Journal of Applied Veterinary Sciences, 5 (3):14 - 30 (2020).
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

Immunomodulating Efficacy of Different Adjuvants in Formulation of Foot and Mouth Disease Vaccine Relative to Its Immunogenicity

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ABSTRACT

The ideal adjuvant is one that able to aid in early stimulation of the humeral immune response, and to promote the production of high antibody titers that would persist for long duration as well as stimulate the cellular immune response. This immunological study was conducted to reveal the aluminum hydroxide gel effect with the use of oil adjuvants on the immune response of polyvalent foot and mouth disease (FMD) vaccine in sheep. Twenty five sheep, were divided to five group (five animals/group) where the 1st group was vaccinated with polyvalent FMD ISA 206 oil vaccine, 2nd group was vaccinated with polyvalent FMD ISA 206 + aluminum hydroxide gel vaccine, 3rd group was vaccinated with polyvalent ISA 201 oil vaccine, 4th group was vaccinated with polyvalent ISA 201 + aluminum hydroxide gel vaccine, 5th group was kept as a negative control (non-vaccinated). Blood and Serum samples were collected from vaccinated animals for monitoring the cellular and humeral immune responses. The results showed that sheep groups immunized with the vaccine prepared with ISA 201with aluminum hydroxide gel is considered the best cellular and humeral immunity post vaccinal response then ISA 201 followed by ISA 206 with aluminum hydroxide gel and the last one is the vaccine prepared with ISA 206 alone.It can be concluded that, ISA oils with aluminum hydroxide gel induce the best cellular and humeral immunity.

Keywords: Aluminum hydroxide gel, FMD vaccine, immune response, Montanide ISA201, Montanide ISA206.

Original Article:
DOI:HTTPS://DX.DOI.ORG/10.21608/JAV 2020.98318

Received :21 April, 2020.
Accepted :02 June, 2020.
Published in:July, 2020.

J. Appl. Vet. Sci., 5(3): 14-30.

INTRODUCTION

Foot and Mouth disease (FMD) is a highly infectious disease of ungulates primarily cattle, sheep, goats and pigs. It also affects wild animals such as buffalo and deer **(Paton *et al.*, 2009 and Dara *et al.*, 2013)**. Foot and Mouth disease virus (FMDV) is the etiologic agent of such devastating disease. Infection with FMDV causes an acute disease that spreads very rapidly and is characterized by fever, lameness, vesicular lesions on the feet, tongue, snout and teats, with high morbidity but low mortality **(Depa *et al,* 2012 and Juleff *et al.*, 2012)**. Seven types of FMDV have been identified such as O, A, C, SAT1, SAT2, SAT3 and Asia1 **(Franki *et al.*, 1991 and OIE, 2017)**.

Foot and Mouth disease (FMD) is enzootic in Egypt since 1950; it remains a serious threat to cattle and buffaloes population (**Depa *et al.*, 2012; Pattnaik *et al.*, 2012 and Abd El-Rhman *et al.*, 2015)**. Serotype O has a long history in Egypt with many topotypes and lineages, serotype A was reported in 2006 followed by SAT2 serotype in 2012**, (Sobhy *et al.*, 2014; Valdazo *et al.*, 2015 and Soltan *et al.*, 2017)**. In Egypt, outbreaks have been reported since 1950, 1960, 2006, 2012 and 2018 for Serotype O **(Farag *et al.*, 2005 and Satya, 2009)**. Serotype A from live animals importation where sever clinical signs were recorded among cattle and buffaloes (**Abd El-Rahman *et al.*, 2006)**. Serotype SAT2/2012 was recorded in Egypt (**Shawky *et al.*, 2013 and Nader *et al.*, 2014)** and SAT2/2018 **(Abd El-Rhman *et al.*, 2020).**

Vaccination is a major tool for FMD control to mitigate the impact of clinical disease, or to reduce and eventually eliminate virus circulation as outlined in the Progressive Control Pathway for FMD (**Food and Agriculture Organization, 2011)**.

The prevention strategy to avoid FMD outbreaks occurrence in Egypt is vaccination using locally produced (O Panasia-2&A Iran-05&SAT2/ EGY-2012) trivalent inactivated vaccine (**El-Bagoury *et al.,* 2014)**. Recently vaccination in Egypt was done using polyvalent inactivated FMD vaccine after field isolation of SAT2/EGY/2018 as typing of field circulated virus. Detection of the causative agent and preparation of vaccine against it was regarded as a necessity to adjunct disease control (**Longjam *et al.,* 2011).**

At present, no effective treatment is available for FMDV infected livestock, vaccination against FMD is the best option to prevent and control this disease. The inactivated vaccine has made significant contributions to prevent and control FMD since the 1990s. However, the potential risk of the virus escaping from the vaccinated herd may cause the spread of the disease (**Rodriguez and Grubman, 2009).**

Adjuvant is a substance added to vaccine to improve the immunogenicity of antigens, and it can induce stronger immune responses and reduce the dosage and production cost of vaccine in populations responding poorly to vaccination. Adjuvants increase either humeral or cell-mediated immune response **(Barnett *et al.,* 2003; Lombard *et al.,* 2007 and Park, 2013)**. Adjuvants in development or in use mainly include aluminum salts, oil emulsions, saponins, immune-stimulating complexes, liposomes, microparticles, nonionic block copolymers, polysaccharides, cytokines, and bacterial derivatives.

The oil adjuvant has the capability for generating a rapid, high, long-lasting immune response. Generally, the Montanide series of oil adjuvants (SEPPIC, France) has a clear immunological effect for inactivated vaccine in different susceptible animals **(Fakhry *et al.,* 2012; Dara *et al.,* 2013and El-Sayed *et al.,* 2015)**. Recently, SEPPIC has developed a new adjuvants (Montanide ISA-201 and Montanide ISA-206) and claimed that those adjuvants induce better immune responses (particularly CMI responses).

Inactivated FMD vaccines are commonly produced as gel or oil adjuvants. Vaccines containing aluminum hydroxide and saponin as adjuvants have several disadvantages such as the induction of short-lived antibody responses which require relatively frequent revaccinations at intervals of 6 or even 4 months. In contrast, oil-based adjuvant FMD vaccines appear to have several advantages such as the induction of high titers and long-lived antibody responses, resulting in more effective protection (**Aucouturier *et al.,* 2001 and Cloete *et al.,* 2008)**.

In studies of vaccine developments for FMD, it is desirable that the adjuvants are applied directly to susceptible target animals. In this study the effects of experimental vaccines using various kinds of adjuvants were compared in sheep in order to achieve highly effective and potent vaccine in corresponding to onset and duration of the immune response of sheep against different targeted FMDV serotypes.

MATERIALS AND METHODS

1.Animals

Sheep

Twenty five breed native sheep, one year old and weighted between 55 and 60 kg, were found to be free from FMD type O Pan Asia-2, A Iran O5, SAT2/Egypt /2012 and SAT2/Egypt /2018 antibodies as screened by serum neutralization test (SNT) and divided into five groups, (five animals/group) as follow:

- Group-1 (GP1) vaccinated with polyvalent FMD vaccine adjuvant with ISA 206 oil
- Group-2 (GP2) vaccinated with polyvalent vaccine adjuvant with FMD ISA 206 and aluminum hydroxide gel
- Group-3 (GP3) vaccinated with polyvalent FMD vaccine adjuvant with ISA 201 oil
- Group-4 (GP4) vaccinated with polyvalent FMD vaccine adjuvant with ISA 201 and aluminum hydroxide gel
- Group-5 (GP5) was kept non-vaccinated as negative control **.**

Suckling baby mice

Fifty suckling Albino Swiss baby mice, 2-4 days old, (Charles River Strain, USA) were supplied by laboratory animal Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, were used for the safety testing of complete virus inactivation.

2. Samples

Heparinized blood samples were obtained from vaccinated and control non vaccinated animals at 0, 3, 7, 14, 21 and 28 days post vaccination for detecting cellular immune response of vaccinated sheep by determination of Lymphocyte blastogenesis using cell proliferation kit (XTT kit), phagocytic percentage, phagocytic index, and interleukine-6.

Also, serum samples were obtained from blood samples collected weekly post vaccination (WPV) for one month and then every 2 WPV up to 4 months and finally each 4 WPV till the end of the experiment (40WPV) to follow up the humeral antibody response of vaccinated sheep using SNT and enzymes linked immunoadsorbent assay (ELISA).

3. Cell culture

Baby Hamster kidney cell line (BHK21) Clone 13 was obtained from veterinary serum and vaccine research institute (VSVRI) using Eagl's medium with 8-10% bovine serum as described by **(Xuan *et al.*, 2011)** and used for application of SNT, virus titration and vaccine preparation.

4.FMD virus Serotypes

Egyptian isolated FMDV Serotypes O Pan Asia-2, A Iran O5, SAT2/Egypt /2012 and SAT2/Egypt /2018 with a titer 10^9 $TCID_{50}$/ml for each type were supplied by Foot and Mouth Vaccine Research Department (FMDRD), VSVRI, Abbasia, Cairo. The virus Serotype O, A and SAT-2 were confirmed by the World Reference Laboratory for FMD (WRL), Pirbright London, UK. These viruses were used in vaccine preparation and serum neutralization test (SNT).

5. Adjuvants

Montanide ISA206: Montanide ISA 206 was obtained from Seppic, Paris, France.

Montanide ISA201: Montanide ISA 201 was obtained from Seppic, Paris, France.

Aluminum hydroxide gel: 2.5% aluminum hydroxide gel was prepared as an alum-based adjuvant. **(Patil *et al.*, 2002a)**

6.Virus purification

Aseptically, the harvested culture media from FMD virus infected BHK21 cell cultures were centrifuged in a cooling centrifuge at 3000 rpm for 20 min. to remove cell debris.

7. Virus concentration

The tissue culture viral fluids of the three serotypes of FMDV (O pan Asia, A Iran O5, SAT2/EGY/2012 and SAT2/EGY/2018) were centrifuged at 7000 round per minute RPM for 30 min and then concentrated by Poly Ethylene Glycol (PEG)-6000 to reach 1/10 of its original volume **(Shabana, 2014)**.

8.Virus inactivation

Binary ethylenamine (BEI) 1% M in 0.2N NaOH was added to the virus suspension to give final concentration of 0.001 M of BEI. The virus and BEI mixtures were mixed well. The virus mixture was placed on a magnetic stirrer in the incubator at 37°C for 18 h .Then sodium thiosulphate was added in a final concentration of 2% to neutralize the BEI action according to **(Ismail *et al.*, 2013).**

9.Vaccine formulations

Four vaccine formulae were prepared as follow:

Formula-1: Polyvalent FMD vaccine adjuvanted with Montanide ISA 206.

Formula-2: Polyvalent FMD vaccine adjuvanted with Montanide ISA 206 and aluminium hydroxide gel.

Formula-3: Polyvalent FMD vaccine adjuvanted with Montanide ISA 201.

Formula-4: Polyvalent FMD vaccine adjuvanted with Montanide ISA 201 and aluminium hydroxide gel.

The concentrated FMDV serotype O Pan Asia-2, A Iran O5, SAT2/Egypt /2012 and SAT2/Egypt /2018 used were diluted using Tris NaCl with pH of 7.6 to be reached a final antigen content 3µg/ dose from each serotypes of FMDV according to (**Daoud *et al.*, 2013)** and then added to each adjuvant used. When we used Montanide ISA 206 and 201adjuvant, the vaccine was formulated according to (**Barnett *et al.*, 1996).** The ratio of the aqueous antigen to the oil adjuvant was 50:50

(w/w) according to (**OIE 2017)**. In the oil/gel adjuvant mixture, 10% of Aluminum hydroxide (AL) was added according to **(Park *et al.*, 2016)**. The amount of antigen per dose of vaccine per serotype was maintained as the same amount of antigen was pre-diluted to the same concentration before mixing it with the target experimental adjuvants. The applied dose of all vaccine formula was 1.5ml inoculated subcutaneously in all experimental animals group.

10. Quality control testing of the prepared vaccine formulae

Sterility test

The prepared vaccines were tested for their freedom of aerobic and anaerobic bacteria, fungal and mycoplasma contaminants by cultured of vaccine sample in thioglycolate broth, Sabouraud's, Nutrient agar; phenol dextrose media and mycoplasma medium **(OIE, 2017 and Code of Federal Regulation of USA, 2019)**.

Safety test

Complete virus inactivation was confirmed in five days Swiss Albino suckling mice according to **(Randall *et al.*, 1964)**.

Potency test

Evaluation of the cellular immune response

Lymphocyte blastogenesis using XTT assay

Blood samples were collected from all sheep groups on the 1st; 3rd; 7th; 14th; 21st and 28th days post-vaccination followed by separation of lymphocytes **(Lucy, 1977 and Lee, 1984)** and subjected to lymphocyte blastogenesis using XTT assay **(Slater *et al.*, 1963 and EL-Naggar, 2012)** and determination of viable cell number **(Mayer *et al.*, 1974).**

Separation and cultivation of mononuclear cells

The preparation of mononuclear cell suspension was separated by Ficollhypaque equilibrium centrifugation method (**Antley and Hazen, 1988**) from sheep peripheral blood cell suspension was adjusted to 10^7 viable mononuclear cells/ml RPMI medium containing 15% Fetal Calf Serum and placed in cell culture 6-wells plate. The monolayer cells were rinsed 3 times gently with RPMI medium to remove non adherent cell. The adherent cells were then covered with RPMI medium containing 15% FSC and incubated for 24 hours in CO2 incubator at 37 °C.

Phagocytic activity of sheep macrophages by using Candida Albicans

The monolayer of adherent mononuclear cells was washed gently 3 times with RPMI medium. Candida albicans cell suspension containing 10^5 cell/ml RPMI medium was incubated with the above monolayers in humidified CO2 incubator at 37 ^{0}C for 1 hour. After incubation, the monolayer cells were washed gently with cold RPMI medium and then fixed with methyl alcohol (0.3 ml/well) for 5 min. The alcohol was discarded and left to dry. The cells were stained with Giemsa stain for 3 minutes. Under the light microscope, using oil immersion lens, 10 fields were examined. The total numbers of phagocytic cells, the number of phagocytes ingested yeast cell and the number of blastospores within individual phagocyte were determined. The percentage of phagocytes containing blastospores was determined by the method of **Harmon and Glisson** which was modified by (**Hussein 1989)** and the mean number of blastospores (more than 2 blastospores) per infected phagocyte (phagocytic index) were calculated by (**Richardson and Smith, 1981)**.

Estimation of interleukin

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

Evaluation of sheep humeral immune response to the prepared vaccines in sheep

Serum samples collected from sheep before and after vaccination (4 times on week intervals then every month up to 40 weeks) were subjected to estimation of antibody titers against the four serotypes of FMDV (O pan Asia, A Iran O5, SAT2/ EGY/2012 and SAT2/ EGY/2018) by SNT using the micro titer technique (**Ferreira, 1976)** and indirect ELISA **(Voller *et al.*, 1976)**.

11. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in the SPSS-12 statistical software package. Multiple comparisons of means were made using Duncan's multiple range tests at $P< 0.05$ %.The results represent the average of five replicates and are presented as the mean ± standard error.

RESULTS

Table 1. Safety test results of the prepared polyvalent FMD vaccines

Prepared Polyvalent FMD Vaccines			
Formula-1	Formula-2	Formula-3	Formula-4
Safe	Safe	Safe	Safe

Table 2. Mean delta optical density of lymphocyte blastogenesis assay in sheep vaccinated with the prepared FMD polyvalent vaccine formulae

Vaccine formulae	Delta optical density of lymphocyte blastogenesis/ day post vaccination (DPV)					
	1st day	3rd day	7th day	14th day	21st day	28th day
Formula-1	0.11	0.36	0.54	0.59	0.61	0.50
Formula-2	0.22	0.65	0.99	0.74	0.75	0.71
Formula-3	0.32	0.70	1.01	0.80	0.80	0.70
Formula-4	0.33	0.88	1.21	0.99	0.99	0.89
Negative non vaccinated control	0.10	0.12	0.13	0.12	0.13	0.10

Table 3. Phagocytic % of sheep vaccinated with the prepared FMD polyvalent vaccine formulae

Sheep groups	Phagocytic percentage					
	1 day	3rd day	7th day	14th day	21st day	28th day
Formula -1	20.1	30.2	49.7	56.1	66.4	54.2
Formula -2	23	33.2	53.8	69.2	66.2	63
Formula -3	23.3	34.2	55.1	70.4	66.3	64.2
Formula -4	29.2	37.5	81.2	92.3	70.4	60.1
Negative non vaccinated control	19	19.5	19.4	19.5	19.5	19.4

Table 4. Phagocytic index of sheep vaccinated with the prepared FMD polyvalent vaccine formulae

Vaccine formulae	Phagocytic index					
	1 day	3rd day	7th day	14th day	21st day	28th day
Formula-1	0.11	0.32	0.49	0.61	0.69	0.45
Formula-2	0.10	0.50	0.61	0.82	0.8	0.58
Formula-3	0.10	0.50	0.67	0.84	0.81	0.59
Formula-4	0.11	0.84	0.98	0.99	0.90	0.68
Negative non vaccinated control	0.12	0.12	0.12	0.13	0.12	0.10

Table 5. Mean delta optical density of Interleukin-6 in sheep vaccinated with the prepared polyvalent FMD vaccine formulae

Vaccine formulae	IL-6 (ng/ml) at DPV					
	1 day	3rd day	7th day	14th day	21st day	28th day
Formula-1	0.85	1.42	2.01	3.74	3.42	3.17
Formula-2	1.22	1.93	3.92	3.72	3.52	3.21
Formula-3	1.41	2.49	4.59	3.96	3.70	3.32
Formula-4	2.42	3.51	4.73	4.12	3.97	3.64
Negative non vaccinated control	0.4	0.39	0.42	0.45	0.43	0.48

Formula-1: Polyvalent FMD vaccine adjuvanted with Montanide ISA 206.
Formula-2: Polyvalent FMD vaccine adjuvanted with Montanide ISA 206 and aluminium hydroxide gel.
Formula-3: Polyvalent FMD vaccine adjuvanted with Montanide ISA 201.
Formula-4: Polyvalent FMD vaccine adjuvanted with Montanide ISA 201 and aluminium hydroxide gel.

Table 6. FMD serotype-O serum neutralizing antibody titer and ELISA (expressed as log_{10}) in different vaccinated sheep groups

WPV	Mean FMD serotype-O antibody titers in sheep vaccinated with							
	Formula-1		Formula-2		Formula-3		Formula-4	
	SNT ±SE	ELISA ±SE	SNT ±SE	ELISA ±SE	SNT ±SE	ELISA ±SE	SNT ±SE	ELISA ±SE
0	0.27 ±0.15^{a}	0.42 ±0.19^{a}	0.3 ±0.088^{a}	0.6 ±0.102^{a}	0.3 ±0.12^{a}	0.6 ±0.03^{a}	0.3 ±0.047^{a}	0.6 ±0.043^{a}
1	1.13 ±0.075^{a,b}	1.35 ±0.01^{a,b}	1.15 ±0.009^{a}	1.32 ±0.099^{a}	1.35 ±0.070^{a,b}	1.52 ±0.14^{a}	1.45 ±0.077^{b}	1.62 ±0.077^{b}
2	1.45 ±0.129^{a}	1.64 ±0.12^{a}	1.54 ±0.082^{a}	1.81 ±0.084^{a}	1.62 ±0.120^{a}	1.8 ±0.090^{a}	1.73 ±0.047^{a}	1.92 ±0.049^{a}
3	1.65 ±0.070^{a}	1.92 ±0.090^{a}	1.72 ±0.095^{a}	1.96 ±0.104^{a}	1.74 ±0.070^{a}	2 ±0.070^{a}	1.86 ±0.037^{a}	2.21 ±0.045^{a}
4	1.95 ±0.060^{b}	2.21 ±0.070^{b}	2.03 ±0.076^{a}	2.25 ±0.087^{a}	2.13 ±0.060^{b}	2.28 ±0.090^{b}	2.34 ±0.067^{a}	2.57 ±0.070^{a}
6	2.42 ±0.090^{c}	2.63 ±0.090^{c}	2.5 ±0.006^{a}	2.81 ±0.075^{a}	2.55 ±0.090^{c}	2.87 ±0.040^{c}	2.72 ±0.122^{a}	2.98 ±0.128^{a}
8	2.55 ±0.056^{b}	2.81 ±0.040^{b}	2.62 ±0.076^{a}	2.91 ±0.088^{a}	2.7 ±0.050^{b}	2.96 ±0.040^{b}	3.31 ±0.067^{a,b}	3.57 ±0.075^{a,b}
10	2.58 ±0.056^{b}	2.83 ±0.040^{b}	2.92 ±0.006^{a}	3.13 ±0.03^{a}	3.1 ±0.050^{b}	3.23 ±0.050^{b}	3.28 ±0.095^{a,b}	3.48 ±0.104^{a,b}
12	2.65 ±0.006^{b}	2.92 ±0.050^{b}	2.85 ±0.037^{a}	3.02 ±0.043^{a}	2.97 ±0.037^{c}	3.23 ±0.040^{c}	3.1 ±0.037^{b}	3.35 ±0.037^{b}
14	2.76 ±0.030^{c}	2.99 ±0.030^{c}	2.79 ±0.006^{a}	2.99 ±0.056^{a}	2.85 ±0.050^{a}	3.11 ±0.050^{a,b}	2.93 ±0.073^{b}	3.2 ±0.066^{b}
16	2.46 ±0.050^{b}	2.69 ±0.040^{b}	2.52 ±0.10^{a}	2.71 ±0.101^{a}	2.67 ±0.06^{a,b}	2.93 ±0.070^{a,b}	2.83 ±0.082^{a}	3.08 ±0.074^{a,b}
20	2.12 ±0.060^{a,b}	2.45 ±0.050^{a,b}	2.22 ±0.102^{a}	2.31 ±0.095^{a}	2.46 ±0.080^{a}	2.72 ±0.090^{a}	2.62 ±0.073^{a,b}	2.9 ±0.066^{a,b}
24	1.84 ±0.080^{a}	2.12 ±0.070^{a}	1.89 ±0.110^{a}	2.12 ±0.099^{a}	2.25 ±0.102^{a,b}	2.51 ±0.115^{a}	2.46 ±0.076^{a,b}	2.72 ±0.066^{a,b}
28	1.65 ±0.010^{a,b}	1.93 ±0.093^{a,b}	1.67 ±0.009^{a}	1.96 ±0.088^{a}	1.95 ±0.122^{b}	2.17 ±0.040^{b,c}	2.19 ±0.142^{b}	2.47 ±0.144^{b}
32	1.52 ±0.120^{b,c}	1.84 ±0.118^{b,c}	1.52 ±0.095^{a}	1.84 ±0.103^{a}	1.83 ±0.050^{c}	2 ±0.07^{c}	1.81 ±0.056^{b}	1.98 ±0.058^{b}
36	1.05 ±0.102^{a,c}	1.28 ±0.047^{a,c}	1.05 ±0.076^{a}	1.28 ±0.081^{a}	1.65 ±0.080^{b}	1.91 ±0.086^{b}	1.65 ±0.047^{a,b}	1.92 ±0.043^{b}
40	0.75 ±0.102^{a,b}	1.01 ±0.086^{a,b}	0.75 ±0.095^{a}	1.01 ±0.103^{a}	1.35 ±0.102^{a}	1.61 ±0.09^{a,b}	1.37 ±0.076^{a,b}	1.68 ±0.086^{a}

SNT=Serum neutralization test, ELISA=Enzyme linked immune sorbent assay, WPV=Week post vaccination, SE=Standard error, FMDV=Foot and mouth disease virus, different letters indicate significant difference between different treatments at $p<0.05$ according to Duncan's multiple range test

Table 7. FMD serotype-A serum neutralizing antibody titer and ELISA (expressed as log10) in different vaccinated sheep groups

WPV	Mean FMD serotype-A antibody titers in sheep vaccinated with							
	Formula-1		Formula-2		Formula-3		Formula-4	
	SNT	ELISA	SNT	ELISA	SNT	ELISA	SNT	ELISA
0	0.27 ±0.073^{a}	0.39 ±0.070^{a}	0.27 ±0.088^{a}	0.55 ±0.099^{a}	0.27 ±0.010^{a}	0.55 ±0.036^{a}	0.3 ±0.047^{a}	0.6 ±0.059^{a}
1	1.02 ±0.110^{a}	1.24 ±0.111^{a}	1 ±0.010^{a}	1.26 ±0.015^{a}	1.02 ±0.060^{a,b}	1.29 ±0.076^{b}	1.48 ±0.075^{b}	1.61 ±0.089^{b}
2	1.33 ±0.080^{a}	1.61 ±0.070^{a}	1.52 ±0.080^{a}	1.8 ±0.082^{a}	1.59 ±0.100^{a}	1.84 ±0.022^{a}	1.71 ±0.035^{a}	1.82 ±0.039^{a}
3	1.52 ±0.060^{a}	1.82 ±0.050^{b}	1.72 ±0.092^{a}	2 ±0.113^{a}	1.77 ±0.072^{a}	2.04 ±0.047^{a}	1.87 ±0.051^{a}	2.3 ±0.069^{a}
4	1.85 ±0.047^{b}	2.21 ±0.041^{b}	1.9 ±0.075^{a}	2.28 ±0.083^{a}	1.92 ±0.065^{a}	2.32 ±0.052^{a}	2.3 ±0.060^{a}	2.53 ±0.069^{a}
6	2.14 ±0.090^{b}	2.19 ±0.082^{b}	2.18 ±0.005^{a}	2.5 ±0.009^{a}	2.2 ±0.068^{a}	2.52 ±0.036^{a}	2.68 ±0.043^{a}	2.9 ±0.048^{a}
8	2.43 ±0.030^{a}	2.61 ±0.022^{b}	2.61 ±0.075^{a}	2.92 ±0.084^{a}	2.65 ±0.052^{b}	2.96 ±0.101^{a}	3.24 ±0.108^{a}	3.45 ±0.111^{a}
10	2.54 ±0.006^{a,b}	2.83 ±0.050^{a}	2.91 ±0.006^{a}	3.17 ±0.008^{a}	3 ±0.054^{b}	3.27 ±0.060^{a,b}	3.18 ±0.074^{a}	3.38 ±0.084^{a}
12	2.65 ±0.040^{b}	2.96 ±0.045^{b}	2.81 ±0.013^{a}	3.04 ±0.019^{a}	2.91 ±0.037^{c}	3.18 ±0.092^{a,b}	3.1 ±0.098^{b}	3.3 ±0.099^{b}
14	2.76 ±0.006^{a}	3.01 ±0.110^{a}	2.81 ±0.105^{a}	3.04 ±0.109^{a}	2.89 ±0.042^{a}	3.12 ±0.032^{b}	2.9 ±0.038^{b}	3.15 ±0.049^{b}
16	2.45 ±0.130^{a}	2.76 ±0.006^{a}	2.55 ±0.112^{a}	2.86 ±0.120^{a}	2.62 ±0.009^{a,b}	2.94 ±0.071^{b}	2.71 ±0.087^{b}	3.08 ±0.092^{b}
20	2.21 ±0.050^{b}	2.54 ±0.050^{a}	2.3 ±0.008^{a}	2.63 ±0.010^{a}	2.38 ±0.066^{a}	2.65 ±0.069^{a}	2.42 ±0.074^{a}	2.7 ±0.084^{a}
24	1.91 ±0.040^{a}	2.3 ±0.055^{a,b}	1.99 ±0.090^{a}	2.4 ±0.097^{a}	2.12 ±0.109^{a,b}	2.43 ±0.071^{a,b}	2.21 ±0.076^{a}	2.51 ±0.082^{a}
28	1.75 ±0.050^{b}	2.03 ±0.050^{a}	1.82 ±0.082^{a}	2.13 ±0.087^{a}	1.95 ±0.103^{b}	2.43 ±0.072^{a,b}	2.09 ±0.076^{a}	2.47 ±0.091^{a}
32	1.65 ±0.060^{c}	1.89 ±0.101^{a}	1.66 ±0.081^{a}	1.99 ±0.086^{a}	1.8 ±0.052^{c}	2.19 ±0.111^{b}	1.83 ±0.124^{b}	2.21 ±0.132^{a,b}
36	1.05 ±0.060^{a}	1.24 ±0.060^{c}	1.15 ±0.089^{a}	1.29 ±0.093^{a}	1.73 ±0.083^{c}	1.92 ±0.052^{b}	1.75 ±0.057^{b}	1.95 ±0.069^{b}
40	0.62 ±0.070^{b}	0.96 ±0.007^{b}	0.71 ±0.066^{a}	0.99 ±0.071^{a}	1.4 ±0.101^{a,b}	1.65 ±0.063^{a,b}	1.47 ±0.075^{a}	1.68 ±0.083^{a}

SNT=Serum neutralization test, ELISA=Enzyme linked immune sorbent assay, WPV=Week post vaccination, SE=Standard error, FMDV=Foot and mouth disease virus, different letters indicate significant difference between different treatments at $p<0.05$ according to Duncan's multiple range test

Table 8. FMD serotype-SAT2/2012 serum neutralizing antibody titer and ELISA (expressed as log_{10}) in different vaccinated sheep groups

WPV	Mean FMD serotype-SAT2/2012 antibody titers in sheep vaccinated with							
	Formula-1		Formula-2		Formula-3		Formula-4	
	SNT	ELISA	SNT	ELISA	SNT	ELISA	SNT	ELISA
0	0.15 ±0.006^{a}	0.4 ±0.077^{a}	0.41 ±0.052^{a}	0.66 ±0.064^{a}	0.51 ±0.015^{a}	0.76 ±0.032^{a}	0.3 ±0.042^{a}	0.6 ±0.049^{a}
1	0.23 ±0.080^{b}	1.52 ±0.1^{b}	1.22 ±0.081^{a}	1.48 ±0.092^{a}	1.32 ±0.060^{a,b}	1.57 ±0.019^{a}	1.48 ±0.065^{b}	1.61 ±0.062^{a}
2	1.35 ±0.006^{b}	1.67 ±0.072^{b}	1.67 ±0.101^{b}	1.86 ±0.105^{b}	1.71 ±0.126^{a}	1.96 ±0.096^{a}	1.87 ±0.043^{a}	2.2 ±0.046^{a}
3	1.55 ±0.052^{b}	1.91 ±0.063^{b}	1.87 ±0.062^{a}	2.12 ±0.06^{a}	1.92 ±0.060^{a}	2.17 ±0.060^{a}	2.32 ±0.021^{a}	2.55 ±0.023^{a}
4	1.84 ±0.005^{b}	2.16 ±0.003^{b}	2.1 ±0.104^{a}	2.35 ±0.109^{a}	2.19 ±0.060^{a}	2.44 ±0.080^{a}	2.71 ±0.050^{a}	2.93 ±0.054^{a}
6	2.14 ±0.053^{a,b}	2.36 ±0.062^{a,b}	2.31 ±0.112^{a,b}	2.52 ±0.119^{a,b}	2.37 ±0.094^{c}	2.62 ±0.045^{b}	2.92 ±0.101^{a,b}	3.25 ±0.104^{a,b}
8	2.42 ±0.002^{a}	2.69 ±0.070^{a}	2.71 ±0.139^{a}	2.95 ±0.144^{a}	2.76 ±0.055^{b}	3.01 ±0.042^{b}	3.18 ±0.063^{a}	3.38 ±0.069^{a}
10	2.42 ±0.076^{a,b}	2.69 ±0.091^{a,b}	3.01 ±0.072^{a}	3.26 ±0.072^{a}	3.09 ±0.043^{b}	3.34 ±0.043^{b}	3.33 ±0.092^{a,b}	3.55 ±0.096^{a,b}
12	2.55 ±0.003^{a}	2.83 ±0.030^{a}	2.87 ±0.053^{a}	3.12 ±0.055^{a}	2.97 ±0.035^{c}	3.22 ±0.039^{b}	3.15 ±0.074^{a}	3.36 ±0.077^{a}
14	2.71 ±0.003^{c}	2.93 ±0.025^{c}	2.76 ±0.09^{b}	3 ±0.086^{a}	2.82 ±0.055^{a}	3.07 ±0.054^{a,b}	3 ±0.081^{a}	3.24 ±0.085^{a}
16	2.51 ±0.005^{b}	2.73 ±0.022^{b}	2.61 ±0.071^{b}	2.86 ±0.048^{a}	2.61 ±0.06^{a,b}	2.86 ±0.032^{a,b}	2.71 ±0.077^{a}	3.08 ±0.077^{a}
20	2.33 ±0.004^{b}	2.54 ±0.026^{b}	2.47 ±0.072^{a}	2.62 ±0.049^{a}	2.47 ±0.084^{a}	2.62 ±0.092^{b}	2.49 ±0.101^{a}	2.7 ±0.109^{a}
24	2.14 ±0.006^{b}	2.43 ±0.062^{b,c}	2.14 ±0.10^{a}	2.43 ±0.15^{a}	2.26 ±0.101^{a,b}	2.51 ±0.114^{a}	2.31 ±0.022^{a}	2.61 ±0.029^{a}
28	1.81 ±0.007^{b}	2.16 ±0.042^{b}	1.81 ±0.070^{a}	2.16 ±0.074^{a}	1.95 ±0.113^{b}	2.23 ±0.102^{b,c}	2.02 ±0.06^{a}	2.27 ±0.07^{a}
32	1.65 ±0.009^{b}	1.92 ±0.021^{b}	1.65 ±0.023^{a}	1.92 ±0.029^{a}	1.86 ±0.052^{c}	2.04 ±0.09^{a}	1.89 ±0.021^{a}	2.1 ±0.024^{a,b}
36	1.22 ±0.101^{b}	1.48 ±0.093^{b}	1.22 ±0.114^{a}	1.48 ±0.123^{a}	1.68 ±0.062^{c}	1.97 ±0.069^{a}	1.73 ±0.121^{a,b}	1.99 ±0.130^{a,b}
40	0.75 ±0.122^{a}	1.04 ±0.134^{a}	0.75 ±0.140^{a}	1.04 ±0.145^{a}	1.47 ±0.101^{a,b}	1.72 ±0.06^{a}	1.47 ±0.070^{a,b}	1.68 ±0.075^{a}

SNT=Serum neutralization test, ELISA=Enzyme linked immune sorbent assay, WPV=Week post vaccination, SE=Standard error, FMDV=Foot and mouth disease virus, different letters indicate significant difference between different treatments at $p<0.05$ according to Duncan's multiple range test

Table 9. FMD serotype-SAT2/2018 serum neutralizing antibody titer and ELISA (expressed as log10) in different vaccinated sheep groups

WPV	Mean FMD serotype-SAT2/2018 antibody titers in sheep vaccinated with							
	Formula-1		Formula-2		Formula-3		Formula-4	
	SNT	ELISA	SNT	ELISA	SNT	ELISA	SNT	ELISA
0	0.31 ±0.03^{a}	0.44 ±0.06^{a}	0.42 ±0.09^{a}	0.76 ±0.144^{a}	0.62 ±0.084^{a}	0.86 ±0.073^{a}	0.51 ±0.009^{b}	0.76 ±0.040^{b}
1	1.24 ±0.076^{a,b}	1.46 ±0.094^{a}	1.39 ±0.092^{b}	1.61 ±0.068^{b}	1.41 ±0.118^{a}	1.67 ±0.139^{a}	1.46 ±0.082^{c}	1.77 ±0.008^{b}
2	1.24 ±0.137^{a}	1.46 ±0.139^{a}	1.62 ±0.087^{b}	1.98 ±0.139^{b}	1.68 ±0.088^{a}	2.01 ±0.06^{b}	1.71 ±0.010^{a}	2.2 ±0.019^{a}
3	1.55 ±0.056^{a,b}	1.92 ±0.087^{a}	1.87 ±0.056^{b,c}	2.05 ±0.047^{a,b}	1.91 ±0.045^{a}	2.12 ±0.112^{a,b}	2.01 ±0.050^{a}	2.25 ±0.060^{a}
4	1.95 ±0.102^{a}	2.13 ±0.09^{c}	2.01 ±0.102^{b}	2.22 ±0.139^{a}	2.08 ±0.102^{a}	2.32 ±0.102^{a}	2.19 ±0.069^{a}	2.44 ±0.109^{a}
6	2.01 ±0.087^{b}	2.21 ±0.114^{a}	2.21 ±0.083^{b}	2.43 ±0.083^{b}	2.31 ±0.047^{a,b}	2.53 ±0.034^{b}	2.76 ±0.054^{b}	3.01 ±0.034^{a,b}
8	2.15 ±0.050^{c}	2.25 ±0.122^{a}	2.61 ±0.037^{a}	2.84 ±0.157^{a}	2.7 ±0.066^{b}	2.94 ±0.075^{b}	3.36 ±0.097^{a}	3.58 ±0.139^{a}
10	2.23 ±0.102^{a}	2.31 ±0.06^{a,c}	2.94 ±0.04^{b}	3.17 ±0.050^{b}	3.01 ±0.102^{b}	3.27 ±0.114^{a}	3.19 ±0.132^{a}	3.38 ±0.056^{b,c}
12	2.31 ±0.141^{a,b}	2.51 ±0.056^{b,c}	2.76 ±0.083^{b}	3.04 ±0.111^{b}	2.83 ±0.073^{b}	3.12 ±0.139^{a}	3.02 ±0.101^{a,b}	3.25 ±0.112^{b}
14	2.54 ±0.056^{a}	2.8 ±0.144^{a}	2.81 ±0.047^{a,b}	3.07 ±0.071^{a}	2.81 ±0.090^{a}	3.07 ±0.05^{b}	2.92 ±0.066^{a}	3.15 ±0.102^{b}
16	2.42 ±0.064^{a}	2.61 ±0.053^{a}	2.61 ±0.050^{a,b}	2.81 ±0.084^{a}	2.61 ±0.120^{a,c}	2.81 ±0.091^{a,b}	2.72 ±0.015^{a}	2.89 ±0.03^{b}
20	2.1 ±0.036^{b}	2.2 ±0.073^{a}	2.11 ±0.112^{a}	2.21 ±0.042^{a}	2.31 ±0.050^{c}	2.51 ±0.076^{a}	2.47 ±0.050^{a,b}	2.68 ±0.09^{a}
24	2.1 ±0.076^{a,b}	2.2 ±0.102^{b}	2.16 ±0.056^{a}	2.29 ±0.060^{c}	2.26 ±0.077^{b}	2.49 ±0.114^{a}	2.36 ±0.084^{a}	2.59 ±0.084^{a}
28	1.65 ±0.037^{a}	1.94 ±0.114^{a}	1.7 ±0.139^{a}	2.26 ±0.008^{a}	2.01 ±0.103^{a}	2.26 ±0.03^{b}	2.15 ±0.009^{a,b}	2.33 ±0.114^{a}
32	1.51 ±0.102^{a}	1.81 ±0.110^{a,b}	1.76 ±0.141^{a,b}	1.87 ±0.010^{a}	1.86 ±0.110^{a}	2.09 ±0.1^{b}	1.86 ±0.120^{c}	2.18 ±0.04^{b}
36	1.24 ±0.137^{a}	1.59 ±0.09^{a}	1.48 ±0.056^{b,c}	1.68 ±0.047^{b}	1.71 ±0.010^{a,b}	2.04 ±0.062^{b,c}	1.86 ±0.048^{b}	2.18 ±0.141^{c}
40	0.61 ±0.03^{b}	0.93 ±0.139^{b}	1.42 ±0.101^{c}	1.61 ±0.022^{b}	1.42 ±0.037^{c}	1.61 ±0.076^{a,b}	1.47 ±0.123^{b}	1.72 ±0.09^{b}

SNT=Serum neutralization test, ELISA=Enzyme linked immune sorbent assay, WPV=Week post vaccination, SE=Standard error, FMDV=Foot and mouth disease virus, different letters indicate significant difference between different treatments at $p<0.05$ according to Duncan's multiple range test

DISCISSION

Foot and Mouth Disease (FMD) is an acute disease caused by Foot and Mouth Disease Virus (FMDV) which causes important economic losses **(Orsel *et al.*, 2007)**. The control of FMD in animals is considered to be important and effective in limiting the spread of infection through effective vaccination using safe and potent FMD vaccine with local circulating FMDV serotypes to give high and early onset of protection with long duration especially in endemic areas as Egypt. FMD vaccines can be defined as a fixed formulation of specific amount of chemically inactivated virus strains mixed with suitable adjuvant. Selecting the suitable vaccine formulation is dependent on several factors as the onset of protection, the duration of protection against FMD and the target species being vaccinated.

The effective formulation of inactivated FMD vaccines requires adjuvant as aluminum hydroxide gel and mineral oils-based formulations which have been widely employed in experimental studies to obtain a vaccine that stimulates a rapid and long-lasting protective immune response and it must be safe for animal use. From this concern, continuous researches must be applied to reach a highly potent with long lived post vaccinal immunogenicity with safety issue .So, this research is interested in comparing between different vaccine formulations differ in the adjuvant added as four vaccine formulations were prepared using Montanide ISA 206 (Formula-1) ,Montanide ISA 206 mixed with Aluminium hydroxide gel (Formula-2), Montanide ISA 201 (Formula-3) and Montanide ISA 201 mixed with aluminium hydroxide gel(Formula-4). Firstly safety test of the prepared vaccines was done in mice and the results showed that the four prepared vaccines were safe for animal use during the whole experiment time and agreed with the requirements of vaccine preparation of **OIE (2017)**, Table (1).

Regarding the cellular immune response of sheep to different FMD vaccine formulae, evaluation of the cellular immunity included were done through estimation of the lymphocyte blastogenesis, phagocytic percentage, phagocytic index in addition to IL-6 levels. The efficient induction of early protection against contact infections by FMDV relies on the rapid assimilation of appropriate innate immune defense, probably leading to the enhanced induction of specific immune responses **(Barnett *et al.*, 2002).** Table (2) showed that the cellular immune response of sheep to the inactivated FMD ISA 206 oil vaccine (Formula-1) revealed increasing mean delta optical density of lymphocyte blastogenesis assay at day 1, 3, 7, 14, 21 and 28 DPV from 0.11 at the day 1 to reach its maximum value (0.61) at the 21st DPV then declined at the 28st DPV (0.50), but in sheep vaccinated with inactivated FMD 206 oil with aluminum hydroxide gel vaccine (Formula-2), showed an increase in the mean value from (0.22) at the day 1 to reach its maximum value (0.99) at the 7th DPV then declined at the 14th DPV (0.74) while the inactivated FMD Montanide 201 oil (Formula-3) revealed increasing from 0.32 at the day 1 to reach its maximum value (1.01) at the 7th DPV then declined at the 14th DPV (0.80), finally, sheep vaccinated with inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine (Formula-4) showed mean delta optical density of lymphocyte blastogenesis assay increasing from 0.33 at the day 1 to reach its maximum value (1.21) at the 7th DPV then declined at the 14th DPV (0.99), but the control sheep remain mean delta optical density of lymphocyte blastogenesis assay around 0.10 to 0.13 allover the time of estimation.

Tables (3,4) showed that the cellular immune response of sheep to the inactivated FMD ISA 206 oil vaccine (Formula-1) revealed increasing phagocytic % and Phagocytic index at day 1, 3, 7, 14, 21 and 28 DPV from (20.1 and 0.11 respectively) at the day 1 to reach its maximum value (66.4, 0.69 respectively) at the 21st DPV then declined at the 28st DPV (54.2 and 0.45 respectively) but in sheep vaccinated with inactivated FMD 206 oil with aluminum hydroxide gel vaccine (Formula-2), showed an increase in the mean value of phagocytic % and Phagocytic index from (23 and 0.10 respectively) at the day 1 to reach its maximum value (69.2 and 0.82 respectively) at the 14th DPV then declined at the 21st DPV (66.2 and 0.8 respectively) while the inactivated FMD Montanide 201 oil (Formula-3) revealed increasing in the mean value of phagocytic % and Phagocytic index from (23.3 and 0.10 respectively) at the day 1 to reach its maximum value (70.4 and 0.84 respectively) at the 14th DPV then declined at the 21st DPV (66.3, 0.81respectively) , Finally sheep vaccinated with inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine (Formula-4) showed phagocytic % and Phagocytic index increasing from (29.2 and 0.11 respectively) at the day 1 to reach its maximum value (92.3 and 0.99 respectively) at the 14th DPV then declined at the 21st

DPV (70.4 and 0.90 respectively), but the control sheep remain phagocytic % and Phagocytic index around (19 to 19.5 and 0.10 to 0.13 respectively) all over the time of estimation.

Table (5) showed that the cellular immune response through estimation of interleukin-6 (IL6) of sheep to the inactivated FMD ISA 206 oil vaccine (Formula-1) revealed increasing of mean value of IL6 at day 1, 3, 7, 14, 21 and 28 DPV from (0.85) at the day 1 to reach its maximum value (3.74) at the 14th DPV then declined at the 21st DPV (3.42), but in sheep vaccinated with inactivated FMD 206 oil with aluminum hydroxide gel vaccine (Formula-2), showed an increase in the mean value of IL6 from (1.22) at the day 1 to reach its maximum value (3.92) at the 7th DPV then declined at the 14th DPV (3.72). While the inactivated FMD Montanide 201 oil (Formula-3) revealed increasing in the mean value of IL6 from (1.41) at the day 1 to reach its maximum value (4.59) at the 7th DPV then declined at the 14th DPV (3.96). Sheep vaccinated with inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine (Formula-4) showed increasing in the mean value of IL6 from (2.42) at the day 1 to reach its maximum value (4.73) at the 7th DPV then declined at the 14th DPV (4.12), While the control negative non vaccinated sheep group, the mean value of IL6 remain around (0.39 to 0.48) allover the time of estimation.

From the above results and the statistical analysis of cellular immunity, it revealed that the FMD vaccine adjuvanated with Montanide ISA oils either 201 or 206 with aluminum hydroxide gel showed a higher post vaccinal cellular immune response than that without mixing with gel and so the addition of gel has a great impact on the post vaccinal cellular immune response **(Park *et al.*, 2014)**. These results were in agreement with (**Knudsen *et al.*, 1979; Sharma *et al.*, 1984)** who reported that cell mediated immune response was a constitute of immune response against FMD virus, and in agreement in some points with (**Knudsen *et al.*, 1979; Mercedes *et al.*, 1996; Elwatany *et al.*, 1999; Sonia *et al.*, 2010; Fakhry *et al.*, 2012 and Mossad *et al.*, 2014)** who mentioned that the Delta optical density of lymphocyte blastogenesis assay and interleukin-6 at day 0, 3, 7, 14, 21 and 28 days post vaccination (DPV) showed that a significant difference between vaccinated and control groups started at 3rd DPV and increased gradually till 21st DPV using trivalent FMD Montanide inactivated vaccine.

Evaluation of the humeral immune response against FMDV serotype (O) antibody titer in vaccinated sheep with different prepared oil adjuvant vaccine formulae were done using SNT and ELISA data (Table-6) showed differences in the onset, intensity and duration of the FMD serotype O antibodies. Concerning the onset of protective antibody titer, it is clear that inactivated FMD ISA 206 oil vaccine induced titers of (1.65±0.070^{a} by SNT and 1.92 ±0.090^{a} log_{10} by ELISA) in the 3rd WPV and inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine induced titers of (1.54 ±0.082^{a} by SNT and 1.81±0.084^{a} log_{10} by ELISA) in the 2nd WPV while inactivated FMD ISA 201 oil vaccine showed earlier immune response in the 2nd WPV (1.62±0.120^{a} by SNT, 1.8±0.090^{a} log_{10} by ELISA). On the other side, the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced protective type (O) antibody titer (1.73±0.047^{a} by SNT and 1.92±0.049^{a} log_{10} by ELISA) in the 2nd WPV.

It is clear that peak of the protective antibody titers induced by the inactivated FMD ISA 206 oil vaccine (2.76±0.030^{c} by SNT and 2.99±0.030^{c} log_{10} by ELISA) appeared in the 14th WPV and by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (2.92±0.006^{a} as SNT and 3.13±0.003^{a} log_{10} as ELISA) in the 10th WPV while the inactivated FMD ISA 201 oil vaccine induced the peak of antibody titers in the 10th WPV (3.1±0.050^{b} by SNT, 3.23±0.050^{b} log_{10} by ELISA). On the other side, the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced peak protective antibody titers (3.31±0.067^{a,b} by SNT and 3.57±0.075^{a,b} $log_{10}$by ELISA) in the 8th WPV.

Regarding the duration of the protective type (O) antibody titers , it is clear that inactivated FMD ISA 206 oil vaccine showed protective titers of (1.52±0.120^{b,c} by SNT and 1.84±0.118^{b,c} log_{10} by ELISA) up to the 32th WPV and also those induced by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (1.52±0.095^{a} as SNT and 1.84±0.103^{a} log_{10} as ELISA) up to the 32th WPV while inactivated FMD ISA 201 oil vaccine showed later protective antibody titers in the 36th WPV (1.65±0.080^{b} by SNT and 1.91±0.086^{b} log_{10} by ELISA). Also, it was noticed that the protective type (O) antibody titers induced by inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine (1.65±0.047^{a,b} by

SNT and 1.92±0.043[b] log_{10} by ELISA) up to the 36th WPV.

From the above results and the statistical analysis of humeral antibody titers against FMDV serotype (O) revealed that the Montanide oils 201 with aluminum hydroxide gel is the best vaccine formula then Montanide oils 201 induced earlier, long lasting immunity then Montanide oils 206 with aluminum hydroxide gel and finally Montanide oils 206.These results came in parallel to those described by **(Dong *et al.*, 2013)** who mentioned that the ELISA antibodies against FMDV type O were compared as induced by Montanide oils 201and 206 showing that the antibody titer induced by oil 201-vaccine were higher than those induced by the oil 206 vaccine on 3dpv, 7dpv, 14dpv, 21dpv and 28dpv. This means that the immune stimulating effect of 201oil is better than that of 206-vaccine.

Regarding to FMDV serotype (A) antibody titers induced in vaccinated sheep the different prepared vaccine formulae are determined by using SNT and ELISA data (Table-7) showed differences in the onset, intensity and duration of the FMD serotype A antibodies. Concerning the onset of protective antibody titer, it is clear that inactivated FMD ISA 206 oil vaccine induced titers of (1.52±0.060[a] by SNT and 1.82 ±0.050[b] log_{10} by ELISA) in the 3rd WPV and inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine induced titers of (1.52±0.080[a] by SNT and 1.8±0.082[a] log_{10} by ELISA) in the 2nd WPV while inactivated FMD ISA 201 oil vaccine showed earlier immune response in the 2nd WPV (1.59±0.100[a] by SNT, 1.84±0.022[a] log_{10} by ELISA). On the other side the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced protective type (A) antibody titer (1.71±0.035[a] by SNT and 1.82±0.039[a] log_{10} by ELISA) in the 2nd WPV.

It is clear that peak of the protective antibody titers induced by the inactivated FMD ISA 206 oil vaccine (2.76±0.006[a] by SNT and 3.01±0.110[a] log_{10} by ELISA) appeared in the 14th WPV and by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (2.91±0.006[a] as SNT and 3.17±0.008[a] log_{10} as ELISA) in the 10th WPV while the inactivated FMD ISA 201 oil vaccine induced the peak of antibody titers in the 10th WPV (3.0±0.054[b] by SNT, 3.27±0.060[a,b] log_{10} by ELISA). On the other side the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced peak protective antibody titers (3.24±0.108[a] by SNT and 3.45±0.111[a] $log_{10}$by ELISA) in the 8th WPV.

Regarding the duration of the protective type (A) antibody titers , it is clear that inactivated FMD ISA 206 oil vaccine showed protective titers of (1.65±0.060[c] by SNT and 1.89±0.101[a] log_{10} by ELISA) up to the 32th WPV and also those induced by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (1.66±0.081[a] as SNT and 1.99±0.086[a] log_{10} as ELISA) up to the 32th WPV (WPV) while inactivated FMD ISA 201 oil vaccine showed later protective antibody titers in the 36th WPV (1.73±0.083[c] by SNT and 1.92±0.052[b] log_{10} by ELISA). Also, it was noticed that the protective type (A) antibody titers induced by inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine (1.75±0.057[b] by SNT and 1.95±0.069[b] log_{10} by ELISA) up to the 36th WPV. From the above results and the statistical analysis of humeral antibody titers against FMDV serotype A revealed that the Montanide oils 201 with aluminum hydroxide gel is the best vaccine formula then Montanide oils 201 induced earlier, long lasting immunity then Montanide oils 206 with aluminum hydroxide gel and finally Montanide oils 206.

Demonstration of FMD type SAT2/Egypt/2012 antibody titers induced in vaccinated sheep with the prepared different oil vaccine formulae using SNT and ELISA data (Table-8) showed differences in the onset, intensity and duration of the FMD serotype SAT2/Egypt/2012 antibodies. Concerning the onset of protective antibody titer, it is clear that inactivated FMD ISA 206 oil vaccine induced titers of (1.55±0.052[b] by SNT and 1.91±0.063[b] log_{10} by ELISA) in the 3rd WPV and inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine induced titers of (1.67±0.101[b] by SNT and 1.86±0.105[b] log_{10} by ELISA) in the 2nd WPV also inactivated FMD ISA 201 oil vaccine showed earlier immune response in the 2nd WPV (1.71±0.126[a] by SNT, 1.96±0.096[a] log_{10} by ELISA). On the other side, the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced protective type SAT2/Egypt/2012 antibody titer (1.87±0.043[a] by SNT and 2.20±0.046[a] log_{10} by ELISA) in the 2nd WPV.

It is clear that peak of the protective antibody titers induced by the inactivated FMD ISA 206 oil vaccine (2.71±0.003[c] by SNT and 2.93±0.025[c] log_{10} by

ELISA) appear in the 14th WPV and by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (3.01±0.072[a] as SNT and 3.26±0.072[a] log_{10} as ELISA) in the 10th WPV also the inactivated FMD ISA 201 oil vaccine induced the peak of antibody titers in the 10th WPV (3.09±0.043[b] by SNT, 3.34±0.043[b] log_{10} by ELISA). On the other side, the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced peak protective antibody titers (3.33±0.092[a,b] by SNT and 3.55±0.096[a,b] $log_{10}$by ELISA) in the 10th WPV.

Regarding the duration of the protective type-SAT2/Egypt/2012 antibody titers , it is clear that inactivated FMD ISA 206 oil vaccine showed protective titers of (1.65±0.009[b] by SNT and 1.92±0.021[b] log_{10} by ELISA) up to the 32th WPV and also those induced by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (1.65±0.023[a] as SNT and 1.92±0.029[a] log_{10} as ELISA) up to the 32th WPV while inactivated FMD ISA 201 oil vaccine showed later protective antibody titers in the 36th WPV (1.68±0.062[c] by SNT and 1.97±0.069[a] log_{10} by ELISA).It was noticed that the protective type SAT2/Egypt/2012 antibody titers induced by inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine (1.73±0.121[a,b] by SNT and 1.99±0.130[a,b] log_{10} by ELISA) up to the 36th WPV. From the above results and the statistical analysis of humeral antibody titers against FMDV serotype SAT2/Egypt/2012 revealed that the Montanide oils 201 with aluminum hydroxide gel is the best vaccine formula then Montanide oils 201 induced earlier, long lasting immunity then Montanide oils 206 with aluminum hydroxide gel and finally Montanide oils 206.

Demonstration of FMD type SAT2/Egypt/2018 antibody titers induced in vaccinated sheep with the prepared different oil vaccine formulae by using SNT and ELISA data (Table-9) showed differences in the onset, intensity and duration of the FMD serotype SAT2/Egypt/2018 antibodies. Concerning the onset of protective antibody titer, it is clear that inactivated FMD ISA 206 oil vaccine induced titers of (1.55±0.056[a,b] by SNT and 1.92±0.087[a] log_{10} by ELISA) in the 3rd WPV and inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine induced titers of (1.62±0.087[b] by SNT and 1.98±0.139[b] log_{10} by ELISA) in the 2nd WPV also inactivated FMD ISA 201 oil vaccine showed earlier immune response in the 2nd WPV (1.68±0.088[a] by SNT, 2.01±0.06[b] log_{10} by ELISA). On the other side, the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced protective type SAT2/Egypt/2018 antibody titer (1.71±0.010[a] by SNT and 2.2±0.019[a] log_{10} by ELISA) in the 2nd WPV.

It is clear that peak of the protective antibody titers induced by the inactivated FMD ISA 206 oil vaccine (2.54±0.056[a] by SNT and 2.8±0.144[a] log_{10} by ELISA) appear in the 14th WPV and by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (2.94±0.04[b] as SNT and 3.17±0.050[b] log_{10} as ELISA) in the 10th WPV also the inactivated FMD ISA 201 oil vaccine induced the peak of antibody titers in the 10th WPV (3.01±0.102[b] by SNT, 3.27±0.114[a] log_{10} by ELISA). On the other side the inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine induced peak protective antibody titers (3.36±0.097[a] by SNT and 3.58±0.139[a] $log_{10}$by ELISA) in the 8th WPV.

Regarding the duration of the protective type-SAT2/Egypt/2018 antibody titers , it is clear that inactivated FMD ISA 206 oil vaccine showed protective titers of (1.51±0.102[a] by SNT and 1.81±0.110[a,b] log_{10} by ELISA) up to the 32th WPV and also those induced by the inactivated FMD ISA 206 oil with aluminum hydroxide gel vaccine (1.76±0.141[a,b] as SNT and 1.87±0.010[a] log_{10} as ELISA) up to the 32th WPV while inactivated FMD ISA 201 oil vaccine showed later protective antibody titers in the 36th WPV (1.71±0.010[a,b] by SNT and 2.04±0.062[b,c] log_{10} by ELISA).It was noticed that the protective type A antibody titers induced by inactivated FMD ISA 201 oil with aluminum hydroxide gel vaccine (1.86±0.048[b] by SNT and 2.18±0.141[c] log_{10} by ELISA) up to the 36th WPV. From the above results and the statistical analysis of humeral immunity, it revealed that the Montanide oils 201 with aluminum hydroxide gel is the best vaccine formula then Montanide oils 201 induced earlier, long lasting immunity then Montanide oils 206 with aluminum hydroxide gel and finally Montanide oils 206.

Our results came in parallel with the result obtained by **(EL-Sayed *et al.*, 2015)** who indicated that vaccines emulsified using Montanide ISA 201 adjuvant elicited a protective humoral immune response from the 2nd WPV for ISA 201 oil by SNT and ELISA titers of (1.62±0.047[a] and 1.8±0.049[a]); (1.59±0.076[a] and 1.836±0.077[a]) and (1.71±0.06[b] and 1.96±0.074[b]) by SNT and ELISA for serotypes O, A, SAT2,

respectively and ISA 206 showed antibody titer by SNT and ELISA of (1.5 ± 0.082^{a} and 1.84 ± 0.084^{a}); (1.56 ± 0.037^{a} and 1.818 ± 0.052^{a}) and ($1.5\pm0.106^{a,b}$ and $1.81\pm0.104^{a,b}$) for FMD virus serotypes O, A and SAT2, respectively. And also came in parallel with **(Wisniewski *et al.*, 1972)** they explained that the SNT measures those antibodies which neutralize the infectivity of FMD virion. The peak of antibody titre in all groups at 10-12 weeks post vaccination and continues with protective level till 32th WPV. Our results also were consistent with the statement of **(Hamblin *et al.*, 1986)** who explained that the SNT measures those antibodies which neutralize the infectivity of FMD virion, while ELISA probably measure all classes of antibodies even those produced against incomplete and non-infectious virus.

The selection of adjuvants in FMD vaccine formulation is important for both early and long-lasting immunity and protection (**Park *et al.*, 2014)**. The aluminum hydroxide gel is the most commonly used adjuvant in commercial vaccines (**Rimaniol and Gras, 2004)**. A previous report showed that aluminum hydroxide gel induces Th2-type responses in animal models, facilitating the dissemination of antibodies from the injected region (**Gupta *et al.*, 1995 and Brewer *et al.*, 1996)**. In addition, the aluminum hydroxide gel was shown to play an important role in memory responses by inducing the differentiation of macro- phages. Gel-adjuvanated FMD vaccines are currently used only in cattle, because they offer only a short period of immunity, making them unsuitable for use in pigs (**Park, 2013)**. Moreover, the immune responses in sheep and goats are poorer than those of oil-based vaccines **(Patil *et al.*, 2002 a,b)**. The combined components of oil and aluminum hydroxide gel have been used to protect against rabies in bovines (**Reddy and Srinivasan, 1996)**.

CONCLUSIONS

FMD polyvalent vaccines prepared with ISA 201 with aluminum hydroxide gel could be considered the best vaccines inducing both cellular and humeral immunity then vaccine formulation prepared with ISA 201 then ISA 206 with aluminum hydroxide gel and the lastly vaccines prepared with ISA 206 alone. Also we can conclude that aluminum hydroxide gel improves the effects of ISA adjuvants.

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How to cite this article:
El-Sayed, E. I. ; Mossad, W.G.;Hassanin A. I. and Shabana, W. 2020. Immunomodulating Efficacy of Different Adjuvants In Formulation of Foot and Mouth Disease Vaccine Relative to Its Immunogenicity. Journal of Applied Veterinary Sciences, 5(3): 14 - 30.
DOI:HTTPS://DX.DOI.ORG/10.21608/JAVS.2020.98318