Study of zeolite as immune stimulant in foot and mouth disease trivalent oil adjuvant vaccine in sheep

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ABSTRACT

Comprehensive sero-immunological study was conducted to reveal the adjuvant's effect of zeolite on the immune response of oil adjuvanted trivalent Foot and mouth disease (FMD) vaccine in sheep. The objective of this study was to evaluate the efficacy of Zeolite (Zeolith UF) as an adjuvant using inactivated trivalent foot and mouth disease virus, to induce protection against foot and mouth disease. This study was conducted in three sheep groups ; the first group was vaccinated intramuscularly (I/M) with trivalent foot and mouth disease zeolite (1 µg/dose) vaccine, The second group was vaccinated with foot and mouth disease oil vaccine and Third group was vaccinated with foot and mouth disease (oil + zeolite) vaccine. The cellular immune responses were monitored in different tested groups that revealed the immune responses in sheep continuous with the permissible protective level is (0.280) Delta Optical Density till 8th for zeolite and oil vaccine vaccine while till 10th week post vaccination with foot and mouth disease zeolite + oil vaccine. The humeral immune responses were monitored in different tested groups that revealed the immune responses in sheep vaccinated with trivalent foot and mouth disease vaccine adjuvanted with zeolite similar to results of trivalent foot and mouth disease oil vaccine continue up to 32 week . While addition of zeolite to foot and mouth disease Montanoide oil vaccine increases immune responses up to 40 week . Our results show that the incorporation of zeolite into foot and mouth disease oil vaccine induces an early & long period of high specific protective immune response in sheep. In conclusion we recommended to use a ground zeolite alone or the best to use with oil as a potential adjuvant in foot and mouth disease vaccine .

INTRODUCTION

Foot and mouth disease (FMD) is an acute contagious viral disease of cloven footed animals *(Orsel et al., 2007).*The causative agent is a single stranded positive- sense RNA virus that belongs to the genus Aphthovirus in the family Picornaviridae . There are seven immunologically distinct serotypes of foot and mouth disease virus, namely ; O, A, C, , SAT1, SAT2, SAT3 and Asia1 (*Belsham, 1993*). In Egypt, the disease is enzootic and outbreaks have been reported since 1950 *(Mousa et al., 1974)*. Type ″ O ″ was the most prevalent since 1960 (*Zahran 1960,* and *Farag et al., 2005).* Sero type ″ A ″ foot and mouth disease virus virus was introduced to Egypt through live animals importation *(Abdel-Rahman et al.,2006),* Recently foot and mouth disease virus serotype SAT2 was recorded in Egypt (*Shawkey et al., 2013)* . The control of foot and mouth disease virus in animals was considered to be important to effectively contain the disease in endemic areas, so that vaccination of animals is effective in limiting the spread of foot and mouth disease virus (*Nair and Sen, 1992*). Most foot-and-mouth disease vaccines are made of binary Ethyleneinmine (BEI) inactivated virus that is adjuvanted with oil adjuvant . Adjuvants, also can prolong the immune response and stimulate specific components of the immune response either humeral or cell mediated

immunity (*Barnett 2003, Pluimers, 2004 and Lombard et al. 2007).*

Zeolite is a mineral micro particle that in earlier studies has shown adjuvant activity against different antigens. Clinoptilolite zeolite is safe and effective (Garces (1999 and Rhodes 2010) .

Zeolites play an important role in regulating the immune system. *Ueki et al. (1994)and Aikoh et al. (1998)* have reported that silica, silicates, and aluminosilicates act as nonspecific imunostimulators similarly to super antigens . Super antigens are a class of immunostimulatory and disease-causing proteins of bacterial and viral origin with the ability to activate relatively large fractions (5-20%) of the T-cell population, as well as humoral immune responses .

The objective of this study was to evaluate the efficacy of Zeolite (Zeolith UF) as an adjuvant using inactivated trivalent foot and mouth disease virus , to induce protection against foot and mouth disease .

MATERIALS AND METHODS

1. Animals :

a-Sheep

21 sheep were classified into five groups, Five animals per each group for first three groups. Three animal were used as negative control (non vaccinated). Three sheep were used for safety test. Sheep were clinically healthy and free from antibodies against foot and mouth disease virus as proved by serum neutralization test.

b.Unweaned baby mice :

30 Swiss Albino suckling mice (three to five days old were) classified into six groups, used in safety test of inactivated virus and vaccines and supplied by the Lab. animals farm of Veterinary Serum and Vaccine Research Institue, Abassia, Cairo, Egypt.

2. FOOT AND MOUTH DISEASE virus Strains :

Foot and mouth disease virus local strains **(**O /pan Asia2 , A/ Iran 05 and SAT2/ Egypt 2012) were locally isolated and were identified by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. and confirmed by world reference laboratory Pirbright FMD-WRL), United Kingdom. Each virus had a titer of 10^8 $TCID_{50}$/ml. These viruses were used as virus mitogens in the lymphocyte proliferation assay, vaccine preparation and serum neutralization test .

3-Adjuvants

i. Montanoid Oil:

ISA 206 Montanoid Oil was obtained from Seppic, Paris, France.

Ii .Zeolite (Zeolith UF **) .**

The fine powder of natural clinoptilolite (Zeolith UF) ,

Micronisiertes Klinoptilolith –Zeolith UF < 10

Hochwertigs Naturmineral –Puder ohne Weitere Zusatza , Zeolith Bentonit-Versand de EichendorffstraBe35,D-09131 Chemnz

4- Trivalent FOOT AND MOUTH DISEASE oil adjuvant vaccine

Briefly, trivalent local strains of foot and mouth disease were propagated in BHK-21 cell line and inactivated by Binary Ethylenemine (BEI), Vaccine formula according to *(Barnett et al.,1999)* .

i. **Preparation of the oil adjuvant vaccines** : The inactivated and clarified virus harvest was concentrated with 8% (w : v) polyethylene glycol (PEG-6000) . The inactivated oil adjuvenated foot and mouth disease vccine was formulated according to *(Barnett et al., 1999 , Hiam and Eman 2010 and Farag et al., 2011)* . The ratio of the aqueous antigen to the oil adjuvant was 50:50 and the emulsions were produced by recycling the aqueous antigen-oil mixture several times. Sterility and safety of the

prepared vaccines were done according to (*OIE 2000).*

ii. Preparation of the Zeolites adjuvant vaccines

Vaccine: formulated using 1 ug/doses of Zeolite .

iii.Preparation of the oil and Zeolites adjuvant vaccines

Vaccine: formulated using 1 ug/doses of Zeolite + oil adjuvant vaccine

5-Quality control of the prepared vaccines:

i. Sterility test : It was applied to confirm that vaccine is free from any bacterial or fungal contaminations. Sterility of the examined vaccine was done by culturing of the tested vaccine on nutrient agar, thioglycolate broth and Sabauraud's dextrose agar (OIE 2000).

ii. Safety test for the formulated

vaccines : The inactivated foot and mouth disease virus was tested for safety in vitro on BHK-21 cell line and the whole prepared vaccines in vivo in susceptible sheep and baby mice (OIE 2000).

Sterility and safety of the prepared vaccines were done according to (*Code of Federal regulation of USA .1986 , Henderson 1970 and OIE 2000).*

6-Cell titer 96 Aqueous One Solution Cell Proliferation Assay (MTS)

Cell titer 96 Aqueous One Solution Cell Proliferation Assay (MTS) that contain a novel Tetrazoliun compound (MTS) and an electron coupling reagent (henazine ethosulfate,PES)] (Promega corporation, USA) was used following the manufacturer's instruction . MTS Cell Proliferation Colorimetric Assay has been applied as a method to evaluate cell mediated immune response .

i-**Phytohaemaglutinin (PHA)**

PHA was obtained from Sigma Company and used as T-lymphocyte mitogen.

EXPERIMENTAL DESIGN

21 sheep were classified into five groups, Five animals for each group for first three groups . The first group was vaccinated with 1 ml of the tested trivalent Foot and mouth disease ″FMD ″ zeolite vaccine, the second group was vaccinated with 1.5 ml of the tested trivalent foot and mouth disease oil vaccine, third group was vaccinated with 1 ml of the tested trivalent foot and mouth disease (oil + zeolite) vaccine , while the fourth group (three animal) were non vaccinated used as negative control and fifth group (three animal) were used for safety test .

Blood samples were collected from all sheep on heparin solution for evaluation of cell mediated immunity, at the 3^{rd}, 7^{th} , 10^{th} and 14^{th} days post vaccination , then every week until the 10^{th} week post vaccination.

Serum samples were collected weekly post vaccination for one month then every 2 weeks post-vaccination till the end of experiment. .

The immune response was evaluated through the estimation of cellular and humoral immune level using Cell titer 96 Aqueous One Solution Cell Proliferation Assay (MTS) assay and serum neutralization test .

Vaccination of sheep:

Each sheep was injected with 1.5 ml vaccine (I /M) on the shoulder region. The sheep were bled weekly for one month, and then every 2 weeks until the end of experiments .

Serum Neutralisation Test (SNT):

Neutralizing antibody titers against foot and mouth disease virus strains (O /pan Asia2 , A/ Iran 05 and SAT2/ Egypt 2012) in serum samples were measured using the neutralization assay as described previously (*Voller et al., 1976 &OIE 2012)* **.**

End-point titers were calculated as the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of foot and mouth disease virus in 50% of the wells.

ii-Evaluation of cell-mediated immunity *in vitro* by Lymphocyte Proliferation test using Cell titer 96 Aqueous One Solution Cell Proliferation (MTS) Assay

The test was carried out as previously described by several authors ***(Riss and Moravec 1992 , Juan et al., 2003 ,Alvero et al., 2007 and Sonia et al., 2010)***

a-**Separation and enumeration of circulating mononuclear cells from peripheral blood according to *(Boymi, 1984).***

b- Separation of lymphocytes according to *Lucy (1984).*

- Viable lymphocyte count followed according to *Mayer et al.(1974)* .

Cell number and viability were determined using the trypan blue exclusion test. The cells were diluted in 0.1% trypan blue, loaded on the haemocytometer and the cells were counted in 32 small squares .

The cells number /ml was calculated according to the following formula:

$$\text{Count /ml} = \frac{\text{Cells count in 32 squares x10x1000 dilution}}{4}$$

Plates were read at 490 nm wave length.

- Calculation:

ΔOD sample (using PHA) : OD of PHA - OD of cells.

ΔOD sample (using virus) : OD of virus - OD of cells.

RESULTS

The result of culturing sterility test revealed that the vaccines free from any pathogenic or non-pathogenic microorganisms .

The results of Safety of inactivated virus in tables (1&2) where these results indicate that no viable viral residues of all serotypes used in vaccine preparation , so the vaccines were safe to use.

Table (1) : The safety test of inactivated Foot and Mouth Disease virus strains .

samples	Safety test of inactivated virus			
	CPE in BHK cell line in different passages			Baby
	1st passage	2nd passage	3rd passage	ce
Inactivated FMD virus type O	NO CPE	NO CPE	NO CPE	No death
Inactivated FMD virus type A	NO CPE	NO CPE	NO CPE	No death
Inactivated FMD virus type SAT 2	NO CPE	NO CPE	NO CPE	No death

FMD =Foot and mouth disease CPE = cytopathic effect BHK=Baby hamster kidney cells

Table (2): The safety test of prepared vaccines .

Tested vaccine	Safety test of prepared vaccines	
	sheep	Baby mice
FMD zeolite vaccine	No local or general symptoms or lesions	No death
FMD oil vaccine		
FMD zeolite + oil vaccine		

FMD = Foot and mouth disease

Table 3: Delta optical density of the cell -mediated immune response of sheep vaccinated with trivalent Foot and mouth disease vaccines measured by (MTS) Proliferation Colorimetric Assay

sheep vaccinated groups	Mitogen and virus used	Mean Delta optical density of samples with different mitogenes at first 10th WEEK POST VACCINATION												
		0 day	3rd day	7th day	10th day	2nd Week	3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week	9th Week	10th Week
Group (1) *FMD zeolite vaccine	**PHA	0.076	0.234	0.294	0.320	0.362	0.401	0.389	0.363	0.284	0.255	0.198	0.162	0.132
	***V(O)	0.047	0.304	0.342	0.448	0.494	0.560	0.525	0.434	0.340	0.322	0.301	0.270	0.199
	V(A)	0.044	0.311	0.348	0.450	0.496	0.566	0.528	0.434	0.348	0.330	0.308	0.273	0.198
	(SAT2)	0.044	0.306	0.342	0.420	0.489	0.548	0.520	0.421	0.334	0.324	0.298	0.265	0.187
Group (2) FMD oil vaccine	PHA	0.076	0.174	0.234	0.260	0.349	0.408	0.384	0.291	0.274	0.248	0.192	0.176	0.121
	V(O)	0.049	0.244	0.282	0.388	0.486	0.554	0.519	0.429	0.320	0.318	0.300	0.259	0.187
	V(A)	0.042	0.251	0.288	0.390	0.479	0.549	0.514	0.422	0.331	0.321	0.298	0.276	0.201
	SAT2)	0.052	0.246	0.284	0.360	0.458	0.533	0.488	0.412	0.330	0.317	0.287	0.261	0.198
Group (3) FMD zeolite + oil vaccine	PHA	0.077	0.314	0.344	0.370	0.410	0.460	0.430	0.370	0.352	0.332	0.317	0.292	0.267
	V(O)	0.050	0.354	0.392	0.489	0.534	0.594	0.564	0.504	0.484	0.451	0.412	0.389	0.290
	V(A)	0.043	0.361	0.396	0.494	0.540	0.600	0.570	0.512	0.492	0.462	0.410	0.394	0.298
	SAT2)	0.061	0.356	0.391	0.473	0.520	0.548	0.520	0.421	0.393	0.364	0.368	0.321	0.281
Group (4) Non vaccinated control group	PHA	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067
	V(O)	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064
	V(A)	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066
	SAT2)	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060

*FMD = Foot and mouth disease ** PHA = Phytohaemaglutinin

***V = virus

N.B.: The permissible protective level is 0.280 Delta Optical Density.

Cell mediated immune response in vaccinated sheep were estimated by Cell titer-Aqueous One Solution Cell Proliferation (MTS) Assay showed in table (3) : the Delta Optical Density (ΔOD) of trivalent vaccine adjuvanted with zeolite , Oil and zeolite oil vaccine measured with (MTS) assay were (0.304 ,0.244 ,0.314) at 3rd DPV against type O and (0.311.0.277,0.354) against type A and (0.306,0.246,0.356) against type SAT2 respectively . Peak of ΔOD were recorded at 3rd week post vaccination (0.560 , 0.554 , 0.594), (0.566 , 0.549 , 0.600) & (0.548 , 0.533, 0.548) against foot and mouth disease virus strains (O, A &SAT2) respectively and continuous with the permissible protective level is 0.280 Delta Optical Density till 8th for Group 1 and Group 2 while till 10th week post vaccination with Group (3) zeolite + oil vaccine.

Results in tables (4) revealed that Serum neutralization test (SNT) for Foot and mouth disease zeolite vaccine (group1) and foot and mouth disease zeolite + oil vaccine (group 3) reach the protective level at 2nd week post vaccination early than group (2) foot and mouth disease oil vaccine which reach protective level at 3rd week post vaccination . The peak of antibody titre in all groups at 10-12 week post vaccination and continues with protective level till 32th week post vaccination in foot and mouth disease zeolite vaccine(group1) and foot and mouth disease oil vaccine (group2) while in foot and mouth disease zeolite + oil vaccine (group3) till 40th week post vaccination .

Table (4) : Mean of serum antibody titers against type (O) , (A) & SAT 2 in sheep vaccinated with trivalent Foot and mouth disease vaccines using serum neutralization test expressed log_{10}

Weeks post vaccination	Sheep groups vaccinated with trivalent FOOT AND MOUTH DISEASE vaccines									Non vaccinated Group(4)
	*FMD zeolite vaccine Group (1)			FMD oil vaccine Group (2)			FMD zeolite+ oil vaccine Group (3)			
	Mean antibody titer against FOOT AND MOUTH DISEASE virus strains									
	FMD (O)	FMD (A)	FMD (SAT2)	FMD (O)	FMD (A)	FMD (SAT2)	FMD (O)	FMD (A)	FMD (SAT2)	
**Pre vacc	0.15	0	0.3	0.15	0.27	0.27	0.15	0.3	0.3	0.3
1	1.1	1.05	1.2	0.9	0.9	0.9	1.2	1.05	1.2	0.3
2	1.65	1.8	1.8	1.14	1.29	1.38	1.65	1.8	1.8	0.3
3	2.1	2.1	1.95	1.71	1.8	1.77	1.8	2.1	2.15	0.3
4	2.4	2.4	2.4	1.95	2.1	1.8	2.4	2.4	2.55	0.3
6	2.7	2.7	2.7	2.34	2.25	2.1	2.7	2.7	2.85	0.3
8	2.85	2.85	2.85	2.58	2.7	2.37	3.0	3.0	3.0	0.3
10	2.85	3.15	3.0	2.82	2.82	2.7	3.3	3.15	3.15	0.3
12	2.55	2.85	3.0	3.0	3.0	3.0	3.3	3.15	3.3	0.3
14	2.55	2.7	2.85	2.8	2.8	2.70	3.0	3.0	3.0	0.3
16	2.4	2.4	2.55	2.6	2.6	2.49	2.85	2.85	2.9	0.3
20	2.1	2.1	2.4	2.5	2.4	2.37	2.6	2.7	2.85	0.3
24	1.8	1.8	2.1	2.37	2.13	2.25	2.4	2.55	2.7	0.3
28	1.65	1.65	1.8	2.13	2.04	2.16	2.25	2.4	2.4	0.3
32	1. 5	1. 5	1.65	1.83	1.77	1.7	2.1	2.1	2.1	0.3
36	1.05	1.05	1.2	1.35	1.17	1.20	1.65	1.8	1.8	0.3
40	0.75	0.6	0.75	0.9	0.75	0.6	1.5	1.65	1.5	0.3

* FMD =Foot and mouth disease **Pre vacc = pre vaccination
Antibody titers expressed as log_{10} $TCID_{50}$ protective antibody titer not less than 1.5

DISCUSSION

Foot and Mouth Disease (FMD) is an acute disease caused by Foot and Mouth Disease Virus which causes important economy losses *(Orsel et al., 2007).*

The control of foot and mouth disease in animals was considered to be important to effectively contain the disease in endemic areas, so that vaccination of animals is effective in limiting the spread of foot and mouth disease .

The effective formulation of foot and mouth disease inactivated vaccines requires adjuvant zeolite and Montanoid ISA 206 mineral oil-based formulations have been widely employed in experimental studies to obtain a vaccine that stimulates a rapid and long-lasting protective immune response .

From result of tables (1&2) vaccines free from any pathogenic and no viable viral residues of all serotypes so the vaccines were safe to use . These results were in agreement with *(OIE 2000)* foot and mouth disease vaccine must be free from any living virus.

The formulation Zeolites- foot and mouth disease virus is non toxic with adjuvant activity *(Batista et al.,2010)* .Previous results demonstrated that the adjuvant effect of natural zeolite microparticles of clinoptilolite (Cliptox™) using two classic T dependent antigens (ovoalbumin and sheep red blood cells) induce an increase in dendritic cells, macrophages and monuclear phagocytes in spleen, in mice injected in two intramuscular doses elicited high titters of specific antibodies

without side effects in the site of inoculation *(Batista,et al.,2010*).

In this work we studied the effect of natural zeolite particles to induce specific and protective immune response against foot and mouth disease. The results of

Cell mediated immune response were supported by (Soos et al.,1984, Sharma et al.,1984)) who reported that cell mediated immune response was a constitute of immune response against Foot and mouth disease virus. And in agreement in some points with (Mercedes et al.,1996 and Samir ,2002) that foot and mouth disease vaccine stimulated the cellular immune response and lymphocyte stimulation by Foot and mouth disease virus was greater than by mitogens (PHA) and appeared increased in 1st and 2nd weeks post vaccination.

Delta Optical Density continues till 8th for Group (1) and Group (2) while till 10th week post vaccination with Group (3) zeolite + oil vaccine . The obtained results were in agreement with Sun et al., (2009) they stated that adjuvant act as an activator of the TH1 response. The Th_1 type is characterized by the production of antigen-specific IgG2a Th_1 and the secretion of gamma interferon, interleukins which favor cellular immunity.

Our results also were supported by Rhodes (2010) who mentioned that zeolite (Zeolith UF) enhance cell mediated immune response.

Serum neutralization titre for Foot and mouth disease (zeolite vaccine) and (zeolite +oil vaccine) reach the protective level at 2nd week while(oil vaccine) reach protective level at 3rd week post vaccination . go in hand with the results obtained are consistent with the statement of Wisniewski et al., (1972) they explained that the serum neutralization test measures those antibodies which neutralize the infectivity of foot and mouth disease virion. . The peak of antibody titre in all groups at 10-12 week post vaccination and continues with protective level till 32th week post vaccination in foot and mouth disease(zeolite vaccine) and (oil vaccine) groups while continues till 40th week in zeolite + oil vaccine group . The results agreed with Kreimir et al 2000 and Rhodes (2010) who showed that Zeolite –inactivated foot and mouth disease vaccine increased the specific antibodies levels and the protection against the virus in sheep.

Results supported also by (Batista, et al., 2010) they found that zeolite help the vaccine work more effectively, increasing antibody production.

All these results demonstrate that zeolite could be a good candidate for the formulation of foot and mouth disease vaccine .

Vaccine formulations containing the adjuvant could promote the presentation of the virus so it could increase the immune response and the protection (Barnett 1999 , Batista, et al., 2010 and Hiam et al., 2012)

Finally, we can conclude that the usage of Zeolite (Zeolith UF) as an adjuvant in inactivated foot and mouth disease vaccine improve both cellular and humoral immunity and gave earlier and more long lasting immunity when used with oil and improved the potency of vaccine . The choice of zeolite is safe for vaccine production.

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