>
<td>0/12</td>
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<td>00.00</td>
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<td>00.00</td>
</tr>
<tr>
<td>5</td>
<td>Montanide ISA 70/PBS/Saponin</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>0/12</td>
<td>00.00</td>
</tr>
<tr>
<td>6</td>
<td>Montanide ISA 71/PBS/Saponin</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>0/12</td>
<td>00.00</td>
</tr>
<tr>
<td>7</td>
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<td>100*</td>
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<td>0</td>
<td>12/12</td>
<td>100*</td>
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<tr>
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<td>2</td>
<td>10/12</td>
<td>83.30*</td>
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<td>12</td>
<td>4</td>
<td>4</td>
<td>8/12</td>
<td>66.60*</td>
</tr>
</tbody>
</table>

* Statistically significant as compared to control group, P < 0.05.