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## Research Article

# Cross Neutralization Between Vaccinal Strain of Commercial Bovine Ephemoral Fever Virus Vaccines and Egyptian Isolate 2018 with Serum Samples of Vaccinated Cattle

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**ABSTRACT**

Bovine Ephemeral Fever (Three-day Sickness) is an arthropod-born viral disease of cattle. It still causes a risk to the cattle industry in Egypt. In recent study, we isolated Bovine Ephemoral Fever virus (BEFV) strain during the recent outbreak in Egypt 2018 from El-Wadi El-Geded governorate. Buffy coats from the infected animals were used for Isolation, identification and characterization of recent isolates of BEFV by using virus neutralization tests (VNT), RT-PCR and sequencing analysis. Local and imported live attenuated BEF virus vaccines at Animal Health Research Institute in Doki, Giza, Egypt. Recent isolate was propagated and titrated on Vero cell culture. Ten calves were allotted into two groups of five animals for each where the first group was vaccinated with local live attenuated BEF virus vaccine, while the second group was vaccinated with imported live attenuated BEF virus vaccine, booster dose of different type of vaccines were been inoculated 15 days later. Serum samples had been collected at 1st, 2nd, 3rd, 4th weeks post vaccination. The sera were tested against recent isolate and vaccinal strain by using serum neutralization test (SNT). It was found that the imported vaccine showed higher protective antibody titer (2.04 log $_{10}$) than Local one (1.92 log $_{10}$) against isolate strain, while the local vaccine showed higher protective antibody titer (2.1 log $_{10}$) than imported one (1.98 log $_{10}$) against vaccinal strain, It is concluded that the recent circulating field isolate BEF virus can be controlled by current vaccines and there is not any need for vaccine updating.

**Key words:** BEFV, SNT, VNT, $TCID_{50}$, RT-PCR

## INTRODUCTION

Bovine Ephemeral Fever (Three-day Sickness) is an important arthropod-born viral disease of cattle and water buffaloes (Hayama *et al*., 2016) It is caused by Rhabdo virus which is a single stranded RNA genome with a lipid envelope and five structural protein genes (N, P, M, G and L). The envelope glycoprotein G contains type-specific and neutralizing antigenic sites (Peter and Eyal, 2015), So it can be identified by using RT-PCR and Sequencing (Blasdell *et al*., 2013). The disease is characterized by bi-phasic fever, muscle stiffness, ocular and nasal discharge, reduce milk production, ruminal stasis and recumbency due to a vascular inflammatory response (Walker, 2005) that cause highly economic losses in cattle industry. It occurs seasonally in tropical, subtropical areas, Asia, Africa and Middle East. (Kasem *et al*., 2014). Outbreaks of BEF had been reported in Egypt in 2000 (Hassan, 2000) 2004 (Al-Gaabary *et al*., 2005) 2014.

(EL-Bagoury *et al*., 2014) Finally, it reappeared in 2018 (Albehwar *et al*., 2018). Prevention and control of the disease mainly based on eradication of arthropod and good vaccination program. There are two types of BEF virus vaccine (live and Inactivated), but live BEF virus vaccine had been used, it is a freeze- dried vaccine with chilled liquid adjuvant (Saponine) that mixed prior to a administering, and the vaccine should be administered twice 2 weeks apart. It provides long- lasting protection than inactivated BEF vaccine (Albhwar *et al*., 2010 and Orly *et al*., 2012).

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The present work aimed to determine molecular characterization of recent BEFV and determine the efficacy of different commercial BEFV vaccines against the recent isolate.

## MATERIALS AND METHODS

**Isolation of BEF virus from five farms in El-Wadi El-Geded governorate, 2018:** Animals from infected farms showed high fever, muscle stiffness, ocular and nasal discharge, recumbancy and ruminal stasis. Blood samples from infected animals were isolated on Na heparin as anticoagulant inside a sterilized plastic centrifuge tube during hyper thermic phase of the disease that centrifuged at 2400 rpm for 20 minute and obtained the buffy coat (Nawal *et al*., 2001). Twenty samples were obtained, but fifteen of them were gave positive result by RT-PCR.

**RNA extraction:** RNA extraction from samples was performed using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH). Briefly, 140 µl of the sample suspension was incubated with 560 µl of AVL lysis buffer and 5.6 µl of carrier RNA at room temperature for 10 min. After incubation, 560 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 60 µl of elution buffer provided in the kit according to Kasem *et al*. (2014).

**Oligo nucleotide Primers** supplied by (Metabion Germany) and listed in Table (1).

**PCR amplification:** Primers were utilized in a 25 µl reaction containing 12.5 µl of Quantitect probe Rt-PCR buffer (QIAgen, Gmbh), 1 µl of each primer of 20 pmol concentration, 0.25 µl of rt-enzyme5.25 µl of water, and 5 µl of template. The reaction was performed in a Biometra thermal cycler. Reverse transcription was applied at 50 $^{O}C$ for 30 min, a primary denaturation step was done at 95 $^{O}C$ for 5 min, followed by 35 cycles of 94$^{O}C$ for 30 sec, 48$^{O}C$ for 40 sec. and 72$^{O}C$ for 45 sec. A final extension step was done at 72$^{O}C$ for 10 min.

**Analysis of the PCR Products:** The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gelpilot 100 bp DNA ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

**BEF Virus (vaccinal strain):** It was supplied by Bank Strain Department, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB). Titer of the virus was $10^5$ $TCID_{50}$ /ml.

**Na heparin:** It was used as anticoagulant inside a sterilized plastic centrifuge tube (20 IU/ml) used for collection of blood used for virus isolation during hyper thermic phase of the disease according to Lucky, (1977).

**Cell line:** Vero cell culture was supplied by CLEVB. It was propagated at Bank Strain Department. The cells were grown and maintained according to Macpherson and Stocher, (1962). It was used for propagation, titration of BEF virus and SNT.

**Propagation and Titration of Isolated BEF virus** the suspected isolated virus in buffy coat samples was propagated for three passages then titrated on Vero cell. The cell cultures were incubated at $37_C{}^0$ for 5-7 days with daily examination for evidence of cytopathic effect (CPE) of the virus according to Abd El- Aziem, (2008) the titer was expressed by $log_{10}TCID_{50}$ according to Reed and Muench, (1938).

**Imported and local live attenuated BEF commercial vaccines:** It was supplied by CLEVB. The vaccines were live, lyophilized that reconstituted in PBS containing Saponin (PBS with 0.2µg Saponin /ml was used as a diluent) at the time of vaccination according to Albehwar *et al*., (2010).

**Calves and experimental design:** Twelve, six month old cross breed calves, about 200 – 300 kg body weight were purchased by CLEVB. Those calves were clinically healthy and proved to be free from antibodies against BEF virus by using SNT (Sero-negative).

Ten calves were allotted into two groups; five calves were vaccinated with field dose S/C of local live attenuated BEF virus vaccine while other five calves were vaccinated with field dose S/C of imported live attenuated BEF virus vaccine and the two groups revaccinated after 15 day. Third group contains two calves was kept as control negative (non-vaccinated).

**Serum samples** Sera were collected from all animals (vaccinated and control) at Zero (day of vaccination) then at 1$^{st}$, 2$^{nd}$, 3$^{rd}$, 4$^{th}$weeks after first and second dose of vaccination. Sera were examined for antibody response against field isolated strain 2018 and Vaccinal strains of BEF virus by serum neutralization assay (SNT).

**Serum Neutralization Test (SNT):** SNT was carried out against Vaccinal BEF virus strain and field isolated strain 2018, the protective neutralizing serum antibody titer is (1.2 $log_{10}$) according to OIE, (2017). It was performed by the microtechnique described by Mellor, (2001).

**Table 1:** Primers sequences, target genes, amplicon sizes

| Target Gene | Primer sequence (5'-3') | Length of amplified product (bp) | Reference |
|---|---|---|---|
| Glycoprotein-G | AGAGCT TGG TGT GAA TAC CCA ACCTAC AAC AGC AGA TA | 420 bp | Kasem *et al.*, 2014 |

## RESULTS

**Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR):** BEFVs were identified with RT-PCR using specific primers. ThecDNA was amplified producing a clear single band 420 base pairs (bP) in length on the a garose gel stained with ethidium bromide (Figure 1), while the identity percent of recent isolate 92.0% with local vaccine and93.2% with imported one (Table 2).

Partial nucleotides sequences of BEFV was obtained and gave the easiness to select from BEF viruses partial and complete sequences found on gene bank to align (Table-2) and to construct the phylogenetic tree. Phylogenetic analysis of the sequences identity revealed that the obtained recent isolate of BEFV (El-Wadi El-Geded-2018) is closely related as 92.0% to BEFV/ isolate EGY- 2005 (local BEF vaccine) glycoprotein mRNA partial cds, but 93.2% with BB2271-919 strain (Imported BEF Vaccine) glycoprotein G (G) gene partial cds, Phylogenic analysis for the amplified product revealed high similarity of the BEF to the published BEF sequence in gene bank Dakahlia Egy 2017 and Daimietta Egy 2017 within the same cluster (Figure -2)

Humoral immune response of calves vaccinated with different commercial BEF vaccines against field isolated virus strain (2018). Imported vaccine gave higher immune response (2.04 log $_{10}$) than Local one (1.92 log$_{10}$) Table (3). Figure (2) while against Vaccinal strain (Abbasia, 2005), local vaccine gave higher immune response (2.1 log $_{10}$) than Imported one (1.98 log $_{10}$) Table (4), Figure (3).

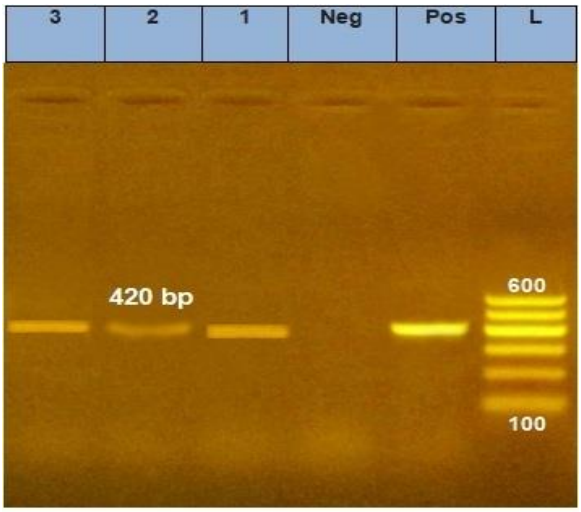


**Fig. 1:** RT-PCR for detection of Bovine Ephemoral fever virus. Positive sample, Sample-1 (Buffy coat), Sample-2 local live attenuated BEF vaccine. Sample -3 imported live attenuated BEF vaccine, Marker: 100 bp ladder.

**Table 2:** Genotyping report between isolate El-Wadi El-Gededgovernorate2018, local and imported live attenuated BEF virus vaccine

| Strains | NO.nt Comp. | Isolate, 2018 | %ID. | % Diff. |
|---|---|---|---|---|
| EGY-2005 (Abbasia strain) | 420 | El-Wadi El-Geded | 92.0% | 8 |
| Imported BEF Vaccine (BB2271-919 strain) | 420 | El-Wadi El-Geded | 93.2% | 6.8 |

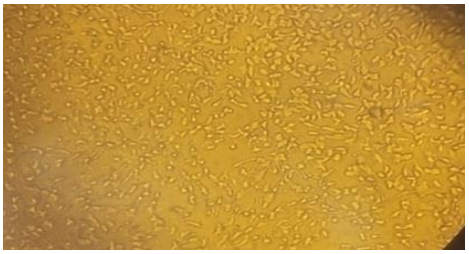

**Photo 1:** CPE of BEF virus on Vero cell culture after 48hours show rounding, granulation then cell detachment showed by inverted microscope.

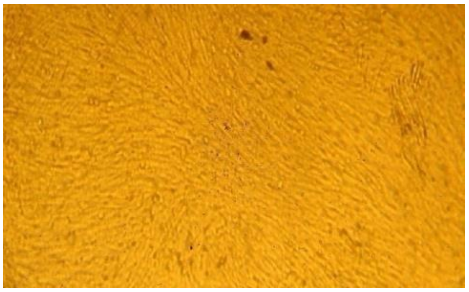

**Photo 2:** Normal Vero cell culture showed by inverted microscope.

**Propagation and Titration of isolated virus:** The virus had a titer of $10^6$ $TCID_{50}$/ml after the 3$^{rd}$ passage and CPE was represented by cell rounding, granulation Photos (1-2).

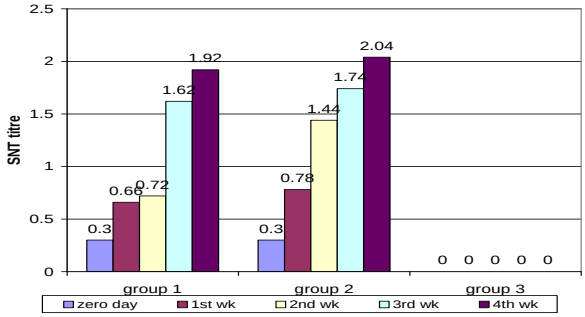


**Fig. 3:** BEF serum neutralizing antibody titer in vaccinated calves with imported and local commercial BEF vaccine against isolated virus. Group I: Calves vaccinated with Local live attenuated BEF virus vaccine. Group II: Calves vaccinated with imported live attenuated BEF virus vaccine. Group III: Calves kept as control negative.

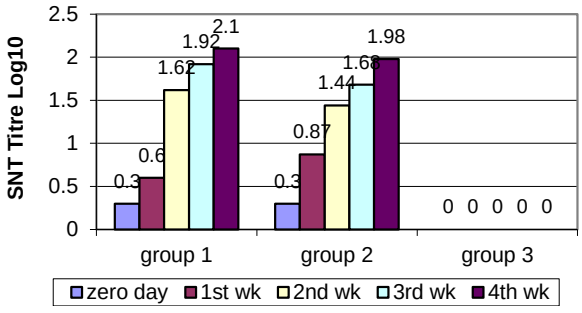


**Fig. 4:** BEF serum neutralizing antibody titer in vaccinated calves with imported and local commercial BEF vaccine against Vaccinal BEF strain. Group 1: Calves vaccinated with Local live attenuated BEF virus vaccine. Group 2: Calves vaccinated with imported live attenuated BEF virus vaccine. Group 3: Control negative.

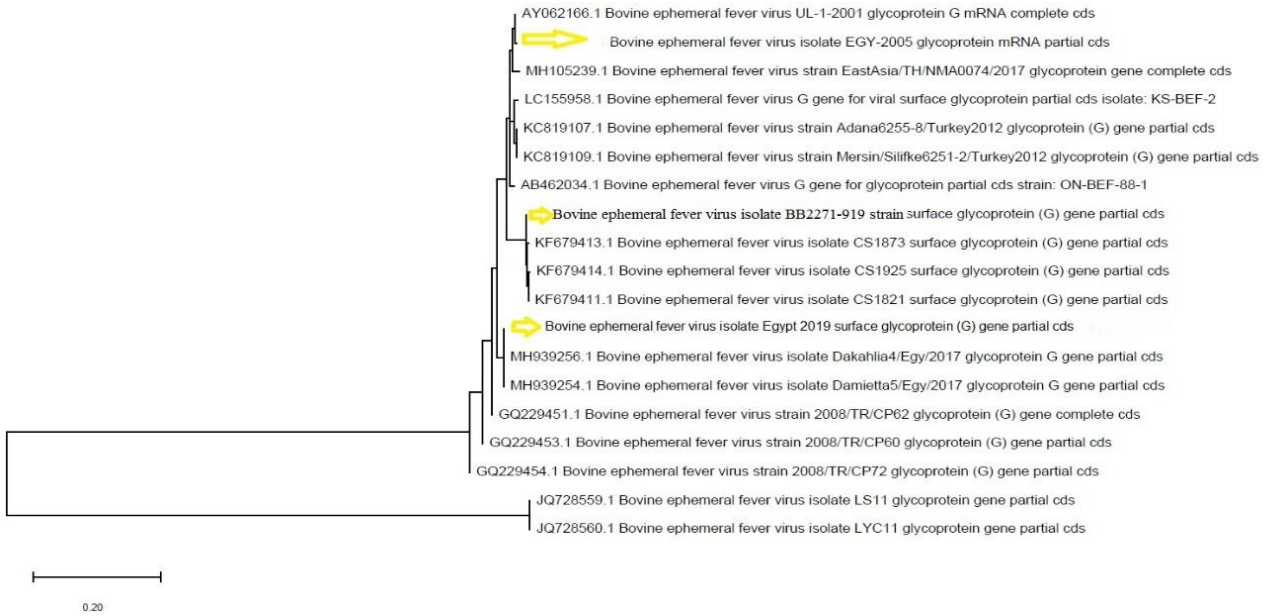


**Fig. 2:** Phylogenetic tree showing the relationship among BEFV depending on the virus partial code gene Sequence.

**Table 3:** BEF serum neutralizing antibody titer in vaccinated calves with imported and local commercial BEF vaccine against isolated virus

| Groups of calves | No. Calves | * SNT Titer ($Log_{10}$) Weeks Post Vaccination | | | | | |
|---|---|---|---|---|---|---|---|
| | | Zero day | 1st | 2nd | | 3rd | 4th |
| Group I | 1 | 0.3 | 0.6 | 0.6 | | 1.5 | 1.8 |
| | 2 | 0 | 0.6 | 0.9 | | 1.8 | 2.1 |
| | 3 | 0 | 0.6 | 0.6 | | 1.5 | 1.8 |
| | 4 | 0.3 | 0.9 | 0.9 | | 1.8 | 2.1 |
| | 5 | 0 | 0.6 | 0.6 | | 1.5 | 1.8 |
| Mean | | 0.3 | 0.66 | 0.72 | | 1.62 | 1.92 |
| Group II | 6 | 0 | 0.6 | 1.2 | 2nd dose | 1.5 | 1.8 |
| | 7 | 0.3 | 0.9 | 1.5 | | 1.8 | 2.1 |
| | 8 | 0.3 | 0.9 | 1.5 | | 1.8 | 2.1 |
| | 9 | 0 | 0.9 | 1.5 | | 1.8 | 2.1 |
| | 10 | 0 | 0.6 | 1.5 | | 1.8 | 2.1 |
| Mean | | 0.3 | 0.78 | 1.44 | | 1.74 | 2.04 |
| Group III | 11 | 0 | 0 | 0 | | 0 | 0 |
| | 12 | 0 | 0 | 0 | | 0 | 0 |
| Mean | | 0 | 0 | 0 | | 0 | 0 |

*Log10 Serum neutralizing antibody titer, Protective titer =1.2; Group I: Calves vaccinated with Local live attenuated BEF virus vaccine. Group II: Calves vaccinated with imported live attenuated BEF virus vaccine. Group III: Calves kept as control negative.

**Table 4:** BEF serum neutralizing antibody titer in vaccinated calves with imported and local commercial BEF vaccine against Vaccinal BEF strain

| Group of calves | No. Calves | * SNT Titer ($Log_{10}$) Weeks Post Vaccination | | | | | |
|---|---|---|---|---|---|---|---|
| | | Zero day | 1st | 2nd | | 3rd | 4th |
| Group I | 1 | 0 | 0.9 | 1.5 | | 1.8 | 2.1 |
| | 2 | 0.3 | 0.9 | 1.8 | | 2.1 | 2.1 |
| | 3 | 0 | 0.9 | 1.8 | | 2.1 | 2.1 |
| | 4 | 0 | 0.9 | 1.5 | | 1.8 | 2.1 |
| | 5 | 0.3 | 0.9 | 1.5 | | 1.8 | 2.1 |
| Mean | | 0.3 | 0.9 | 1.62 | | 1.92 | 2.1 |
| Group II | 6 | 0 | 0.6 | 1.2 | 2nd dose | 1.5 | 1.8 |
| | 7 | 0.3 | 0.9 | 1.5 | | 1.8 | 2.1 |
| | 8 | 0 | 0.9 | 1.8 | | 1.8 | 2.1 |
| | 9 | 0 | 0.9 | 1.2 | | 1.5 | 1.8 |
| | 10 | 0.3 | 0.9 | 1.5 | | 1.8 | 2.1 |
| Mean | | 0.3 | 0.87 | 1.44 | | 1.68 | 1.98 |
| Group III | 11 | 0 | 0 | 0 | | 0 | 0 |
| | 12 | 0 | 0 | 0 | | 0 | 0 |
| Mean | | 0 | 0 | 0 | | 0 | 0 |

*Log10 Serum neutralizing antibody titer, Protective titer =1.2. Group I: Calves vaccinated with Local live attenuated BEF virus vaccine. Group II: Calves vaccinated with imported live attenuated BEF virus vaccine. Group III: Calves kept as control negative.

## DISCUSSION

Bovine Ephemoral fever virus (BEF) is an important viral disease that still causes a risk in cattle industry in Egypt, However there is an effective vaccine used against the disease that represented by biphasic fever, lamness, stiffness, recumbancy, drop in milk production, nasal and ocular discharge (Walker, 2005) aboration and decrease fertility of bulls(Nandi and Negi 1999).It is an acute vector- born viral disease of cattle and water buffaloes, It is caused by Rhabdovirus. The disease was recorded in Egypt in 2000, 2004, 2014 and 2018 (Hassan, 2000; Al-Gaabary *et al*., 2005 EL-Bagoury *et al*., 2014), So Vaccination and eradication of arthropod is very important ways to control the disease. There are two types of BEF virus vaccines (live and inactivated) but live vaccine had been used as it provide prolonged immunity than inactivated due to saponine action as it has antiviral activity, inhibits virus penetration and protein synthesis, It also doesn't have cytopathic effects on target cell at concentration that achieve antiviral effect of saponine (Chenq *et al*., 2006).

In this study BEF could be isolated virus during hyper thermic phase of the disease as described by Nawal *et al*., (2001). The isolated BEF virus strain propagated on Vero cells for three passages yielded a titer of $10^6$ $TCID_{50}$/ml. Similar results were obtained by Zaghawa *et al*., (2017) who isolated BEF virus on Vero cell line showing that isolated virus induced specific CPE characterized by cell rounding, cell aggregation followed by detachment of the cell sheet as show in photos (1,2) in agreement with what was reported that BEF virus isolated and propagated on different cell cultures as Vero cells as described by Abd El- Aziem, (2008, Zheng *et al*. (2011), OIE, (2015) and Albehwar *et al*. (2018).

Rt - PCR amplification of the G glucoprotein gene revealed the highly conserved 420-bp of BEF virus for local live attenuated BEF vaccine. Imported live attenuated BEF vaccine and local isolated BEF virus based on the length of the amplicons. Similar results were obtained by Zaghawa *et al*. (2017) and Albehwar *et al*., (2018) who reported that RT-PCR is a superior test for the provision of rapid and specific data for BEFV detection targeting the G glycoprotein gene. The conventional RT-PCR is sensitive, specific and rapid test for detection of BEFV in clinical samples Moreover it was found that RT-PCR assay to be useful for testing RNA samples extracted from peripheral blood mononuclear cells and so it could be an important tool for the screening of BEF infection. Sequencing of the G glucoprotein gene was performed on BEF local and imported vaccines as well as local isolated strain revealed that the identity between the isolated virus and imported vaccine was 93.2% while it was 92.0% with the local produced vaccine On the other hand, the difference between the local isolated virus and the imported BEF vaccine was 6.8% while it was 8% with the local produced BEF vaccine and Similar results were obtained by Zaghawa *et al*., (2017) who found that the identity values among the nucleotide sequences of amplified part of bovine ephemeral virus isolated in 2017, G Glycoprotein gene determined a range from 84.7% to 100% and shared 90.4-96.5% sequence identity. The isolated virus belonged to the 3rd cluster that includes the Australian strains. This close relation with the Australian sequence is difficult to interpret. Oguzoglu *et al*., (2015) reported that variations were observed in other amino acids in regions that had been mapped previously to sites G1, G2 or G3 did not affect the neutralization phenotypes of epitopes targeted by the MAb.so from the relevant results.

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 1.69663082 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. This analysis involved 19 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1872 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [3] (Saitou and Nei 1987, Tamura *et al*., 2004 and Kumar *et al*., 2018). Phylogenic tree performed on glycoprotein (G) gene partial cds of isolated virus revealed that the isolated virus clustered with the same group with other isolated virus in Gene Bank (accession numbers MH939256 Dakahlia Egy 2017 and MH939254 Damietta Egy, 2017)

Humoral immune response in vaccinated calves with different commercial BEF vaccines against field isolated virus strain (2018) recorded that imported vaccine gave higher immune response (2.04 $\log_{10}$) than local one (1.92 $\log_{10}$) at four weeks post vaccination as shown in Table (3) and figure (2).While immune response of vaccinated calves with commercial BEF vaccines against vaccinal strain showed that the local vaccine gave higher immune response (2.1 $\log_{10}$) than imported one (1.98 $\log_{10}$) as shown in Table (4) and figure (3)which is non-significant in protection percent of vaccinated cattle) as it can be (not less than 1.2-1.5 $\log_{10}$ on the basis of homolgus neutralization titers higher than heterlogus neutrization titer amongst. There is also anecdotal evidence that vaccines developed in several countries using BEFV strains isolated more than 40 years ago remain effective against currently circulating strains and that vaccines developed against a strain of the virus from one region are effective against viruses currently circulating in other regions of the world (Walker and Klement., 2015) as BEFV is considered to exist as a single serotype worldwide. Various neutralization tests conducted using isolates from Australia, China, Japan, Kenya, Nigeria and South Africa has demonstrated strong antigenic cross reactions (Trinidad *et al*., 2015).

It could be concluded that two types of commercial live attenuated BEF virus vaccines (Local, Imported) induced high protective level against Vaccinal and isolated strain 2018, so it is not recommended to add it to BEF virus vaccine.

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