



Effect of Vegetable Oils as Adjuvants on Immune Response to Polyvalent Foot and Mouth Disease Inactivated Vaccine

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ABSTRACT

Foot and mouth disease (FMD) is one of the most important viral diseases in Egypt and the main way for its control is sufficient vaccination. A vaccine that could improve early and long-lasting immunity by selecting the best adjuvants is the main target for veterinarians. In this study, different formulae from polyvalent inactivated FMD vaccine were prepared using different vegetable oils (Peanut oil, Olive oil, and Sunflower oil) supplemented with Ginseng saponin and compared with locally used Montanide ISA206 as alternative adjuvants. Evaluation of such formulae was carried out through the international quality control protocol for vaccine evaluation, vaccination of calves groups to follow up their cell-mediated immunity using lymphocytic proliferation assay and level determination of interleukine-6, interleukin12 by the fourth week post-vaccination. Humeral immune response was evaluated by recording serum neutralizing antibodies' protective values by the 6th week. All the prepared vaccine formulae were found to be potent for vaccinated calves, except the Olive oil vaccine showed week performance. Our data suggest that Peanut oil and Sunflower oil supplemented with Ginseng saponin could be used as adjuvants in polyvalent FMD vaccine with comparable results to conventionally used mineral oil Montanide ISA206.

Keywords: Adjuvants, FMD, Montanide ISA206, Vegetable oils.

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INTRODUCTION

Foot-and-mouth disease (FMD) is an acute, febrile, and contagious vesicular disease affecting cloven-hoofed animals causing huge economic losses caused by FMD virus (FMDV) which is a member of the genus *Aphthovirus* in the family *Picornaviridae* within seven distinct serotypes throughout the world (A, O, C, Asia1 and South African Territories SAT 1-3) (Doel 2003).

Regarding Egypt, serotype O₁ had been isolated yearly since 1960 while serotype A confirmed in March 2006 (Abd El-Rhman *et al.*, 2006), serotype SAT2 was recently introduced in 2012 through live animals' importation (Abd El-Aty *et al.*, 2013). The disease is characterized clinically by the formation of vesicles on the mouth, teats and feet of animals resulted in huge economic losses due to decreased animal production and reproduction and mortality in calves (James and Rushton 2002).

The major tool for FMD control, especially in developed countries to reduce the impact of clinical disease and eliminate virus circulation, is the vaccination of susceptible animals with inactivated whole virus vaccine as outlined in the Progressive Control Pathway for FMD control (Food and Agriculture Organization, 2011). Adjuvants as chemicals boost the immune response against the associated antigens reduce the required amount of antigen and the multiple immunization protocol required to provide a protective immune response (Cao, 2014).

Both aluminum hydroxide gel and oil emulsion adjuvanted vaccines have been used for FMD vaccines preparation, noting that oil-based adjuvant vaccines appear to induce high titers of antibodies showing more effective protection (Aucouturier *et al.*, 2001 and Cloete *et al.*, 2008). Montanide ISA 206, the mineral-based oil, which readily forms (water-in-oil-in-water) emulsion with its low viscosity and ability to enhance

rapid and long-term immune response was used with many inactivated vaccines (Doel *et al.*, 1994; Barnett *et al.*, 1996 and Aucouturier *et al.*, 2001).

Currently, a variety of vegetable oils is being used as adjuvants having the major advantage over mineral oils because they are easily biodegradable compounds, and they induce little or no adverse reactions. They are easily available, cheap, pure and safe. It has been proved that vegetable oil adjuvants can enhance the host's immune response. Moreover; the combination with Ginseng saponins may amplify the adjuvant effect (Gupta *et al.*, 1993; Song *et al.*, 2009).

The present work was designed to prepare different formulae of inactivated polyvalent FMD vaccine with vegetable oils adjuvants (Peanut oil, Olive oil, and Sunflower oil) in the form of W/O emulsion to induce an early immune response compared to routinely used mineral oil (Montanide ISA206). Cytokines production, cellular, and humeral immune responses were followed up till reaching the protective level.

MATERIALS AND METHODS

1. FMD virus strains

Local FMD virus strains O pan Asia, A Iran O5, SAT2/EGY/2012 and SAT2/ EGY/2018 were obtained from the Department of Foot and Mouth Diseases Research, Veterinary Serum and Vaccine Research Institute (DFMDR/VSVRI), with virus titer calculated by the formula of (Reed and Muench 1938) 10^8 TCID₅₀/ml for each virus strain.

2.1. Calves

The entire experiment was conducted on FMD antibodies free unvaccinated twenty-five native breed calves maintained under field conditions in a private farm. These animals were divided into five groups (5 calves/ group) as follow:

***Group-1:** vaccinated with Montanide ISA 206 oil adjuvanted vaccine

***Group-2:** vaccinated with Peanut oil adjuvanted vaccine

***Group-3:** vaccinated with Sunflower oil adjuvanted vaccine

***Group-4:** vaccinated with Olive oil adjuvanted vaccine

The used dose of each vaccine formula was 3ml/ calf inoculated S/C. while the fifth group (5calves) was kept without vaccination as a control negative. Also, four calves for safety testing.

2.2. Suckling baby mice

Suckling Swiss baby mice, two to four days old, (Charles River Strain, USA) (OIE, 2017) supplied by Laboratory Animal Department (LAD/VSVRI) were used for testing virus inactivation.

3. Cell culture

Baby Hamster kidney cell line (BHK21) supplied by VSVRI using Eagle's Minimum Essential Medium supplemented with 8-10% newborn calf serum (Xuan *et al.*, 2011) was used for the preparation

of FMD virus suspensions for vaccine preparation; virus titration and serum neutralization test.

4. Blood and sera samples

Samples of heparinized blood and serum were collected from the vaccinated and control calves at 0, 3, 7, 14, 21 and 28 days post-vaccination to assess the prepared FMD vaccine formulae's cellular immune response by Lymphocyte blastogenesis using a cell proliferation kit (XTT kit), interleukine-6 and interleukine-12. Also, serum samples for the serum neutralization test were obtained from all calf groups at the time of vaccination (zero time) and every week till they reach the protective antibody level up to 6 weeks post-vaccination.

5. FMD virus inactivation

Clarification of FMD virus serotypes was done by chloroform at a concentration of 1.5% (Volume/ Volume). Binary ethyleneimine 1M in combination with 0.04% formalin (BEI-FA) was used as an inactivating agent to each virus suspension for 24 hours at 37°C according to (Barteling and Cassim 2004) and (Ismail *et al.*, 2013). 20% Sodium thiosulphate was added in a final concentration of 2% were added to neutralize the excess of BEI and formalin residues.

6. Adjuvants and surfactants

6.1. Vegetable oils: Sigma Aldrich supplied products Number P2144, O1514 and 1642347 for Peanut oil, Olive oil, and Sunflower oil, respectively

6.2. Standardized Ginseng saponin (GS) (Zhang *et al.*, 2018) was obtained from Hongjiu Ginseng Industry Co. Ltd. (Jilin, China).

6.3. Montanide ISA206: Seppic, Paris, France supplied Montanide ISA206

6.4. Other materials: Span 80 as oil-soluble surfactant supplied by MP Biomedical Inc., France, Lot. No. 1553J and Tween 80 as the aqueous soluble surfactant supplied by Sigma-Aldrich, Germany, Lot No. 085k0096 were used for the preparation of inactivated FMD polyvalent vaccine as emulsifier agent for vegetable oils.

7. Preparation of different formulae of oil inactivated polyvalent FMD vaccine

The four inactivated virus suspensions were mixed in equal volumes to form one suspension then divided into four portions to prepare four different vaccine formulae as follow:

7.1. Using vegetable oil (Peanut, Olive and Sunflower oil) adjuvants:

Peanut, sunflower and Olive oils combined with Ginseng were used as adjuvants of FMD vaccine by mixing 4 µg of GS with oil phase containing 6% Span-80, using dimethyl sulfoxide (DMSO) co-solvent. The aqueous antigenic phase contains 3 %Tween 80 emulsified with the oil phase at 1:1 (vol/vol) with a dispersing device according to (Zhang *et al.*, 2014).

7.2. Preparation of Montanide ISA 206 adjuvanted vaccine

Montanide ISA 206 oil adjuvant was used as 50:50 (V/V) to the virus suspension and stirred to form a water-in-oil-in-water blend. To obtain an extremely stable emulsion, slow-shear mixing at 300 rpm for 5 min was performed and was followed by a brief cycle of mixing at the same speed for 24 h at 4°C according to (Patil *et al.*, 2002)

8. Evaluation of prepared FMD vaccine formulae

8.1. Physical parameters: Physical parameters of the vaccines like viscosity, stability and emulsion type were studied as described by Stone (1988).

8.2. Sterility test: Sterility assays of all prepared FMD vaccine formulae were performed on thioglycolate broth, Sabouraud's agar, Nutrient agar, phenol dextrose media and mycoplasma medium according to (OIE 2017).

8.3. Safety test: The safety of each prepared vaccine formulae was tested by subcutaneous inoculation of double dose in the calve dewlap. In addition to intraperitoneal inoculation of suckling baby mice (OIE 2017)

8.4. Evaluation of cellular immune response

8.4.1. Lymphocyte blastogenesis using XTT assay

It was carried out according to (Slater *et al.*, 1963) and (EL-Naggar, 2012) through the separation of lymphocytes as described by (Lucy, 1977) and (Lee, 1984) and determination of viable cell number according to (Mayer *et al.*, 1974).

8.4.2. Estimation of Interleukin

Estimation of interleukin in the sera of vaccinated and control calves including IL-6 and IL-12 levels was carried out using calve IL-6 and IL-12 ELISA Kit Catalog No. EKE51028 supplied by Biomatik Company, Wilmington, Delaware, USA.

8.5. Serological evaluation of the humoral immune response

FMD (O pan Asia, A Iran O5, SAT2/ EGY/2012 and SAT2/Egypt/2018) serum neutralizing antibody titers were monitored in vaccinated calves using serum neutralization assay carried out on serum samples obtained pre and on week intervals up to 6 weeks post-vaccination. Such samples were exposed to heat inactivation at 56°C for 20 minutes in a water bath for deactivating the complement and nonspecific inhibitors. Serum neutralizing antibody titers were determined against FMD serotypes using the technique described by Ferreira, (1976). The serum neutralization index was calculated according to (Reed and Muench, 1938).

9. Statistical analysis

P Values of outcome data were calculated and analyzed using SPSS program version 21 (2012). Values of < 0.05 were considered statistically significant.

RESULTS

Table 1: Mean delta optical density of lymphocyte blastogenesis assay in calves vaccinated with the prepared vaccine formulae

Animal Groups	Delta optical density of lymphocyte blastogenesis					
	1 st DPV	3 rd DPV	7 th DPV	14 th DPV	21 st DPV	28 th DPV
Group 1	0.25	0.42	0.64	0.80	0.93	0.85
Group 2	0.31	0.60	0.72	0.83	0.88	0.71
Group 3	0.17	0.36	0.58	0.72	1.02	0.91
Group 4	0.20	0.27	0.35	0.53	0.59	0.43
Control	0.24	0.20	0.14	0.19	0.20	0.21

Table 2: Interleukin-6 immune response expressed as mean delta optical density of calves vaccinated with the prepared vaccine formulae

Animal Groups	IL-6 (ng/ml) at DPV					
	1 st DPV	3 rd DPV	7 th DPV	14 th DPV	21 st DPV	28 th DPV
Group 1	0.43	1.24	2.17	3.88	4.41	3.84
Group 2	0.82	1.71	2.98	4.14	4.90	3.32
Group 3	0.36	0.94	2.58	4.82	5.71	4.3
Group 4	0.34	0.88	1.27	2.83	3.24	2.31
Control	0.30	0.36	0.41	0.32	0.28	0.41

Table 3: Interleukin-12 immune response expressed as mean delta optical density of calves vaccinated with the prepared vaccine formulae

Animal Groups	IL-12 (ng/ml) at DPV					
	1 st DPV	3 rd DPV	7 th DPV	14 th DPV	21 st DPV	28 th DPV
Group 1	4.00	4.27	4.93	5.82	7.18	5.3
Group 2	4.63	4.71	5.23	5.74	6.88	5.92
Group 3	3.86	3.98	5.53	7.28	8.00	6.89
Group 4	3.74	4.18	4.32	5.00	5.13	3.52
Control	3.93	3.31	3.67	3.55	3.72	3.44

Table 4: Mean FMD type O serum neutralizing antibody titers expressed in log₁₀ in different vaccinated calves' groups

Animal Groups	FMD type O serum neutralizing antibody titers/WPV						
	0 WPV	1 st WPV	2 nd WPV	3 rd WPV	4 th WPV	5 th WPV	6 th WPV
Group 1	0.39	0.96	1.29	1.62	1.71	1.74	1.92
Group 2	0.27	1.02	1.38	1.5	1.59	1.68	1.89
Group 3	0.48	0.87	1.23	1.56	1.62	1.71	1.83
Group 4	0.54	0.78	0.93	1.17	1.26	1.32	1.53
Control	0.63	0.48	0.39	0.51	0.33	0.42	0.54

Table 5: Mean FMD type A serum neutralizing antibody titers expressed in log₁₀ in different vaccinated calves' groups

Animal Groups	FMD type A serum neutralizing antibody titers/WPV						
	0 WPV	1 st WPV	2 nd WPV	3 rd WPV	4 th WPV	5 th WPV	6 th WPV
Group 1	0.42	1.17	1.56	1.59	1.74	1.8	1.98
Group 2	0.33	1.08	1.47	1.62	1.71	1.77	2.04
Group 3	0.51	1.02	1.32	1.59	1.68	1.86	1.92
Group 4	0.48	0.96	0.81	1.05	1.26	1.35	1.62
Control	0.39	0.66	0.48	0.27	0.33	0.30	0.42

Table 6: Mean FMD type SAT2/2012 serum neutralizing antibody titers expressed in log₁₀ in different vaccinated calves' groups

Animal Groups	FMD type SAT2/2012 serum neutralizing antibody titers/WPV						
	0 WPV	1 st WPV	2 nd WPV	3 rd WPV	4 th WPV	5 th WPV	6 th WPV
Group 1	0.48	0.87	1.26	1.56	1.68	1.83	1.89
Group 2	0.63	0.9	1.32	1.5	1.59	1.77	1.8
Group 3	0.51	0.96	1.17	1.56	1.62	1.62	1.68
Group 4	0.33	0.75	0.87	1.08	1.2	1.29	1.5
Control	0.48	0.39	0.51	0.33	0.57	0.36	0.45

Table 7: Mean FMD type SAT2/2018 serum neutralizing antibody titers expressed in log₁₀ in different vaccinated calves' groups

Animal Groups	FMD type SAT2/2018 serum neutralizing antibody titers/WPV						
	0 WPV	1 st WPV	2 nd WPV	3 rd WPV	4 th WPV	5 th WPV	6 th WPV
Group 1	0.66	0.84	1.29	1.62	1.65	1.74	1.77
Group 2	0.39	0.99	1.32	1.59	1.59	1.68	1.71
Group 3	0.27	0.78	1.23	1.5	1.62	1.74	1.83
Group 4	0.48	0.69	0.81	1.05	1.26	1.35	1.62
Control	0.36	0.45	0.39	0.51	0.42	0.6	0.42

Group 1 vaccinated with Montanide ISA 206 oil adjuvanted vaccine

Group 2 vaccinated with Peanut oil adjuvanted vaccine.

Group 3 vaccinated with Sunflower oil adjuvanted vaccine.

Group 4 vaccinated with Olive oil adjuvanted vaccine.

DISCUSSION

Regarding the control of FMD and its importance as disease-causing huge economic losses, there are many efforts to improve the commercial vaccines aiming to reach the highest levels of immunity required to protect susceptible livestock. One of such trials is the use of oil adjuvants which enhance the vaccine potency. The vegetable oils can be used as vaccine adjuvants representing an exciting matter for the vaccine specialists. The present work aims to investigate the benefit of such oils when used as adjuvants for the polyvalent inactivated FMD vaccine compared with conventionally used mineral oil Montanide ISA 206.

All prepared inactivated polyvalent FMD vaccines showed homogenous appearance and stable; emulsion type was confirmed by drop test. The vaccines were free from aerobic and anaerobic bacteria; fungi and mycoplasma where tested on specific media and safe inducing no abnormalities in inoculated mice. There was no noticeable toxicity or prolonged pyrexia was observed in the vaccinated calves. Moreover, none of the vaccinated calves showed a localized reaction. Where as, the Peanut oil vaccine resulted in localized swelling in the inoculation site, which spontaneously relieved after a maximum of 48 hours of vaccination., in agreement with the recommendation of (Stone 1988) and (OIE 2017). This finding also corroborates the study conducted by (Freitas *et al.*, 2013). On the other hand, (Mustafa *et al.*, 2016) demonstrated that sunflower oil caused local reaction at the inoculation site, which may be attributed to using different vaccination protocols and animal models.

It is well known that the cellular immune response is associated with early protection against FMD and is involved in forming the adaptive immune response to FMDV infection (Grubman *et al.*, 2008; Summerfield *et al.*, 2009). Through the present work, the cellular immunity in vaccinated calves with the prepared four inactivated polyvalent FMD vaccine was evaluated by assessing the IL-6 and IL-12 levels in addition to lymphocyte blastogenesis. Table (1, 2 &3) showed that all used oil adjuvants could induce acceptable levels of IL-6 and IL-12 and lymphocyte proliferation levels with the same pattern. The highest delta optical density of IL-12 was 8.00 induced by inactivated polyvalent FMD vaccine adjuvanted with Sunflower oil followed by those induced by Montanide ISA-206 oil (7.18); Peanut oil (6.88), and Olive oil (5.13) by the third-week post-vaccination. On the other side, IL-6 (table-2) revealed that Sunflower oil-induced the highest optical density (5.71) followed by that induced by Peanut oil (4.90); Montanide ISA-206 oil (4.41) than Olive oil (3.24).

The unvaccinated control showed the lowest values for IL-12 and IL-6 (3.72 & 0.28, respectively). Also, Sunflower oil in the prepared FMD vaccine showed the highest optical density lymphocyte blastogenesis (1.02) followed by that of ISA-206 oil (0.93), Peanut oil (0.88) and last that of Olive oil (0.59). According to a determination of P-value; it appears that there is no significant difference between the IL-6 and 12 and lymphocyte blastogenesis values obtained by vaccine formulae with Montanide ISA-206; peanut and sunflower oils while there is a significant difference between such values and those induced by olive oil which induces the lowest values cellular immune response parameters.

These results came in line with some previous studies on vegetable oil adjuvants (Song *et al.*, 2009; Li *et al.*, 2012 and Zhang *et al.*, 2014) who reported that vegetable oil (Rapeseed oil) in combination with Ginseng significantly enhanced gamma interferon (IFN- γ) and interleukin 5 (IL-5) levels, splenocyte proliferative responses, and the numbers of IgG-secreting plasma cells in the bone marrow.

Results of unvaccinated control calves showed the lowest value (0.28) as shown in table (3). Similar results were obtained by (Mossad *et al.*, 2014) and agree with (Knudsen *et al.*, 1979; Mercedes *et al.*, 1996; El-watany *et al.*, 1999; Sonia *et al.*, 2010 and Fakhry *et al.*, 2012) who reported that the Delta optical density of lymphocyte blastogenesis assay and interleukin6, 12 showed a significant difference between vaccinated and control groups started at 3rd DPV and increased gradually till 21st DPV using FMD oil inactivated vaccine.

Serum neutralization test applied on serum samples obtained from calves vaccinated with inactivated polyvalent FMD vaccine with the four oil adjuvants, revealed that both of ISA 206, Peanut oil and Sunflower oil adjuvant vaccine formulae induced protective serum neutralization index by the third-week post-vaccination recording the values of 1.62; 1.59; 1.56 and 1.62 by ISA-206 oil for type O; A; SAT2-2012 and SAT2-2018 respectively, 1.5; 1.62; 1.5 and 1.59 for the four types respectively by Peanut oil; 1.56; 1.59; 1.56 and 1.5 for the four types respectively by Sunflower oil. Longer duration (6weeks) was detected to reach a protective NI by olive oil with values of 1.53; 1.62; 1.5 and 1.62 for type O; A; SAT2-2012 and SAT2-2018, respectively.

Results revealed that there is no significant difference between the obtained NI among the different calve groups vaccinated with the vaccine formulae with Montanide ISA206; Peanut oil and Sunflower oil with Ginseng saponin, while the lowest NI was obtained by Olive oil, showing the significant difference with other oils as determined by the P-value. The obtained NI for

the four types of FMD antibodies showed higher values than the protective value (1.5) (OIE 2017). Although it is known that Montanide ISA206 induces higher levels of FMD antibodies in animals vaccinated with the FMD vaccine (El-Bagoury *et al.*, 2014) and (Ibrahim *et al.*, 2015), The present findings suggest the ability of these vegetable oils to enhance the humoral immune response in agreement with (Sartor *et al.*, 2011) who attributed that to the up-regulation of Th2 response.

These results go beyond previous reports, showing that Olive oil possesses inferior adjuvant activity as compared to the peanut oil in the case of Newcastle disease (ND) vaccine (Ezeifeke *et al.*, 2008) and (Wanasawaeng *et al.*, 2009), in agreement with (Freitas *et al.*, 2013), who demonstrated the adjuvant action of peanut, rice and cotton oils for ovalbumin.

CONCLUSION

Depending on the present study results, both Peanut oil and Sunflower oil supplemented with Ginseng saponin appear to be a good candidate for use as an alternative adjuvant for polyvalent foot and mouth vaccine.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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