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Investigation of honey bee venom effect on the immunogenicity of foot-and-mouth disease vaccine in sheep

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Abstract

Background: Foot-and-mouth disease (FMD) is one of the most highly contagious and economically significant diseases of cloven-hoofed animals worldwide. FMD virus (FMDV) is the cause of the disease. The virus has seven serological types, identified as; O, A, C, SAT1, SAT2, SAT3, and Asia1. The aim of this study enhancement of FMD vaccine immunogenicity is the unique way to control FMD in Egypt.

Aim: Our research studied the effect of bee venom (BV) as simultaneously inoculated with the commercial vaccine on the immune response of experimentally vaccinated sheep in comparison with the inoculation of the vaccine alone through evaluation of the cellular and humoral immune response.

Methods: Estimation of cellular immunity using phagocytic activity, phagocytic percentage, lymphocyte blastogenesis, interleukin-6 (IL-6), and interleukin-12 (IL-12) and estimation of humoral immunity using serum neutralization test (SNT) and enzyme-linked immunosorbent assay (ELISA).

Result: Evaluation of the cellular immunity expressed in lymphocyte blastogenesis, phagocytic activity, phagocytic percentage, IL-6, and IL-12 showed higher levels in sheep vaccinated by the trivalent FMD vaccine (serotypes O pan Asia, A Iran O5, and SAT2/EGY/2012) with BV comparable to those induced by the vaccine alone. Following up the humoral immune response of vaccinated sheep revealed that FMDV antibodies serotypes O pan Asia, A Iran O5, and SAT2/EGY/2012 as measured by SNT and ELISA assay induced by FMD with BV were higher than those induced by inactivated FMD alone.

Conclusion: The inoculation of BV with FMD vaccine simultaneously is of high benefit inducing high level of specific immunity which could be of long duration.

Keywords: FMD, Bee venom, Cellular immunity, Humoral immunity.

Introduction

Foot-and-mouth disease FMD is one of the most troubles viral diseases among livestock especially cloven-footed domestic and wild animals including cattle, buffaloes, sheep, goats, and pigs (Depa *et al*., 2012). It is a highly contagious viral disease among livestock in the world in the terms of economic impact and hindering on the trade of animals on national and international level and restriction of people movement which affect the tourism sector (Knight *et al*., 2017). FMD has the ability to cause great economic losses due to reduced milk yields, abortions, delayed conception, perinatal mortality, and premature culling and also due to hindering on the trade of animals both locally and internationally, and restrictions on the movement of people which affect the tourism sector (James and Rushton, 2002).

During 2016–2019, different strains of FMD virus (FMDV) (O, A, SAT2) were circulated in Egypt that were detected from cattle and buffalo from various Egyptian governorates using enzyme-linked immunosorbent assay (ELISA) test. The three serotypes have different ratios for their expansion in Egypt where infected animals were infected with FMDV serotype O (79.3%), A (16.3%), and SAT2 (4.4%) (Ibrahim *et al*., 2018; El-Mayet *et al*., 2020).

Controlling of FMD in susceptible animals such as cattle, sheep, and goats attained by vaccination through single vaccination is efficient in decreasing viral transmission between animals as a supplemental control measure (Orsel *et al*., 2007).

In sheep, quadrivalent double emulsion (Montanide ISA206) vaccines were tested, and revealed that the oil adjuvant characterized by promotion of faster immune response than aluminum hydroxide gel vaccine. The neutralizing antibody titers in the animals were maintained at >3 $\log_{10}$ for 90 days (Patil *et al*., 2002).

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Serum neutralization test (SNT) and ELISA showed that the average protective FMD serum antibody titers in calves vaccinated with Montanide ISA 206 was begun at the 3rd week after vaccination and at the 10th week it achieved their higher level continuous protective level until 32nd week (Gamil, 2010).

As a result of being humoral antibody titer does not give sufficient details about vaccine-stimulated immunity against FMD, a comparison among immunity conferred after vaccination and cell-mediated immune responses showed a positive relationship between viral neutralizing antibodies and cell-mediated immunity (Carr *et al*., 2013).

Bee venom (BV) therapy (Apitherapy) has been used since ancient times (Kim *et al*., 2015). The venom has many scientific names as Apis Venenum Purum, Apitoxin, Bald-Faced Hornet, Bumblebee Venom, Honeybee Venom, Mixed Vespids, Pure BV, Wasp Venom, White-Faced Hornet, Yellow Hornet, and Yellow-Jacket Venom. Also, *Apis mellifera* (Honeybee); *Bombus terrestis* (Bumblebee); *Vespula maculata* (Hornet, Wasp) Family: Apidae; Vespidae (Meier and White, 1995).

The major components of BV, melittin and phospholipase A2, acquire their predominance from their broad beneficial action.

This work was designed to spot the light on honey BV as a natural material on the immune response of sheep to the trivalent FMD vaccine aiming to enhance the vaccine immunogenicity.

Materials and Methods

FMD virus

Locally isolated (FMDV) strains O PanAsia-2, A Iran O5, and SAT2/EGY/2012 of calves origin were typed and subtyped at the FMD Research Department (FMDRD), Veterinary Serum and Vaccine Research Institute (VSVRI), Abasia, Cairo and confirmed by the World Reference Laboratories, Pirbright, UK. These viral serotypes were in serological tests (SNT and ELISA). These viruses had the titer of 8.5, 8, and 7.0 $TCID_{50}$/ml, respectively.

FMD vaccine

Trivalent FMD vaccine was supplied by VSVRI and used to vaccinate the experimental sheep under the effect of BV.

Honey BV

Honey BV of *Apis mellifera lamarckii* was obtained from Bee Keeping Department, Agriculture Research Center, Egypt. A stock solution of BV was prepared in sterile distilled water at 0.1% and sterilized by filtration through 0.2 µ pore-size filter as described previously (Kamal, 2016).

Cell culture

Baby Hamster Kidney cell line (BHK_{21} $clone_{13}$) was kindly supplied by the Animal Research Institute, Pirbright, UK and propagated at FMDRD using minimum essential medium (MEM) with Eagle's salts supplemented with 10% new born calf serum according to Farag *et al*. (2006). These cells were maintained and propagated according to Macpherson and Stocker (1962); using MEM supplied by Gibco (G80 Gibco Limited, Parisley, UK) supplemented with 10% new born calf serum and antibiotics (100 µgs of streptomycin and 100 IU of penicillin-G sodium/ml). These cells were used for study the safety of BV and its antiviral activity and SNT.

Sheep

Thirty native breed sheep, of 1-year-old and 55 and 60 kg body weight, were divided into three groups (10 animals/group) as follow:

-Group 1 (GP1) vaccinated with polyvalent FMD ISA 206 oil vaccine alone using a dose of 1 ml/animal inoculated subcutaneously.

-Group 2 (GP2) vaccinated with polyvalent FMD ISA 206 using the same dose with 2 ml/animal as each 1 ml contains 3.9 µg of BV inoculated subcutaneously (Mansour *et al*., 2016).

-Group 3 (GP3) was kept without any inoculation as negative control.

Samples

Heparinized blood samples were obtained from all animals at 0-, 3-, 7-, 14-, 21-, and 28-days post-vaccination (DPV) for testing their cellular immune response using lymphocyte blastogenesis assay using cell proliferation kit (XTT kit), phagocytic activity and index.

Serum samples for estimation of the interleukin-6 (IL-6) and IL-12 were obtained from all animals at the same time points shown above.

Serum samples for monitoring of the humoral antibody response of the vaccinated sheep were collected weekly post-vaccination (WPV) for 1 month then every two WPV post-vaccination till the 4th month and finally every four WPV till the end of experiment (40th WPV).

Evaluation of the cellular immune response

Lymphocyte blastogenesis using XTT assay

Lymphocyte blastogenesis assay was carried out using XTT assay according to EL-Naggar (2012) through separation of lymphocytes as described by Lucy (1977) and Lee (1984) and determination of viable cell number according to 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 six-wells plate. The monolayer cells were rinsed three times gently with RPMI medium to remove non-adherent cell. The adherent cells were then covered with RPMI medium containing 5% fetal calf serum (FCS) and incubated for 24 hours in CO_2 incubator at 37 °C.

Phagocytic activity of sheep macrophages by using Candida albicans

The monolayer of adherent mononuclear cells was washed gently three 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 °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 minutes. 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 number 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 two blastospores) per infected phagocyte (phagocytic index) was calculated by Richardson and Smith (1981).

Estimation of IL

Estimation of the level of IL in the sera of experimental sheep including IL-6 and IL-12 was carried out using sheep IL-6 ELISA Kit Catalog No. EKE51028 supplied by Biomatik Company, Wilmington, DE, IL-12 ELISA Kit Catalog No. EKE925701 supplied by Biomatik Company, Wilmington, DE.

Evaluation of sheep humoral immune response

Serum samples collected from sheep before and after vaccination were subjected to estimation their antibody titers against the three serotypes of FMDV (O pan Asia, A Iran O5, SAT2/EGY/2012 and SAT2/EGY/2018) by SNT using the micro titer technique described by Ferreira (1976) and indirect ELISA according to Voller *et al*. (1976).

Statistical analysis

The obtained data were analyzed using analysis of variance in the Statistical Package for the Social Sciences version 12 statistical software package for PCS Multiple comparisons of means were made using Duncan's multiple range tests at $p < 0.05$%.

Results

Lymphocyte blastogenesis assay revealed that mean optical density of lymphocyte blastogenesis carried out on blood samples collected from vaccinated sheep reached its highest value by the 14th DPV showing higher levels in sheep received BV with FMD vaccine (1.10 ± 0.22^b) than in sheep received the vaccine alone (0.95 ± 0.13^b) and reached the lowest values (0.78 ± 0.21^c) and (0.76 ± 0.12^{bc}), respectively, by the 35th DPV as tabulated in Table 1.

Phagocytic activity assay revealed that mean phagocytic percentage and index carried out on blood samples collected from vaccinated sheep reached its highest value by the 14th DPV that was higher in sheep received BV with FMD vaccine (92.3, 0.99) than that in sheep received the vaccine alone (66.4, 0.90) which was at 21st DPV showing the lowest values (32.2, 0.5) and (24.1, 0.3), respectively, by the 35th DPV as tabulated in Tables 2 and 3.

Estimation of IL-6 and IL-12 in vaccinated sheep showed higher levels in sheep vaccinated with FMD vaccine with BV than in sheep vaccinated with FMD vaccine alone by the 14th DPV (3.97 ± 0.32^b and 3.78 ± 0.16^b for IL-6 and 6.8 ± 0.32^b, and 6.2 ± 0.19^b for IL-12, respectively. By the 35th DPV the lowest

Table 1. Mean delta optical density of lymphocyte blastogenesis assay of vaccinated sheep.

Sheep groups	Delta optical density of lymphocyte blastogenesis/DPV*						
	1st DPV	3rd DPV	7th DPV	14th DPV	21st DPV	28th DPV	35th DPV
GP-1	0.32 ± 0.14	$0.45 \pm 0.24^{a,c}$	0.67 ± 0.13^b	0.95 ± 0.13^b	$0.81 \pm 0.26^{b,c}$	$0.76 \pm 0.14^{b,c}$	$0.76 \pm 0.12^{b,c}$
GP-2	0.41 ± 0.23	$0.82 \pm 0.16^{b,c}$	1.15 ± 0.25^b	1.10 ± 0.22^b	0.97 ± 0.1^c	0.80 ± 0.18^c	0.78 ± 0.21^c
GP-3	0.10 ± 0.22	0.12 ± 0.23^a	0.13 ± 0.16^a	0.12 ± 0.16^a	0.14 ± 0.19^a	0.12 ± 0.15^a	0.13 ± 0.17^a

DPV: days post vaccination.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests.

Table 2. Phagocytic % of vaccinated sheep.

Sheep groups	Phagocytic percentage/DPV*						
	1st DPV	3rd DPV	7th DPV	14th DPV	21st DPV	28th DPV	35th DPV
GP-1	20.1	30.2	49.7	56.1	66.4	54.2	24.1
GP-2	29.2	37.5	81.2	92.3	70.4	60.1	32.2
GP-3	19	19.5	19.4	19.5	19.5	19.4	19

DPV: days post vaccination.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests.

Table 3. Phagocytic index of vaccinated sheep.

Sheep groups	Phagocytic index/DPV*						
	1st DPV	3rd DPV	7th DPV	14th DPV	21st DPV	28th DPV	35th DPV
GP-1	0.11	0.32	0.49	0.61	0.69	0.45	0.3
GP-2	0.11	0.84	0.98	0.99	0.90	0.68	0.5
GP-3	0.12	0.12	0.12	0.13	0.12	0.10	0.12

DPV: days post vaccination.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests.

Table 4. Mean concentration of IL-6 (ng/ml) in vaccinated sheep.

DPV*	Mean concentration level of IL-6 (ng/ml) in sheep sera		
	Sheep groups		
	Unvaccinated control	Received FMD vaccine alone	Received FMD vaccine with BV
0	0.40 ± 0.21	0.89 ± 0.15	1.43 ± 0.13
3rd	0.35 ± 0.29^{a}	1.44 ± 0.34^{a,c}	2.51 ± 0.26^{b,c}
7th	0.32 ± 0.26^{a}	2.11 ± 0.23^{b}	4.78 ± 0.27^{b}
14th	0.3 ± 0.18^{a}	3.78 ± 0.16^{b}	3.97 ± 0.32^{b}
21th	0.39 ± 0.39^{a}	3.62 ± 0.16^{b,c}	3.81 ± 0.21^{c}
28th	0.4 ± 0.25^{a}	3.17 ± 0.18^{b,c}	3.43 ± 0.11^{c}
35th	0.38 ± 0.18^{a}	3.12 ± 0.15^{b,c}	3.33 ± 0.51^{c}

DPV: days post vaccination.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests.

Table 5. Mean concentration of IL-12 (ng/ml) vaccinated sheep.

DPV*	Mean concentration level of IL-12 (ng/ml) in sheep sera		
	Sheep groups		
	Unvaccinated control	Received FMD vaccine alone	Received FMD vaccine with BV
0	4.1 ± 0.23	4.4 ± 0.12	4.6 ± 0.13
3rd	4.3 ± 0.25^{a}	4.61 ± 0.14^{a,c}	5.6 ± 0.26^{b,c}
7th	4.1 ± 0.19^{a}	5.15 ± 0.13^{b}	7.5 ± 0.27^{b}
14th	4.2 ± 0.17^{a}	6.2 ± 0.19^{b}	6.8 ± 0.32^{b}
21th	4.3 ± 0.12^{a}	5.3 ± 0.17^{b,c}	5.5 ± 0.21^{c}
28th	4.1 ± 0.14^{a}	4.9 ± 0.12^{b,c}	5.3 ± 0.11^{c}
35th	4.1 ± 0.14^{a}	4.8 ± 0.19^{b,c}	5.3 ± 0.51^{c}

DPV: days post vaccination.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests.

recorded levels of IL-6 and IL-12 were 3.33 ± 0.51^{c} and 3.12 ± 0.15^{bc} and 5.3 ± 0.51^{c} and 4.8 ± 0.19^{bc}, respectively, in sheep received BV with FMD vaccine and in sheep received the vaccine alone as shown in Tables 4 and 5.

Monitoring of sheep immune response to FMD vaccine alone or with BV through application of SNT and solid phase indirect ELISA; revealed that vaccinated sheep exhibited FMD serotype O protective antibody titers (1.55 ± 0.09 and 1.77 ± 0.1 $\log_{10}$ by SNT and ELISA, respectively, by the second week post-vaccination with FMD vaccine alone. These values were higher with administration of BV (1.70 ± 0.09 and 1.93 ± 0.13 $\log_{10}$ by SNT and ELISA, respectively) by the first week post-vaccination. These titers recorded their peaks (2.97 ± 0.03 $\log_{10}$ by SNT and 3.13 ± 0.03 $\log_{10}$ by ELISA) on the 12th WPV and 3.2 ± 0.05 $\log_{10}$ by SNT and 3.43 ± 0.03 $\log_{10}$ by ELISA on the 10th WPV using the vaccine alone and with BV, respectively. FMD antibodies were gradually decreased to reach

its lowest protective levels (1.55 ± 0.9 log_{10} by SNT and 1.77 ± 0.06 log_{10} by ELISA) on the 34th WPV with FMD vaccine alone while in the case of administration of BV these values were (1.55 ± 0.09 log_{10} by SNT and 1.78 ± 0.03 log_{10} by ELISA) on the 38th WPV. Non-protective FMD serotype O antibody titers (less than 1.5 and 1.8 log_{10} by SNT and ELISA, respectively) were recorded by the 36th and 40th WPV with FMD vaccine alone and with BV, respectively. These results are demonstrated in Table 6.

Administration of FMD vaccine alone and with BV resulted in induction of specific FMD serotype A antibodies as determined by application of SNT and solid phase indirect ELISA. These tests revealed that vaccinated sheep exhibited FMD serotype A protective antibody titers (1.55 ± 0.09 and 1.8 ± 0.1 log_{10} by SNT and ELISA, respectively, by the second week post-vaccination with FMD vaccine alone and (1.75 ± 0.09 log_{10} by SNT and 2 ± 0.06 log_{10} by ELISA) with administration of BV by the first week post-vaccination. These titers recorded their peaks (3.05 ± 0.03 log_{10} by SNT and 3.30 ± 0.01 log_{10} by ELISA) on the 12th WPV and 3.2 ± 0.05 log_{10} by SNT and 3.45 ± 0.04 log_{10} by ELISA on the 10th WPV using the vaccine alone and with BV, respectively. Such antibodies were gradually decreased to reach its lowest protective levels (1.55 ± 0.9 log_{10} by SNT and 1.77 ± 0.03 log_{10} by ELISA) on the 34th WPV with FMD vaccine alone while in the case of administration of BV these values were (1.60 ± 0.04 log_{10} by SNT and 1.95 ± 0.12 log_{10} by ELISA) on the 38th WPV. Non-protective FMD serotype O antibody titers (less than 1.5 and 1.8 log_{10} by SNT and ELISA, respectively)

Table 6. Mean FMD serotype O serum neutralizing antibody and ELISA titers in vaccinated sheep.

WPV	Mean FMD O antibody titers (log10 ± SD/WPV*)					
	Sheep groups					
	Unvaccinated control		Received FMD vaccine alone		Received FMD vaccine with BV	
	SNT	ELISA	SNT	ELISA	SNT	ELISA
0	0.2 ± 0.09^{a}	0.43 ± 0.04^{a}	0.25 ± 0.09^{a}	0.47 ± 0.03^{a}	0.2 ± 0.09^{a}	0.43 ± 0.15^{a}
1	0.25 ± 0.09^{a}	0.48 ± 0.05^{a}	1.1 ± 0.09^{b}	1.32 ± 0.16^{b}	1.70 ± 0.09^{c}	1.93 ± 0.13^{c}
2	0.3 ± 0.15^{a}	0.56 ± 0.1^{a}	1.55 ± 0.09^{b}	1.77 ± 0.1^{c}	1.85 ± 0.09^{d}	2.08 ± 0.03^{d}
3	0.35 ± 0.09^{a}	0.58 ± 0.04^{a}	1.75 ± 0.09^{b}	1.97 ± 0.13^{c}	2 ± 0.09^{b}	2.23 ± 0.03^{d}
4	0.2 ± 0.09^{a}	0.43 ± 0.04^{a}	2.05 ± 0.09^{b}	2.27 ± 0.1^{bc}	2.35 ± 0.17^{b}	2.58 ± 0.1^{c}
6	0.2 ± 0.09^{a}	0.43 ± 0.12^{a}	2.3 ± 0.09^{b}	2.52 ± 0.1^{b}	2.65 ± 0.08^{c}	2.88 ± 0.06^{c}
8	0.35 ± 0.09^{a}	0.57 ± 0.09^{a}	2.45 ± 0.09^{b}	2.67 ± 0.1^{b}	2.8 ± 0.15^{c}	3.03 ± 0.06^{c}
10	0.2 ± 0.09^{a}	0.4 ± 0.11^{a}	2.75 ± 0.09^{b}	2.97 ± 0.1^{b}	3.2 ± 0.05^{c}	3.43 ± 0.13^{c}
12	0.25 ± 0.17^{a}	0.41 ± 0.09^{a}	2.97 ± 0.03^{c}	3.13 ± 0.03^{b}	3.1 ± 0.03^{c}	3.46 ± 0.02^{c}
14	0.30 ± 0.15^{a}	0.53 ± 0.13^{a}	2.75 ± 0.09^{c}	2.92 ± 0.08^{c}	2.95 ± 0.09^{c}	3.33 ± 0.01^{d}
16	0.2 ± 0.05^{a}	0.43 ± 0.12^{a}	2.5 ± 0.9^{c}	2.72 ± 0.13^{c}	2.75 ± 0.09^{c}	2.98 ± 0.03^{c}
18	0.2 ± 0.03^{a}	0.43 ± 0.04^{a}	2.35 ± 0.09^{c}	2.55 ± 0.1^{c}	2.65 ± 0.09^{d}	2.88 ± 0.06^{d}
20	0.25 ± 0.05^{a}	0.48 ± 0.16^{a}	2.25 ± 0.0^{c}	2.47 ± 0.08^{c}	2.5 ± 0.09^{c}	2.73 ± 0.06^{d}
22	0.25 ± 0.09^{a}	0.48 ± 0.16^{a}	2.05 ± 0.09^{c}	2.27 ± 0.13^{c}	2.4 ± 0.015^{c}	2.63 ± 0.08^{c}
24	0.25 ± 0.09^{a}	0.47 ± 0.12^{a}	2 ± 0.09^{c}	2.22 ± 0.06^{c}	2.35 ± 0.15^{d}	2.58 ± 0.06^{d}
26	0.2 ± 0.09^{a}	0.43 ± 0.16^{a}	1.9 ± 0.09^{c}	2.12 ± 0.13^{c}	2.20 ± 0.09^{d}	2.43 ± 0.06^{c}
28	0.2 ± 0.09^{a}	0.42 ± 0.05^{a}	1.85 ± 0.09^{c}	2.07 ± 0.06^{c}	2.15 ± 0.09^{d}	2.38 ± 0.13^{d}
30	0.25 ± 0.09^{a}	0.48 ± 0.11^{a}	1.7 ± 0.09^{c}	1.92 ± 0.06^{c}	2.05 ± 0.09^{d}	2.28 ± 0.06^{d}
32	0.15 ± 0.0^{a}	0.37 ± 0.08^{a}	1.6 ± 0.09^{c}	1.82 ± 0.03^{c}	1.9 ± 0.09^{d}	2.13 ± 0.1^{c}
34	0.2 ± 0.09^{a}	0.42 ± 0.04^{a}	1.55 ± 0.09^{c}	1.77 ± 0.06^{c}	1.8 ± 0.0^{c}	2.03 ± 0.08^{d}
36	0.15 ± 0.00^{a}	0.38 ± 0.02^{a}	1.40 ± 0.09^{b}	1.66 ± 0.04^{c}	1.7 ± 0.09^{c}	1.93 ± 0.03^{d}
38	0.2 ± 0.09^{a}	0.41 ± 0.11^{a}	1.1 ± 0.09^{b}	1.32 ± 0.1^{b}	1.55 ± 0.09^{c}	1.78 ± 0.03^{c}
40	0.2 ± 0.09^{a}	0.41 ± 0.12^{a}	0.8 ± 0.09^{b}	1.02 ± 0.1^{b}	1.35 ± 0.15^{c}	1.62 ± 0.06^{c}

WPV: Week post vaccination; SD: Standard deviation.
p-value = 0.000.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests. Protective serum antibody titer by SNT = 1.5 and by ELISA = 1.8 log_{10} according to OIE (2017).

Table 7. Mean FMD serotype A serum neutralizing antibody and ELISA titers in vaccinated sheep.

WPV	Mean FMD A antibody titers (log_{10} ± SD/WPV*)					
	Sheep groups					
	Unvaccinated control		Received FMD vaccine alone		Received FMD vaccine with BV	
	SNT	ELISA	SNT	ELISA	SNT	ELISA
0	0.2 ± 0.09[a]	0.43 ± 0.1[a]	0.25 ± 0.09[a]	0.5 ± 0.05[a]	0.2 ± 0.09[a]	0.45 ± 0.13[a]
1	0.25 ± 0.09[a]	0.41 ± 0.12[a]	1.15 ± 0.09[c]	1.4 ± 0.13[b]	1.75 ± 0.09[b]	2 ± 0.06[c]
2	0.3 ± 0.15[a]	0.52 ± 0.14[a]	1.55 ± 0.09[b]	1.8 ± 0.1[b]	1.85 ± 0.09[c]	2.1 ± 0.12[b]
3	0.35 ± 0.09[a]	0.51 ± 0.04[a]	1.7 ± 0.09[b]	1.95 ± 0.05[b]	2.05 ± 0.09[c]	2.32 ± 0.06[c]
4	0.2 ± 0.09[a]	0.39 ± 0.04[a]	1.9 ± 0.09[b]	2.15 ± 0.1[b]	2.5 ± 0.09[c]	2.70 ± 0.04[c]
6	0.2 ± 0.09[a]	0.43 ± 0.11[a]	2.05 ± 0.09[c]	2.30 ± 0.05[b]	2.65 ± 0.09[d]	2.9 ± 0.06[c]
8	0.35 ± 0.09[a]	0.51 ± 0.16[a]	2.35 ± 0.09[b]	2.60 ± 0.05[b]	2.8 ± 0.0.9[c]	3.05 ± 0.06[c]
10	0.2 ± 0.09[a]	0.42 ± 0.07[a]	2.7 ± 0.15[b]	2.95 ± 0.13[b]	3.2 ± 0.05[c]	3.45 ± 0.04[c]
12	0.25 ± 0.17[a]	0.51 ± 0.18[a]	3.05 ± 0.03[c]	3.30 ± 0.1[b,c]	3.2 ± 0.03[c]	3.6 ± 0.06[c]
14	0.30 ± 0.15[a]	0.46 ± 0.05[a]	2.8 ± 0.09[b]	3.05 ± 0.1[c]	2.95 ± 0.17[c]	3.43 ± 0.1[d]
16	0.2 ± 0.09[a]	0.36 ± 0.19[a]	2.5 ± 0.9[c]	2.75 ± 0.1[c]	2.75 ± 0.09[d]	3.13 ± 0.17[c]
18	0.2 ± 0.09[a]	0.36 ± 0.04[a]	2.35 ± 0.09[c]	2.6 ± 0.1[c]	2.7 ± 0.15[d]	2.95 ± 0.1[d]
20	0.25 ± 0.09[a]	0.51 ± 0.04[a]	2.3 ± 0.09[c]	2.55 ± 0.1[c]	2.5 ± 0.09[c]	2.78 ± 0.04[c]
22	0.25 ± 0.09[a]	0.49 ± 0.07[a]	2.1 ± 0.15[c]	2.35 ± 0.18[b,c]	2.4 ± 0.015[d]	2.65 ± 0.15[c]
24	0.25 ± 0.09[a]	0.41 ± 0.09[a]	2.05 ± 0.09[c]	2.30 ± 0.05[c]	2.35 ± 0.09[d]	2.6 ± 0.06[d]
26	0.2 ± 0.09[a]	0.40 ± 0.11[a]	1.9 ± 0.09[c]	2.15 ± 0.1[c]	2.20 ± 0.09[d]	2.45 ± 0.06[c]
28	0.2 ± 0.09[a]	0.36 ± 0.04[a]	1.85 ± 0.09[c]	2.1 ± 0.05[c]	2.15 ± 0.09[d]	2.4 ± 0.04[d]
30	0.25 ± 0.09[a]	0.41 ± 0.09[a]	1.7 ± 0.09[c]	1.95 ± 0.05[c]	2 ± 0.09[d]	2.25 ± 0.12[c]
32	0.15 ± 0.0[a]	0.38 ± 0.03[a]	1.6 ± 0.09[c]	1.85 ± 0.05[b,c]	1.9 ± 0.09[d]	2.15 ± 0.14[c]
34	0.2 ± 0.09[a]	0.36 ± 0.04[a]	1.55 ± 0.09[c]	1.77 ± 0.03[c]	1.85 ± 0.09[d]	2.14 ± 0.04[d]
36	0.15 ± 0.00[a]	0.34 ± 0.07[a]	1.40 ± 0.09[c]	1.59 ± 0.05[c]	1.7 ± 0.09[d]	1.95 ± 0.12[c]
38	0.2 ± 0.09[a]	0.42 ± 0.11[a]	1.1 ± 0.09[b]	1.35 ± 0.1[b]	1.60 ± 0.09[c]	1.85 ± 0.06[c]
40	0.2 ± 0.09[a]	0.39 ± 0.14[a]	0.75 ± 0.15[b]	1.0 ± 0.13[b]	1.4 ± 0.09[c]	1.65 ± 0.04[c]

WPV: Week post vaccination; SD: Standard deviation.
p-value = 0.000.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests. Protective serum antibody titer by SNT = 1.5 and by ELISA = 1.8 log_{10} according to OIE (2017).

were recorded by the 36 and 40 WPV with FMD vaccine alone and with BV, respectively, as demonstrated in Table 7. Following up the levels of FMD serotype SAT2 antibodies in vaccinated sheep with FMD vaccine alone and with BV; it was found that such antibodies were detectable by application of SNT and solid phase indirect ELISA which showed that vaccinated sheep exhibited FMD serotype SAT2 protective antibody titers (1.55 ± 0.09 and 1.77 ± 0.1 log_{10} by SNT and ELISA, respectively, by the second week post-vaccination with

Table 8. Mean FMD serotype SAT2 serum neutralizing antibody and ELISA titers in vaccinated sheep.

WPV	Mean FMD SAT2 antibody titers (log_{10} ± SD/WPV*)					
	Sheep groups					
	Unvaccinated control		Received FMD vaccine alone		Received FMD vaccine with BV	
	SNT	ELISA	SNT	ELISA	SNT	ELISA
0	0.2 ± 0.09[a]	0.43 ± 0.04[a]	0.25 ± 0.1[a]	0.47 ± 0.03[a]	0.2 ± 0.09[a]	0.43 ± 0.15[a]
1	0.25 ± 0.09[a]	0.48 ± 0.05[a]	1.15 ± 0.09[c]	1.32 ± 0.16[b]	1.75 ± 0.09[b]	1.93 ± 0.13[c]
2	0.3 ± 0.15[a]	0.56 ± 0.1[a]	1.55 ± 0.09[b]	1.77 ± 0.1[b]	1.85 ± 0.09[c]	2.08 ± 0.03[c]
3	0.35 ± 0.09[a]	0.58 ± 0.04[a]	1.7 ± 0.09[b]	1.97 ± 0.13[b]	2.05 ± 0.09[c]	2.23 ± 0.03[c]
4	0.2 ± 0.09[a]	0.43 ± 0.04[a]	1.95 ± 0.09[b]	2.27 ± 0.03[b]	2.45 ± 0.17[c]	2.58 ± 0.1[c]
6	0.2 ± 0.09[a]	0.43 ± 0.12[a]	2.15 ± 0.09[b]	2.52 ± 0.1[b]	2.65 ± 0.08[c]	2.88 ± 0.06[c]
8	0.35 ± 0.09[a]	0.57 ± 0.09[a]	2.35 ± 0.09[b]	2.67 ± 0.1[b]	2.8 ± 0.09[c]	3.03 ± 0.06[c]
10	0.2 ± 0.09[a]	0.4 ± 0.11[a]	2.7 ± 0.09[b]	2.97 ± 0.1[b]	3.25 ± 0.09[c]	3.43 ± 0.13[c]
12	0.25 ± 0.17[a]	0.41 ± 0.09[a]	3.05 ± 0.09[c]	3.13 ± 0.03[b]	3.2 ± 0.09[c]	3.46 ± 0.016[c]
14	0.30 ± 0.15[a]	0.53 ± 0.13[a]	2.8 ± 0.09[c]	2.92 ± 0.08[c]	2.95 ± 0.09[c]	3.33 ± 0.01[d]
16	0.2 ± 0.05[a]	0.43 ± 0.12[a]	2.55 ± 0.9[c]	2.72 ± 0.13[c]	2.75 ± 0.09[c]	2.98 ± 0.03[c]
18	0.2 ± 0.03[a]	0.43 ± 0.04[a]	2.35 ± 0.09[c]	2.55 ± 0.1[c]	2.6 ± 0.09[d]	2.88 ± 0.06[d]
20	0.25 ± 0.05[a]	0.48 ± 0.16[a]	2.3 ± 0.0[c]	2.47 ± 0.08[c]	2.5 ± 0.09[c]	2.73 ± 0.06[d]
22	0.25 ± 0.09[a]	0.48 ± 0.16[a]	2.2 ± 0.09[c]	2.27 ± 0.13[c]	2.45 ± 0.09[d]	2.63 ± 0.08[c]
24	0.25 ± 0.09[a]	0.47 ± 0.12[a]	2.05 ± 0.09[c]	2.22 ± 0.06[c]	2.3 ± 0.09[d]	2.58 ± 0.06[d]
26	0.2 ± 0.09[a]	0.43 ± 0.16[a]	1.9 ± 0.09[c]	2.12 ± 0.13[c]	2.20 ± 0.09[d]	2.43 ± 0.06[c]
28	0.2 ± 0.09[a]	0.42 ± 0.05[a]	1.85 ± 0.09[c]	2.07 ± 0.06[c]	2.15 ± 0.09[d]	2.38 ± 0.13[c]
30	0.25 ± 0.09[a]	0.48 ± 0.11[a]	1.7 ± 0.09[c]	1.92 ± 0.06[c]	2.05 ± 0.09[d]	2.28 ± 0.06[d]
32	0.15 ± 0.0[a]	0.37 ± 0.08[a]	1.6 ± 0.09[c]	1.82 ± 0.03[c]	1.9 ± 0.09[d]	2.13 ± 0.1[d]
34	0.2 ± 0.09[a]	0.42 ± 0.04[a]	1.45 ± 0.09[c]	1.77 ± 0.06[c]	1.85 ± 0.0[d]	2.03 ± 0.08[d]
36	0.15 ± 0.00[a]	0.38 ± 0.02[a]	1.40 ± 0.09[c]	1.66 ± 0.04[b]	1.75 ± 0.09[d]	1.93 ± 0.03[c]
38	0.2 ± 0.09[a]	0.41 ± 0.11[a]	1.1 ± 0.1[b]	1.32 ± 0.1[b]	1.65 ± 0.15[c]	1.78 ± 0.03[c]
40	0.2 ± 0.09[a]	0.41 ± 0.12[a]	0.75 ± 0.2[b]	1.02 ± 0.1[b]	1.3 ± 0.09[c]	1.62 ± 0.06[c]

(WPV: Week post vaccination; SD: Standard deviation.
p-value = 0.000.
Different letters indicate significant difference between different treatments at $p < 0.05$ according to Duncan's multiple range tests. Protective serum antibody titer by SNT = 1.5 and by ELISA = 1.8 log_{10} according to OIE (2017).

FMD vaccine alone and (1.75 ± 0.09 log_{10} by SNT and 1.95 ± 0.13 log_{10} by ELISA) with administration of BV by the first week post-vaccination. These titers recorded their peaks (3.05 ± 0.03 log_{10} by SNT and 3.13 ± 0.03 log_{10} by ELISA) on the 12th WPV and 3.25 ± 0.09 log_{10} by SNT and 3.45 ± 0.04 log_{10} by ELISA on the 10th WPV using the vaccine alone and with BV, respectively. Such antibodies were gradually decreased to reach its lowest protective levels (1.60 ± 0.04 log_{10} by SNT and 1.82 ± 0.03 log_{10} by ELISA) on the 32nd WPV with FMD vaccine alone while in the case of administration of BV these values were (1.65 ± 0.15 log_{10} by SNT and 1.75 ± 0.03 log_{10} by ELISA) on the 38th WPV. Non-protective FMD serotype O antibody titers (less than

Table 9. Cumulative table showing the immune modulator effect of BV on the immune response of sheep to the trivalent FMD vaccine.

Items	Mean FMD antibody titers (log_{10} ± SD/WPV*)			
	Vaccination program			
	Received FMD vaccine alone		Received FMD vaccine with BV	
	SNT	ELISA	SNT	ELISA
Serotype O				
Start	1.55 ± 0.09	1.77 ± 0.01	1.76 ± 0.04	1.93 ± 0.13
	2nd WPV		1st WPV	
Peak	2.79 ± 0.03	3.13 ± 0.03	3.2 ± 0.05	3.45 ± 0.12
	12th WPV		10th WPV	
Duration	1.55 ± 0.44	1.77 ± 0.06	1.55 ± 0.09	1.78 ± 0.03
	34th WPV		38th WPV	
Serotype A				
Start	1.55 ± 0.09	1.8 ± 0.01	1.75 ± 0.09	2.0 ± 0.06
	2nd WPV		1st WPV	
Peak	3.05 ± 0.03	3.30 ± 0.01	3.2 ± 0.05	3.45 ± 0.04
	12th WPV		10th WPV	
Duration	1.55 ± 0.09	1.77 ± 0.03	1.60 ± 0.04	1.95 ± 0.12
	34nd WPV		36th WPV	
Serotype SAT2				
Start	1.55 ± 0.09	1.77 ± 0.01	1.75 ± 0.09	1.95 ± 0.13
	2nd WPV		1st WPV	
Peak	3.05 ± 0.09	3.13 ± 0.03	3.25 ± 0.09	3.45 ± 0.03
	12th WPV		10th WPV	
Duration	1.6 ± 0.04	1.68 ± 0.03	1.65 ± 0.15	1.75 ± 0.03
	32nd WPV		38th WPV	

WPV: Week post vaccination; SD: Standard deviation.
p-value = 0.000.
Protective serum antibody titer by SNT = 1.5 and by ELISA = 1.8 log_{10} according to OIE (2017).

1.5 and 1.8 log_{10} by SNT and ELISA, respectively) were recorded by the 36 and 40 WPV with FMD vaccine alone and with BV, respectively as demonstrated in Table 8, and the immune modulator effect of BV is shown in Table 9

Discussion

The results of cellular immunity were coming as stated by Knudsen *et al*. (1979), who reported that cell-mediated immune response was a constitute of immune response against FMDV, and Garcia *et al*. (1996), Elwatany *et al*. (1999), Sonia *et al*. (2010), and Fakhry *et al*. (2012) mentioned that the delta optical density of lymphocyte blastogenesis assay and IL-6 at 0, 3, 7, 14, 21, and 28 -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. Also, our result come in parallel to El-Din *et al*. (2014) who concluded that the Egyptian trivalent FMD oil vaccine showed a maximum cellular immune response at 21 DPV.

The results of humoral immunity came in parallel with the result obtained by Ibrahim *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 ± 0.106[a,b] and 1.81 ± 0.104[a,b]) for FMDV serotypes O, A, and SAT2, respectively. 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. In addition, these results agree with those obtained by Selim *et al*. (2010) who reported that the mean antibody titers against FMD vaccine strain O1/3/93 were detected in sheep sera vaccinated with Alumhydroxide gel vaccine following one WPV by SNT, whereas, the mean peak titers (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 titer reached a protective level starting from the 4th WPV to record peak titer 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 titer 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 titer remained within the protective level up to 34 WPV. El-Din *et al*. (2014) concluded that the Egyptian trivalent FMD oil vaccine-induced protective humoral immune response extended for 32 WPV.

The obtained high levels of FMD immune response in vaccinate sheep receiving BV could be attributed to effect of BV as immunomodulator which stimulates immune system to protect the body against infection by its stimulation of prostaglandin generation which have biological activities resulting in stimulation of IL-10, TNF alpha, and CD8 which result in regulation in IgE which responsible for histamine release (Rekka *et al*., 1990). Eiaka *et al.* (2016) found that following up rabies antibodies in vaccinated dogs using SNT revealed that BV induced the highest levels of antibodies (128) when inoculated before and simultaneously with vaccination by rabies vaccine. Rabies vaccine alone or before inoculation of BV induced lower titers of antibodies (32 and 64, respectively) by the fourth week post-vaccination. Also, ELISA confirming came parallel to those of SNT revealing that BV induced the highest levels of antibodies (7 and 6 $\log_2$, respectively) when inoculated before and simultaneously with vaccination by rabies vaccine and rabies vaccine alone or before inoculation of BV induced lower ELISA titers (5 $\log_2$) by the fourth week post-vaccination.

Depending on the demonstrated data it could be concluded that BV enhances the immune response of vaccinated sheep to the trivalent FMD vaccine resulted in earlier induction of specific FMD serotypes antibodies than in the case of administration of the vaccine alone reaching their peak on earlier time with prolonged duration of immunity.

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