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Original Article

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. Although the capsular type A of *Pasteurella multocida* is mostly involved, other capsular types are occasionally incriminated. The present study aimed at investigating the effect of some adjuvants on immunogenicity and protectivity of *P. multocida* bacterin in chickens, compared to an Iranian commercial vaccine. Eight-week-old chicken pullets were double vaccinated with an interval of three weeks. Vaccine immunogenicity testing was conducted using an in-house indirect enzyme-linked immunosorbent assay 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 7days after the exposure. The findings showed that oil adjuvants Montanide ISA 70-and Montanide ISA 71-containingvaccines (with or without saponin) caused a powerful immune reaction than the aluminum adjuvanted vaccine and commercial vaccine ($P<0.05$). Significant protection against challenge was merely induced by the oil adjuvanted vaccines ($P<0.05$). The majority of the studied chickens showed inflammation at the injection site (yellow) throughout 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.

Keywords: Adjuvants, Chicken, *Pasteurella multocida*, Vaccine

1. Introduction

Fowl cholera is an important disease affecting domestic and wild bird species; however, turkeys are highly vulnerable. It can be occurred by the strains of capsular type A of *Pasteurella multocida* though other capsular types are occasionally incriminated (1). Fowl cholera is typically occurred as a fulminating disease with tremendous bacteraemia 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 (2). 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 (3).

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 enhance the immunogenicity of these vaccines is the nature of the adjuvant (4). Adjuvants are substances that stimulate the immune system and increase the host reaction against an antigen with no particular antigenic impact. Since there are not any universal adjuvants and their function has not yet been completely obvious, they must be adapted according to multiple criteria, such as antigens, the route of application, target species, and kind of immune reaction or the immunity period (5). 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 (6, 7).

Aluminum 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 field conditions (8). However, these vaccines have disparate defects, such as the induction of short-term immune responses which require relatively repetitious revaccinations (5).

Oil-based adjuvants, such as water-in-oil (W/O) emulsions have been widely employed to formulate inactivated avian vaccines. Such emulsions are powerful adjuvants inducing strong and long-term immune responses, leading to more effective protection (9). Mineral oil-oriented emulsions have been effective; however, in some cases, they cause local inflammatory responses with reactive antigens at the inoculation site (5).

The immunostimulatory effect of adjuvants may be synergized by the application of co-adjuvants. Saponin and its derivatives are widely used as vaccine adjuvants to stimulate antibody and cell-mediated immune responses. Although studies on oil-based adjuvants and saponin, as co-adjuvant, are limited, experimental trials found synergistic immune responses (10).

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 (5, 11). Two formulations (solutions) made by mineral oil include a highly refined emulsifier achieved by mannitol, as well as purified oleic acid collected from vegetables (12, 13). ISA 70 and ISA 71 are similar; however, the former includes an enriched light mineral oil that can stimulate Th1-type cell-mediated immunity (14). Compared to traditional oil emulsions, these adjuvants are very stable and easy to inject; moreover, they have high immunopotentiation capacity and show fewer side effects (15, 16). Furthermore, they have been used for enhancing the immune reaction to diseases affecting poultry, cattle, and small ruminants (14).

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 (17, 18).

The current study 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; moreover, the probable best adjuvant that can stimulate protection to experimental fowl cholera was identified in this study.

2. Material and Methods

2.1. Animals

In total, 288 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, Ahvaz, Iran.

2.2. Antigens

The used antigens were obtained by the 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 (Merck, Germany) via the 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). The suspension's optical density (OD) was set at 1.5 (Equivalent to 8×10^9 organisms per mL) spectrophotometrically (Ultraspec 2000, Pharmacia Biotech, USA) at 540 nm.

2.3. 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, 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 ata low speed agitating mixer at RT. The adjuvants and vaccines were stored at 4°C.

Table 1. Mean±SD of antibody titers against *P. multocida* (SP%) obtained in Lohmann selected leghorn laying chickens at different days post-immunization with various adjuvant or vaccine formulations

Group	Adjuvant or vaccine formulation	Anti- *P. multocida* antibody titer (SP%) at different days post-immunization				
		1	7	14	21	35
1	PBS (control)	27.50±4.50	32.00±4.00^{a}	31.70±9.50^{a}	118.00±19.30^{b}	73.70±17.00^{a}
2	Aluminum hydroxide/PBS		33.70±5.30^{a}	15.20±1.70^{a}	48.00±3.90^{a}	55.80±2.50^{a}
3	Montanide ISA 70/PBS		60.80±9.20^{a}	20.40±2.90^{a}	65.80±7.90^{a}	66.40±9.70^{a}
4	Montanide ISA 71/PBS		56.00±12.50^{a}	26.30±5.20^{a}	60.20±5.40^{a}	72.40±6.60^{a}
5	Montanide ISA 70 /PBS/Saponin		36.70±6.40^{a}	36.00±11.40^{a}	123.50±6.10^{b}	102.50±9.70^{a}
6	Montanide ISA 71 /PBS/Saponin		62.30±9.60^{a}	28.00±12.60^{a}	94.80±11.50^{a}	60.30±12.00^{a}
7	Commercial vaccine		69.70±10.10^{a}	66.20±9.80^{a}	141.80±16.90^{b}	171.00±16.00^{c}
8	Aluminum hydroxide/ Bacterin		121.50±17.20^{b}	130.80±4.10^{b}	156.60±4.50^{c}	207.50±6.40^{c}
9	Montanide ISA 70/ Bacterin		109.50±10.60^{b}	196.30±3.20^{c}	223.70±2.30^{d}	250.50±2.90^{d}
10	Montanide ISA 71/ Bacterin		152.90±12.40^{c}	198.20±2.50^{c}	224.10±4.90^{d}	253.00±2.50^{d}
11	Montanide ISA 70 /Saponin/ Bacterin		110.90±10.00^{b}	199.80±3.40^{c}	233.50±4.30^{d}	260.90±2.40^{d}
12	Montanide ISA 71 /Saponin/ Bacterin		107.40±10.10^{b}	202.10±2.20^{c}	226.60±3.90^{d}	254.70±3.90^{d}

^{abcd} Different superscripts in the same column indicate significant differences among groups ($P<0.05$).

2.4. 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. Moreover, 24 chickens were subjected to 1 mL of sterile PBS injection alone.

2.5. 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.

2.6. 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 1×10^2 colony forming units perbird 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).

Table 2. Protection of various adjuvant or vaccine formulations in different treatment groups of Lohmann selected leghorn laying chickens after intramuscular challenge with $2\times LD_{50}$ (equivalent to 1×10^2 CFU/bird) of virulentPM3927 strain of *P. multocida*two weeks post-secondary immunization

Group	Adjuvant or vaccine formulation	Total number of challenged chickens	Number of died chickens	Number of infectious samples	Survived/ challenged	% Protection
1	PBS (control)	12	12	12	0/12	00.00
2	Aluminum hydroxide/PBS	12	12	12	0/12	00.00
3	Montanide ISA 70/PBS	12	12	12	0/12	00.00
4	Montanide ISA 71/PBS	12	12	12	0/12	00.00
5	Montanide ISA 70 /PBS/Saponin	12	12	12	0/12	00.00
6	Montanide ISA 71 /PBS/Saponin	12	12	12	0/12	00.00
7	Commercial vaccine	12	7	7	5/12	41.66
8	Aluminum hydroxide/Bacterin	12	8	8	4/12	33.30
9	Montanide ISA 70/Bacterin	12	0	0	12/12	100*
10	Montanide ISA 71/Bacterin	12	0	0	12/12	100*
11	Montanide ISA 70 /Saponin/Bacterin	12	2	2	10/12	83.30*
12	Montanide ISA 71 /Saponin/Bacterin	12	4	4	8/12	66.60*

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

2.7. 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 and 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) (19). 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 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 the 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 (tetramethylbenzidine 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:

SP% =(OD sample-OD negative control/OD positive control-OD negative control)×100

2.8. Statistical Analysis

The data from immunized chickens with those from control groups were analyzed in SPSS software (version 19.0, SPSS Inc., USA) through ANOVA. Moreover, the values were compared using Dunnett's C test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. 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 that received tested vaccines (groups 7-12) increased quickly at days 7, 14, 21, and 35 post-immunization, and there was a considerable difference in the 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 to the other groups ($P<0.05$).

3.2. Protection against Challenge

The protection level of different adjuvants and vaccines in immunized chickens (groups 2-12) and the PBS control group with $2\times LD_{50}$ 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 the 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 rates of these OAVs plus saponin were 83.30% and 66.60%, respectively. According to the results, only the oil adjuvanted vaccines could induce significant protection against challenge ($P<0.05$). Viable organisms were isolated from the liver, spleen, and heart blood of dead challenged chickens. Surviving chickens which had been euthanized at day 7 post-challenge did not show any signs of disease or infection.

3.3. Safety of Vaccines

Daily monitoring of the chickens that had been inoculated with tested adjuvants and vaccines showed that in most birds under study, especially in groups that were inoculated with saponified adjuvants or vaccines, inflammation was observed at the injection site (yellow) through the trial. However, no modification was observed in animals' behavior.

4. Discussion

Several adjuvants have been shown with vaccine properties; however, the majority of the commercially veterinary vaccines are supplemented with a classical adjuvant, such as aluminum salts and oil emulsions (20). 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 (12). Safe W/O emulsion adjuvantscan be used to formulate effective inactivated poultry vaccines. Since only a few reports of ISA 70 and ISA 71 adjuvanted fowl cholera vaccines could be found, it was hypothesized that these new oil-based adjuvants, with or without saponin, might induce a better immune response and protection in chicken, compared to a conventional alum-based adjuvant vaccine.

The results of the measurement of serum anti-*P. multocida* antibody titer in the chickens under study, which was performed by ELISA showed that the immune response in the groups only receiving adjuvant had a slight increase on the post-immunization weeks. Moreover, similar to some other studies (9, 10), the antibody level of the control group did not considerably increase at days 7, 14, 21, and 35 post-immunization.

Generally, adjuvants' action relies on various mechanisms, including (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, such as macrophages and lymphocytes, (IV) stimulating the lymphocytes accumulation in draining lymph nodes and altering recirculation, thereby facilitating cell association, and (V) the induction of specific cytokines according to the type of emulsion (5).

In the present study, among the groups which were immunized with vaccines under study, the highest level of antibody titers was related to oil-based ISA 70- and ISA 71- containing vaccines. In agreement with our findings, Belloc, Laurent (21) examined various adjuvants impacts on the 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*4 and 8weeks post-immunization. Similarly, Mudassar, Habib (22) 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, compared totwo 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, had more robust immune responses in the hosts, and antibody titer induced by them were significantly higher than those by others. Furthermore, trials of researchers on other organisms have confirmed strong antibody response obtained from oil-based adjuvants used in the present study (9, 13, 23).

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. Consistentwiththis result, Kumar, Chaturvedi (10) reported that the OAV made by saponin caused potent humoral and cellular immune reactions toward hemorrhagic septicemia among mice, as well as calves. Therefore, adding saponinto oil-based adjuvants can increase the antibody titer of the vaccines.

According to the results, the antibody response of almost all chickens to the second immunization was much greater than that of the first, which is consistent with the results reported by Hilgers, Nicolas (20). It

seems that a considerable immunological stimulus had been evoked by the second exposure.

In the 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 line with the present results, Arous, Deville (9) announced Montanide ISA 71 VG as a beneficial adjuvant to formulate poultry vaccine, which can induce an effective immune reaction in a Newcastle disease model. Similarly, immunity induced by inactivated oil-based haemorrhagic septicaemia vaccine adjuvanted with Montannide ISA 70 could protect 100% of calves under study up to 150 days post-immunization. Moreover, Rajagopal, Nair (24) 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. Moreover, they can provide 10% more protection after exposure with $200\times LD_{50}$ and $100\times LD_{50}$ of the virulent strain of the organism. Similarly, (13), Jang, Kim (23) 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, Esmaelzadeh (18) 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, Chaturvedi (10) reported that after the challenge of several groups of mice with $1000\times LD_{50}$ of live *P. multocida*, serotype B, the S-OAV group had 80% protection, compared to 60% protection by OAV. However, in the current study, the protection level ofnon-saponified ISA 70- and ISA 71- containing vaccines was 100%, compared to 83.30% and 66.60% by the OAVs plus saponin, respectively.

In the present study, in most tested chickens, especially in groups that were immunized with saponified oil adjuvant vaccines, inflammation was observed at the injection site (yellow) throughout 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 the inoculation site than do viral oil-adjuvanted vaccines (25). Belloc, Laurent (21) also stated that Montanide ISA 774 is an adjuvant including both mineral and non-mineral oils caused fewer local responses in laying chickens, compared to other tested adjuvants (Montanide ISA 70, Montanide IMS 1112, and W/O emulsion prepared with Tween/span). Furthermore, Belgian researchers' studies have shown that formalin-killed oil-adjuvanted *Pasteurella* vaccine led to local tissue irritation, as well as lesions 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, and the surfactant quality (21).

Overall, the results of this study showed that oil-based adjuvant vaccines (ISA 70- and ISA 71- containing vaccines) induced high levels of immune responses, compared to alum-based adjuvant vaccines (aluminum hydroxide adjuvanted vaccine and commercial vaccine). In addition, only the oil adjuvanted vaccines could induce 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 good alternatives to alum-based adjuvant vaccines.

Authors' Contribution

Study concept and design: R. Gh.
Acquisition of data: M. Gh.
Analysis and interpretation of data: D. Gh.
Drafting of the manuscript: M. M.
Critical revision of the manuscript for important intellectual content: A. R. J.
Statistical analysis: R. Gh.
Administrative, technical, and material support: R. Gh.

Ethics

All the procedures were approved by the Ethics Committee at the Research Deputy of Shahid Chamran University of Ahvaz, Ahvaz, Iran. Under the project number (95471133).

Conflict of Interest

The authors declare that they have no conflict of interest.

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