Introduction

Foot-and-mouth disease (FMD) is one of the most important animal diseases in the world. Although there are several methods to combat the disease, the preferred method in endemic regions is vaccination. FMD vaccines are composed of inactivated whole virus particles and oil or alum adjuvants. Inactivated FMD virus (FMDV) antigen alone is a weak immunogen; as such, many adjuvant candidates have been investigated to improve the immune response. Nevertheless, only two adjuvant formulations are in use today: aluminium hydroxide gel \([\text{Al(OH)}_3]\) with saponin, and an oil-based adjuvant [1]. Alum vaccines result in a weaker immune response compared to oil adjuvant vaccines, especially in pigs [2] and have a tendency to result in maternal antibody interference [3]. Although alum-based vaccines are generally considered safe [4], granulomas sometimes arise when the subcutaneous route is used for delivery rather than...
intramuscular injection [5]. Another side effect of alum adjuvants are increased IgE production [4], which can result in allergenicity [6] and neurotoxicity [7].

Saponins are steroids or triterpenoid glycosides found in wild or cultured plants and bacteria. Triterpenoid saponins are found in many plants, including soybean, bean, pea, allium, tea, spinach, sunflower, and chestnut. Saponins have been used widely as adjuvants in many veterinary vaccines and are frequently used in combination with alum adjuvants in foot and mouth disease vaccines. The triterpenoid saponin Quil A, obtained from the barks of Quillaja saponaria tree grown naturally in the Andes Mountains of Peru, has been studied extensively due to its adjuvant activity [8]. Quil A is composed of more than 23 different saponins [9]. Although it is a natural extract of the Quillaja saponaria tree, it is toxic to humans. In addition to local reactions, severe hemolysis of erythrocytes can sometimes occur [10,11]. However, Quil A has long been used successfully in veterinary medicine [11].

Because few safe and effective adjuvants exist for human use, QS-21, one of the 23 saponins found in Quil A, has been the preferred adjuvant in many recent clinical human trials and vaccine studies, including those for cancers [12] as well as infectious and neurodegenerative diseases such as acquired immune deficiency syndrome [13], hepatitis B [14], and Alzheimer’s disease [15].

Saponins exhibit both specific and non-specific stimulatory immune effects, such as inflammation. The exact mechanism of this immune-stimulating effect is not clearly understood [16], but it is thought that saponin induces the production of cytokines, including interleukins and interferons, that invoke other immune system elements [9].

In general, oil-based vaccines provide longer immunity and elicit less interference from maternal antibodies and earlier protection in cattle and pigs [1,3]. The addition of saponin in the form of Quil A as an oil adjuvant significantly enhances immune responses to FMD vaccines [17].

The goal of this study was to investigate the effect of QS21 on humoral immunity via a water-in-oil-in-water (w/o/w) type emulsion of the Montanide ISA206 (Seppic/France) FMD vaccine. Cattle were used as the target animal to evaluate the serum virus neutralizing antibody response, which is the best indicator of the protection conferred. The results showed that a strong neutralizing antibody response was initiated at the first week after vaccination with QS-21, suggesting a safe alternative to Quil A.

Materials and Methods

Control vaccine (ISA206)
A commercial oil adjuvanted vaccine (Turvac oil 14/18) was produced at the FMD (SAP) Institute Ankara, Turkey and contained O TUR07 and ATUR11 strains and Montanide ISA206 as an oil adjuvant. In brief, the viruses used to generate the vaccine were propagated in BHK-21 suspended cell culture. Binary ethylene-imine was used for inactivation. Viruses were concentrated and semi-purified using polyethylene glycol, then were combined with Montanide ISA206 to formulate a double oil emulsion. It has been shown via animal challenge that the potency of the vaccine is 6PD50 for each antigen, according to the World Organisation for Animal Health (OIE) manual [18]. The cut-off values for the neutralizing antibody titres necessary for protection were pre-determined for each strain. The logarithmic values of the serum neutralization titres above 1.20 and 1.04 were considered protective for the OTUR07 and ATUR11 homologue viruses, respectively.

Vaccine containing QS-21 (ISA206+QS-21)
QS-21 was purchased from Dessert King International (San Diego, CA, USA) at >98% purity. QS-21 powder was added directly to the ready oil emulsion vaccine and mixed by gentle shaking to obtain 750 µg QS-21 per cattle dose (2 mL) as a final concentration. The formulation with QS-21 was freshly prepared on the day of immunization.

Cattle
Nine-month-old Holstein-Friesian FMD antibody seronegative calves were used in the study. Each group consisted of 6 animals. Two non-vaccinated animals were used as negative controls. The animals were kept in closed containments during the study.

Immunization and sampling
Two milliliter vaccines were administered via the deep intramuscular route on the necks of animals.

Blood samples were collected on days 0, 1, 3, 8, 14, 28, 45, 60, and 90. The animals were monitored every day for body temperature, local temperature, lesions, and appetite. Sera samples were stored at -20°C until tests were performed.

Serological assays

Virus neutralization test
A virus neutralization test was performed according to the
OIE manual [18]. Briefly, sera were heat inactivated in a water bath. Two-fold serial dilutions of sera samples from 1:4 to 1:512 were prepared. The diluted sera samples were then incubated with 100 TCID<sub>50</sub> homolog virus for 1 hour at 37°C. After one hour, the BHK-21 cell suspension was added to all wells. Forty-eight hours later, the endpoint titres were determined using the results of the cytopathic effect formation.

**Liquid phase blocking enzyme-linked immunosorbent assay**

The FMDV specific total antibody response was determined by liquid phase blocking enzyme-linked immunosorbent assay (ELISA). The polyclonal antibodies used in the test were produced in-house against the OTUR07 and ATUR11 vaccine strains. The test was performed as described by Hamblin et al. [19]. Briefly, 96-well microplates were coated with specific rabbit anti-FMDV type O and type A polyclonal antibodies overnight. The next day, sera samples were incubated with a homologue virus suspension of equal volume for 1 hour in a 37°C incubator. This mixture was then transferred to the polyclonal antibody coated plates and incubated again for one hour at 37°C. After washing three times with phosphate buffered saline containing Tween 20, specific guinea pig anti-FMDV antibodies were added. After a washing step, a goat anti-guinea pig antibody conjugated with horse radish peroxidase (1:2,000 dilution) was added to each well. After one-hour incubation and subsequent washing, 50 µL peroxidase substrate [10 mg o-phenylenediamine and 37.5 µL of 30% (v/v) H<sub>2</sub>O<sub>2</sub> solution in 25 mL 0.1 M citrate-phosphate buffer, pH 8.0] was added to each well, and plates were incubated at room temperature for 15 minutes. The reaction was stopped using sulfuric acid, and the optical density at 492 nm wavelength was measured using an ELISA reader.

**Data analysis**

Data analysis was performed using SPSS version 19.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). The Mann-Whitney U test was used for the comparison of data from two groups. Differences were evaluated as significant when p < 0.05.

**Ethics statement**

The experiment was conducted in accordance with the International Harmonization of Animal Care and Use guidelines. The study protocol was approved by the ethics committee of the Sap Institute.

**Results**

**Reactogenicity**

A drop of blood was observed at the injection site for animals in the QS-21 group. There was an evident increase in local temperature at the injection site that lasted several days. Body temperatures fell within physiological range for both groups. No other adverse effect was observed during the study. Post-mortem examination of the injection site revealed no macroscopic differences between the two groups of animals.

**Serological responses**

**Virus neutralization test results**

For type O, virus neutralizing antibodies were detected from the third day on in both groups (4/6 for QS-21 and 3/6 for control vaccine). On the eighth day post-vaccination (dpv), a strong neutralizing antibody titre was measured in the QS-21 group; this rapid increase continued until 28 dpv. Thereafter, a slight decrease in antibody titre was observed in this group and remained high until day 90 dpv. In the control vaccine

![Fig. 1. The mean serum virus neutralizing antibody titres for the groups (type O). VNT, virus neutralization test.](http://www.ecevr.org/) 

140  
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group, there was a neutralizing antibody response slightly above the protective levels at 8 dpv (2 animals were below the protective level) that continued to rise until 45 dpv and reached a plateau before 90 dpv. Both groups had protective levels of neutralizing antibodies on day 90. For type A, while the virus neutralization antibody titres reached protective levels by 8 dpv in the QS-21 group, the same protective levels were achieved on 28 dpv in the control group. Virus neutralization test (VNT) results are shown in Figs. 1-4. The differences between the two groups for VNT type O and VNT type A at days 8, 14,
were significant. There was no significant difference between the two groups at days 45 and 90 in VNT value for both types.

**Liquid phase blocking ELISA results**

For type O, a robust specific antibody response was obtained for the QS-21 group at day 8. It reached a maximum level on 14 dpv, and then gradually declined towards day 90. In the oil

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**Fig. 4.** The virus neutralizing antibody titres of individual animals in both groups (type A). (A) Montanide ISA206+ QS-21. (B) Montanide ISA206. VNT, virus neutralization test.

**Fig. 5.** The mean serum enzyme-linked immunosorbent assay (ELISA) antibody titres of the two groups (type O).
adjuvant control group, the response began on day 8 but reached a peak on 45 dpv. Similarly, for type A, a marked early response was detected from day 8 for the formulation containing QS-21 and reached a peak on 28 dpv. In contrast, in the control group, no antibody titre was detected until day 28, with the exception of one animal on 14 dpv.

Liquid phase blocking ELISA test results are shown in Figs. 5-8.

**Fig. 6.** The enzyme-linked immunosorbent assay (ELISA) antibody titres of individual animals in both groups (type O). (A) Montanide ISA206+QS-21. (B) Montanide ISA206.

**Fig. 7.** The mean serum enzyme-linked immunosorbent assay (ELISA) antibody titres of the two groups (type A).
Discussion

Current FMD vaccines generally do not protect cattle herds from outbreaks, even after frequent vaccination [20]. Thus, there is a need for the development of new vaccines that induce a rapid and specific innate response characterized by B-cell activation [21]. Oil adjuvant vaccines are known to be better inducers of a humoral antibody response than gel vaccines [22,23]. Although the combination of saponin and oil is not common, a synergy between saponin and oil for immunopotentiation has been reported in some studies. Gerber [24] observed better immune responses in guinea pigs, pigs and cats vaccinated against canine parvovirus, pseudorabies virus, and feline infectious virus, respectively, when Quil A together with oil was used as an adjuvant than when Quil A or oil was used alone. Martinez-Fernandez et al. [25] reported an enhanced immune response induced by a Fasciola hepatica oil emulsion vaccine containing saponin from Quillaja extract in sheep and mice.

There is considerable variation in saponin quality as a natural product [26]. This might be associated with a variation in the incidence of adverse reactions between vaccine batches [26,27]. QS-21 is much better tolerated than raw saponin, which may cause red blood cell hemolysis, pain at the site of injection, and high local reactogenicity [28]. It is not common to find an oil adjuvant and saponin derivatives together in FMD vaccines. One of the few studies in this field showed increased IgG levels following the use of oil adjuvant with Quil A compared to oil adjuvant alone in piglets [17]. However, ELISA and indirect hemagglutination assay were utilized rather than a VNT assay. Another study carried out by Smitsaart et al. [2] evaluated the inclusion of saponin with the oil adjuvant in an FMD vaccine in cattle compared to the response in different species. Again, liquid phase ELISA was used for the evaluation of the response to the vaccine and the calculation of the expected percentage of protection. Another study [29] showed
that *Cochinchina momordica* seed extracts, a possible saponin source [30], increased FMD-VP-1 antibodies in an oil adjuvanted vaccine in a guinea pig model.

Early evaluations of QS-21 as an adjuvant showed that it was linked with high CD8+ T cell responses in mice [31] and high antigen-specific antibody responses in humans [32]. Despite the negative result produced by QS-21 in a human study [33], in the present study, we found that its use in combination with an oil adjuvant in a FMD vaccine formulation enhanced antibody response. Furthermore, no adverse reaction was observed in cattle during this study. A local rise in temperature in the QS-21 group was considered a normal reaction of the immune system. It was thought that the negative result produced with QS-21 in that study might have been related to the dose of QS-21 used. Only a single dose was used in the human influenza study, and insufficient information about the method of production and purity of QS-21 used was provided. Conversely, in another study comparing various adjuvants with beta amyloid in mice, the best results were obtained with QS-21 compared to Alum, TiterMax Gold, and incomplete Freund’s adjuvant/complete Freund’s adjuvant [15]. Similarly, in a study of Quil A, the best specific antibody response was obtained with Quil A compared to other adjuvants used as antigens, including nanoparticle formulations of chitosan, lipopolysaccharide, and ovalbumin in mice [34]. In a human immunodeficiency virus vaccine study of QS-21, MPLA, Alum, and ISCOMATRIX in mice, the highest CD4+ response was observed with the QS-21 formulation.

Different adjuvants in the same formulation may not always improve the response, as was the case in a bovine virus diarrhoea vaccine study [35]: two strong adjuvants—Quil A and silica vesicles together—did not yield the expected synergistic effect. However, Song et al. [36] reported that Ginseng stem-leaf saponin and mineral oil acted synergistically. Similarly, the results of this study suggest that Montanide ISA 206 and QS-21 could work harmoniously. It was assumed that oil provides a slow release of the antigen and that QS-21 summons immune cells to the injection site, as speculated by Song et al. [36].

Neutralizing antibodies play an important role in protection against FMD [37,38]. It is well-known that there is strong correlation between neutralizing antibody titre and protection [39]. In contrast, antibodies detected by ELISA may not be correlated with neutralizing antibody titre and protection [37]. In this study, QS-21 improved the antibody response within the first month in the target species. In particular, a dramatic increase in neutralizing antibody response as early as the eighth day was observed in the QS-21 group, a good indicator of protection against FMD. The ELISA results also support a remarkable early increase in specific total antibody in the sera of the QS-21 group. This early rise in antibody level is important in the protection of a susceptible population from diseases with high morbidity such as FMD. The early antibody response produced by a ring vaccination may help to contain the circulation of the virus, but it is necessary to determine whether this early response was the result of adjuvancy or the surfactant effect of QS-21. Because QS-21 might have disrupted emulsion, this might allow the rapid release of antigen into tissue. In any case, this marked early response is undoubtedly critical in the fight against FMDV infection.

The disadvantages of QS-21 include its high price and ecological footprint. These problems can be overcome by using a synthetic version of QS-21 or by replacing it with an abundant substance from another plant source showing the same effect.

In conclusion, an early response characterized by strong neutralizing antibody titres against FMDV was detected beginning the first week after immunization in the QS-21 group, whereas the oil adjuvant control vaccine induced a slower increase in specific antibody response. Although both responses were nearly equalized on 45 dpv, this finding may be useful for emergency or ring vaccinations where a rapid response is required.

In this study, because the target animals were used directly, a dose-response study could not be carried out. Different doses or combinations with different adjuvants should be tested for better protection. Furthermore, other routes of administration such as subcutaneous or intranasal should be investigated to identify the optimum response.

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**References**

1. Doel TR. Optimisation of the immune response to foot-
and-mouth disease vaccines. Vaccine 1999;17:1767-71.
2. Smitsaart E, Mattion N, Filippi JL, et al. Enhancement of
the immune response induced by the inclusion of saponin
in oil adjuvanted vaccines against foot-and-mouth
disease. In: Session of the Research Group of the Standing
Technical Committee of EuFMD; Chania, Crete, Greece;
2004 Oct 12-15. p.255-62.
3. Spath EJ, Smitsaart E, Casaro AP, et al. Immune response
of calves to foot-and-mouth disease virus vaccine emulsi-
fied with oil adjuvant: strategies of vaccination. Vaccine
1995;13:909-14.
4. Lindblad EB. Aluminium compounds for use in vaccines.
Immunol Cell Biol 2004;82:497-505.
5. Straw BE, MacLachlan NJ, Corbett WT, Carter PB, Schey
HM. Comparison of tissue reactions produced by Hae-
mophilus pleuropneumoniae vaccines made with six dif-
ferent adjuvants in swine. Can J Comp Med 1985;49:149-
51.
6. Goto N, Kato H, Maeyama J, Eto K, Yoshihara S. Studies
on the toxicities of aluminium hydroxide and calcium phos-
phate as immunological adjuvants for vaccines. Vaccine
1993;11:914-8.
7. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state
and future trends. Immunol Cell Biol 2004;82:488-96.
8. Francis G, Kerem Z, Makkar HP, Becker K. The biological
action of saponins in animal systems: a review. Br J Nutr
2002;88:587-605.
9. Kensil CR, Patel U, Lennick M, Marciani D. Separation
and characterization of saponins with adjuvant activity
from Quillaja saponaria Molina cortex. J Immunol 1991;
146:431-7.
10. Woolhouse ME, Haydon DT, Pearson A, Kitching RP. Fail-
ure of vaccination to prevent outbreaks of foot-and-mouth
disease. Epidemiol Infect 1996;116:363-71.
11. Golde WT, de Los Santos T, Robinson L, et al. Evidence of
activation and suppression during the early immune re-
sponse to foot-and-mouth disease virus. Transbound Emerg
Dis 2011;58:283-90.
12. Park ME, Lee SY, Kim RH, et al. Enhanced immune re-
sponses of foot-and-mouth disease vaccine using new
oil/gel adjuvant mixtures in pigs and goats. Vaccine 2014;
32:5221-7.
13. Patil PK, Bayry J, Ramakrishna C, Hugar B, Misra LD, Na-
tarajan C. Immune responses of goats against foot-and-
mouth disease quadrivalent vaccine: comparison of dou-
ble oil emulsion and aluminium hydroxide gel vaccines
in eliciting immunity. Vaccine 2002;20:2781-9.
14. Gerber JD. Vaccine formulation. United States patent U.S.
4,806,350. 1989 Feb 21.
15. Martinez-Fernandez AR, Nogal-Ruiz JJ, Lopez-Aban J,
et al. Vaccination of mice and sheep with Fh12 FABP from
Fasciola hepatica using the new adjuvant/immunomod-
ulator system ADAD. Vet Parasitol 2004;126:287-98.
16. Strobbe R, Charlier G, Debecq J, Van Aert A. Studies about
the adjuvant activity of saponin fractions in foot and mouth
Can Çokçalışkan et al • QS-21 enhances the early antibody response to oil adjuvant FMD vaccine in cattle

34. Wen ZS, Xu YL, Zou XT, Xu ZR. Chitosan nanoparticles act as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice. Mar Drugs 2011; 9:1038-55.

35. Mody KT, Mahony D, Zhang J, et al. Silica vesicles as nanocarriers and adjuvants for generating both antibody and T-cell mediated immune responses to bovine viral diarrhoea virus E2 protein. Biomaterials 2014;35:9972-83.

36. Song X, Bao S, Wu L, Hu S. Ginseng stem-leaf saponins (GSLs) and mineral oil act synergistically to enhance the immune responses to vaccination against foot-and-mouth disease in mice. Vaccine 2009;27:51-5.

37. Cedillo-Barron L, Foster-Cuevas M, Belsham GJ, Lefevre F; Parkhouse RM. Induction of a protective response in swine vaccinated with DNA encoding foot-and-mouth disease virus empty capsid proteins and the 3D RNA polymerase. J Gen Virol 2001;82(Pt 7):1713-24.

38. McCullough KC, Crowther JR, Butcher RN, et al. Immune protection against foot-and-mouth disease virus studied using virus-neutralizing and non-neutralizing concentrations of monoclonal antibodies. Immunology 1986;58:421-8.

39. Pay TW, Hingley PJ. A potency test method for foot and mouth disease vaccine based on the serum neutralizing antibody response produced in cattle. Vaccine 1992;10:707-13.

32. Livingston PO, Adluri S, Helling F; et al. Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine 1994;12:1275-80.

33. Mbawuike I, Zang Y, Couch RB. Humoral and cell-mediated immune responses of humans to inactivated influenza vaccine with or without QS21 adjuvant. Vaccine 2007; 25:3263-9.

27. Yeruham I, Yadin H, Haymovich M, Perl S. Adverse reactions to FMD vaccine. Vet Dermatol 2001;12:197-201.

28. Evans TG, McElrath MJ, Matthews T; et al. QS-21 promotes an adjuvant effect allowing for reduced antigen dose during HIV-1 envelope subunit immunization in humans. Vaccine 2001;19:2080-91.

29. Xiao C, Rajput ZI, Liu D, Hu S. Enhancement of serological immune responses to foot-and-mouth disease vaccine by a supplement made of extract of cochinchna momordica seeds. Clin Vaccine Immunol 2007;14:1634-9.

30. Song X, Hu S. Adjuvant activities of saponins from traditional Chinese medicinal herbs. Vaccine 2009;27:4883-90.

31. Wilson NS, Yang B, Morelli AB; et al. ISCOMATRIX vaccines mediate CD8+ T-cell cross-priming by a MyD88-dependent signaling pathway. Immunol Cell Biol 2012;90:540-52.

Disease vaccine. III. Comparison of the irritant, adjuvant and hemolytic activities of six commercial saponins and their hemolytic fractions obtained by chromatography on sephadex G 100. Arch Exp Veterinarmed 1976;30:173-81.

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