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# PREPARATION OF INACTIVATED BOVINE EPHEMERAL FEVER VIRUS VACCINE (BEF) FROM LOCALLY ISOLATED STRAIN USING DIFFERENT ADJUVANT

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## SUMMARY

Inactivated tissue culture BEF virus vaccine was prepared from the 8th passage from a locally isolated BEF virus from native breed cattle during BEF outbreak in 2000 in Middle Egypt. The virus was inactivated using Binary ethylene amine (BEI) at concentration of 1% for 3.5 hours. The adjuvant contained mixture of Negilla Sativa oil, mineral oil and vitamin E and selenium with different ratio in tow different formula (A-vaccine and B-vaccine). The prepared vaccines were characterized using different techniques including, the drop test, emulsion stability and viscosity .In addition its provide to be sterile and safe. These vaccines were compared with aluminum hydroxide gel inactive BEF virus vaccine. B-vaccine induce immune response in vaccinated cattle more than A-vaccine and aluminum hydroxide gel BEF vaccine, while control non-vaccinated groups did not develop any immune response against BEF virus when measure by SPA, VNT, and ELISA .Exposure of vaccinated cattle to natural infection with BEF virus revealed no clinical signs of BEF syndrome, while control non-vaccineted cattle groups developed clinical manifestation of BEF syndrome.

## INTRODUCTION

Bovine ephemeral fever (BEF) is an acute febrile infection of cattle and water buffaloes caused by an arthropod-borne Rhabdovirus. The infection occurs in many tropical and subtropical regions of the world, excluding Europe and Americans and USSR (St. George 1984, and Nandi and Negi, 1999). The morbidity may be high but the mortality is low. The disease causes great economic losses such as moralities, abortion, decreased body weight, disruption of markets, drop in milk production ranges from 34-94 with an average of 46% and cows milk yield did not reach to pre-

illness level on convalescence and lowered fertility of bulls as well as the expenses of care or treatment and vaccination (St-George, 1984, Nandi and Negi, 1999). Live attenuated BEF virus vaccine produced in mouse brain or cell culture have been partially effective in preventing the disease (Uren et al 1991, Tzipori and Spradbrow 1978) and such vaccines are commercially available in South Africa, Japan, Taiwan and Australia. However, these vaccines are expensive to produce and require a cold chain, some must be mixed with adjuvant immediately before use, and there are reports of vaccine break down in the field (Uren et al., 1994). So far, attempts to develop non living vaccine using virus inactivated with formalin or B-porpiolactone , resulted in poor and unreliable immunity (Inaba et al,. 1973, Della Porta and Snowdon 1979, and Uren et al., 1994). Inactivated vaccines that considered safe and more economic especially when vegetable plant extract has the ability to induce cellular immune response non specially were done using ,Negellia Sativa oil as adjuvant (Madbouly et al 2000). This work aimed to prepare inactivated BEFV vaccine from a locally isolated strain using different adjuvants

## MATERIAL AND METHODS

### Material:

**1-Vaccine:**

Inactivated virus BEFV vaccine adjuvated with ALOH was obtained from Serum and vaccine research institute, Abbasia, Cairo.

**Virus:**

Local isolated virus (BEF) from native breed cattle at 2000-2001,isolated from buffy of natural infected native breed cattle by I/C of suckling mice Madbouly et al., ( 2006)

**2-Sera and antisera :**

**Sera.**

Fetal calf serum and newborn calf serum were obtained from Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia and used mainly for tissue culture cells (purchased from GIBCO )

**2-2 Antisera:**

**2-2-1** Reference BEF virus anti serum was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

**2-2-2** Hyper- immune serum against (BEF) virus prepared in rabbit according to Davis and Walker, (1974). The prepared hyper immune serum was inactivated at 56°C for 30 minutes and stored at-20°C in small aliquots until used.

2-3 Antibovine conjugated with Horseradish peroxides, Sigma, USA.

**3-Experimental animals:**

**Cattle:**

Friesian cattle 9-36 months old free from specific antibodies against BEFV were used in this work. These animal were reared in the farm of Fac. Vet. Med. Beni-Suef University.

**4-Media:**

**4-1 Eagleís minimum essential medium. (MEM) :**

Commercially MEM (Flow laboratories, U.K

**4-2 Tissue culture Medium (E199):**

Commercially E199 is purchased from GIBCO limited.

**a. Growth medium :**

Used in tissue culture procedure. Eagleís minimum essential media , with Earl's balanced salt solution containing 10% calf serum.

**b. Maintenance medium:**

as growth medium without lactalbumin hydrolysate and foetal calf serum (FCS) was reduced to 1-5%.

**5- Media for detection of bacterial and fungal contamination was prepared according to Curickshank et al.,( 1975).**

**6- Tris buffer (TBS) pH 7.8:**

Was prepared according to Powell and Watson (1975) , used for BEF virus purification by sucrose gradient ultracentrifugation.

**7- Inactivating agent:**

Bromethylenamine hydrobromide 95%: A working solution of 1% BEI was prepared according to Bahnemann,( 1975).

**8- Sodium thiosulphat solution (20%):**

. used as a residual BEI neutralizing agent.

**9-Adjuvants:**

**1- Marcol 52 (ESSO)**

Mineral oil was obtained from Serum & Vaccine research Institute, Abbasia, Cairo and used for preparation of vaccine.

**2- Nigella Sativa oil adjuvant:**

Nigella sativa oil was prepared as adjuvant according to the method described by Madbouly et al., (2000).

**3- Vitamins E and Selenium solution:**

Purchased from AGRI-Vet (HELM) Germany

**Methods:**

**1-Vaccine preparation:**

**1-1.Propagation of local isolated BEFV from native breed cattle at 2000 in $Vero_{121}$** cell line for vaccine preparation: In 25 ml capacity (flask) of confluent sheet of vero121 cells, the growth medium was decanted and 0.2ml of local isolated BEFV suspension was inoculated. After adsorption for 60 minutes at 37°C a maintenance medium consisting of E199 plus 1% FCS was added. The culture was examined daily for 5 days to observe developed CPE.

**1-2.BEFV titration on Vero121 cells:** Virus titration was done according to Theodoridis (1975). The end point was determined and the virus infectivity titer was calculated according to the method of Reed and Muenche (1938).

**1-3.Virus inactivation by 1% BEI.**

Inactivation of BEFV was done as described by Daoud et al., (2001).

**1-4.Safety test of the BEI treated virus:**

**A-In Tissue culture:**

Collected sample of BEI inactivated BEFV, were

inoculated in confluent sheet of $Vero_{121}$ cells. The flasks were incubated at 37°C in 5% $CO_2$ incubator and examined daily for the presence of CPE.

**B-In Mice:**

The inactivated virus suspension, was inoculated intracerbrally into suckling mice, then observed daily for any nervous manifestation or death until 7 days.

**1-4. Preparation of oil adjuvants:**

**1-4-1. Preparation of Nigella Sativa Adjuvant:**

It was prepared according to Madbouly et al., (2000). Nigella Sativa oil was mixed with Arlacel A in A ratio of nine parts oil to one part Arlacel A weight to volume , with thoroughly mixing before sterilization by passing them through a Seitz filter, the oil Arlacel A mixture was stored at room temperature and used within few weeks of preparation.

**1-4-2.Preparation of Marcol 52 Adjuvant:**

It was prepared by mixing of Marcol 52 to one part span 80 ,after thoroughly mixing, it was sterilized by Seitz filter; the oil span mixture was stored at 4°C until used.

**1-4-3. Preparation of vitamin E and Selenium oil:**

Vitamin E and Selenium oil was mixed with tween 80 % then thoroughly mixed until stable emulsion oil was obtained, under the sterile conduction in laminar flow, the mixture stored at 4C° until used.

**1-5.Preparation of oil emulsion vaccine:**

**1-5-1.Preparation of Marcol 52 oil adjuvant inactivated BEFV vaccine:**

The stable emulsion was prepared by thoroughly mixing of aqueous phase and oil phase.

One part of aqueous phase containing 99% inactivated BEFV suspension mixed with 1% tween 80 was mixing with 3 part prepared Marcol 52 adjuvant with continuous mixed by using of sterile syringe until production of stable emulsion.

**1-5-2. Preparation of Nigella Sativa oil adjuvant inactivated BEFV vaccine:**

prepared aqueous phase by thoroughly mixed of the (inactivated BEFV with 1%tween 80) then homogenization with Nigella sativa Arlacela oil mixture previously prepared until obtained stable emulsion.

**1-5-3 Preparation of Vitamin E and Selenium oil adjuvant inactivated BEFV vaccine:**

The oil phase was throughy mixing with aqueous phase until stable emulsion was obtained.

**1-5-4. Prepared of Combined Marcol 52, Nigellia Sativa oil and Vitamin E and Selenium oil adjuvant inactivated BEFV vaccine:**

Different formula of oil emulsion was prepared, as shown in table (1). In all instance, the water phase was added gradually to the oil phase during vigorous mixing in blender, until satisfactory emulsion was obtained the physical features of the emulsions are given in table (4).

**Table (1) :** preparation of the vaccine.

| Type of vaccine | Oil phase | | | Aqueous**** phase |
|---|---|---|---|---|
| | Marcol* +span | Nigella sativa **+ Arlacel A | Vit.E and Sel ***+tween | Antigen + tween |
| A | 2.25 | 100 | 75 | 100 |
| B | 133.4 | 133.3 | 133.3 | 100 |

* Marcol 52 + Span 80= 90:10.

** Nagila Sativa oil and Arlacel A = 90: 10

*** Vit. E and selenium 9 tween = 99: 1

**** Inactivated BEFV by BEI + tween 80 = 99: 1

**1-6. Characterization of oil prepared vaccines:**

The prepared oil vaccines were subjected to the following tests

**1-6-1 Drop test**: It was done according to Roshdy, (1996),

**1-6-2 Emulsion stability:** It was done According to Roshdy, (1996),

**1-6-3 Viscosity**: It was applied according to Cessi and Nardelli, (1974).

**1-7.Quality control of the prepared vaccines:**

**1-7-1. Sterility Test:**

The vaccine batches were inoculated in Nutrient Agar, MacConky Agar, and Sabaroud broth then incubated at 37° for 72 hours for detection of bacterial colonies and at 25°C for 7 days for detection fungal growth.

**1-7-2 Safety Test:**

**1-7-2-1. In rabbits (Roshdy, 1996):**

Infectivity of each vaccine was checked by subcutaneous inoculation of 15 ml into 3 rabbits (5 ml for each) the vaccine being safe when no local or generalized lesions were developed. Besides absence of fever or any systemic reaction over one week observation period.

**1-7-2-2 In Cow:**

Infectivity of each vaccine was checked by the subcutaneous inoculation of 10 ml into one susceptible cow, the vaccine being safe when no local or generalized lesions were developed. Besides absence of fever, over one week observation period.

**1-8. Experimental Design:**

A-Twenty four Friesian cattle aged from 9- to 36 month old were seronegative for BEFV antibodies by SPA, VNT and ELISA, classified into three main groups (eight animals for each).

B-Eight Friesian cattle 9-12 months old were divided into 4 groups, two per each, subgroups1, 2 & 3 were vaccinated with A, B, & Abbasia vaccines each animal received two doses S/C 4 weeks apart and group4 was left as control non

vaccinated. The same experimental design was applied in the another two groups of cattle aged 18-24 months and over 36 months (each group of them was subdivided into 4 subgroup).All animals were kept under observation for one year and their sera were collected monthly vaccination for 9months

**1-9. Evaluation of humoral immune response in vaccinated animals:**

The collected sera from vaccinated and control animals were tested by SPA,VNT and ELISA.

**1-9-1 SPA agglutinated test**: it was done according to Madbouly., (1987 )

**1-9-2 Virus neutralization test (VNT) :**

Virus neutralization was done according to Cybinski (1987).

**1-9-3 Enzyme linked Immuno Sorbent Assay (ELISA):**

Standardization of ELISA test. (checker board titration) was done according to Zakrzewski et al., (1992): Negative control sera were included.

Cutoff value (CV) was obtained by calculating the mean ODs plus 2SD.

Antibody titration by ELISA: It was done according to Zakrzewski et al. (1992).

## RESULTS

**(1): Inactivation of BEFV (8th passage on $Vero_{121}$ cell) by BEI at 37°C.**

The 8th passage of BEFV on Vero121 cell, has lost it's infectivity after treatment with BEI for 2, 3.5, 5.5 hours at concentration 1.5, 1, and 0.5 hours at 37°C, respectively.

**(2): Safety test of inactivated BEF virus in $Vero_{121}$ and mice.**

The BEI inactivated BEFV was safe by inoculated in $Vero_{121}$ cell culture and inoculated I/C in mice, the inactivated BEFV was safe it did not produce any CPE in $Vero_{121}$ and any death or neurological signs in mice.

**Table (2): Sterility Test of the inactivated BEFV vaccine**

| Culture media | Incubation temperature | Time of incubation | Results |
|---|---|---|---|
| Nutrient Agar medium | 37°C | 3-7 days | No colonies |
| MacConkyagar medium | 37°C | 3-7 days | No colonies |
| Sabaroud broth | 25°C | 3-15 days | No turbidity |

**Table (3): Toxicity test for each type of used adjuvant and emulsifier predicated for vaccine preparation**

| Oil adjuvant | Dead | Mice | |
|---|---|---|---|
| | | Abnormal sign | Inflammatory reaction |
| Nigella sative oil | - | - | + |
| Marco / 52 | - | - | + |
| Vite & selenum | - | - | + |
| Span 80 | - | - | + |
| Tween 80 | - | - | + |

+ Moderate inflammatory reaction

The data present in table( 3) showed that no death or abnormal clinical sings noticed on the inoculated a adult mice with the used adjuvants (Nigella Sativa, Marcol 52 and Vitamin E and Selenium) and emulsifiers ($Span_{80}$ and $Tween_{80}$). All these adjuvant and emulsifiers induced slight inflammatory reaction at the site of injection.

**Table (4): Physical characters of BEFV inactivated vaccines:**

| Type of vaccine | Viscosity | Stability at 4°C for 8 months | | | Freezing and thawing (3 cycle) | Emulsification |
|---|---|---|---|---|---|---|
| | | 2M | 4M | 8M | | |
| A | + | Stable | Stable | Stable | Stable | Good homogenized |
| B | + | Stable | Stable | Stable | Stable | Good homogenized |

+ Easily flow
+++ Creamy
ND = Not done

The characters of prepared vaccines were studied for viscosity, emulsification and stability. The vaccines type A & B were easily flow throw pipette (2ml), stable for 8months at 4 C, even when freeze and thaw for 3cycle and with good emulsion.

**Table (5): Safety test for inactivated BFD oil emulsion vaccines**

| Designation | Safety test | | | |
|---|---|---|---|---|
| | Rabbits | | Cow | |
| | MI/Dose | Inflammatory reaction | MI/Dose | Inflammatory reaction |
| A | 5 | Mild | 10 | Mild |
| B | 5 | Mild | 10 | Mild |

Concerning safety the first four type of vaccine when injected in rabbit and in cow Vaccine A and B induced mild inflammatory reaction in rabbit and cow.

**Table (6): Evaluation of humeral immune response in vaccinated cattle by SPA agglutination test.**

| Type of vaccine and age | | Mean SPA log 2 antibody titer / monthly post vaccination | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1* | 2 | 3 | 4 | 5 | 6 | 7** | 8 | 9 |
| Aluminum hydroxide vaccine | | | | | | | | | | |
| | 9-12 M | 2 | 5.5 | 4.5 | 3.5 | 3 | 2 | 1.5 | 5.5 | 5 |
| | 14-24M | 3.5 | 6 | 5.5 | 4.5 | 3.5 | 2.5 | 2 | 6 | 5.5 |
| | 36-48M | 2.5 | 6.5 | 6 | 4.5 | 3.5 | 1.5 | 1.5 | 6. | 5.5 |
| A- Vaccine | | | | | | | | | | |
| | 9-12 M | 5.5 | 6.5 | 6 | 4.5 | 3.5 | 3 | 2.5 | 5.5 | 5 |
| | 14-24M | 3 | 6 | 5.5 | 5.5 | 4.5 | 3.5 | 3 | 5.5 | 4.5 |
| | 36-48M | 4.5 | 7 | 6.5 | 6 | 5.5 | 4.5 | 3.5 | 5.5 | 4.5 |
| B. Vaccine | | | | | | | | | | |
| | 9-12 M | 5.5 | 7.5 | 7 | 5.5 | 5.5 | 4.5 | 4 | 6 | 4.5 |
| | 14-24M | 4.5 | 8 | 7.5 | 7.5 | 7 | 6.5 | 5 | 6.5 | 4.5 |
| | 36-48M | 4 | 7.5 | 8 | 7.5 | 7 | 7 | 5.5 | 6 | 5 |
| Control | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 5 |

Data depicted in table (6) showed clearly that the neutralizing antibody titer in vaccinated animal were increased gradually from first month PV till the 3rd month and then become stationary or another 3 months. (4, 5, 6 months) then decreased at the 7th month PV and increased abruptly at the 8th month PV followed by decrease in the 9th months PV. The antibody titer induce by

type B vaccine is higher than those induce by commercial vaccine Aluminum hydroxide gel vaccine (Abbasia vaccine) and type A-vaccine.

The antibody was increased titer in 2nd and 3rd month PV (4-8) in type B-vaccine, while in type A-vaccine (3-7) and type Aluminum hydroxide gel (Abaisia vaccine) (2.5-6.5.) , may be due to the booster dose after the first month and increased antibody titer at 8th month due to exposure of vaccinated cattle to natural infection with BEFV at 7th months P.V .The vaccinated cattle did not show any clinical symptoms of BEF syndrome.. The Aluminum hydroxide gel (Abbasia vaccine) did not induce antibody titer more than 6log2 even after the booster dose; control animal did not show any neutralizing antibody titer until 7th month appear clinical symptoms of BEF syndrome (fever , ocular and nasal discharge , stiffness ) and rose neutralizing antibody titer due to natural infection with BE F virus.

Data depicted in table (7) showed clearly that the antibody titer in vaccinated animal were increased gradually from first month PV till the 3rd month PV and then became stationary at months. (4, 5, 6 PV) then decreased at the 7th month PV and increased upreabtily at the 8th month PV and decreased 9th month PV. The antibody titer induced by type B vaccine is higher than those induced by commercial vaccine Aluminum hydroxide gel vaccine (Abbasia vaccine) and type A- vaccine. Control animal did not show any neutralizing antibody titer until 7th month appear clinical symptoms of BEF syndrome (fever, ocular and nasal discharge, stiffness) and rose neutralizing antibody titer due to natural infection with BE F virus.

**Table (7): Level of neutralizing antibody of befv invaccinated cattle as tested by VNT**

| Type of vaccine and age | | Mean neutralization antibody titer / monthly post vaccination | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1* | 2 | 3 | 4 | 5 | 6 | 7** | 8 | 9 |
| Aluminum hydroxide vaccine | | | | | | | | | | |
| | 9-12 M | 1.5 | 4 | 3.5 | 2.5 | 2.5 | 1 | 1 | 4.5 | 4 |
| | 14-24M | 2.5 | 4 | 3.5 | 3.5 | 3 | 2.5 | 1.5 | 4 | 4 |
| | 36-48M | 1.5 | 4 | 4 | 3.5 | 2 | 1 | 1 | 4.5 | 4. |
| A- Vaccine | | | | | | | | | | |
| | 9-12 M | 3.5 | 5 | 5.5 | 4.5 | 2.5 | 2.5 | 2.5 | 4.5 | 4 |
| | 14-24M | 2.5 | 5 | 5 | 4 | 4 | 3.5 | 2.5 | 5 | 3.5 |
| | 36-48M | 3 | 5.5 | 6 | 5.5 | 5 | 4 | 4 | 5 | 3 |
| B. Vaccine | | | | | | | | | | |
| | 9-12 M | 4.5 | 6 | 6 | 5 | 5 | 4.5 | 4 | 5 | 4 |
| | 14-24M | 4 | 6.5 | 6 | 6 | 5.5 | 4.5 | 4 | 5.5 | 5 |
| | 36-48M | 3.5 | 6.5 | 6.5 | 6.5 | 6 | 5 | 4.5 | 5.5 | 4 |
| Control | | >1 | >1 | >1 | >1 | >1 | >1 | >1 | 4 | 5 |

**(3) Mean ELISA optical density for BEFV antibodies in sera vaccinated and control cattle.**

The antibody titer in vaccinated animal were measured by ELISA increased gradually from first month PV till the 3rd month PV and then became stationary at months. (4, 5, 6 PV) then decreased at the 7th month PV and increased upreabtily at the 8th month PV followed decreased 9th month PV. The antibody titer induced by type B vaccine is higher than those induced by commercial vaccine Aluminum hydroxide gel vaccine (Abbasia vaccine) and type A-vaccine control animal did not show any neutralizing antibody titer until 7th month appear clinical symptoms of BEF syndrome (fever ,ocular and nasal discharge stiffness) and rose neutralizing antibody titer due to natural infection with BE F virus as in (Fig 1,2,3).

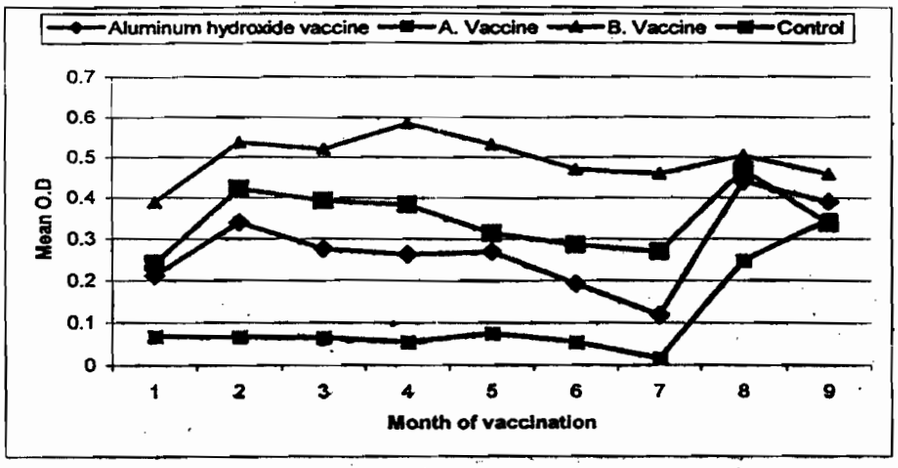


**Fig. (1):** Mean optical density measured by ELISA for detecting BEFV antibodies in sera of cattle 9-12 months with two dose in activated BEFV oil emulsion vaccine.

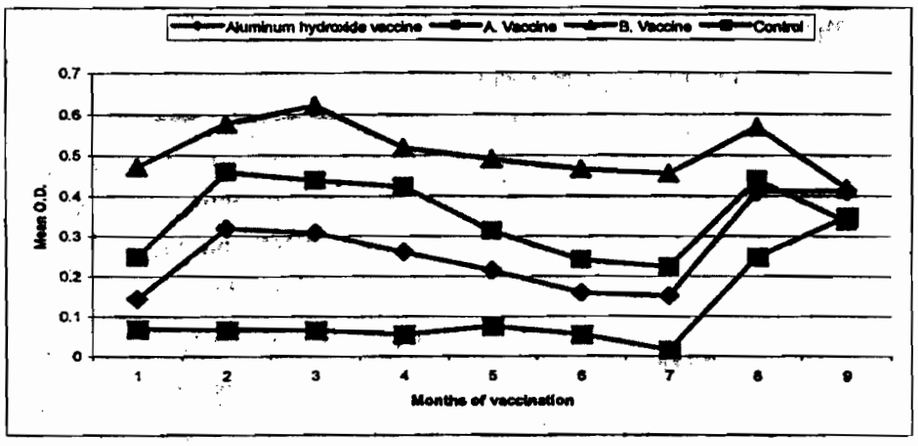


**Fig. (2):** Mean optical density measured by ELISA for detecting BEFV antibodies in sera of cattle 14-24 months with two dose in activated BEFV oil emulsion vaccine.

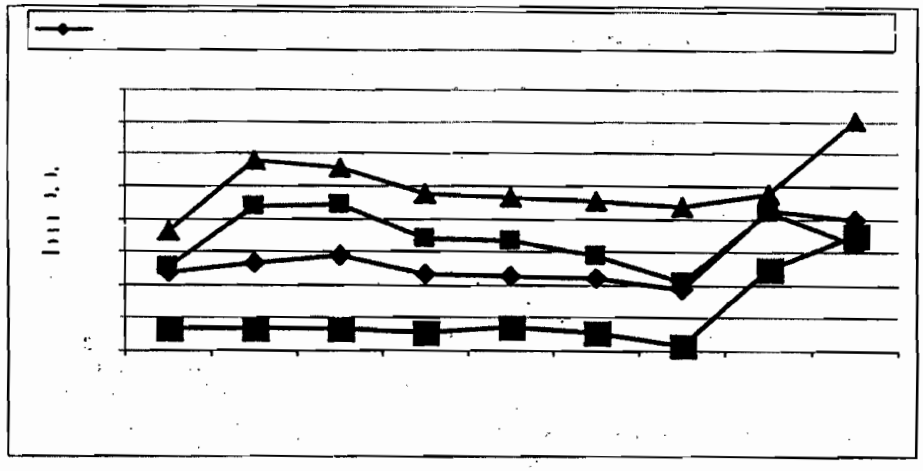

**Fig. (3):** Mean optical density measured by ELISA for detecting BEFV antibodies in sera of cattle 36-48 months with two dose in activated BEFV oil emulsion vaccine.

## DISCUSSION

Because of the great economic important of BEF to cattle industry, there was a need for an effective vaccine which would be suitable for commercial production.

A BEF vaccines with adjuvant, Freund's complete adjuvant or white oil adjuvant develops immunity but of short duration ( 50% at six month ) and no protection at nine months (Vanselow et al 1985, 1995). The present work aimed to produce inactivated BEF oil emulsion vaccine prepared from locally isolated virus strain and adjuvanted with different adjuvants .

A trial was done to produce an inactivated BEFV by using BEI as used by (Daoud et al., 2001). The inactivation of BEF virus by BEI 0.5, 1 and 1.5% which prepared in the same manner with a final concentration of 0.01 M this preparation was run in parallel with that prepared by Smith et al. (1993) for inactivation of Newcastle disease virus. Complete BEFV inactivation was achieved after 5, 3.5, 2 hours at BEI final concentration 0.5, 1 and 1.5, respectively at 37°C. The concentration of choice was 1% to the final concentration 0.01M as it resulted incomplete viral inactivation within 3.5 hours (Suitable time), where the infectivity titer $\log^{10} TCID_{50}$ reached from 6.5 to zero.

Meanwhile, to avoid any chemical toxic effect and expensive coast by using high concentration of BEI However, the inactivation of BEF virus by BEI, steadily decrease in direct relation to the time of inactivation of reaction this result agree with (Behneman, 1975) who used BEI, 1% final inactivation of FMD virus and the prepared

vaccine was immunogenic and potent when compared with other inactivator &( Daoud et al., 2001) who used BEI, 1% final concentration to inactivate BEF virus strain, propagated Vero121 cell culture without alteration on its immunogenic properties.

For evaluating the safety of inactivated BEFV, it was inoculated in using cell culture system ($Vero_{121}$) and Mice 1-3 day old, the inactivated BEF virus was safe as it didn't produce any CPE on the $Vero_{121}$ and no death or neurological signs in mice Moreover the batch of inactivated BEF virus subjected for testing its sterility, by cultivating it on nutrient agar, MacConcky and Sabaurods broth. The obtained results in table (2) revealed that the prepared inactivated BEF virus was sterile as it was free from any bacterial and fungal contaminates there was no any colonies or turbidity on the agar media or used broth for this purpose. Different types of adjuvant were selected for preparing the oil phase like, crude nigella Sativa oil (Madbouly et al, 1999 and 2000). Vitamin E and Selenium (Smith et al,. 1993, Li and Wang 1995) and Mineral oil (Reddy et al., 2003). For preparing the BEF virus vaccines different formula were used by combination of oil adjuvant (El. Kady et al., 1990, Madbouly et al., 1999 and 2000) for enhancing the immune response with production of high antibodies standing for long time these prepared vaccines compared with other commercial inactivated Aluminum hydroxide gel BEF virus vaccine. The prepared vaccines were, stable, safe and sterile as in table (2,3,4). The safeties of the prepared vaccines were checked by subcutaneous inoculated of one dose (5ml and 10ml) for rabbit and susceptible cow respectively.

The inoculated rabbit and cow didn't develop local or general lesions and the temperature after vaccination was ranged form 38-39°C in vaccinated cow. The physical character of inactivated BEF virus vaccine with different emulsion adjuvant are summarized in table (4) the prepared vaccines were stable for 8 months at 4°C and stable after three cycles freezing and thawing. The oil and aqueous phase didn't show evidence of separation.

The lower viscosity is an important characteristic for oil emulsion vaccines because it helps the vaccination process by lowering fatigue of the workers and saving time in work when large number of animals are vaccinated .Vaccine type A and B are easily flow throw pipette (2ml), stable for 8 months at 4°C even when freezed and thawed for 3 cycle and with good emulsification.

Addition of hydrophilic emulsifier as Tween 80 and Spain 80 was associated with increased emulsion stability, decrease emulsion viscosity and increase serological response. The obtained results were in parallel with those obtained by Smith et

al. (1993) who noticed good effect with Tween 80 in studies with clostridial oil emulsion vaccines. Reddy et al., (2003) found that the type of emulsion obtained appear to be influenced by method of emulsification or by aqueous 10 oil ratio.

BEFV is not satisfactory immunogenic to produce adequate of protective neutralization antibodies in the vaccinated animal. (Tozipori and Spradbrow 1978) in the present study, we used Nagilla Sativa combination with mineral oil and vitamin E and Selenium for enhancing the immune response . Experimental inoculation of inactivated BEFV vaccine locally prepared (Adjuvant with combination Nagilla sativa, mineral oil, vitamin E and Selenium) in 24 cows with different ages were done for evaluating the immune response of these vaccines (A-vaccine, B vaccine ) and comparing them with Aluminum hydroxide gel vaccine (Abbasia vaccine).

Sera obtained from vaccinated and non vaccinated animal were tested for the presence of antibodies by SPA, VNT and ELISA. The results related to SPA test in table (6) showed clearly that the antibody titers in vaccinated animal were increase gradually from first month of PV till the 3rd month PV and then became stationary for other 3 months (4, 5 & 6) PV then decreased at the 7th month PV and increase abruptly at the 8th month PV followed decrease at 9th month PV. The antibody titer induced by the B-vaccine is higher than those induced by type A& commercial vaccine Aluminum hydroxide gel vaccine (Abbasia vaccine), while the type A-vaccine is more higher than the commercial one Increase of the immune response in vaccinated cattle due to increase age but decrease immune response in 24 month aged cattle due to pregnancy which consider stress factor.

The increased antibody titer expressed $\log_2$ at the 2nd and 3rd months of age (4-8) in type B-vaccine, (3-7) in type A-vaccine (2.5-6.5) type Aluminum hydroxide gel (Abbasia vaccine) may be due to the booster dose at the first month. The Aluminum hydroxide gel (Abbasia vaccine) did not induce antibody titer more than 6 $\log_2$ even after booster dose. The control animal not showed any neutralizing antibody titer until 7 month then rose due to natural infection with BEF virus ( vaccinated animals did not show the clinical disease )

For confirming these results by testing the obtained sera by VNT and ELISA, we found the same result as in VNT, increase neutralizing antibody titer at, 2nd and 3rd months of age (3.5-6.5) in type B-vaccine, (2.5-5) in type A-vaccine and (2.5-4) in type Aluminum hydroxide gel (Abbasia vaccine). Aluminum hydroxide gel (Abbasia vaccine) did not induce antibody titer more than 4 $\log_2$ even after booster dose. The control animal did not show any neutralizing antibody titer until 7 month then rose antibody due natural infection

.Confirmation of these results with ELISA as in (Fig 1,2,3 ) showed clearly that ODs in vaccinated animal were increased gradually from first month PV as in SPA and VNT All the three types of BEFV vaccines succeeded in inducing satisfactory level of neutralizing antibody in vaccinated animal, when exposure natural infection with BEFV at 7th month did not show any clinical symptoms or rise temperature, while control non vaccinated cattle developed fever, salivation, nasal discharge, stiffness & lameness.

From these obtained results, it becomes clear that the type B prepared vaccine is more effective than type A & the commercial one vaccines. The type A is more effective than the commercial vaccine and this may be contributed to the used adjuvant (which is composed from mineral oil, Nigella Sativa and Vitamin E and selenium with same amount for each one (133.3) with constant aqueous phase (100 ml). The ability of Nigella Sativa oil and Vitamin E and Selenium for stimulating the immune system non specifically were recorded by (Cessi and Nardelli 1974; El-Kady et al., 1990, and Madbouly et al., 1999 and 2000). Beside Anti-inflammatory and antioxidant activity, growth promoting effect, Antimicrobial effect, beside the safe natural oil. The mechanism of immune stimulation of Nigella Sativa is though its effect on the antigen presenting cells (APC) .

Oil (Madbouly et al, 1999 and 2000).Activation status of APCs could stimulate either by the expression of the major. histocombitability antigens or secretion cytokines . the type of immune response in greatly depended on both the viral antigen and the type of adjuvant included in inactivated vaccine : thus, Nigella Sativa, Vitamin E & Selenium and mineral oil could direct the type of immune response to include such superior response.

In spite that, the commercial vaccine (Abbasia) has higher antigenic amount (50%) than the prepared vaccines in this study (20%), these prepared vaccines elicited antibody titer higher than those elicited by Abbasia vaccine and this trigger to potential effect of the used adjuvant in their preparation.

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