Original Article

Compared to Aluminum Hydroxide Adjuvant, Montanide ISA 206 VG Induces a Higher and More Durable Neutralizing Antibody Response against FMDV in Goats

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ABSTRACT

Foot-and-mouth disease (FMD) has a high prevalence in cloven-hoofed animals. It is also highly contagious and remains a serious threat to livestock worldwide. Despite the widespread vaccination program in Iran, outbreaks of FMD continue to occur. Vaccination is one of the most effective methods of preventing FMD. The vaccines used in Iran are of the inactivated type and contain several serotypes. Since inactivated vaccines without adjuvants do not induce a high and durable antibody response, it is necessary to use adjuvants. Montanide ISA 206 VG is a mineral oil-based adjuvant that produces a water-in-oil-in-water (w:o:w) emulsion in vaccine preparations. However, a large number of manufacturers in Iran and around the world still use alum adjuvant (with or without saponin) to produce the FMD vaccine. This study used Montanide ISA 206 and alum adjuvants to administer the O2010 serotype of the FMD virus to goats. A total of six goats were divided randomly into three groups. Vaccines were administered subcutaneously twice, at a one-month interval. Blood sampling was done at different times, and the micro-neutralization method was used to measure the neutralizing antibody titer in each serum. Seven days after the second vaccination, the alum group's antibody titer was higher but not statistically significant. However, from the 28th day after the second injection until the end of the study, the Montanide ISA 206 group's antibody titer was significantly higher than that of the alum group. Six months after the second injection, the antibody titer in the ISA 206 group remained at the peak level, while in the alum group, it decreased and reached the minimum protective level. Nine months after the second injection, the antibody titer remained at its peak level in the ISA 206 group, whereas it dropped significantly in the alum group. Based on the findings, ISA 206 VG is capable of generating long-term humoral immunity in goats against the FMD serotype O2010 and could replace aluminum hydroxide adjuvants in FMD vaccine preparations.

Keywords: Foot and Mouth Disease; Montanide ISA 206 VG; Aluminium Hydroxide; O2010 serotype

1. Introduction

Foot-and-mouth disease (FMD) is highly prevalent in cloven-hoofed animals. As an aphthovirus of the Picornaviridae family, the FMD virus causes FMD (1), a highly contagious disease and a serious threat to livestock worldwide. There are seven distinct FMD serotypes, including A, O, Asia1, C, and South African Area Types 1, 2, and 3, as well as several (sub)lineages. The World Reference Laboratory (WRL) first reported FMD in Iran in 1956 as a serotype O virus (1). The Asia1 and A serotypes were isolated in 1956 and 1960, respectively (WRLFMD, Iran, 1956-1960) (2). Despite the widespread vaccination program in Iran, outbreaks of FMD continue to occur (3).

Vaccination is one of the most effective methods for preventing FMD. In Iran, vaccines are of the inactivated type and contain several serotypes (4). In traditional inactivated vaccines, the virus is produced in suspension cultures of a Baby Hamster Kidney (BHK) cell line and is then inactivated (5). Since inactivated vaccines without adjuvants have low immunogenicity, adjuvants are essential to induce a higher and longer antibody response and reduce the number of immunizations needed to initiate a protective immune response. Consequently, vaccine availability increases and vaccinations are cost-effective (6). The effectiveness of the FMD vaccine largely depends on the choice of an appropriate adjuvant.

Inactivated FMD vaccines are usually formulated with aluminum hydroxide (Al[OH]3) or mineral oil-based adjuvants. Aluminum hydroxide and mineral oil adjuvants are the most widely used adjuvants in FMD vaccines (7). However, vaccine formulations containing aluminum hydroxide and crude saponin as adjuvants have toxic effects and short-lived antibody responses.

One of the characteristics of adjuvants based on mineral oil is their ability to create depot formation at the injection site, which causes a slow release of antigens (8). Montanide ISA 206 VG is a mineral oil-based adjuvant manufactured by SEPPIC (France) that contains anhydro mannitol and octadecenoic acid esters. A water-in-oil-in-water (w:o:w) emulsion is produced by this adjuvant, and in many Asian and South American countries, it is commonly used to formulate FMD vaccines (8).

A large number of manufacturers in Iran and around the world still use alum adjuvant (with or without saponin) to produce the FMD vaccine. Although this type of vaccine causes a relatively rapid increase in the antibody titer, the antibody titer usually drops within a few months, necessitating a booster dose after 4-6 months. Several studies have demonstrated that oil adjuvants produce stronger and longer immune responses than Al(OH)3 adjuvants (8). Besides affecting the level of the immune response, oil adjuvants also direct the immune system along different pathways. When oil-in-water adjuvants are used, the immune system is generally directed toward Th1, whereas alum adjuvants direct it toward Th2 (9). Furthermore, mineral oil-based adjuvants generally produce a more durable immune response than aluminum adjuvants (10).

Studies examining the effectiveness of the FMD vaccine are generally conducted on cattle. Since every animal has different physiological and immune characteristics, it is imperative to test each vaccine on different target animals to determine its effectiveness (11).

This study aimed to investigate the effect of two different commercial adjuvants on the amount and longevity of the neutralizing antibody titer against the FMD serotype O2010 in goats. This study used Montanide ISA 206 VG and alum adjuvants to administer the O2010 FMD vaccine to goats. The immune response of goats was assessed by measuring the neutralizing antibodies generated by the Virus Neutralization Test (VNT) for up to nine months.

2. Materials and Methods

2.1. Preparation of Vaccine

The cell cultures of BHK21 were used to propagate the virus, both in monolayers and suspensions. It was

then harvested and centrifuged to remove all cell debris. TCID50 was used to determine the virus concentration titer, followed by inactivation with Binary Ethyleneimine (BEI) at 4 mM w/v for 30 h at 30°C. Sodium thiosulfate (2 mM) was added to neutralize and remove the residues of BEI (12). The sterility test was conducted by inoculating a small amount of inoculum into bacteriological media and observing whether or not any kind of bacteria could be identified. It was confirmed through the use of BHK-21 cell culture that the virus had been completely inactivated (13). In the final step, 6.4×10^6 TCID50/ml of the inactivated FMD virus serotype O2010/IR was utilized as the antigen payload per dose. As a result, vaccines were prepared with ISA 206 VG as follows: in formulation 1, inactivated monovalent (O2010) FMD vaccine was mixed with ISA 206 VG (SEPPIC, France) adjuvant, and in formulation 2, it was mixed with 2.5% aluminum hydroxide gel. In these two formulations, the adjuvant was formulated with the aqueous vaccine in a ratio of 50:50.

2.2. Immunization of the Animals

A total of six goats, aged 1-2 years, were kept in the experimental room of the animal facility of the Razi Vaccine and Serum Research Institute. The goats had not received any FMD vaccines before the study. During the study, no symptoms of FMD infection were observed in any of the goats. We randomly divided the goats into three groups. Vaccines were administered subcutaneously twice at a one-month interval to goats in each group using individual syringes in the middle of the cervical area with 1 ml of one of the formulated vaccines. ISA 206 VG was administered to goats in Group 1, and alum was administered to goats in Group 2. Goats in Group 3 were left unvaccinated as the control group.

2.3. Blood Sample Collection

The neutralizing antibodies of vaccinated and non-vaccinated goats were measured at the beginning of the study, four weeks after the first vaccination, as well as one week, four weeks, six months, and nine months after the second vaccination. A sample of approximately 5 ml of blood was collected from the jugular vein of all experimentally immunized and control subjects.

2.4. Virus Neutralization Test

In this study, the micro-neutralization method was used to measure the neutralizing antibody titer in each serum (14). A thermal inactivation was performed on the serum at 56°C for 30 min. We prepared serial dilutions of samples according to a two-fold dilution method. The diluted serum was incubated with 100 TCID50 of the virus for 1 h. IB-RS-2 cells were added to each 96-well plate. The cytopathic effect (CPE) was determined microscopically and calorimetrically after 72 h at 37°C in 5% CO2. Using Karber's (1931) method (15), serum titers were calculated at 50%. Based on the reciprocal of the serum dilution that neutralized 100 TCID50 of the virus in 50% of the wells, antibody titers were calculated as log10 of the reciprocal of the final serum dilution (15)(١٤) .

2.5. Statistical Analysis

Data from the VNT results were recorded. Data analysis was performed using the SPSS software (version 25). To determine the statistical significance between the adjuvant formulation and its immune response, an analysis of variance (ANOVA) was performed. Tukey's post-hoc test was also used for pairwise comparison of the mean antibody titer at different times. A *P*-value of 0.05 was used to express statistical significance at a 95% confidence interval.

3. Results

According to the sterility test conducted on the vaccine formulations, the vaccines were free from the presence of aerobic and anaerobic bacteria or fungal and mycoplasma contaminants. To confirm complete virus inactivation, we assessed the CPE absence in BHK-21 cells. Consequently, the vaccine formulations were considered safe for animal experimentation following the OIE requirements (13).
The amount of neutralizing antibody titer in VNT was measured in the studied goats before and four weeks

after the first injection, as well as one week, four weeks, six months, and nine months after the second injection (Figure 1). It is important to note that the goats had no history of FMD vaccination, and the antibody titer before the start of vaccination also indicated no history of previous vaccinations or FMD (Figure 1). The neutralizing antibody titer after the antigen injection with ISA 206 VG had an upward trend until the fourth week, but from the fourth week onward, it remained almost constant. After the antigen injection with aluminum hydroxide, the antibody titer increased until the fourth week but then declined (Figure 2). The amount of the neutralizing antibody titer one week after the second injection showed that the antibody induction in the alum group was faster than in the group injected with Montanide ISA 206 VG adjuvant. However, the difference was not significant. The induction of antibody titer was faster in goats injected with the alum adjuvant than in those of the ISA 206 VG group (Figure 2). The neutralizing antibody titer was also checked before the second injection (four weeks after the first injection) in both groups. The statistical analysis of the antibody titer in the two groups did not show a significant difference (Table 1). However, the average antibody titer in the ISA 206 VG group was slightly higher than in the alum group.

Tukey's post-hoc test was used for pairwise comparison of the average antibody titer at different times. Based on the results before the second injection, there was no significant difference between the ISA 206 VG and the alum groups in the average antibody titer. However, the average antibody titer in the negative control group was significantly different from that in the ISA 206 VG and the alum groups during the study. One week after the second injection,

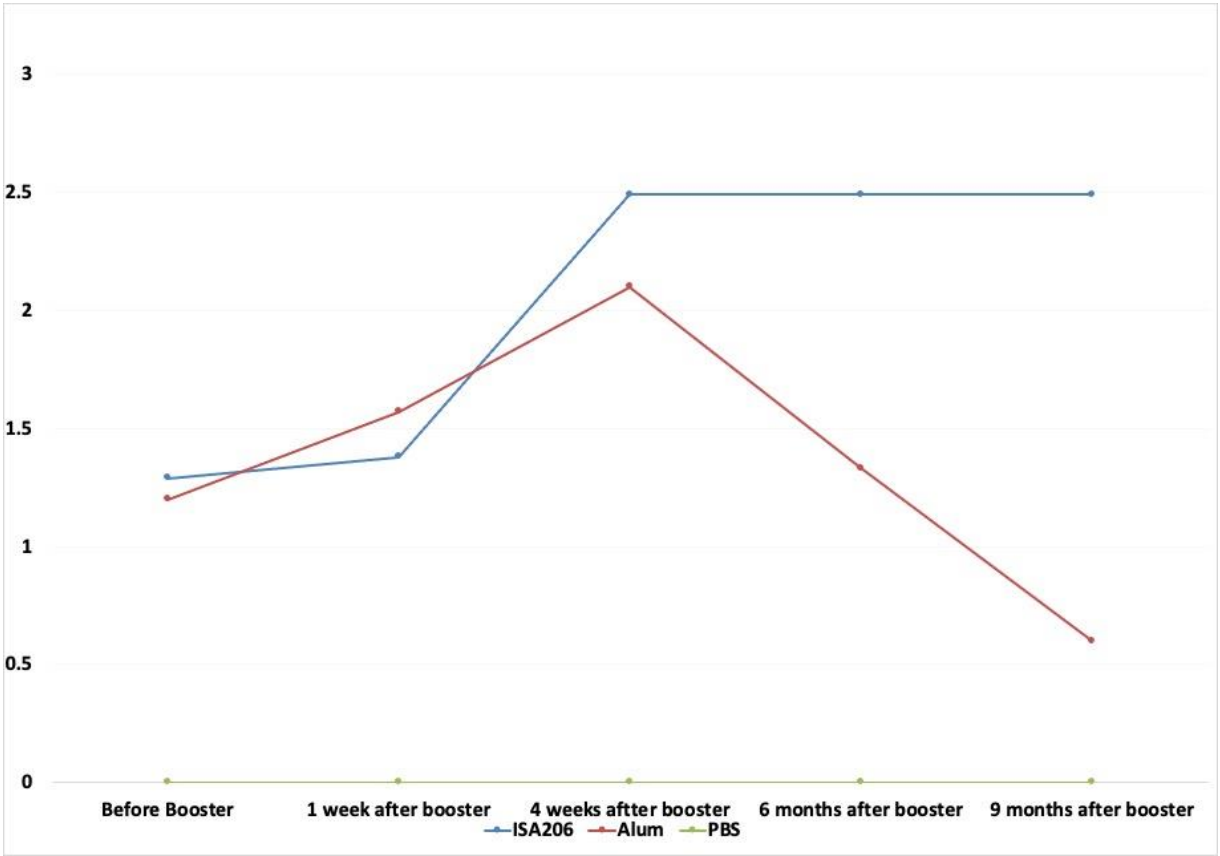

Figure 1. Linear diagram of the geometric mean of the antibody titer (Log10)

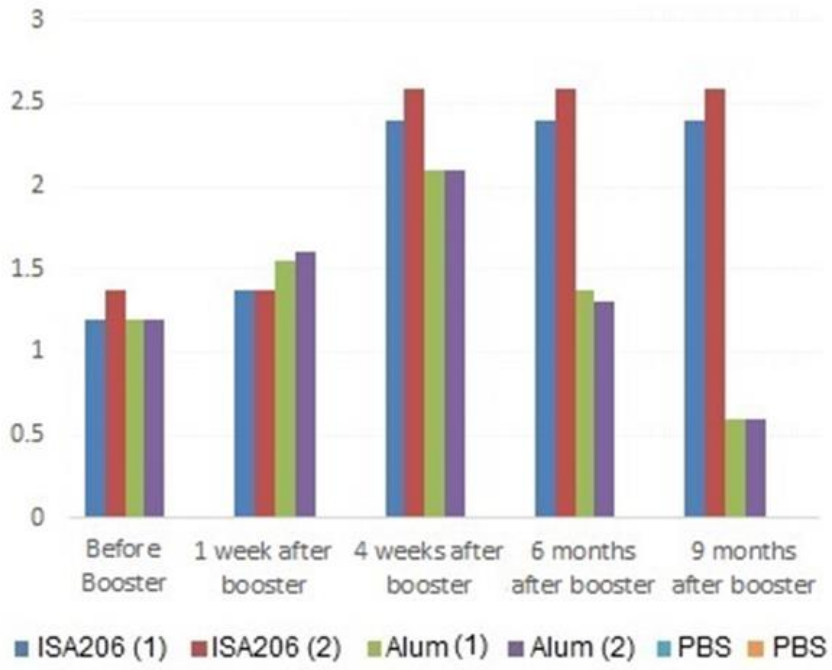

Figure 2. Antibody titer of individual animals at different sampling time

the average antibody titer did not differ significantly between the ISA 206 VG group and the alum group. However, four weeks after the second injection, there was a significant difference in the average antibody titer between the ISA 206 VG group and the alum group. Six and nine months after the injection, similar results were obtained to four weeks after the second injection (Table 1).

The examination of the neutralizing antibody titer before the second injection (four weeks after the first injection) showed no significant difference between the two groups, although the average antibody titer was slightly higher in the ISA 206 VG group than in the alum group. The amount of antibody titer in the group injected with alum on the seventh day after the second injection was higher than that in the ISA 206 VG group, but the difference was not significant (P>0.5). However, the amount of antibody titer in the group injected with Montanide ISA 206 VG on the 28th day after the second injection was significantly higher than that of the alum group (Table 1). Four weeks after the second injection, the neutralizing antibody titer reached its peak in all four goats in the two experimental groups. However, the peak antibody titer was significantly higher in goats in the ISA 206 VG group than in the alum group. Both groups had a significantly higher titer than the control group (Table 1). The amount of antibody titer six months after the

Table 1. Statistical analysis of antibody response of goats against FMDV serotype O2010 between groups vaccinated with ISA 206 VG or Alum

Sampling time	Mean Difference	Std. Error	Sig.
Before 2nd vaccination	0.09	0.07	0.518
One week after 2nd vaccination	0.19	0.19	0.621
Four weeks after 2nd vaccination	0.39	0.07	0.026
6 months after 2nd vaccination	1.15	0.21	0.025
9 months after 2nd vaccination	1.89	0.07	00.00

second injection in the ISA 206 VG group remained at the level of the peak antibody titer recorded four weeks after the second injection. However, in the group injected with alum, the amount of antibody titer decreased within six months after the second injection, and the amount of neutralizing antibody titer after six months was at the level of protective titer (1/16). Nine months after the second injection, the amount of antibody titer remained at the peak titer level in the ISA 206 VG group, while it dropped significantly in the alum group (Figure 1).

4. Discussion

In endemic areas, commercial and common FMD vaccinations with alum adjuvant result in short-term immunity in cattle, requiring a booster dose every 4-6 months to ensure adequate protection (7). A variety of adjuvants are used to enhance the effectiveness of FMD vaccination (7, 16). Different studies have shown that Montanide ISA 206 VG oil-emulsified FMD vaccines induce long-lasting immunity in animals (17).

Since neutralizing antibodies are the most important factor in the immune response against FMD, measuring them is crucial in determining the protective immune response (18). The points mentioned indicate that an adjuvant must be able to rapidly create a high level of specific neutralizing antibodies against its accompanying antigen for a long time to be effective. The results of numerous studies conducted in this field have demonstrated that oil adjuvants can create a rapid and long-lasting antibody response (16).

In this study, Montanide ISA 206 VG and alum adjuvants were used to administer O2010 FMD vaccines to goats with a nine-month follow-up of antibody titers. A peak level of antibody titer was observed in the group injected with Montanide ISA 206 VG adjuvant, which was higher than the required minimum protection level. Around six months after injecting alum, the antibody titer dropped below the level needed to protect goats. It is generally believed that oil adjuvants cause side effects, such as hemolysis, swelling, and necrosis at the injection site (19). Aside from transient local swelling, no clinical or pathological signs were observed in animals injected with the virus and Montanide ISA 206 VG adjuvant.

Various studies have shown that aluminum hydroxide combined with various antigens shifts the immune response toward Th2, which results in a stronger production of antibodies and weaker cellular immunity, compared to the Th1 pathway. The Th2 pathway also produces a high level of IgE (20). Saponin adjuvants were combined with aluminum hydroxide as a means of compensating for the lack of cellular immunity in various studies (21). The humoral immunity induced by FMD is short-lived but is rapidly induced even with viruses that have been killed and emulsified with adjuvants (22).

Prior to injections, neutralizing antibodies were measured to determine the titer. It was then examined before the second injection, as well as one week, four weeks, six months, and nine months after the second injection. Before the second injection, there was no significant difference in antibody titers between the two groups. ISA 206 VG, however, had a slightly higher antibody titer than the alum. According to the findings, antibody titers were higher when ISA 206 VG was used rather than alum. At 28 days after the second injection, the antibody titer of the Montanide ISA 206 VG group was significantly higher than that of the alum group. All four goats in both groups reached their peak neutralizing antibody titer four weeks after the second injection, but the ISA 206 VG goats had significantly higher peaks of antibody titers than the alum goats. In both groups, the titer was significantly higher than in the control group.

The obtained results showed that in the aluminum hydroxide adjuvant group, the antibody titer against the FMD virus developed faster than the antibody titer

in the Montanide ISA 206 VG group, and the antibody titer in the Montanide ISA 206 VG group reached its peak value within four weeks. Approximately four weeks after the second injection, the peak antibody titer in the Montanide ISA 206 VG group was significantly higher than that in the alum group. After six months, the antibody titer was approximately at the protective level in the alum group but dropped below the protective level (1/16) after nine months, indicating the need to administer a booster dose approximately six months after the second injection. Even nine months after the second injection, the amount of antibody titer in the Montanide ISA 206 VG group remained at its peak. The amount was much higher than what was required to protect the animal from FMD. This study demonstrated that Montanide ISA-201 (206) is effective in generating neutralizing antibodies against the FMD virus. Based on this study, the mean neutralizing antibody titers of the vaccinated goats and the time at which they reached their maximum were consistent with those found in other studies (23, 24).

As a result of these findings, Montanide ISA 206 VG is superior to alum-based adjuvants in terms of the amount and duration of the induced antibody titers in vaccinated animals (23, 24). As FMD virus serotypes differ greatly and protection from some serotypes is incomplete, new FMD virus serotypes are frequently discovered, and a vaccine may not be effective in the long term. Therefore, the selection of vaccine strains *in vivo* or *in vitro* is an essential step in ensuring that they are effective and appropriate. According to the results of this study, the amount and duration of the protective titers were higher in the vaccine composed of serotype O when used with Montanide ISA 206 VG oil adjuvant, compared to alum. It has been found that the inactive vaccine formulated with Montanide ISA 206 VG can control and reduce FMD more effectively than the inactive vaccine prepared with alum.

This study investigated the effect of using Montanide ISA 206 VG in the administration of O2010 FMD in goats. This study showed that complete FMD virus serotype O2010, along with alum and ISA 206 VG adjuvants, separately induced protective antibody titers in both groups. In both goats, the alum-adjuvant induced antibody titers faster one week after the second injection (Figure 2). The peak antibody titer was observed in both groups four weeks after the second injection. The antibody titer was significantly higher in goats administered with ISA 206 VG than in the alum group. In addition, the antibody titer in the ISA 206 VG group was still at its peak up to nine months after the second injection, which is higher than the amount necessary to protect. This study showed that ISA 206 VG adjuvant is a suitable adjuvant for the administration of the FMD virus and for use in a multivalent serotype FMD vaccine in goats. Since the neutralizing titer in goats in the ISA 206 VG group had high persistence, a booster injection is likely required after more than six months, which is cost-effective. To determine the amount of virus required to induce a suitable response in goats, along with the use of ISA 206 VG adjuvant, further research is recommended. Due to the high ability of the ISA 206 VG adjuvant in triggering an immune response, it is possible to achieve a high protective titer with acceptable durability with a lower amount of virus in combination with the ISA 206 VG adjuvant.

Authors' Contribution

Study concept and design: M. D. and M. T.

Acquisition of data: M. D. and SM. A.

Analysis and interpretation of data: M. D., SM. A., and M. T.

Drafting of the manuscript: M. D.

Critical revision of the manuscript: M. T and M. D.

Statistical analysis: M. D. and M. T.

Ethics

It is declared that all ethical considerations were taken into account in the preparation of the submitted manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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