



## DURATION OF IMMUNE RESPONSE IN CALVES FOR INACTIVATED DOUBLE OIL EMULSION BOVINE EPHEMERAL FEVER VIRUS VACCINE.

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### ABSTRACT

In this study a double oil emulsion inactivated BEF vaccine mixed with Montanide ISA 206 was prepared. Studying the dynamics of serum antibody in vaccinated calves using of SNT and ELISA showed that antibody titers reached level considered protective within two weeks and lasted for about 40 weeks. In conclusion, results of present study indicated that the vaccine formulated according to our procedures could provoke long lasting protective immune response after single dose administration, without inducing any adverse reactions.

**Keywords:** BEF vaccine, Montanide ISA 206, SNT, ELISA.

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### 1. INTRODUCTION

**B**ovine ephemeral fever (BEF) is an arthropod borne disease of cattle and buffalos, characterized by sudden onset of fever, stiffness, lameness, nasal and ocular discharges, (St George, 1988).

Major economic losses were due to drop in milk production, reduction in condition of prime animals and disruption of stock movements and markets (Walker, 2005). Bovine ephemeral fever (BEF) virus belonged to genus Ephemerovirus, family Rhabdoviridae. The virus structure consisted of a negative-sense, single-stranded RNA genome and 5 structural proteins, including nucleoprotein (N), polymerase-associated protein (P), matrix protein (M), large RNA-dependent RNA polymerase (L), and surface glycoprotein (G), which induces the production of protective neutralizing antibody (Uren et al., 1994).

BEF spread in tropical and subtropical zones of Asia, Australia, and Africa (Bai et al., 1991). In Egypt, several outbreaks of

BEF were recorded in Lower Egypt (Hessian et al., 1991), Bahr El-Bakar (Hassan, 2000), Dakahlia and Damita (Daoud et al., 2001), Menofeia (Soad et al., 2001); Assiut (Abd El-Rahman et al., 2002), Sharkia (Hamoda et al., 2002), Kafr El-Sheik (Al-Gaabary et al., 2005), Dakahlia and Damita (Daoud et al., 2005), Menoufia Governorate (Nayel, 2006), Damita (Degheidy et al., 2011) and Sharkia (Zaher and Wahid, 2011). Although BEF virus is transmitted via insect vectors (Dhillon et al., 2000), the exact vector of BEF has not been identified, so prevention efforts were mainly aimed at efficient vaccination of susceptible animals. The earliest BEF vaccines were based on field isolates of BEF virus which were attenuated by repeated passages in suckling mice and/or cell cultures (Van der Westhuizen, 1967). Many of the live attenuated (LA) vaccines produced a long-lasting neutralizing antibody response which lasted more than 12 months (Tzipori and Spradbrow, 1973), their use is discouraged by some due to their potential lack of safety. The fact that these vaccines contain

attenuated live viruses carries the risk that these viruses might back-mutate to their virulent form, especially considering the relatively high mutation rate of RNA viruses (Lee *et al.*, (2012). Furthermore, LA vaccines include their potential for causing adverse clinical reactions (Della-Porte and Snowdon, 1977) and their potential sensitivity to impairment by heat or light. Thus, the use of inactivated vaccines is considered a safer approach. These vaccines were prepared with various adjuvants such as Freund's complete or incomplete adjuvant, aluminum hydroxide, dextran sulfate, or Quil A. The ability of commercial inactivated gel adjuvant vaccines to produce long lasting protective immunity is doubtful, and it may need to be boosted (Amani, 2006).

Therefore the need for using an inactivated BEF vaccine that provides longer duration of protective immune response so the aim of our study was to prepare an experimental inactivated BEF vaccine mixed with Montanide ISA 206 adjuvant. In this work we studied the safety of this vaccine and the dynamics of antibody response following single dose application in calves.

## 2. MATERIALS AND METHODS

### 2.1. Materials:

**2.1.1. Bovine Ephemeral Fever (BEF) virus:**  
Locally isolated Bovine Ephemeral Fever Virus (BEF/AVS/2000) was propagated in BHK-21 monolayer cell. The titer of virus used for vaccine production was  $10^8$  TCID<sub>50</sub>/ ml (Reed and Muench, 1938) and 32 complement fixing unit titer of antigenicity using CFT (Traub and Mansu, 1944). It was also used for SNT.

### 2.1.2. Calves:

Twenty one local breed calves (6 – 8 months old) weighing about 250-300 Kg, were apparently healthy and free from antibodies against BEF virus as proved by Serum Neutralization Test (SNT). These calves were divided into three groups: Group one consisted of 15 calves, each was

inoculated subcutaneously (S/C) with 2 ml of the prepared vaccine, Group two consisted of three calves remained as control non vaccinated and Group three consisted of three calves for safety testing of the prepared vaccine.

### 2.1.3. Serum samples:

Serum samples were collected from vaccinated and non-vaccinated calves weekly for 4 weeks post vaccination and then every 2 weeks till the end of the experiment (40-46 weeks). Sera were stored at – 20°C and inactivated at 56°C for 30 minutes before being used for evaluation of the humoral immune response using SNT and Enzyme Linked Immuno-Sorbent Assay (ELISA).

### 2.1.4. Baby Hamster Kidney cells (BHK 21 clone 13):

These cells were supplied by the Animal Virus Institute, Pirbright, UK and propagated at FMD Department, Abbassia, Cairo, using Minimum Essential Medium (MEM) with Earl's salts and sterile newborn calf serum 10% for the growth of cells or 5% for maintenance of cells according to the technique described by Macpherson and Stocher (1962). These cells were used for virus titration and serum neutralization test.

## 2.2. Methods:

### 2.2.1. Preparation of inactivated oil adjuvant BEF vaccine:

### 2.2.2. Virus inactivation by Binary Ethyleneimine (BEI):

It was used for inactivation of BEF virus of 3<sup>rd</sup> passage on the BHK 21 clone 13 cell line. 0.1 M BEI in 0.2 N NaOH was added to the virus suspension to give a final concentration of 0.1 % M of BEI. The virus and BEI mixture were mixed well and the pH adjusted to 8.0 by sodium bicarbonate. The virus was placed in the incubator at 37°C for 6 hrs with continuous stirring for inactivation to occur. Sodium thiosulphate was added to give a final concentration of 2% to neutralize the action of BEI.

*2.2.3. Vaccine formulation with Montanide ISA-206:*

Equal volumes of BEF antigen suspension and MONTANIDE ISA 206 oil (Seppic, France) were mixed. Only low shear mixing 300 rpm for 5 min followed by a further brief mixing cycle for 24 hours later to form extremely stable water in oil in water emulsion (double phase emulsion) according to Barnett et al., (1996).

*2.2.4. Evaluation of prepared inactivated BEF vaccine:*

*2.2.4.1. Sterility evaluation:*

Samples from the prepared inactivated BEF vaccine were cultured on Tryptose Phosphate, Thioglycolate broth, Sabouraud's agar and PPLO media. If any viable microorganisms were detected, the vaccine was unsafe for use, according to Code of Federal Regulation of USA (1986).

*2.2.4.2. Safety evaluation:*

Three calves were injected with 10 doses of the prepared vaccine, S/C in different sites and observed for 10 days for development of any clinical signs or local reaction according to Manal (2005).

*2.2.4.3. Potency evaluation:*

Humoral immune response of calves vaccinated with prepared vaccine was evaluated using SNT as described by Ferreira (1976) and ELISA as described by Voller et al. (1976).

### **3. RESULTS**

*3.1. Sterility and safety of the vaccine:*

The prepared inactivated oil adjuvant BEF vaccine was proved to be free from foreign contaminants (aerobic and anaerobic bacteria; fungi and mycoplasma) and safe in vaccinated animals where such animals remained healthy all over the experimental period without local reaction at the site of inoculation.

*3.2. Evaluation of humoral immune response of calves to the inactivated BEF vaccine:*

*3.2.1. Using SNT:*

The mean specific BEF neutralizing antibody titers were detectable by the 1<sup>st</sup> week post vaccination in vaccinated animal group as 1.15 log<sub>10</sub> neutralizing antibody titers, with the peak antibody titers were recorded by the 8<sup>th</sup> week post vaccination as 2.28 log<sub>10</sub> neutralizing antibody titers. The specific BEF neutralizing antibodies lasted for 40<sup>th</sup> week post vaccination with good levels in vaccinated calves as shown in table (1) and figure (1).

*3.2.2. Using ELISA:*

Results came in a parallel manner with that of SNT confirming each other showing that the mean specific BEF antibody titers were detectable by the 1<sup>st</sup> week post vaccination in vaccinated animal group as 1.47 log<sub>10</sub> serum antibody titers with the peak antibody titers were recorded by the 8<sup>th</sup> week post vaccination as 2.57 log<sub>10</sub> serum antibody titers. The specific BEF antibodies lasted for 40<sup>th</sup> post vaccination with good levels in vaccinated calves as shown in table (2) and figure (2).

### **4. DISCUSSION**

The progress in vaccine production is directed towards the selection of the proper adjuvant that can elaborate high and long lasting immunity (Dalsgaard, 1990). The effective immunity of a vaccine depends on the physical association between antigen and adjuvant (Allison and Byars, 1992). Double oil emulsion (Water in Oil in Water) posses the advantages of both water and oil based vaccines, with the outer aqueous antigens stimulating the immediate production of reactive antibodies and also stimulation of prolonged immunity (Hsieh et al., 2006). In this study, the advantageous of using double oil emulsion inactivated BEFV vaccine adjuvanted with Montanide ISA 206 in providing not only sustained immune response but also without producing any adverse reaction in calves after single dose application. Prepared vaccine proved to be sterile after cultivation on different media, safe and well tolerated

Table (1): Mean neutralizing antibody titers in sera of calves vaccinated with inactivated BEF vaccine.

Weeks post vaccination	Mean neutralizing antibody titers against BEF virus	
	Vaccinated calves	Non vaccinated calves
0	0	0
1 <sup>st</sup>	1.15	0.1
2 <sup>nd</sup>	1.6	0.1
3 <sup>rd</sup>	1.95	0
4 <sup>th</sup>	2.00	0
6 <sup>th</sup>	2.15	0.1
8 <sup>th</sup>	2.28	0.25
10 <sup>th</sup>	2.00	0.1
12 <sup>th</sup>	1.95	0.1
14 <sup>th</sup>	1.8	0.1
16 <sup>th</sup>	1.8	0
18 <sup>th</sup>	1.8	0.1
20 <sup>th</sup>	1.75	0
22 <sup>nd</sup>	1.7	0.1
24 <sup>th</sup>	1.7	0
26 <sup>th</sup>	1.6	0.1
28 <sup>th</sup>	1.6	0.2
30 <sup>th</sup>	1.55	0
32 <sup>nd</sup>	1.55	0
34 <sup>th</sup>	1.55	0
36 <sup>th</sup>	1.50	0.2
38 <sup>th</sup>	1.50	0.2
40 <sup>th</sup>	1.50	0
42 <sup>nd</sup>	1.25	0.1
44 <sup>th</sup>	1.1	0
46 <sup>th</sup>	0.9	0.1

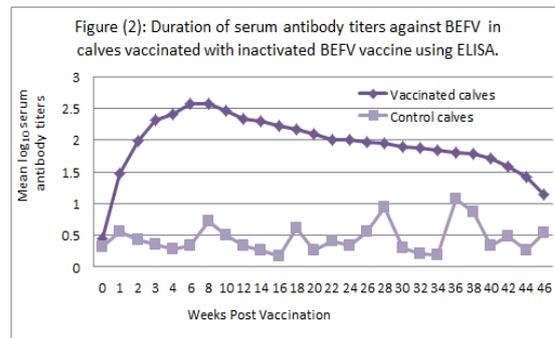
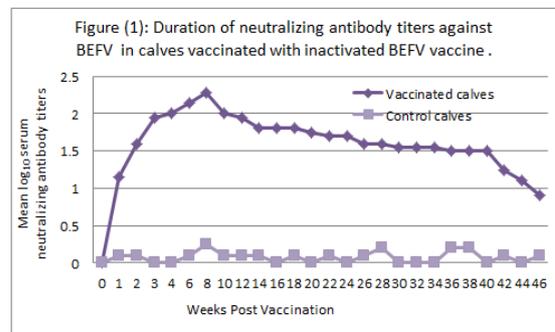
Mean BEF serum neutralizing antibody titer= the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 TCID<sub>50</sub> of BEF virus.  
 Protective serum neutralizing antibody titer=1.5 log<sub>10</sub> according to Wang *et al.* (2001).

Table (2): Mean serum antibody titers in calves vaccinated with inactivated BEF vaccine evaluated using ELISA.

Weeks post vaccination	Mean serum antibody titers against BEF virus	
	Vaccinated calves	Non vaccinated calves
0	0.44	0.32
1 <sup>st</sup>	1.47	0.56
2 <sup>nd</sup>	1.99	0.43
3 <sup>rd</sup>	2.32	0.35
4 <sup>th</sup>	2.4	0.28
6 <sup>th</sup>	2.58	0.33
8 <sup>th</sup>	2.57	0.72
10 <sup>th</sup>	2.46	0.51
12 <sup>th</sup>	2.33	0.34
14 <sup>th</sup>	2.29	0.26
16 <sup>th</sup>	2.22	0.18

18 <sup>th</sup>	2.17	0.61
20 <sup>th</sup>	2.09	0.26
22 <sup>nd</sup>	2.01	0.41
24 <sup>th</sup>	2.00	0.34
26 <sup>th</sup>	1.96	0.55
28 <sup>th</sup>	1.95	0.94
30 <sup>th</sup>	1.9	0.30
32 <sup>nd</sup>	1.88	0.21
34 <sup>th</sup>	1.83	0.19
36 <sup>th</sup>	1.81	1.07
38 <sup>th</sup>	1.78	0.87
40 <sup>th</sup>	1.72	0.33
42 <sup>nd</sup>	1.59	0.48
44 <sup>th</sup>	1.42	0.27
46 <sup>th</sup>	1.14	0.53

Mean BEF serum antibody titer using ELISA calculated as log<sub>10</sub> titer.



when injected subcutaneously, with no noticeable toxicity or prolonged pyrexia moreover none of vaccinated calves showed localized reaction at site of inoculation. These observations were recorded also by Phuong *et al.* (1999) and Catrucci *et al.* (1993). Humoral immune response to the prepared vaccine was investigated using SNT and ELISA after single dose application. The vaccinated calves were followed up for a period of 46 weeks. The mean specific BEF antibody titers were detectable by the 1<sup>st</sup> week post vaccination in vaccinated animal group, reached a good titer at 2<sup>nd</sup> week post vaccination, with the

peak antibody titers were recorded by the 8<sup>th</sup> week post vaccination. The specific BEF antibodies lasted for 40<sup>th</sup> post vaccination with good levels in vaccinated calves. These results agreed with the studies found that serum neutralizing antibody titers level increased by 8 to 128 fold in one month post vaccination with inactivated BEFV vaccine; Oil based inactivated BEF vaccine stimulated neutralizing antibody titers to reach 2.4 log<sub>10</sub> after 8 weeks post-vaccination (Hsieh et al., 2005 and 2006) which were not far from our results. Protective serum neutralizing antibody titer is 1.5 log<sub>10</sub> according to Wang et al. (2001). The same result was revealed by Manal (2005) who found that neutralizing antibody titers reached level considered to be protective at 2<sup>nd</sup> week post vaccination with mean BEF neutralizing antibodies titers attained maximum level (2.28 log<sub>10</sub>) at 6 weeks, and Theodariasis et al. (1973) who found that BEF oil vaccine could induce high neutralizing antibodies detectable for at least one year. The obtained results disagreed with that of Boaron et al. (2013) who reported that neutralizing antibody titers reached 2.1 to 2.5 log<sub>10</sub> after 13-15 days after using booster dose, with the longest consecutive follow-up period without challenge interference after vaccination by oil based inactivated BEF vaccine was 6 months. Evaluation of humoral immune response of calves

vaccinated by the inactivated BEF vaccine using ELISA showed results came in a parallel manner with that of SNT confirming each. The same finding were obtained by Lorenz and Wittmann (1983) and Hamblin et al. (1986) who reported that ELISA gave similar results to SNT but with that obtained by Lombard and Petermann (1982), and Barnett et al. (1996) who stated that ELISA results were in contrast to the neutralizing profile. Meanwhile, other workers reported different periods of protection of oil based BEF vaccines as Boaron et al., (2013) who recommended at least three to four vaccinations to confer long lasting immunity all over the year and Manal (2005) who found that aluminum hydroxide gel BEF vaccine gave a good protection for 40 to 44 week with boosting 4 weeks after preliminary vaccination. In this study using Montanide ISA 206 as an oil adjuvant for inactivated BEF vaccine gave longer-term dynamics of the antibody response without need to booster dose.

In conclusion, results of present study indicated that inactivated oil BEF vaccine adjuvanted with Montanide ISA206 oil and formulated according to previous procedures could provoke protective immune response within two weeks and lasted for about 40 weeks, after single dose administration without induce any adverse reactions.

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