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Humeral and cellular immune response of Egyptian trivalent foot and mouth disease oil vaccine in sheep

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Abstract

This work deals with the cellular and humeral immune response in sheep vaccinated with a trivalent inactivated oil Foot and Mouth Disease (FMD) vaccine produced locally in Egypt. Thirty sheep were vaccinated with the Egyptian FMD inactivated trivalent oil vaccine using a dose of 1.5 ml/animal inoculated subcutaneously while ten sheep were kept as control. Delta optical density of lymphocyte blastogenesis assay at day 0, 3, 7, 14, 21 and 28 days post vaccination (DPV) showed that there was a significant difference between vaccinated and control groups started at 3 DPV and increased gradually till 21 DPV. The phagocytic percentage started at 3 DPV and reached its highest ($P<0.05$) at 21 DPV. Regarding the phagocytic index, there was a significant difference ($P<0.05$) between vaccinated and control sheep groups starting at 3 DPV with its highest value at 21 DPV. At day 0, 3, 7, 14, 21 and 28 DPV an increase in the mean IL-6 and IL-12 concentration with a significant difference between the vaccinated group and the control group at 3 DPV and gradual increase ($P<0.05$) at 21 DPV was observed. In addition, serum neutralization and ELISA tests revealed that all vaccinated sheep exhibited detectable antibody levels against the three serotypes of FMDV from the first week post vaccination (WPV) and became protective at 3rd WPV. We concluded from the results that immune response was improved by the Egyptian FMD vaccine.

Keywords: FMD trivalent vaccine, sheep, cellular immunity, lymphocyte blastogenesis, phagocytic activity, IL-6, IL-12, SNT, ELISA

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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). Foot and Mouth disease virus (FMDV) is the etiologic agent of such devastating disease that can affect cloven-hoofed livestock. Infection with FMDV causes an acute disease that spreads very rapidly and is characterized by fever, lameness and vesicular lesions on the feet, tongue, snout and teats, with high morbidity but low mortality (Juleff et al., 2012). A total of seven types of FMDV have been identified such as O, A, C, SAT_1, SAT_2, SAT_3 and Asia1 (Franki et al., 1991; OIE, 2010).

Regarding Egypt, the disease is enzootic and many outbreaks have been reported since 1950. FMD serotypes SAT2, A and O were reported in years 1950, 1972 and 2000 respectively (Aidaros, 2002). The type O was the most prevalent since 1960 and onwards (Farag et al., 2005; Parida, 2009). Serotype A was recorded in Egypt recently when it was introduced to Egypt during 2006 through live animal's importation where sever clinical signs were recorded among cattle and buffaloes (Abd El-Rahman et al., 2006). In addition, serotype SAT-2 of FMD virus was later introduced to Egypt during 2012 through live animal's importation, isolated and typed by VSVRI and confirmed by world reference Laboratories, Pirbright, United Kingdom (Shawky et al., 2013).

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Control of FMD in animals was considered to be corner stone to eliminate the disease outbreaks in endemic areas, through effective vaccination for limiting the spread of FMD (Depa et al., 2012). Vaccine adjuvant prolongs the immune response and stimulate specific component either humeral or cell mediated immunity (Lombard et al., 2007).

The link between increased protection and increased antibody was reaffirmed and a significant link between IL-6 levels and antibody levels was shown. It was conclude that quantifying the levels of IL-6 in serum could provide additional means of qualifying whether a vaccine will afford clinical protection or not (Cox et al., 2011). It was demonstrated that the vaccine did not induce a systemic inflammatory response, or a systemic elevation of T lymphocyte activity. Although the IL-6 and IL-8 did not relate to protection, IL-12 production was highest in the protected vaccinated pigs. Thus, the induction of monocytic cell activity, demonstrable by the production of IL-6, IL-8 and IL-12, appears to play a critical role in induction of the innate immune defence which relate to early protection against FMD (Barnett et al., 2002).

The efficacy of several adjuvants to induce such protection showed that the aqueous IMS1313 plus inactivated FMDV induce a higher protective immune response than the vaccine with inactivated virus alone at seven DPV. Mice inoculated with this formula showed higher lympho-proliferative index values and higher IL-2, IL-4 and IFN gamma levels than the control (Quattrocchi et al., 2004).

The present work was designed to evaluate the immune response of sheep to Egyptian trivalent FMD vaccine through estimation of humeral and cellular immunity.

Materials and Methods

Animals

Thirty Egyptian sheep of about one year old and an average weight of 55 kg free from antibodies against FMDV serotypes O Pan Asia-2, A Iran O5 and SAT2/Egypt/2012 were vaccinated with the Egyptian FMD inactivated trivalent oil vaccine using a dose of 1.5 ml/animal subcutaneously while ten sheep were kept without vaccination as control.

FMDV strains

Egyptian isolated FMDV type O Pan Asia-2, A Iran O5 and SAT2/Egypt /2012 with a titre 10^9 $TCID_{50}$/ml for each type were supplied by Foot and Mouth Vaccine Research Department (FMDRD), Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. The virus type O, A and SAT-2 were confirmed by the World Reference Laboratory for FMD (WRL) Pirbright London, UK. These viruses were used for production of the trivalent FMD vaccine.

Preparation of inactivated trivalent FMD vaccine

FMD virus was propagated in BHK21 cell line in roller bottles (Huang et al., 2011) and inactivated with mixture of Binary Ethyleneimine (BEI) and formalin as described by Soliman et al. (2013). The vaccine formulation was carried out according to the method described by Gamil (2010) where the oil phase consisted of Montanide ISA-206 mixed with the inactivated virus in equal parts of an aqueous and oil phase (weight/weight) and mixed thoroughly. The vaccine was prepared on the base that each dose of vaccine contains not less than 10^8 $TCID_{50}$ and 2.1µg 146S/dose of each virus type (Daoud et al., 2013).

Sampling

Heparinized blood samples were obtained from experimental animals at day 0, 3, 7, 14, 21 and 28 post vaccination for assay of cell mediated immunity. Serum samples were obtained weekly post vaccination for 4 weeks then every 2 weeks post vaccination up to 40 weeks for evaluation of humoral immunity and estimation of IL-6 and IL-12.

Evaluation of cellular immunity

Lymphocyte blastogenesis using XTT assay

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

$$Count\ /\ ml\ = \frac{cells\ counted\ in\ 32\ squares\ x\ 10\ x\ 1000\ x\ dilution}{4}$$

According to the viable cell count, the viable lymphocytes were adjusted to a concentration of 5 x 10^6 cells/ml suspended in RPMI medium containing 10% foetal calf serum (FCS). This step was followed by setting up of lymphocyte and using cell proliferation kit (XTT kit) according to Scudiero et al. (1988).

Phagocytic activity of sheep macrophages by using Candida Albicans separation and cultivation of mononuclear cells

The preparation of mononuclear cell suspension was separated by ficol hypaque equilibrium centrifugation method (Antley and Hazen, 1988) from sheep peripheral blood as the cell suspension was adjusted to 10^7 viable mononuclear cells/ml RPMI medium containing 15% FCS 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 CO_2 incubator at 37^0C.

Evaluation of phagocytic activity

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 CO_2 incubator at 37^0C 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 a period of 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 and the mean number of blastospores (more than 2 blastospores) per infected phagocyte (phagocytic index) were calculated as follow:

Phagocytic percentage

It was performed by the method of Harmon and Glisson (1989) which was modified by Hussein (1989).

$$phagocytic\ percentage = \frac{no\ of\ phagocytes\ which\ ingest\ Candida}{total\ no\ of\ phagocytes} \times 100$$

Phagocytic index

It was done according to Richardson and Smith (1981)

$$phagocytic\ index = \frac{total\ no\ of\ phagocytes\ which\ ingest\ more\ than\ two\ Candida}{total\ no\ of\ phagocytes\ which\ ingest\ Candida}$$

Estimation of interleukin level in the serum of vaccinated and control sheep

Estimation of IL-6 and IL-12 levels was carried out using Sheep IL-6 ELISA Kit Catalog No. MBS701893 and Sheep IL-12 ELISA Kit Catalog No. MBS738336 supplied by Biosource Company, San Diego, California, USA.

Estimation of humeral immunity

Estimation of serum neutralizing antibody titre against each FMDV using serum neutralization test (SNT)

SNT was carried out using the micro titre technique (Ferreira, 1976).

Estimation of FMD antibody titre using indirect ELISA

It was done according to Hamblin et al. (1986).

Statistical Analysis

Data were analyzed using the statistical software (SPSS, 15). Data was shown as mean and standard deviation (SD). Independent T-test was used to compare the mean value between the control and vaccinated sheep. Significance was declared at $P<0.05$.

Results and Discussion

Current testing regimes for FMD vaccines including those used for emergency purposes are often quantified for potency in accordance to the E.P. This relies on the viral challenge of vaccinated cattle using groups of at least five animals inoculated with reduced doses of vaccine to achieve 50% protection (Anon, 2008). However, this approach has both logistic and practical concerns, foremost being unprotected animals suffering the painful clinical manifestations of FMD and the need to use secured containment facilities. There has been concerted effort to develop alternative approaches for quantifying vaccine efficacy and the E.P. has supported this move. In addition, Cox et al. (2011) stated that serologically based approaches have certain advantages including insignificant discomfort and subsequent blood sampling. They allow several FMD vaccines to be tested simultaneously and/or a number of serotypes to be examined in a single polyvalent vaccine and can significantly improve the accuracy of the result. For this reason many research workers developed and evaluated a variety of serological tests and assessed the correlation of the results with protection. However, a variable number of animals with low or no detectable neutralizing antibody will resist challenge with live virus while others which have acceptable titres will still succumb to disease. These observations highlight the importance of developing robust and challenge free models for the assessment of FMD vaccine efficacy and the need to identify other correlations of protection rather than relying on neutralizing antibody titre alone in order to improve the margin of error observed with *in vitro* approaches.

For this reason, the present study was carried out to find alternative pathways for the evaluation of the efficacy of the produced vaccine. As a new assay, evaluation of the cellular immunity will include estimation of the IL-6 and IL-12 levels in addition to lymphocyte blastogenesis and phagocytic activity.

The efficient induction of early protection against contact infections by FMDV relies on the rapid assimilation of appropriate innate immune defence, probably leading to the enhanced induction of specific immune responses (Barnett et al., 2002).

Regarding the cellular immune response of sheep to the trivalent inactivated FMD vaccine, delta optical density of lymphocyte blastogenesis assay (Table 1) at day 0, 3, 7, 14, 21 and 28 DPV showed an increase in the mean lymphocyte from 0.12± 0 at the day 0 to reach its maximum at 21 DPV (0.87±0.08) in vaccinated sheep and then declined after at 28 DPV (0.73±0.09). There was significant difference between the vaccinated and control groups at all the vaccination day except zero day. The phagocytic activity was also estimated at day 0, 3, 7, 14, 21 and 28 DPV which was expressed in the form of phagocytic percentage and phagocytic index (Table 2&3).

The vaccinated group showed a mean increase in both the phagocytic percentage and its index at zero day with a maximum mean at 21 DPV. Results showed no significant difference at day 0 while there is a significant difference between the vaccinated group and the control group in the phagocytic percentage started at 3 DPV and reached its highest significant difference at 21 DPV. Regarding the phagocytic index, there is a significant difference between vaccinated and control sheep started at 3 DPV and reached its highest value at 21 DPV. These results agree with those of El-Watany et al. (1999), Mansour (2001) and Samir (2002) who reported that FMD vaccine stimulates the cellular immune response and lymphocyte stimulation on 1st and 2nd weeks post vaccination. Also this result is supported by Patch et al. (2011) which reported that the induction of cytotoxic T-cell (CTL) killing responses following vaccination of swine, which essentially signify that these responses may have a biological effect for controlling FMDV infection *in vivo*. Pervaiz et al. (2013) found that cell mediated immune response expressed by lymphocyte proliferative assay showed its highest level at 14th DPV which may be attributed to the higher antigenic content of virus particles per 2 ml cattle dose, moreover, FMDV-specific lympho proliferative response, a surrogate measure of cell mediated immune (CMI) activation, was observed comparatively higher in the Montanide oil vaccine.

Estimation of IL-6 at day 0, 3, 7, 14, 21 and 28 DPV (Table 4) showed an increase in the mean concentration from the day 0 and reached maximum at 21 DPV then started decline at 28 DPV in vaccinated sheep. The results also showed that in the control group at day 0 showed no significant difference while there is a significant difference between the vaccinated group and the control group started at 3 DPV and increased gradually till reaching its highest significance at 21 DPV.

Estimation of IL-12 at day 0, 3, 7, 14, 21 and 28 DPV (Table 5) in the vaccinated group showed an increase in the mean IL-12 concentration at the day 0 reached maximum at 21 DPV then decline at 28 DPV. In the control group at day 0, there is no significant difference while there is a significant difference between the vaccinated group and the control group started at 3 DPV and increased gradually till reaching its highest significance at 21 DPV. In this respect, Barnett et al. (2002) concluded that although the IL-6 and IL-8 did not relate to protection, IL-12 production was highest in the protected vaccinated pigs. Thus, the induction of monocytic cell activity, demonstrable by the production of IL-6, IL-8 and IL-12 appear to play a critical role in FMDV emergency vaccine induction of the innate immune defense which relate to early protection against FMD. Also Cox et al. (2011) showed that such analyses confirmed that systemic IL-6 levels increased with the administered vaccine and that the odds of protection against challenge increased as IL-6 levels increased. The link between increased protection and increased antibody was reaffirmed and a significant link between IL-6 levels and antibody levels was shown. We, therefore, concluded that quantifying the levels of IL-6 in serum could provide additional means of qualifying a vaccine in the absence of an actual challenge and thus offer the possibility of improved vaccine potency testing in both in terms of animal welfare as well as cost.

Table 1: Lymphocyte blastogenesis assay expressed as delta optical density in sheep vaccinated with trivalent FMD oil vaccine

Parameters	Delta optical density of lymphocyte blastogenesis											
	Zero day		3 Dpv*		7 Dpv		14 Dpv		21 Dpv		28 Dpv	
	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS
Mean	0.12	0.12	0.12	0.51	0.12	0.67	0.12	0.77	0.12	0.87	0.12	0.73
S.D	0	0	0	0.06	0	0.07	0	0.08	0	0.08	0	0.09
Minimum	0.10	0.10	0.10	0.41	0.10	0.52	0.10	0.63	0.11	0.70	0.10	0.52
Maximum	0.12	0.15	0.12	0.69	0.12	0.79	0.12	0.88	0.13	0.99	0.12	0.86
Statistical data	T= 1.504 P > 0.05 NS		T= 19.164 P < 0.05 S		T= 22.266 P < 0.05 S		T= 26.684 P < 0.05 S		T= 29.27 P < 0.05 S		T= 19.99 P < 0.05 S	

S.D: Standard Deviation CS: Control Sheep (n = 10) VS: Vaccinated Sheep (n= 30); S: Significant; NS: Non Significant; *Dpv: Days post vaccination

Table 2: Phagocytic percentage of sheep vaccinated with trivalent FMD oil vaccine

Parameters	Phagocytic percentage											
	Zero day		3 Dpv*		7 Dpv		14 Dpv		21 Dpv		28 Dpv	
	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS
Mean	19.72	20.23	19.9	30.92	19.82	51.33	19.8	71.18	19.82	81.8	19.82	69.86
S.D	1.92	1.43	1.41	2.95	1.87	5.77	1.74	5.59	1.50	4.30	1.57	3.66
Minimum	17.6	17.4	17.9	25.6	17.2	41.5	17.8	60.5	17.6	71.2	17.4	62.9
Maximum	22.6	22.9	21.9	38.6	22.7	65.3	22.4	78.9	21.7	88.6	22.2	75.9
Statistical data	T= 0.903 P > 0.05 NS		T= 11.293 P < 0.05 S		T= 16.832 P < 0.05 S		T= 28.382 P < 0.05 S		T= 44.264 P < 0.05 S		T= 41.677 P < 0.05 S	

S.E: Standard Deviation CS: Control Sheep (n = 10) VS: Vaccinated Sheep (n= 30); S: Significant; NS: Non Significant; *Dpv: Days post vaccination

Table 3: Phagocytic index of sheep vaccinated with trivalent FMD oil vaccine

Parameters	Phagocytic index											
	Zero day		3 Dpv*		7 Dpv		14 Dpv		21 Dpv		28 Dpv	
	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS
Mean	0.10	0.10	0.10	0.36	0.11	0.46	0.12	0.60	0.11	0.77	0.11	0.69
S.D	0	0.01	0	0.05	0	0.05	0	0.04	0.01	0.06	0.01	0.05
Minimum	0.09	0.07	0.1	0.29	0.1	0.39	0.11	0.5	0.09	0.66	0.09	0.6
Maximum	0.12	0.14	0.12	0.45	0.13	0.56	0.14	0.67	0.13	0.89	0.13	0.78
Statistical data	T= 0.312		T= 15.959		T= 20.348		T= 31.273		T= 33.698		T= 32.488	
	P > 0.05		P < 0.05		P < 0.05		P < 0.05		P < 0.05		P < 0.05	
	NS		S		S		S		S		S	

S.D: Standard Deviation CS: Control Sheep (n = 10) VS: Vaccinated Sheep (n= 30); S: Significant; NS: Non Significant; *Dpv: Days post vaccination

Table 4: Interleukin-6 immune response expressed as delta optical density of sheep vaccinated with trivalent FMD oil vaccine

Parameters	IL-6 (pg/ml) at days post vaccination											
	Zero day		3 Dpv*		7 Dpv		14 Dpv		21 Dpv		28 Dpv	
	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS
Mean	0.39	0.44	0.22	1.15	0.32	2.02	0.28	3.22	0.39	4.27	0.35	3.58
S.D	0.22	0.20	0.09	0.61	0.07	0.57	0.07	0.48	0.15	0.47	0.1	0.45
Minimum	0.1	0.2	0.1	0.2	0.2	1.2	0.2	2.4	0.2	3.4	0.2	2.9
Maximum	0.7	0.9	0.4	1.9	0.4	2.7	0.4	3.8	0.6	4.8	0.6	4.4
Statistical data	T= 0.706		T= 4.723		T= 9.423		T= 19.028		T= 25.34		T= 22.271	
	P > 0.05		P < 0.05		P < 0.05		P < 0.05		P < 0.05		P < 0.05	
	NS		S		S		S		S		S	

S.D: Standard Deviation CS: Control Sheep (n = 10) VS: Vaccinated Sheep (n= 30); S: Significant; NS: Non Significant; *Dpv: Days post vaccination

Table 5: Interleukin-12 immune response expressed as delta optical density of sheep vaccinated with trivalent FMD oil vaccine

Parameters	IL-12 (pg/ml) at days post vaccination											
	Zero day		3 Dpv*		7 Dpv		14 Dpv		21 Dpv		28 Dpv	
	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS	CS	VS
Mean	4.32	4.40	4.36	4.61	4.41	5.15	4.4	5.60	4.37	6.30	4.27	5.58
S.D	0.20	0.18	0.19	0.19	0.17	0.35	0.14	0.33	0.13	0.28	0.14	0.32
Minimum	3.9	4.1	3.9	4.2	4.1	4.5	4.2	5	4.2	5.8	4.1	5.1
Maximum	4.6	4.8	4.6	4.9	4.6	5.7	4.6	6.2	4.6	6.8	4.6	6.2
Statistical data	T= 1.187		T= 3.553		T= 6.384		T= 11.067		T= 20.769		T= 12.319	
	P > 0.05		P < 0.05		P < 0.05		P < 0.05		P < 0.05		P < 0.05	
	NS		S		S		S		S		S	

S.D: Standard Deviation CS: Control Sheep (n = 10) VS: Vaccinated Sheep (n= 30); S: Significant; NS: Non Significant; *Dpv: Days post vaccination

Humeral immune response of sheep vaccinated with the Egyptian trivalent inactivated oil FMD vaccine is shown in Table 6. All the vaccinated animals exhibited detectable specific antibodies against the used types of FMD virus by the first WPV, the protective level at 3rd WPV and reaching peak titres by the 10-12th WPV. These values began to decline gradually recording the levels of unprotection at 32-34 WPV using SNT and ELISA respectively. These results agreed with those obtained by Selim et al. (2010) who reported that the mean antibody titres against FMD vaccine strain O_1/3/93 were detected in sheep sera vaccinated with Alum-hydroxide gel vaccine following one WPV by SNT, whereas, the mean peak titres (1.9 $\log_{10}$) by SNT were detected by the 6th week post vaccination. Our results also agreed with Mohamed et al. (2013) who used FMD ISA 206 oil bivalent vaccine alone and noticed that the specific FMD neutralizing antibody titre reached a protective level starting from the 4th WPV to record peak titre by the 16th WPV and then declined gradually afterward. El-Sayed et al. (2012) reported that vaccination of calves with the locally produced bivalent FMD adjuvant vaccine induced higher antibody titre than the recommended protective level (1.5 $\log_{10}$ for SNT and 1.8 $\log_{10}$ for ELISA) for type A and O estimated by SNT and ELISA. This antibody titre remained within the protective level up to 34 WPV. Our results disagreed with Pervaiz et al. (2013) who mentioned that the significant rise in the SNT following vaccination with FMD ISA 206 oil vaccine was at 14 DPV. This result could be attributed to the use of 146S quantity (11.5 µg) of virus particles per 2 ml.

Table 6: Mean antibody titre in vaccinated sheep with FMD trivalent inactivated oil vaccines using SNT and ELISA

WPV	Mean antibody titre (log_{10}/ml)					
	SNT			ELISA		
	Type A	Type O	Type SAT-2	Type A	Type O	Type SAT-2
0	0.3	0.3	0.45	0.6	0.6	0.75
1	0.9	1.05	1.05	1.1	1.25	1.25
2	1.2	1.35	1.5	1.45	1.6	1.75
3	1.5	1.8	1.65	1.71	2.01	1.86
4	1.8	2.1	1.95	2.1	2.4	2.25
6	1.95	2.4	2.4	2.25	2.7	2.7
8	2.25	2.7	2.55	2.45	2.9	2.75
10	2.25	2.7	2.7	2.55	3	3
12	2.4	2.55	2.55	2.66	2.92	2.81
14	2.4	2.55	2.25	2.64	2.79	2.55
16	2.1	2.25	2.25	2.4	2.59	2.49
18	1.95	2.25	1.95	2.24	2.54	2.35
20	1.95	2.1	1.95	2.25	2.45	2.3
22	1.8	1.95	1.8	2.1	2.38	2.18
24	1.65	1.95	1.8	2.05	2.3	2.1
26	1.65	1.8	1.8	2.0	2.21	2.05
28	1.5	1.8	1.65	1.9	2.1	2.0
30	1.5	1.8	1.65	1.9	2.05	1.96
32	1.5	1.5	1.65	1.82	1.9	1.93
34	1.5	1.5	1.5	1.71	1.71	1.81
36	1.35	1.5	1.5	1.65	1.8	1.72
38	1.2	1.2	1.5	1.43	1.43	1.73
40	0.9	0.9	1.2	1.2	1.2	1.5

Conclusion

The tested Egyptian trivalent FMD oil vaccine showed a maximum cellular immune response at 21 DPV and a protective humeral immune response extended for 32 WPV.

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