

Research Article

Preparation and Comparative Evaluation of Vegetable oil Adjuvanted Foot-and-Mouth Disease Vaccines in Rabbits

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ABSTRACT

The main objective of the present study was to prepare and comparatively evaluate foot-and-mouth disease (serotype O) vaccines having different vegetable oils (peanut, sunflower and olive oil) as adjuvants and determine the possible adverse reactions, they induce. For that purpose, female rabbits (n=30) were divided, randomly into 5 groups, group A, B, C and D were inoculated with aluminium hydroxide, peanut oil, sunflower oil and olive oil respectively (@ 0.4 ml/ml). Booster dose were given (group A to D) 21 days following priming dose. Group E was kept as negative control (given 0.4 ml normal saline). Collection of serum samples were done at the day of vaccination (day 0) and then 7, 14, 21, 28, 42, 56 and 70 days following priming dose of vaccination. Measurement of antibody titers was done by indirect haemagglutination assay. It was found that following the priming dose of vaccination, antibody titers attained by group A were found to be higher as compared to the animals vaccinated with the peanut (group B) and sunflower adjuvanted (group C) vaccines, although this difference was not found to be significant statistically (P>0.05), however, titers of group A, B and C were significantly higher (P<0.05) as compared to the group E. Following the booster dose of immunization, increase in titers of group B, C and D were found to be higher as compared to the group A. However, statistically, GMTs of group A, B, C and D were not found to be significantly different (P>0.05), although they differed significantly (P<0.05) from group E all the times following the booster dose. None of the vaccines induced any localized or systemic reactions (deaths and rise in body temperature) following priming vaccination although they induced localized reactions of mild type following booster dose of the vaccination. The present study recommends the adjuvant action of vegetable oils for foot and mouth vaccine.

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INTRODUCTION

Foot and mouth disease (FMD) is an economically important, acute and highly contagious disease of even-toed animal's like buffalo, cattle, goat, sheep and many other domestic and zoo animals (Bastos et al., 2003). The disease manifests itself clinically by the formation of vesicles on mouth, teats and feet of animals. It results in significant economic losses to dairy farmer in form of decreased production of milk, abortions and weight loss. The disease is a major constraint in the trade of animals (and their products) internationally (James and Rushton, 2002).

The causative agent of the disease is a virus; Aphthovirus belonging to the family *Picornaviridae* (Bablanian and Grubman, 1993). This is a naked, single stranded RNA, positive sense virus having genome size of 8500 base pairs. This virus possess an icosahedral symmetry and four structural proteins (VP1, 2, 3 and 4); among them VP1 is a major immunogenic protein (Cloette et al., 2008).

Proper control of the disease can be achieved by a variety of different methods like controlling the movement of animals and slaughtering of suspected and diseased animals (John, 2002). However, in countries like Pakistan where the disease is endemic in nature, and often appears as outbreaks, it is controlled by the proper vaccination of the animals (Orsel et al., 2007). Currently vaccine is being prepared in the University of veterinary and animal Sciences (UVAS), Lahore and is found to be very effective in terms of post vaccination humoral immune response as well as the protection from clinical disease (Altaf et al., 2012).

Both aluminium hydroxide and saponin (AS) and oils are currently being used as adjuvants of FMD. It is a well-known fact that the oil adjuvants offer a better protection as compared to the AS adjuvanted vaccines. Oil adjuvants provide higher titers of antibodies that tend to persist for a longer duration of time as compared to the AS adjuvanted vaccines (Sadir et al., 1988). Those animals which were immunized with the oil adjuvanted FMD vaccines showed a better protection against homologous virus challenge than

those vaccinated with the AS adjuvanted FMD vaccines (Bahnmann et al., 1987). The main disadvantage associated with the use of oil adjuvanted vaccines is that sometimes they result in the severe local reactions such as cyst and granuloma formation. Several factors may be responsible for such reactions, the principle factor involved is the impurities present within oil used as adjuvant. So it is suggested that only those oils should be used which have lower viscosity and the chain length of that hydrocarbon compound should be 12–30 (Bomford, 1977). Hydrocarbons having lower chain length induce more inflammation (Gupta et al., 1993).

So, in view of above mentioned findings, it is better to use that oil as adjuvant of FMD vaccine which not only provides persistent antibody titers against FMD but also does not produce any local adverse reactions. Now a day, a variety of vegetable oils is being used as adjuvants. The major advantage of vegetable oils over mineral oils is that being easily biodegradable compounds, they induce little or no adverse reactions. Moreover, they are easily available, cheap, pure and safe. It has been proved that the vegetable oil adjuvants have potential to enhance the immune response of the host (Gupta et al., 1993). Recently conducted studies showed that corn, rice, canola, soya bean, olive, linseed, rapeseed, peanut, sesame and cashew nuts have potential to stimulate both immune response of the host (Barnett et al., 1996).

In the present study, it was shown that the emulsions made from the peanut oil and sunflower oil were able to enhance the specific humoral response against FMD vaccine.

MATERIALS AND METHODS

FMDV type O strain was procured from WTO– Quality Operations laboratory, University of veterinary & animal Sciences (UVAS), Lahore. Reed and Munch, (1938) method was used to calculate the biological titer of virus and final concentration of the virus was adjusted at 10^6 units of TCID₅₀ (Altat et al., 2012). Binary ethyleneimine (0.01 M) was used as inactivation agent of antigen. Virus inactivation was done at 37°C for 48 hours (Bahnmann, 1975).

Vegetable oils (Peanut, sunflower and olive oil) were used as adjuvants of FMD vaccine and Tween 80 and Span 80 were used as surfactants. Both vegetable oils and surfactants were sterilized by autoclaving at 121°C for 15 minutes. A total of 10% of aqueous phase (Tween–80) and oil phase (Span 80) were added to the vegetable oils (peanut, sunflower and olive oil) as surfactants, so the final hydrophilic lipophile balance (HLB) of the oil adjuvanted FMD vaccine was adjusted at 7.0. One part of inactivated antigen was added into four parts of sterilized vegetable oils (peanut, sunflower and olive oil) containing surfactants to make water-in-oil (W/O) emulsion at aqueous to oil ratio of 1:4 (Ezeifeke et al., 2008) (Table 1). Physical parameters of the vaccine like color, viscosity, stability and emulsion type were also studied as described by Stone (1988).

Viral suspension (serotype O)	Type of adjuvant	Oil emulsion (Adjuvant with surfactant)			Total volume
		Oil	Span-80	Tween-80	
25 ml	Peanut oil	100 ml	8.3 ml	2.7 ml	136 ml
25 ml	Sunflower oil	100 ml	8.3 ml	2.7 ml	136 ml
25 ml	Olive oil	100 ml	8.3 ml	2.7 ml	136 ml

Table 1: Composition of different vegetable oil adjuvanted FMD vaccines

Table 2: Vaccination schedule of rabbits	Group	No. of animals	Adjuvants used	Vaccine dose	Route of administration
	A	6	Aluminium hydroxide and saponin	0.4 ml	Intramuscular
B	6	Peanut oil	0.4 ml	Intramuscular	
C	6	Sunflower oil	0.4 ml	Intramuscular	
D	6	Olive oil	0.4 ml	Intramuscular	
E	6	Normal saline	0.4 ml	Intramuscular	

Sterility of all of these vaccines was observed by inoculating each vaccine on different bacteriological media, i.e nutrient agar, MacConkey's agar, blood agar and Sabouraud's dextrose agar at 37°C for a period of 24–48 hours (Mahboob et al., 1996). For safety testing, 0.1 ml of each vaccine was inoculated into the tongue of the goats (intradermal), followed by inoculation of 2 ml of vaccine on neck of the animals after 4 days. The animals were monitored for a further 6 days for the presence of any of FMD signs i.e vesicles on mouth, feet and teats (Cloette et al., 2008).

Experimental Model

A total of 30 female rabbits of 6–12 months of age were used. Only those animals were selected which were not vaccinated with FMD vaccine previously. These animals were divided into five groups, each having six animals. These animals were vaccinated with the priming dose of vaccine first and then booster dose was given 21 days after the priming dose, with vaccines containing different

adjuvants as follows (Table 2). Group A was used as positive control (given a commercially procured FMD vaccine having AS as adjuvant) while group E was kept as negative control.

Measurement of Humoral Immune Response

Blood samples without anticoagulants were collected from the jugular vein of the rabbits by inserting 24 gauge needle fitted with 3 ml disposable syringe on day 0, 7, 14, 21, 28, 42, 56, and 70 days post vaccination. After the collection of blood samples, it was immediately transferred to sterile test tubes to the stand on slant position and kept in refrigerator overnight. The serum was separated from each sample and stored at –20 °C till used. Antibody titer was determined by using indirect haemagglutination (IHA) test as described by Xiao et al. (2007). Other parameters like: Body temperature, presence of swelling at the site of inoculation of vaccine and mortality of rabbits were recorded in each group for 7 days following priming and booster dose of vaccination.

Indirect Haemagglutination Assay (IHA)

IHA was performed to determine the humoral immune response against different FMD vaccines inoculated to rabbits.

Inactivation of Serum

Serum samples collected from the animals were subjected to inactivation by keeping them in water bath at 56°C for 45 minutes, in order to inactivate complement and non-specific inhibitors, prior to be used in IHA.

Erythrocytes

Sheep blood was collected, 10 ml in quantity, using a sterile disposable plastic syringe, containing 1 ml of 4% sodium citrate solution. After collection of blood, the syringe was gently rotated for proper mixing of blood with anticoagulant. The needle was removed; the citrated blood was poured into the centrifuge tube and subjected to centrifugation at 1500 revolutions per minute (rpm) for 5 minutes. The supernatant (plasma and buffy coat) was discarded using a sterile pipette. The packed erythrocytes were suspended in 10 ml of phosphate buffered saline (PBS) and re-centrifuged at 1500 rpm for 5 minutes. The supernatant was pipetted off and packed erythrocytes were re-suspended in 10 ml of PBS and re-centrifuged at 1500 rpm for 5 minutes. After three washings, packed erythrocytes, as 10% suspension were used in the PBS, were used for the sensitization with FMD O serotype.

Procurement of Antigen

Live FMD virus (serotype O) was procured from the WTO, Quality Operations Laboratory, University of Animal and Veterinary Sciences, Lahore. Virus was stored at -20°C till use.

Sensitization of Sheep Erythrocytes

The washed sheep erythrocytes were sensitized with the FMD serotype O. For sensitization of erythrocytes, two

volumes of the FMD virus having concentration of 40 micrograms of virus was mixed with one volume of 10% erythrocytes suspension in PBS followed by 1 volume of 1% glutaraldehyde in PBS which was freshly prepared from stock solution (25 %) in each test. The mixture was agitated every 15 min. during incubation for 60 minutes at room temperature (25°C). The sensitized erythrocytes were washed two times with PBS (pH 7.2) and were centrifuged at 1500 rpm for 5 minutes. After the removal of supernatant fluid, the erythrocytes were finally re-suspended in 20 volumes of PBS in order to make the final concentration of 0.5%, to be used in IHA (Xiao et al., 2007).

Procedure for Indirect Haemagglutination Assay

IHA antibody titers of all of the serum samples were determined against FMD serotype O by micro titration technique as described by the Xiao et al. (2007). The results of each serum sample, thus obtained were recorded, and geometric mean titer was calculated.

Statistical Analysis

The geometric mean titers (GMT) and cumulative mean titers (CMT) were calculated using the procedure described by Thrusfield (1986) and the statistical differences among GMTs and CMTs of different groups within each experiment were estimated using the analysis of variance (ANOVA) of means applying Duncan's multiple range (DMR) test (Duncan, 1955; Steel and Torrie, 1980).

RESULTS

Various parameters (color, viscosity, emulsion type and stability) of the different vegetable oil adjuvanted vaccines were noted and were as shown in the table 3. Regarding sterility of the vaccines, all of the vaccines were found to be sterile when inoculated on different bacteriological media (Table 4).

Regarding safety of the vaccines, all of the inoculated animals failed to produce any sign of disease. Thus all of the vaccines were safe.

Table 3: Various parameters of different vaccines

Adjuvant used	Vaccine color	Viscosity (Seconds)	Emulsion type	Stability test (at 4 and 37 °C)
Peanut oil	Light creamy white	5	W/O	Stable
Sunflower oil	Dark creamy white	6	W/O	Stable
Olive oil	Light creamy white	4.5	W/O	Stable

Table 4: Sterility test for different vaccines

Media	Commercial vaccine (having AS adjuvant)	Peanut oil adjuvanted vaccine	Sunflower oil adjuvanted vaccine	Olive oil adjuvanted vaccine
MacConkey's agar	-	-	-	-
Nutrient agar	-	-	-	-
Sabouraud agar	-	-	-	-
Blood agar	-	-	-	-

+ = Growth found on the media 24–48 hours following inoculation (at 37°C)

- = No growth found on the media 24–48 hours following inoculation (at 37°C)

Humoral Immune Response of Rabbits following FMD Vaccination

IHA was done in order to measure the humoral immune response against FMD serotype O. On the day of vaccination (day 0), different groups had IHA titers ranging from 2 to 4 and statistically, the difference in the GMTs of different groups of rabbits was found to be non-significant. Afterwards, at day 7 of vaccination, the IHA titers of different groups ranged from 2 to 16 and the statistical analysis showed that group A had a significant difference ($P<0.05$) in the GMTs as compared to the group D and E while no significant difference ($P>0.05$) was found among group A, B and C. At the 14th day of vaccination, IHA titers of different groups ranged from 2 to 32 and GMTs of the group A and B showed a significant difference ($P<0.05$) statistically as compared to the group E while no significant difference ($P>0.05$) was found among group A, B, C and D. At 21st day of vaccination, the IHA titers of different groups ranged from 2 to 64 and the statistical analysis of the GMTs revealed that group A and B had a significant difference ($P<0.05$) as compared to the group E while statistically, no significant difference ($P>0.05$) was found between groups A, B, C and D. After the booster dose of vaccination, at day 28th initial vaccination, the IHA titers of different groups ranged from 2 to 64 and the statistical analysis of the GMTs revealed that group A and B had a significant difference ($P<0.05$) as compared to the group E while statistically, no significant difference ($P>0.05$) was found among groups A, B, C and D. At 42nd day of vaccination, the IHA titers of different groups ranged from 2 to 128 and the statistical analysis of the GMTs revealed that group A, B, C and D had a significant difference ($P<0.05$) as compared to the group E while statistically no significant difference ($P>0.05$) was found among groups A, B, C and D. At 56th day of vaccination, the IHA titers of different groups ranged from 2 to 128 and the statistical analysis of the GMTs revealed that group A, B, C and D had a significant difference ($P<0.05$) as compared to the group E while statistically, no significant difference ($P>0.05$) was found among groups A, B, C and D. Same trend was observed on the day 70 of vaccination (Table 5).

Table 5: GMTs of FMD in rabbits inoculated with different vaccines

Days PV	A	B	C	D
7	7.13a	5.04ab	4.49ab	2.52b
14	17.96a	12.70ab	10.08bc	5.04c
21	35.92a	28.51ab	22.63b	8.98c
28	71.84a	64.00a	57.02ab	35.92b
42	80.64a	71.84a	64a	57.02a
56	90.51a	90.51a	80.64a	71.84a
70	101.59a	114.04a	101.59a	90.51a

– Means sharing the same small letters do not differ at $P<0.05$

Statistical analysis of CMT showed that no significant difference among different vaccinated groups but CMT of all vaccinated groups differed significantly than from non-vaccinated (group E) animals (Table 6).

Table 6: CMTs of different groups of rabbits

Group	CMT
A	42.37 ^a
B	36.51 ^a
C	31.47 ^a
D	20.49 ^{ab}
E	3.13 ^b

Determination of Adverse Reactions of Different Vaccines of FMD

Adverse reactions of different vaccines were studied for a 7 days after the initial and booster shot of the vaccination. The various parameters which were studied included body temperature, swelling at the site of inoculation of vaccination and mortality. Following the initial shot of vaccination, body temperature of the group A, B, C, D and E (taken as °F) ranged from the 101.8 to 103.6, 101.6 to 103.8, 101.8 to 103.6, 102.0 to 103.8 and 101.4 to 103.2 respectively while the mean temperature of these groups were 102.4, 102.3, 102.5, 102.4 and 102.5 respectively. While following the booster shot of vaccination body temperature of the group A, B, C, D and E (taken as °F) ranged from the 101.6 to 103.6, 102.0 to 103.8, 102.0 to 103.6 and 102.0 to 103.4 respectively while the mean temperature of these groups were respectively 102.6, 102.5, 102.7, 102.7 and 102.4. Statistical analysis of the means of the temperature showed that there was no significant difference ($P>0.05$) in the mean body temperature of different groups neither following priming nor booster vaccination of FMD.

Following priming and booster dose of vaccination, local reactions were also studied at the site of inoculation and none of the groups showed the localized reactions at the site of inoculation, while following the booster vaccination, 5/6, 4/6, 6/6, 0/6 and 0/6 animals of group A, B, C, D and E showed a localized reactions at the site of inoculation.

Following priming and booster dose of vaccination, mortality was also observed in rabbits for 7 days following each shot. All the rabbits remained in a healthy condition and there was no deaths occurred during this time.

DISCUSSION

The present study strongly recommends the use of different vegetable oils (peanut, olive and sunflower) as adjuvants of FMD. The use of these oils as adjuvants of FMD vaccine showed a compatible humoral response activity as compared to the AS adjuvanted commercial FMD vaccine. After boosting day 21 following first shot, antibody titers increased at a much rapid rate as compared to the AS adjuvanted FMD commercial vaccine. These findings suggest the ability of these vegetable oils to enhance the humoral immune response by the up-regulation of Th2 response. But till now, the exact mechanism, by which different vegetable oils tend to increase the activity of humoral immune response, is yet to be determined but two possibilities may be: oils tend to create a depot effect locally by sequestering the antigen and thus permitting a sustained but slow release of any antigen. This leads to a prolonged stimulation of immune system and results in the generation of much higher titers of antibodies due to persistent recruitment of antigen presenting cells which results in the generation of higher level of antibodies (Sartor et al., 2011).

Secondly, it has been also reported that these oils contain a variety of different types of immunomodulators like linoleic acid, oleic acid and vitamin E (Calder, 1998). It has been reported that the peanut oil contains about 62% of oleic acid.

The results of our study are in agreement with the Ezeifeke et al., (2008), which argued that in case of Newcastle disease (ND) vaccine, the peanut oil is found to be the best adjuvant while the olive oil possess inferior adjuvant activity as compared to the peanut oil. The findings of our study are also in agreement with the Wanasawaeng et al., (2009) which supports the adjuvant action of peanut oil for ND vaccine and it also induced localized tissue reactions at the site of inoculation. The findings of the present study are also in agreement with the study conducted by the Freitas et al., (2013), which demonstrated the adjuvant action of peanut, rice and cotton oils for ovalbumen, however, in contrary to the present study, these oil adjuvants were found to be free of any adverse systemic or localized reactions.

The findings of the present study recommend the adjuvant action of variety of locally available vegetable oils for FMD vaccine.

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