

Effect of Carbopol as an Immune-Stimulant in Bovine Vaccination against Foot and Mouth Disease

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Abstract

Three different formulae of inactivated trivalent FMD vaccine serotypes (O Pan Asia1, A Iran O5 and SAT2/EGY/2012) were prepared as formula (1) prepared with Montanide ISA 206 (50% oil to 50%antigen); formula (2) prepared with carbopol (50% carbopol to 50% antigen) and formula (3) prepared with 25% Montanide ISA 206 and 25% carbopol with 50 % antigen. All of such formulae were found to be free from foreign contaminants; safe and potent showing no post vaccinal reactions and high protective levels of specific FMD antibodies. The immunogenicity of each vaccine formula was determined by vaccination of calves groups where cellular immunity was evaluated by Lymphocyte blastogenesis using XTT assay through separation of lymphocytes and determination of viable cell number in addition to estimation of interleukin IL-6 and IL-12 levels revealing higher values on the use of carbopol with Montanide oil than the use of each adjuvant alone. Also monitoring of humeral FMD antibodies in vaccinated calves revealing that obtained results and the statistical analysis of humeral antibody titers against FMDV serotypes (O; A and SAT2) induced by Montanide oils 206 with carbopol is the best vaccine formula followed by Montanide oils 206 and finally Carbopol which give early, short lasting immunity.

Keywords: Carbopol; Immune-Stimulant; Bovine Vaccination; Foot and Mouth Disease

Introduction

Foot and mouth disease (FMD) is the most highly contagious with economically significant diseases of cloven-hoofed animals worldwide [1]. FMD is able to infect cattle, buffalo, goats, pigs and wild cloven-hoofed animals. FMD virus (FMDV) is the real causative agent, there are seven sero-types; A, C, O, SAT1, SAT2, SAT3 and Asia1 [2]. The main clinical signs on animal infected with FMD is fever, vesicular lesions on the tongue, snout, feet and teats, lameness and, with high morbidity and low mortality [3]. The circulating serotypes in Egypt are FMD serotypes O, A and SAT2 [4-6].

Control of foot and mouth disease (FMD) by means of vaccination, the factors influencing the potency of the vaccine and the induction of a protective antibody response, is the integrity of the structural protein and the intact virion using 146S [7].

Adjuvants stimulate the immune response duration. The nature of the adjuvant can determine the particular type of immune response [8].

The Capturing of soluble immune mediators such as cytokines and chemokines could result in good intercellular signaling and into more efficient leukocyte recruitment to the site of vaccine delivery. Alternatively, carbopol action could rely on nonimmune cells, whose role in promoting immunity would be revealed in subsequent time [8]. Carbopol was tested, as previous uses in horses [9,10], pigeons and swine [11]. The criteria of adjuvant of polyacrylic acids, designated by the term carbopols, may vary greatly with the number of carboxyl groups present in the final molecule. It was shown that Carbopol 934 is actually immunogenic and may be a relevant alternative to oil in avian species for which safety is a major concern). Aluminum hydroxide was proved to be less immunogenic than Carbopol and the last was totally safe by vaccination the young goslings with inducing a good serological response [12].

In a trail to improve rabies vaccine's immunogenicity, water-soluble acrylic acid (carbopol) was used as an adjuvant in the prepared vaccine. The potency test of the prepared vaccine revealed that it is potent and efficient [13].

The use of carbopol as an adjuvant can induce robust humoral immunity and T-cell responses to some subunit vaccines [9].

Polyacrylic acid polymers termed carbopols have been evaluated as adjuvants in animal vaccines [8,9,14-17]. These reports suggest that no side effect in mammals using carbopols. To yield potentially high immune response made combination to carbopols with other adjuvant formulations such as MF59 [18,19]. It was studied that effect of Carbopol which giving strong T cell and B cell responses in mice, with protection against influenza infection, also anti-tumor responses and without any sign of toxicity in mice [20].

Adding of carbopol for animals vaccine result in systemic adjuvant activity including proinflammatory T cell sensitization, fast leukocyte recruitment, proinflammatory cytokine secretion with antigen capture fastly by the inflammatory monocytes [21].

Aim of the Study

The main goal of this work is the enhancement of the immunogenicity of the trivalent FMD vaccine using carbopol as adjuvant aiming to provide bovines with high protective immunity with long duration to safe such animals to avoid the great dramatic economic losses caused by the virus infection.

Materials and Methods

Animals

160 healthy calves of 8 months of age in two localities and free from FMD type O/Pan Asia, A/Iran05 and SAT2/Egypt 2012 antibodies as screened by serum neutralization test and indirect ELISA.

FMD virus strains:

Local FMDV serotypes (A Iran 05, O Pan Asia1 and SAT2/EGY/2012) were inoculated to propagate in BHK21 to prepare virus fluid were supplied by FMDV department, VSVRI. All serotypes were confirmed by the World Reference Laboratory for FMD, Pirbright London, UK.

Cell culture

Baby Hamster kidney cell line (BHK21) was propagated and maintained using Eagle's Minimum Essential Medium (MEM) supplied with 8-10% new born calf serum as described by [22] and used for SNT, virus propagation and titration for vaccine preparation.

Virus infectivity and antigenicity

Titration of the virus was carried out for the FMDV serotypes and the infectivity titer was calculated in \log_{10} (TCID₅₀) as described by [23] and the CFT was carried out according to [24].

Virus purification

Centrifugation using cooling centrifuge at 3000 rpm for 20 min was done to the harvested FMDV culture medium infected BHK21 to remove cell debris.

Virus inactivation

FMDV serotypes (A Iran O5, O Pan Asia1 and SAT2/EGY/2012) at their seventh passage on BHK21 cell line with an infectivity titer of 10^8 TCID₅₀/dose were subjected to inactivation process by a combination of 0.04% formaldehyde and 1 mM binary ethyleneimine (BEI) as the method described by [25,26]. To neutralize the effect of BEI was added 20% of sodium thiosulfate in a final concentration of 2% and also to neutralize the excess of formalin was added 20% of sodium bisulfite in a final concentration of 2%

Used adjuvants

Carbopol adjuvant used in this experiment was provided by Lubrizol Co. as a fluffy white powder. It was dissolved in hot water to prepare 0.5% aqueous stock solutions, sterilized by autoclaving at 121°C for 20 minutes, then stored at 4°C until further use (United States Pharmacopeial Convention, 1990).

Montanide ISA206 was supplied from Seppic, Paris, France.

Preparation of inactivated vaccines

Mixing of the inactivated FMDV serotypes for vaccine preparation was carried out confirming that each dose should contains not less than 10^9 TCID₅₀/dose and 2.4µg 146S antigen content from each FMD virus strain.

Formulation of the prepared experimental vaccine batches

Three formulae of trivalent inactivated FMD vaccine were prepared using the mentioned adjuvants as follow:

- Formula (1) was prepared with Montanide ISA 206 (50% oil to 50%antigen).
- Formula (2) was prepared with carbopol (50% carbopol to 50% antigen).
- Formula (3) was prepared with 25% Montanide ISA 206 and 25% carbopol with 50 % antigen.

Quality control testing of the prepared FMD trivalent vaccine formulae

Viscosity testing

The viscosities of the three prepared vaccine formulae were measured according to the work of Stone [27] where vaccine samples were placed out from the storage at 4°C and allowed to equilibrate to room temperature then 1 ml of each sample was drawn into a 1-ml pipette, and then the time required for 0.4 ml of the sample to flow out of the vertically positioned pipette was recorded.

Sterility and safety testing

The prepared vaccine batches were tested for their freedom of aerobic and anaerobic bacteria; fungal and mycoplasma contaminants by culturing the samples on Sabouraud's, Nutrient agar, thioglycolate broth, phenol dextrose media and mycoplasma medium and the safety of such preparation was done in baby mice according to the directions of [28].

Potency test

Calves vaccination

Includes 160 calves at a private farm at El-Fayoum Governorate divided into 4 groups (50 calves/group in the first three group and 10 calves in the 4th group) as follow:

- Group (1) was vaccinated with the trivalent oil FMD.
- Group (2) was vaccinated with the trivalent carbopol FMD vaccine.
- Group (3) was vaccinated with the trivalent oil-carbopol FMD vaccine.

The used vaccine dose of each vaccine formula was 3 ml/animal inoculated subcutaneously.

- While Group (4) was kept without vaccination as negative control group.

Assessment the humeral immune response to the formulated FMD vaccine was carried out on collected serum samples from the all cattle (vaccinated and non-vaccinated) to follow up the antibody titers against the serotypes of FMDV (A Iran O5, O Pan Asia1 and SAT 2/ EGY/2012) using serum neutralization test (SNT) using the microtiter technique as mentioned by [29] and indirect ELISA as described by [30]. SNT and ELISA were carried out on serum samples collected on week intervals up to 4 weeks; on 2 weeks interval up to 16 weeks then on 4 weeks intervals up to 36 weeks post vaccination.

Assessment the cell mediated immunity

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, and estimation of IL-6 and IL-12.

Assessment the cellular immunity Lymphocyte blastogenesis by XTT assay according to [31,32] by separation of lymphocytes as mentioned by [33,34] and estimation to viable cell number according to the following formula cited by [35].

According to the viable cell count, the viable lymphocytes were adjusted to a concentration of 5×10^6 cells/ml suspended in RPMI medium containing 10% fetal calf serum (FCS). This step was followed by using cell proliferation kit (XTT kit) and setting up of lymphocyte as described by [36].

Estimation of interleukin IL-6 and IL-12 levels in the serum of vaccinated and control calves was carried out using bovine IL-6 ELISA Kit Catalog No. MBS701893 and bovine IL-12 ELISA Kit Catalog No. MBS738336 supplied by Biosource Company, San Diego, California, USA.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in the SPSS-12 statistical software package for P.C.S. Duncan's multiple range tests at $P < 0.05\%$.

Results and Discussion

The cellular immune response of calve groups to the prepared three different trivalent FMD vaccine formulae, assessment of the lymphocyte blastogenesis, IL-6 and IL-12 levels revealed induction of early protection against FMDV with rapid assimilation of appropriate innate immune defense, leading to the enhanced of specific immune responses [37]. Table 1 illustrated the cellular immune response of calves to the inactivated FMD ISA 206 oil vaccine (Formula-1) showed increasing mean delta optical density of lymphocyte blastogenesis assay at day 1, 3, 7, 14, 21 and 28 DPV from 0.32 at the day 1 to reach its maximum value (0.95) at the 14th DPV then declined at the 28st DPV (0.76), but in calves vaccinated with inactivated FMD carbopol vaccine (Formula-2), showed an increase in the mean value from (0.31) at the day 1 to reach its maximum value (0.90) at the 14th DPV then declined at the 21st DPV (0.79) while the inactivated FMD Montanide 206 oil and carbopol (Formula-3) revealed increasing from 0.41 at the day 1 to reach its maximum value (1.15) at the 7th DPV then declined at the 14th DPV (1.10), but the control calves remain mean delta optical density of lymphocyte blastogenesis assay around 0.10 to 0.13 all over the time of estimation.

Calves groups	Delta optical density of lymphocyte blastogenesis/DPV*					
	1 st DPV	3 th DPV	7 th DPV	14 th DPV	21 st DPV	28 th DPV
Group-1	0.32	0.45	0.67	0.95	0.81	0.76
Group-2	0.31	0.42	0.64	0.90	0.79	0.72
Group-3	0.41	0.82	1.15	1.10	0.97	0.80
Group-4	0.10	0.12	0.13	0.12	0.13	0.10

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

Table 2 illustrated the cellular immune response through assessment of interleukin-6 (IL6) of calves 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 (0.89) at the day 1 to reach its maximum value (3.78) at the 14th DPV then declined at the 21st DPV (3.62), but in calves vaccinated with inactivated FMD carbopol vaccine (Formula-2), showed an increase in the mean value of IL6 from (0.85) at the day 1 to reach its maximum value (3.71) at the 14th DPV then declined at the 21st DPV (3.57) while the inactivated FMD Montanide 206 oil and carbopol (Formula-3) revealed increasing in the mean value of IL6 from (1.43) at the day 1 to reach its maximum value (4.78) at the 7th DPV then declined at the 14th DPV (3.97). While the control negative non vaccinated calves' group, the mean value of IL6 remain around (0.39 to 0.48) all over the time of assessment.

Calves groups	IL-6 (ng/ml) at DPV*					
	1 st DPV*	3 th DPV	7 th DPV	14 th DPV	21 st DPV	28 th DPV
Group-1	0.89	1.44	2.11	3.78	3.62	3.17
Group-2	0.85	1.40	2.01	3.71	3.57	3.02
Group-3	1.43	2.51	4.78	3.97	3.81	3.43
Group-4	0.40	0.35	0.32	0.3	0.39	0.4

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

From the above results and the statistical analysis of cellular immunity, it is revealed that the FMD vaccine adjuvanted with Montanide ISA 206 and Carbopol showed a higher post vaccinal cellular immune response than that adjuvanted with each adjuvant separately, indicating that addition of carbopol has a great impact on the post vaccinal cellular immune response in agreement with what stated by [21]. Also these results came parallel with [38,39] who mentioned that cell mediated immune response was a constitute of immune response against FMD virus, and in agreement in some points with [38,40-44] who mentioned that the Delta optical density of lymphocyte blastogenesis assay and interleukin6 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 and also agreed with [12] who proved that Carbopol more immunogenic than aluminum hydroxide when administered to young goslings and breeders alike. Carbopol adjuvanted vaccine induced a high serological response.

Calves groups	IL-12 (ng/ml) at DPV*					
	1 st DPV	3 rd DPV	7 th DPV	14 th DPV	21 st DPV	28 th DPV
Group-1	4.4	4.61	5.15	6.2	5.3	4.9
Group-2	4.3	4.56	5.1	6.3	5.2	4.8
Group-3	4.6	5.6	7.5	5.8	5.5	5.3
Group-4	4.1	4.3	4.1	4.2	4.3	4.1

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

*DPV: Days Post Vaccination

Group (1) vaccinated with the trivalent oil FMD.

Group (2) vaccinated with the trivalent carbomer FMD vaccine.

Group (3) vaccinated with the trivalent oil-carbomer FMD vaccine.

Group (4) kept without vaccination as negative control group.

Assessment of the humeral immune response against FMDV serotype (O) antibody titer in vaccinated calves with prepared oil adjuvant vaccine formulae using SNT and ELISA data (Table 4 and 5) showed differences in the intensity, onset and duration of the FMD serotype O antibodies. The onset of antibody protective titer, the inactivated FMD ISA 206 oil vaccine induced titers of (1.75 ± 0.13^a by SNT and $2.05 \pm 0.125^{a,c} \log_{10}$ by ELISA) in the 2nd WPV and inactivated FMD carbopol vaccine induced titers of ($1.52 \pm 0.06^{a,b}$ by SNT and $1.82 \pm 0.05^{a,b} \log_{10}$ by ELISA) in the 1st WPV while inactivated FMD ISA 206 oil with Carbopol vaccine showed earlier immune response in the 1st WPV ($1.62 \pm 0.102^{a,c}$ by SNT, $1.92 \pm 0.105^{a,c} \log_{10}$ by ELISA). The peak of the protective antibody titers by the inactivated FMD ISA 206 oil vaccine ($3.02 \pm 0.60^{a,b}$ by SNT and $3.32 \pm 0.55^{a,b} \log_{10}$ by ELISA) appeared in the 12nd WPV and by the inactivated FMD carbopol vaccine (2.12 ± 0.137^a as SNT and $2.42 \pm 0.122^a \log_{10}$ as ELISA) in the 10th WPV while the inactivated FMD ISA 206 oil carbopol vaccine induced the peak of antibody titers in the 10th WPV (3.44 ± 0.093^c by SNT, $3.74 \pm 0.083^c \log_{10}$ by ELISA). The duration of the type-O antibody protective titers, the inactivated FMD ISA 206 oil vaccine showed protective titers of ($1.6 \pm 0.12^{a,c}$ by SNT and $1.9 \pm 0.17^{a,c} \log_{10}$ by ELISA) up to the 34th WPV and also those induced by the inactivated FMD carbopol vaccine ($1.51 \pm 0.060^{a,b}$ as SNT and $1.81 \pm 0.05^{a,b} \log_{10}$ as ELISA) up to the 24th WPV while inactivated FMD ISA 206 oil carbopol vaccine showed later protective antibody titers in the 36th WPV ($1.5 \pm 0.071^{b,c}$ by SNT and $1.8 \pm 0.051^{b,c} \log_{10}$ by ELISA).

Calves groups	FMD type-O serum neutralizing antibody titers (\log_{10}/ml) /WPV*																
	0WPV \pm SE	1W \pm SE	2W \pm SE	3W \pm SE	4W \pm SE	6W \pm SE	8W \pm SE	10W \pm SE	12W \pm SE	14W \pm SE	16W \pm SE	20W \pm SE	24W \pm SE	28W \pm SE	32W \pm SE	34W \pm SE	36W \pm SE
Group-1	0.3 \pm 0.15 ^a	1.31 \pm 0.08 ^{a,c}	1.75 \pm 0.13 ^a	1.89 \pm 0.06 ^b	2.35 \pm 0.90 ^{a,b}	2.51 \pm 0.056 ^a	2.86 \pm 0.006 ^b	2.94 \pm 0.030 ^c	3.02 \pm 0.60 ^{a,b}	2.84 \pm 0.56 ^a	2.63 \pm 0.73 ^{a,b}	2.42 \pm 0.60 ^c	2.27 \pm 0.80 ^a	2.04 \pm 0.01 ^{a,b}	1.79 \pm 0.64 ^c	1.6 \pm 0.12 ^{a,c}	1.26 \pm 0.105 ^{a,b}
Group-2	0.45 \pm 0.14 ^a	1.52 \pm 0.06 ^{a,b}	1.64 \pm 0.13 ^b	1.71 \pm 0.105 ^{a,c}	1.82 \pm 0.015 ^{a,b}	1.89 \pm 0.15 ^{a,b}	1.92 \pm 0.105 ^{a,c}	2.12 \pm 0.137 ^a	1.98 \pm 0.105 ^{a,c}	1.82 \pm 0.093 ^{a,b}	1.65 \pm 0.047 ^c	1.59 \pm 0.102 ^{a,c}	1.51 \pm 0.060 ^{a,b}	1.32 \pm 0.129 ^a	1.15 \pm 0.090 ^c	0.98 \pm 0.088 ^b	0.45 \pm 0.075 ^{a,b}
Group-3	0.3 \pm 0.13 ^a	1.62 \pm 0.102 ^{a,c}	1.86 \pm 0.076 ^a	1.98 \pm 0.081 ^a	2.45 \pm 0.050 ^{a,c}	2.82 \pm 0.105 ^{a,c}	3.01 \pm 0.066 ^a	3.44 \pm 0.093 ^c	3.15 \pm 0.083 ^c	2.92 \pm 0.114 ^{a,b}	2.71 \pm 0.105 ^{a,c}	2.62 \pm 0.009 ^{a,b}	2.42 \pm 0.075 ^b	2.14 \pm 0.109 ^{a,b}	1.98 \pm 0.114 ^{a,c}	1.88 \pm 0.108 ^a	1.5 \pm 0.071 ^{b,c}
Group-4	0.3 \pm 0.17 ^a	0.3 \pm 0.14 ^a	0.4 \pm 0.15 ^a	0.3 \pm 0.12 ^a	0.4 \pm 0.19 ^a	0.4 \pm 0.14 ^a	0.4 \pm 0.14 ^a	0.3 \pm 0.15 ^a	0.3 \pm 0.16 ^a	0.3 \pm 0.17 ^a	0.3 \pm 0.16 ^a	0.3 \pm 0.14 ^a	0.3 \pm 0.15 ^a	0.3 \pm 0.17 ^a	0.3 \pm 0.18 ^a	0.3 \pm 0.15 ^a	0.3 \pm 0.18 ^a

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

Calves groups	FMD type-O ELISA antibody titer (\log_{10}/ml) /WPV*																
	0WPV* \pm SE	1W \pm SE	2W \pm SE	3W \pm SE	4W \pm SE	6W \pm SE	8W \pm SE	10W \pm SE	12W \pm SE	14W \pm SE	16W \pm SE	20W \pm SE	24W \pm SE	28W \pm SE	32W \pm SE	34W \pm SE	36W \pm SE
Group-1	0.6 \pm 0.15 ^a	1.61 \pm 0.06 ^{a,b}	2.05 \pm 0.125 ^{a,c}	2.19 \pm 0.13 ^a	2.65 \pm 0.22 ^{a,b}	2.81 \pm 0.044 ^a	3.16 \pm 0.009 ^b	3.24 \pm 0.035 ^c	3.32 \pm 0.55 ^{a,b}	3.14 \pm 0.75 ^a	2.93 \pm 0.64 ^{a,b}	2.72 \pm 0.65 ^c	2.57 \pm 0.74 ^a	2.34 \pm 0.05 ^{a,b}	2.09 \pm 0.53 ^{a,c}	1.9 \pm 0.17 ^{a,b}	1.56 \pm 0.10 ^{a,b}
Group-2	0.75 \pm 0.15 ^a	1.82 \pm 0.05 ^{a,b}	1.94 \pm 0.15 ^b	2.01 \pm 0.095 ^{a,c}	2.12 \pm 0.07 ^{a,b}	2.19 \pm 0.10 ^a	2.22 \pm 0.085 ^a	2.42 \pm 0.122 ^a	2.28 \pm 0.04 ^a	2.12 \pm 0.084 ^{a,b}	1.95 \pm 0.03 ^{a,c}	1.89 \pm 0.105 ^{a,c}	1.81 \pm 0.05 ^{a,b}	1.62 \pm 0.13 ^a	1.45 \pm 0.05 ^c	1.28 \pm 0.07 ^b	0.75 \pm 0.06 ^{a,b}
Group-3	0.6 \pm 0.15 ^a	1.92 \pm 0.105 ^{a,c}	2.16 \pm 0.06 ^a	2.28 \pm 0.07 ^a	2.75 \pm 0.06 ^{a,c}	3.12 \pm 0.10 ^{a,c}	3.31 \pm 0.043 ^a	3.74 \pm 0.083 ^c	3.45 \pm 0.06 ^c	3.22 \pm 0.12 ^{a,b}	3.01 \pm 0.10 ^{a,c}	2.92 \pm 0.006 ^{a,b}	2.72 \pm 0.065 ^b	2.44 \pm 0.105 ^{a,b}	2.28 \pm 0.115 ^{a,c}	2.18 \pm 0.105 ^a	1.8 \pm 0.051 ^{b,c}
Group-4	0.6 \pm 0.15 ^a	0.6 \pm 0.12 ^a	0.7 \pm 0.14 ^a	0.6 \pm 0.15 ^a	0.7 \pm 0.13 ^a	0.7 \pm 0.15 ^a	0.7 \pm 0.14 ^a	0.6 \pm 0.15 ^a	0.6 \pm 0.15 ^a	0.6 \pm 0.13 ^a	0.6 \pm 0.17 ^a	0.6 \pm 0.14 ^a	0.6 \pm 0.13 ^a	0.6 \pm 0.12 ^a	0.6 \pm 0.14 ^a	0.6 \pm 0.13 ^a	0.6 \pm 0.15 ^a

Table 5: Mean FMD type-O ELISA titer in different vaccinated calves' groups.

From the above results and the statistical analysis of humeral antibody titers against FMDV serotype O revealed that the Montanide oils 206 with carbopol is the best vaccine formula which induced earlier, long lasting immunity then Montanide oils 206 and finally Carbopol which give early, short lasting immunity.

The antibody titer of FMD is considered high protective using SNT and ELISA as recommended by [28] as $1.5 \log_{10}$ by SNT and $1.8 \log_{10}$ by ELISA.

FMDV serotype (A) antibody titers induced in vaccinated calves with the different prepared vaccine formulae are determined by using SNT and ELISA data (Table 6 and 7) showed differences in the onset, intensity and duration of the FMD serotype A antibodies. The onset of antibody protective titer, inactivated FMD ISA 206 oil vaccine induced titers of (1.62 ± 0.30^b) by SNT and $1.92 \pm 0.33^a \log_{10}$ by ELISA in the 2nd WPV and inactivated FMD carbopol vaccine induced titers of $(1.62 \pm 0.27^{a,b})$ by SNT and $1.92 \pm 0.30^{a,b} \log_{10}$ by ELISA in the 1st WPV while inactivated FMD ISA 206 oil with Carbopol vaccine showed earlier immune response in the 1st WPV ($1.71 \pm 0.17^{a,b}$ by SNT, $2.01 \pm 0.14^{a,b} \log_{10}$ by ELISA). The peak of the type-A antibody protective titers induced by the inactivated FMD ISA 206 oil vaccine ($3.2 \pm 0.30^{a,b}$ by SNT and $3.5 \pm 0.35^{a,b} \log_{10}$ by ELISA) appeared in the 12nd WPV and by the inactivated FMD carbopol vaccine (2.3 ± 0.55^a as SNT and $2.6 \pm 0.48^{a,c} \log_{10}$ as ELISA) in the 10th WPV while the inactivated FMD ISA 206 oil carbopol vaccine induced the peak of antibody titers in the 10th WPV ($3.21 \pm 0.55^{a,b}$ by SNT, $3.51 \pm 0.52^{a,b} \log_{10}$ by ELISA). The inactivated FMD ISA 206 oil vaccine gave protective titers of (1.51 ± 0.70^b) by SNT and $1.81 \pm 0.60^{a,b} \log_{10}$ by ELISA up to the 32th WPV and also those induced by the inactivated FMD carbopol vaccine (1.62 ± 0.21^a as SNT and $1.92 \pm 0.25^{a,b} \log_{10}$ as ELISA) up to the 24th WPV while inactivated FMD ISA 206 oil carbopol vaccine gave later protective antibody titers in the 34th WPV ($1.64 \pm 0.72^{a,b}$ by SNT and $1.94 \pm 0.63^{a,b} \log_{10}$ by ELISA). From the previous results and the statistical analysis of humeral antibody titers against FMDV serotype A revealed that the Montanide oils 206 with carbopol is the best vaccine formula which induced earlier, long lasting immunity then Montanide oils 206 and finally Carbopol which give early, short lasting immunity.

Calves groups	FMD type-A serum neutralizing antibody titers (\log_{10}/ml) /WPV*																
	0WPV* ± SE	1W ± SE	2W ± SE	3W ± SE	4W ± SE	6W ± SE	8W ± SE	10W ± SE	12W ± SE	14W ± SE	16W ± SE	20W ± SE	24W ± SE	28W ± SE	32W ± SE	34W ± SE	36W ± SE
Group-1	0.3 ± 0.14 ^a	1.41 ± 0.40 ^{a,b}	1.62 ± 0.30 ^b	1.86 ± 0.8 ^{b,c}	2.01 ± 0.76 ^b	2.34 ± 0.7 ^{a,b}	2.65 ± 0.6 ^a	2.84 ± 0.8 ^{a,b}	3.2 ± 0.30 ^b	2.71 ± 0.40 ^{a,b}	2.56 ± 0.50 ^{b,c}	2.24 ± 0.8 ^{b,c}	2.01 ± 0.6 ^a	1.78 ± 0.9 ^{a,b}	1.51 ± 0.7 ^b	1.23 ± 0.60 ^{b,c}	0.85 ± 0.8 ^b
Group-2	0.45 ± 0.17 ^a	1.62 ± 0.27 ^{a,b}	1.75 ± 0.40 ^a	1.86 ± 0.6 ^{a,b}	1.95 ± 0.59 ^{a,c}	2.09 ± 0.6 ^a	2.15 ± 0.7 ^{a,b}	2.3 ± 0.55 ^a	2.14 ± 0.69 ^{b,c}	1.95 ± 0.58 ^{a,b}	1.84 ± 0.32 ^a	1.71 ± 0.47 ^a	1.62 ± 0.21 ^a	1.42 ± 0.37 ^{a,b}	1.29 ± 0.53 ^a	0.87 ± 0.42 ^a	0.49 ± 0.37 ^a
Group-3	0.3 ± 0.15 ^a	1.71 ± 0.17 ^{a,b}	1.95 ± 0.60 ^a	2.41 ± 0.40 ^a	2.83 ± 0.40 ^{a,b}	2.95 ± 0.42 ^b	3.01 ± 0.25 ^a	3.21 ± 0.55 ^{a,b}	3.01 ± 0.92 ^{a,c}	2.94 ± 0.66 ^{b,c}	2.76 ± 0.25 ^{a,b}	2.54 ± 0.40 ^{a,b}	2.31 ± 0.56 ^{a,b}	1.97 ± 0.102 ^a	1.73 ± 0.35 ^a	1.64 ± 0.72 ^{a,b}	0.98 ± 0.35 ^a
Group-4	0.3 ± 0.17 ^a	0.3 ± 0.14 ^a	0.4 ± 0.15 ^a	0.3 ± 0.12 ^a	0.4 ± 0.19 ^a	0.4 ± 0.14 ^a	0.4 ± 0.14 ^a	0.3 ± 0.15 ^a	0.3 ± 0.16 ^a	0.3 ± 0.17 ^a	0.3 ± 0.16 ^a	0.3 ± 0.14 ^a	0.3 ± 0.15 ^a	0.3 ± 0.17 ^a	0.3 ± 0.18 ^a	0.3 ± 0.15 ^a	0.3 ± 0.18 ^a

Table 6: Mean FMD type-A serum neutralizing antibody titers in different vaccinated calves' groups.

Claves groups	FMD type-A ELISA antibody titer (\log_{10}/ml) /WPV*																
	0WPV* ± SE	1W ± SE	2W ± SE	3W ± SE	4W ± SE	6W ± SE	8W ± SE	10W ± SE	12W ± SE	14W ± SE	16W ± SE	20W ± SE	24W ± SE	28W ± SE	32W ± SE	34W ± SE	36W ± SE
Group-1	0.6 ± 0.14 ^a	1.71 ± 0.42 ^{a,b}	1.92 ± 0.33 ^a	2.16 ± 0.77 ^{a,b}	2.31 ± 0.70 ^b	2.64 ± 0.66 ^{a,b}	2.95 ± 0.54 ^a	3.14 ± 0.74 ^{a,b}	3.5 ± 0.35 ^{a,b}	3.01 ± 0.45 ^{a,b}	2.86 ± 0.45 ^{b,c}	2.54 ± 0.70 ^{b,c}	2.31 ± 0.65 ^{a,b}	2.08 ± 0.80 ^{a,b}	1.81 ± 0.60 ^{a,b}	1.53 ± 0.60 ^{a,b}	1.15 ± 0.86 ^{a,b}
Group-2	0.75 ± 0.15 ^a	1.92 ± 0.30 ^{a,b}	2.05 ± 0.44 ^{a,b}	2.16 ± 0.57 ^{a,b}	2.25 ± 0.52 ^{a,c}	2.39 ± 0.62 ^{a,b}	2.45 ± 0.67 ^{a,b}	2.6 ± 0.48 ^{a,c}	2.44 ± 0.40 ^{b,c}	2.25 ± 0.50 ^{a,b}	2.14 ± 0.40 ^a	2.01 ± 0.41 ^b	1.92 ± 0.25 ^{a,b}	1.72 ± 0.40 ^{a,b}	1.59 ± 0.57 ^{a,b}	1.17 ± 0.40 ^a	0.79 ± 0.30 ^a
Group-3	0.6 ± 0.13 ^a	2.01 ± 0.14 ^{a,b}	2.25 ± 0.57 ^a	2.71 ± 0.38 ^a	3.13 ± 0.68 ^{a,b}	3.25 ± 0.36 ^b	3.31 ± 0.22 ^a	3.51 ± 0.52 ^{a,b}	3.31 ± 0.84 ^{a,c}	3.24 ± 0.60 ^{b,c}	3.06 ± 0.21 ^{a,b}	2.84 ± 0.39 ^a	2.61 ± 0.52 ^{a,b}	2.27 ± 0.97 ^a	2.03 ± 0.30 ^a	1.94 ± 0.63 ^{a,b}	1.28 ± 0.20 ^a
Group-4	0.6 ± 0.15 ^a	0.6 ± 0.12 ^a	0.7 ± 0.14 ^a	0.6 ± 0.15 ^a	0.7 ± 0.13 ^a	0.7 ± 0.15 ^a	0.7 ± 0.14 ^a	0.6 ± 0.15 ^a	0.6 ± 0.15 ^a	0.6 ± 0.13 ^a	0.6 ± 0.17 ^a	0.6 ± 0.14 ^a	0.6 ± 0.13 ^a	0.6 ± 0.12 ^a	0.6 ± 0.14 ^a	0.6 ± 0.13 ^a	0.6 ± 0.15 ^a

Table 7: Mean FMD type-A ELISA antibody titer in different vaccinated calves' groups.

Demonstration of FMD type SAT2/Egypt/2012 antibody titers induced in vaccinated calves with the prepared different oil vaccine formulae by using SNT and ELISA data (Table 4 and 5) appeared differences in the onset, intensity and duration of the FMD serotype SAT2/Egypt/2012 antibodies. The onset of protective antibody titer, the inactivated FMD ISA 206 oil vaccine induced titers of ($1.62 \pm 0.01^{a,c}$ by SNT and $1.92 \pm 0.12^{a,c} \log_{10}$ by ELISA) in the 3rd WPV and inactivated FMD carbopol vaccine induced titers of (1.54 ± 0.36^a by SNT and $1.84 \pm 0.40^a \log_{10}$ by ELISA) in the 2nd WPV while inactivated FMD ISA 206 oil with Carbopol vaccine showed earlier immune response in the 1st WPV (1.53 ± 0.41^a by SNT, $1.83 \pm 0.47^a \log_{10}$ by ELISA). It is clear that peak of the protective SAT2 antibody titers induced by the inactivated FMD ISA 206 oil vaccine ($2.84 \pm 0.62^{a,c}$ by SNT and $3.14 \pm 0.66^{a,b} \log_{10}$ by ELISA) appeared in the 12nd WPV and by the inactivated FMD carbopol vaccine (2.12 ± 0.32^c as SNT and $2.42 \pm 0.38^c \log_{10}$ as ELISA) in the 10th WPV while the inactivated FMD ISA 206 oil carbopol vaccine induced the peak of antibody titers in the 8th WPV (3.09 ± 0.97^a by SNT, $3.39 \pm 0.97^a \log_{10}$ by ELISA). The protective type- SAT2/Egypt/2012 antibody titers, inactivated FMD ISA 206 oil vaccine showed protective titers of ($1.65 \pm 0.36^{a,c}$ by SNT and $1.95 \pm 0.41^{a,c} \log_{10}$ by ELISA) up to the 32nd WPV and also those induced by the inactivated FMD carbopol vaccine (1.53 ± 0.67^a as SNT and $1.83 \pm 0.71^a \log_{10}$ as ELISA) up to the 24th WPV while inactivated FMD ISA 206 oil carbopol vaccine showed later protective antibody titers in the 34th WPV ($1.62 \pm 0.74^{a,b}$ by SNT and $1.92 \pm 0.81^{a,b} \log_{10}$ by ELISA).

Claves groups	FMD type-SAT2 serum neutralizing antibody titers (\log_{10}/ml) /WPV*																
	0WPV* ± SE	1W ± SE	2W ± SE	3W ± SE	4W ± SE	6W ± SE	8W ± SE	10W ± SE	12W ± SE	14W ± SE	16W ± SE	20W ± SE	24W ± SE	28W ± SE	32W ± SE	34W ± SE	36W ± SE
Group-1	0.3 ± 0.15 ^a	0.92 ± 0.03 ^b	1.43 ± 0.16 ^{a,b}	1.62 ± 0.01 ^{a,c}	2.13 ± 0.35 ^{a,b}	2.26 ± 0.62 ^a	2.41 ± 0.33 ^{a,b}	2.65 ± 0.74 ^a	2.84 ± 0.62 ^{a,c}	2.61 ± 0.51 ^{a,b}	2.57 ± 0.27 ^a	2.34 ± 0.47 ^{a,b}	2.21 ± 0.31 ^{a,c}	1.92 ± 0.51 ^{a,b}	1.65 ± 0.36 ^{a,c}	1.41 ± 0.24 ^{b,c}	0.84 ± 0.65 ^{a,c}
Group-2	0.3 ± 0.13 ^a	1.12 ± 0.22 ^{a,b}	1.54 ± 0.36 ^a	1.62 ± 0.05 ^{b,c}	1.76 ± 0.62 ^b	1.84 ± 0.25 ^{a,c}	2.01 ± 0.41 ^{a,b}	2.12 ± 0.32 ^c	2.05 ± 0.87 ^{a,c}	1.96 ± 0.63 ^{a,b}	1.82 ± 0.40 ^c	1.67 ± 0.27 ^{a,b}	1.53 ± 0.67 ^a	1.14 ± 0.22 ^{a,b}	0.95 ± 0.53 ^c	0.45 ± 0.29 ^b	0.3 ± 0.75 ^{b,c}
Group-3	0.3 ± 0.14 ^a	1.53 ± 0.41 ^a	1.69 ± 0.24 ^a	2.01 ± 0.16 ^{a,c}	2.32 ± 0.43 ^{a,b}	2.74 ± 0.36 ^{b,c}	3.09 ± 0.97 ^a	2.95 ± 0.63 ^b	2.74 ± 0.43 ^{a,c}	2.63 ± 0.63 ^{a,b}	2.31 ± 0.51 ^{a,b}	2.17 ± 0.37 ^{a,c}	1.94 ± 0.82 ^a	1.82 ± 0.49 ^{a,b}	1.62 ± 0.86 ^{a,b}	1.62 ± 0.74 ^{a,b}	1.07 ± 0.32 ^{a,b}
Group-4	0.3 ± 0.17 ^a	0.3 ± 0.14 ^a	0.4 ± 0.15 ^a	0.3 ± 0.12 ^a	0.4 ± 0.19 ^a	0.4 ± 0.14 ^a	0.4 ± 0.14 ^a	0.3 ± 0.15 ^a	0.3 ± 0.16 ^a	0.3 ± 0.17 ^a	0.3 ± 0.16 ^a	0.3 ± 0.14 ^a	0.3 ± 0.15 ^a	0.3 ± 0.17 ^a	0.3 ± 0.18 ^a	0.3 ± 0.15 ^a	0.3 ± 0.18 ^a

Table 8: Mean FMD type-SAT2 serum neutralizing antibody titers in different vaccinated calves' groups.

Claves groups	FMD type-SAT2 ELISA antibody titer (\log_{10}/ml) /WPV*																
	0WPV* ± SE	1W ± SE	2W ± SE	3W ± SE	4W ± SE	6W ± SE	8W ± SE	10W ± SE	12W ± SE	14W ± SE	16W ± SE	20W ± SE	24W ± SE	28W ± SE	32W ± SE	34W ± SE	36W ± SE
Group-1	0.6 ± 0.14 ^a	1.22 ± 0.05 ^b	1.73 ± 0.18 ^{a,b}	1.92 ± 0.12 ^{a,c}	2.43 ± 0.40 ^{a,b}	2.56 ± 0.74 ^a	2.71 ± 0.37 ^{a,b}	2.95 ± 0.81 ^a	3.14 ± 0.66 ^{a,b}	2.91 ± 0.57 ^{a,b}	2.87 ± 0.32 ^a	2.64 ± 0.49 ^{a,b}	2.51 ± 0.35 ^{a,b}	2.22 ± 0.55 ^{a,c}	1.95 ± 0.41 ^{a,c}	1.71 ± 0.35 ^{b,c}	1.14 ± 0.72 ^{a,c}
Group-2	0.6 ± 0.12 ^a	1.42 ± 0.29 ^{a,b}	1.84 ± 0.40 ^a	1.92 ± 0.09 ^{b,c}	2.06 ± 0.68 ^b	2.14 ± 0.29 ^{a,c}	2.31 ± 0.48 ^{a,b}	2.42 ± 0.38 ^c	2.35 ± 0.91 ^{a,c}	2.26 ± 0.69 ^{a,b}	2.12 ± 0.48 ^c	1.97 ± 0.34 ^{a,b}	1.83 ± 0.71 ^a	1.44 ± 0.29 ^{a,b}	1.25 ± 0.57 ^c	0.75 ± 0.34 ^b	0.6 ± 0.82 ^{b,c}
Group-3	0.6 ± 0.15 ^a	1.83 ± 0.47 ^a	1.99 ± 0.31 ^a	2.31 ± 0.20 ^{a,c}	2.62 ± 0.48 ^{a,b}	3.04 ± 0.43 ^{b,c}	3.39 ± 0.97 ^a	3.25 ± 0.68 ^b	3.04 ± 0.49 ^{a,c}	2.93 ± 0.68 ^{a,b}	2.61 ± 0.56 ^{a,b}	2.47 ± 0.41 ^{a,c}	2.24 ± 0.89 ^a	2.12 ± 0.84 ^{a,b}	1.92 ± 0.94 ^{a,b}	1.92 ± 0.81 ^{a,b}	1.37 ± 0.39 ^{a,b}
Group-4	0.6 ± 0.15 ^a	0.6 ± 0.12 ^a	0.7 ± 0.14 ^a	0.6 ± 0.15 ^a	0.7 ± 0.13 ^a	0.7 ± 0.15 ^a	0.7 ± 0.14 ^a	0.6 ± 0.15 ^a	0.6 ± 0.15 ^a	0.6 ± 0.13 ^a	0.6 ± 0.17 ^a	0.6 ± 0.14 ^a	0.6 ± 0.13 ^a	0.6 ± 0.12 ^a	0.6 ± 0.14 ^a	0.6 ± 0.13 ^a	0.6 ± 0.15 ^a

Table 9: Mean FMD type-SAT2 ELISA antibody titer in different vaccinated calves' groups.

*WPV: Week Post Vaccination.

Group (1) vaccinated with the trivalent oil FMD.

Group (2) vaccinated with the trivalent carbomer FMD vaccine.

Group (3) vaccinated with the trivalent oil-carbomer FMD vaccine.

Group (4) kept without vaccination as negative control group.

The enhancing effect of carbopol on the immune response of vaccinated calves with trivalent FMD vaccine adjuvant with carbopol came in agreement with what obtained by [8,45] reported similar findings with EH-1 and rabies vaccines.

Conclusion

Depending on the present obtained results and the statistical analysis of humeral antibody titers against FMDV serotypes (A; O and SAT2) it could be concluded that the Montanide oils 206 with carbopol is the best vaccine formula which induced earlier, long lasting immunity followed by Montanide oils 206 and finally Carbopol which give early, short lasting immunity.

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