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Original Article

Evaluation of Immunogenicity of *Clostridium perfringens* Type B Toxoid and Inactivated FMD (O) Virus with (ISA70-MF59)Adjuvant

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Abstract

Foot and mouth disease (FMD) and enterotoxemia are important diseases of hoofed animals. Vaccination against livestock pathogens, especially these two diseases, plays a key role in the prevention and control of these diseases. The use of combined vaccines with the aim of creating a better immune response and producing cheaper vaccines is a great contribution to Vaccine industry. This research aimed to compare the immunogenicity of FMD (O) and *Clostridium perfringens* type B toxoid along with adjuvant (MF59) and Montanide (ISA70) to create the best immunogenicity. To investigate the immune responses of vaccines, it was injected into an animal model, and the antibody titer was measured by enzyme-linked immunosorbent assay (ELISA) test and VN antibody titer. The results showed that the formulation with MF59 adjuvant brought more stable immunogenicity against FMD and *Clostridium perfringens type* B, and the length of the immunogenicity period also increased significantly. Therefore, the combined vaccine (*Clostridium perfringens* + FMD) could play a major role invaccine industry as an alternative vaccine against *Clostridium perfringens* and FMD in livestock.

Keywords: *Clostridium perfringens*, Combination vaccine, FMD, ISA70, MF59

1. Introduction

In recent years, a lot of research has been done to produce vaccines. However, vaccine development requires a combination of multiple strategies, such as adjuvant investigation. Adjuvants are compounds that enhance the immune response to antigens in vaccines. Among the factors included in the selection of adjuvant are: animal species, pathogen type, vaccine antigen, route and type of immunogenicity (1). Oil-based adjuvants (e.g., W/O, O/W, and W/O/W) are used directly in formulations as powerful adjuvants that provide long-term safety (2). Selecting the right combination of antigens and adjuvants in formula and morestability is the aim of the study. Vaccination program uses the foot and mouth disease (FMD) virus and *clostridium perfringens* bacterial vaccine to control laboratory diseases; however, frequent vaccination causes stress in animals or increases the cost for the farmer. The present study aimed to prepare a combined vaccine of *Clostridium*

perfringens type B toxoid and inactivated FMD virus serotype (O).

Foot and mouth disease is characterized by fever and vesicles in the mouth, breast, and feet, which is highly contagious in livestock. This disease is caused by economic losses from increasing milk and meat production. Usually animals grow, but it usually causes high mortality of young animals. It is a non-enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Aphthovirus* of the *Picornaviridæ* family. It includes seven serotypes (O-A-C-Asia1- SAT1, 2, 3). The capsid virus contains 60 copies of 4 building structures (vp_1, vp_2, vp_3, vp_4) which is considered the main factor of immunogenesis and is capable of inducing neutralizing and protective antibodies against the disease (3, 4). Vaccines available since the early 1900s have helped to eradicate FMD in parts of the world. However, the disease still affects millions of animals worldwide and is a major obstacle to trade in animals and their products. Although current available vaccines, which are administered intramuscularly to animals, are serotype- and subtype-specific in the short term, they fail to provide life-long protective protection (5).

Clostridium perfringens is an anaerobic gram-positive bacterium. Today, more than 200 species of *Clostridium* have been identified that live in nature as well as in the digestive system of humans and animals (which is pathogenic for humans and animals in about 30 species). These bacteria are normally found in the soil and in the digestive tract of healthy sheep and goats as a part of the natural microflora. One of the most important causes of intestinal disease in domestic animals is that it causes inflammation, bleeding, and necrosis of the intestines of sheep, cattle, pigs, goats, and chickens (6). Bacterial exotoxins are closely related to their virulence. Bacterial toxins are divided into five main groups and are named (alpha, beta, epsilon, and iota) and have several extracellular toxins as well. Two other main toxins (enterotoxin and beta-2) can also be produced by all types of Clostridium perfringens (2).

Clostridium perfringens type B has been reported most frequently in lambs, occasionally in calves, and rarely in foals. It occurs mostly in winter and spring and is rarely seen in summer. In soil contaminated by feces, bacteria can survive for months. The disease usually affects infant ruminants (normally less than two weeks old) and causes hemorrhagic mucosal enteritis, which leads to death. Babies die within one to four days or even a few hours (7).

2. Materials and Methods

2.1. Cultivation of *Clostridium perfringens* Type B Bacteria and Preparation of Toxin

(It was performed based on the standard operating producers (SOPs) of the Anaerobic Department of Razi Serum Institute (RVSRI) and Pharmacopoeia standard).

The vaccine strains of *Clostridium perfringens* type B (CN 228) were obtained from the Anaerobic Department of Razi Serum Institute (RVSRI) (8).

The toxin generation medium for type B contains:

1-peptone (4%)

2-Na_2HPO_4 (0.5%)

3-Maltose (1%)

4-Cysteine hydrochloride (0.0.5%)

Final pH = 7.2

In the continuation of bacterial culture, one liter of the culture medium was centrifuged (8000 g=20 min). Then, the supernatant solution was removed, and the production of toxin (Beta) was calculated by calculating the minimum lethal dose (MLD) according to the approved and reviewed standard SOP (RVSRI, ANB.0024.sop). Using the Bradford (9) technique, the protein content of toxins was measured.

Isolation of beta toxin was carried out in several steps.

2.2. Protein Purification

After culturing the bacteria, one liter of the culture medium was centrifuged (8000 g=20 min) to remove the cell debris. Then, the top solution of each type was removed, 50% ammonium sulfate was added to it during two steps and stirred at 4°C for 4 h, a precipitate was obtained. The resulting precipitates were collected together with a buffer (pH=6 -7)Tris-HCl (7).

Next, dialysis was performed at a temperature of 4°C (MWCO 10 KDa, D9402, Sigma). Then, they were

passed through a 0.45 filter and loaded on a G-50 sepharos (sigma) column. The output of the column was collected, and in the next step, polyacrylamide gel electrophoresis (PAGE-SDS) was used to trace beta toxin in the samples [Figure 1, SDS-PAGE was performed using the method of Laemmli (1970)]. The optical absorbance of the fractions was measured at 280 nm. Toxins with higher purity (maximum optical absorption) were selected and combined.

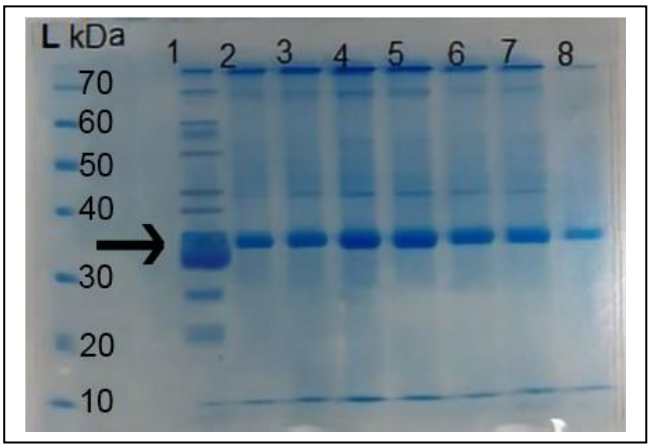

Figure 1. Polyacrylamide gel electrophoresis (SDS-PAGE) on some fractions after Sephadex (G-50) gel column chromatography. L: protein size indicator. Number 1: culture supernatant containing beta toxin, numbers 2-8: fractions after Sephadex G-50 gel show beta toxin

Then, the concentration of toxins in this neighborhood was calculated using the Bradford (9) method.

Finally, the MLD was calculated (BALB/c mice 2±2 g, 6-8 weeks) (7, 10).

2.3. Toxoid Preparation

By adding formaldehyde for 14 days at a temperature of 37°C, we turn the desired toxins into toxoids. Then, they were kept at -20°C until the final formulation (4).

2.4. Virus Antigen Preparation

The virus (O2016/IR) was propagated on BHK21 and incubated for 24 h at 37°C and 5% CO_2 atmosphere. The resulting culture was centrifuged and then concentrated by 6000 polyethylene glycol 8% (11). To determine the titer, the virus concentration was measured by the TCID50 method (12). In the next step, to inactivate the virus, 4mμ Binary Ethyleneimine (BEI) was added per liter at a temperature of 30°C for 30 h, and to neutralize and remove the remaining BEI, 2 mμ (sodium thiosulfate) was added per liter (3). In the end, 2.7 x 10^7 TCID50 inactive virus (O2016/IR) was used for each vaccine dose.

2.5. Vaccine Formulation

According to the standard operating producers) SOPs¬) from from Razi Vaccine and Serum Research Institute (RVSRI)

(Clostridium perfringens type B toxoid) and FMD antigen are added to the desired adjuvants (5, 10). The formulation of the antigens was based on a commercial vaccine dose (13).

2 ml of combined vaccine including ISA70 adjuvant (Seppic France)

Group A: 70% Montanide ISA70 adjuvant (seppic France) + *Clostridium perfringens* type B toxoid + PBS

Group B: 70% Montanide ISA70 adjuvant (seppic France) + FMD vaccine + PBS

Group C: 70% Montanide ISA70 adjuvant (seppic France) + *Clostridium perfringens* type B toxoid + FMD PBS+ vaccine

The adjuvant was added to the aqueous phase (vaccine) at a ratio of 70:30 (w/w), stirred for one hour at room temperature (RT) at a speed of 4,200 rpm, and then kept at 4°C for 24 h (Seppic, France).

2 ml of combined vaccine including adjuvant (Seppic France) MF59

Group D: 50% MF59 adjuvant (Seppic France) + *Clostridium perfringens* type B toxoid + PBS

Group E: 50% MF59 adjuvant (Seppic France) + FMD vaccine + PBS

Group F: 50% MF59 adjuvant (Seppic France) + *Clostridium perfringens* type B toxoid + FMD vaccine + PBS

The adjuvant was added to the aqueous phase (vaccine) at a ratio of 50:50 (w/w) and stirred for one hour at 20°C with a speed of 3,000 rpm and then kept in a refrigerator (Seppic, France).

Group G: *Clostridium perfringens* type (B) toxoid vaccine

Group H: commercial vaccine (bacterin toxoid) positive control

Group I: commercial foot-and-mouth disease (FMD) vaccine, positive control

Group J: the group that was not vaccinated (negative control)

2.6. Grouping and Injection to Sheep

The 40 sheep (6 months old) were divided into ten experimental groups (4 sheep in each group). These sheep have never been infected or vaccinated against FMD virus and enterotoxemia (*Clostridium perfringens*). After vaccination, the sheep were examined for one year. Vaccination of sheep in categorized groups (A-J) was done as subcutaneous injection SC, and the second injection of vaccine was done after 14 days.

2.6.1. Investigation of the Humoral Immune Response

To check the antibody titer, blood was taken from sheep for one year on the designated days. To separate the serum, the blood samples were incubated at 4-8°C for 4-5 h and then centrifuged (2,500 rpm, 20 min, 4°C) (3).

2.7. Indirect Enzyme-Linked Immunosorbent Assay

Dilutions of the toxin were prepared with carbonate buffer, and poured into the wells of 96-well microplates and incubated overnight in the refrigerator. Next, washing was done with a washing solution (PBS 1X and Tween 20). Then, it was blocked with a solution (1% BSA) for 30 min. Antisera were diluted with a diluent solution. Around 100 microliters of serum samples was poured into the wells and incubated for 60 min. The second stage of washing was done. The conjugate was added and incubated for 1 h. The plate was washed and the3,3',5,5'-Tetramethylbenzidine (TMB) was poured into the wells, and was placed in the dark for 15 min. In the end, we added 1 M hydrochloric acid and immediately read the optical density (OD) with an enzyme-linked immunosorbent assay (ELISA) Reader device at a wavelength of 450 nm and analyzed the results (14).

2.8. Serum Neutralization Test

animal serum samples were placed in a water bath at 56°C for 30 min to perform the serum neutralization test (SN) for the purpose of checking the viral antibody titer (OIE 2017).The titer of neutralizing antibodies against FMD-O2016/IR was measured by Microneutralization test (3, 15, 16).

3. Results

3.1. Results of Immunogenicity against Foot and Mouth Disease Virus Serotype (O)

The logarithmic titer of the antibody response of the sera was calculated by the SN Test and the formula (Read and Monarch). All vaccinated groups had a protective titer. A significant difference was observed between the antibody titer in the groups that received the combined vaccine (FMD and *Clostridium perfringens type B* toxoid) compared to the monovalent vaccine ($P<0.05$).

The foot and mouth antibody titer for groups vaccinated with MF59 reached its highest level on day 75 and had a good protective titer for up to 180 days. The groups vaccinated with ISA70 also have good titers compared to the commercial vaccine. These results showed that during the period of immunogenicity, there was a significant difference in FMD and toxoid vaccine with MF59 adjuvant, and it had higher immunogenicity than other groups [$P<0.05$; Figure 2].

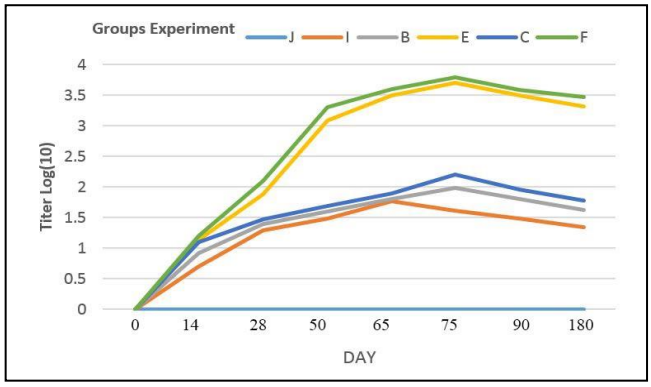

Figure 2. Protective antibody titer against foot-and-mouth disease serotype O (cut-off >1.2)
J (control-), I (commercial vaccine, control+), B (FMD+ ISA70), E (FMD+ MF59). C (Toxoid +FMD+ ISA70), F (Toxoid+FMD+MF59)

3.2. Results of Humoral Immunity against Beta Toxin

The antibodies against beta toxins gradually increased in all immunized groups A significant difference was found between the antibody titers in the groups that received the combined vaccine (FMD and *Clostridium perfringens type B* toxoid) compared to the monovalent vaccine ($P<0.05$).

During the period, the Beta antibody titer in the groups vaccinated with MF59 increased significantly compared to the ISA70 group, which indicates the high efficiency of MF59 adjuvant compared to ISA70 for Beta toxin ($P<0.05$). Comparing the results of the vaccine formulated with ISA70 adjuvant compared to commercial vaccines shows that vaccines formulated with ISA70 are more immunogenic ($P<0.05$). Group G vaccine had lower titer and shorter immunogenicity compared to other groups, and the results indicated that the toxoid vaccine needs adjuvant for higher immunogenicity (Figure 3).

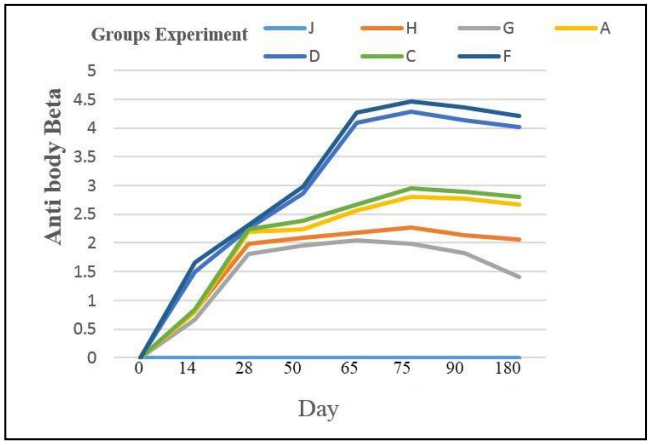

Figure 3. Beta antibody titers (on sampling days) against *Clostridium perfringens type (B)*
J (control-), H (commercial vaccine control+), G (Toxoid vaccine), A (Toxoid+ ISA70), D (Toxoid+MF59),
C (Toxoid +FMD+ISA70), F (Toxoid+FMD+MF59)

4. Discussion

Vaccination against livestock diseases plays an important role in preventing and controlling diseases and economic losses to livestock farmers and the country. Most vaccines are not able to stimulate efficient cellular responses (17). The present study was conducted to develop multiple vaccines (enterotoxemia and foot-and-mouth disease) to increase immunity, control diseases, prevent economic losses, and reduce the frequency of injections to reduce stress in livestock.

The development of vaccines depends on the type of adjuvants and its formulation that can not only stimulate strong humoral responses against a specific pathogen, but also be able to induce a long-lasting and effective immunity to compete with antigens and also with minimal or no adverse effects. There are different types of adjuvants with different functions. Although aluminum salts stimulate Th2 immune cells and can lead to increased production of antigen-specific antibodies, they are unable to stimulate strong Th1 or cytotoxic responses (18).

To overcome such limitations, adjuvant (MF59) has been used to enhance antigen-specific immune responses more and faster. The MF59 is the first oil-in-water emulsion adjuvant licensed for human vaccines after alum and can enhance vaccine immune responses through multiple mechanisms (19).

This adjuvant can lead to an increase not only in humoral responses, but also in cellular immune responses. This leads to the improvement of vaccine efficiency against intracellular pathogens. Vaccination programs using FMD viral and *Clostridium perfringens* bacterial vaccines effectively controlled the disease; however, frequent vaccinations cause stress in animals or increase costs for the rancher.

Therefore, the present study aimed to develop a combined FMD enterotoxemia vaccine using an inactivated FMD vaccine and enterotoxemia toxoid.)

In examining the results of ELISA and VNT of sheep vaccinated with ISA70 and MF59, this study revealed that although the compatibility of the combination of immunogens did not show any interference in immunogenicity between FMD antigens and *Clostridium perfringens* type B toxoid, it created the long-term and stable immune response. In addition, the formulation with MF59 oil adjuvant creates a stronger

and longer response against bacteria and viruses, and the length of the immunogenicity period also increases. The group that had the combined vaccine with MF59 adjuvant produced the highest antibody titer, and the immunogenicity of the vaccine was higher than the other groups. The immunogenicity of ISA70 adjuvant was acceptable according to the standard of pharmacopoeia and the method recommended by the World Organization for Animal Health (OIE) manual.

In 2016, by examining the influenza vaccine with adjuvant MF59 and alum in the mouse animal model, it was stated that MF59 can more effectively induce antibody production, and these adjuvants induce humoral immunity and Th2 cytokine production in viral infection (20).

In 1993, by evaluating the inactivated Newcastle virus with complete and incomplete Freund's adjuvants (IFA) and ISA70 and aluminum phosphate gel, which was investigated for 16 weeks in chickens, the ISA70 antibody titer peaked after two weeks after the injection. It was found that the effectiveness of ISA70 on antigen (NDV) was similar to IFA (21).

Tariq, Anjum (21) announced in 2021 that the results of Bacterin Toxoid on ISA70 oily adjuvant base had a higher serum titer compared to Alum, and 100% protective effect was observed in the challenge test. Moreover, from the examination of injection of *leishmaniasis* protein vaccine with ISA70 subcutaneously, it was showb that this vaccine induced the proliferation of lymphocytes (21).

Using combination vaccines instead of monovalent vaccines to improve vaccine quality along with economic and time savings can be a suitable method for the production of enterotoxemia toxoid and FMD vaccines in the industrial production phase and in high volume. This type of combination vaccine reduces the need for repeat vaccination. In this research, efficacy of enterotoxemia and FMD vaccine was incorporated with different immunogenic formulations. The results of our investigation also showed that the combined immunogenicity of the vaccine (*Clostridium perfringens* toxoid and FM) with MF59 adjuvant was higher than ISA70.

With the findings of this study, it can be concluded that the knowledge obtained from this research can effectively improve the production of these types of vaccines. Vaccines have an export value and are suitable tools for earning foreign exchange income of the country, because they can create a suitable immunity against other patients.

Authors' Contribution

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

Ethics

All ethical procedures were approved by the ethics committee of the Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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