

Molecular Characterization of Recent Isolates of Bep Virus in Egypt

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Abstract

The present work was conducted to identify and characterize recent isolate of bovine ephemeral fever virus (BEFV) in Egypt. 103 serum samples were obtained from 1600 vaccinated animals in El-Salhia dairy farm during the period of July to August 2015. The 103 animals were found to have non-protective low titers of BEF antibodies ($\leq 2-8$) as tested by serum neutralization test (SNT), showing BEF signs (fever; hurried respiration; lameness and recumbency). Three buffy coat and five blood plasma samples from the 103 suspected infected dairy cattle were collected. Trials of virus isolation in baby mice brain and BHK₂₁ cell culture revealed that three samples of buffy coat and five blood plasma samples were suspected to contain BEFV. Virus identification using virus neutralization tests (VNT) and direct fluorescent antibody technique (FAT) confirmed the presence of BEFV. In addition, PCR, sequencing analysis and phylogenetic tree showed that the obtained isolate is closely related to Egypt-2005 strain. These findings indicated that the locally produced vaccine is highly immunogenic inducing a protection rate of 93.6%. Appearance of disease signs in cattle with low immune levels could be due to parturition and high lactation stress factors in addition to suspected other infection leading to poor immune response (immune suppression) to the vaccine.

Keywords: Bovine ephemeral fever (BEFV), Isolation, Identification, PCR, FAT.

Introduction

Bovine ephemeral fever virus (BEFV) is an arthropod-borne rhabdovirus which causes a disabling febrile infection of cattle and water buffalo. The disease is common in tropical and subtropical regions of Africa, Asia, Australia and the Middle East and is of major economic importance to dairy and grazing industries. The disease is characterized by sudden onset of fever, depression, difficult swallowing, serous ocular and nasal discharge, dyspnea and stiffness and lameness[1].

BEFV is the type species of the genus Ephemero-virus which also presently includes Adelaide River virus, Berrimah virus, tentatively Kimberley and Malakal and Puchong viruses [2]. All of these viruses have been isolated from insects or associated with insect vectors. However, only BEFV is known to cause disease in vertebrates. Like other rhabdoviruses, BEFV has a (-ve) ssRNA genome and five structural proteins including a nucleoprotein (N), a polymerase-associated protein (P), a matrix protein (M), a large RNA-dependent RNA polymerase (L) and a surface glycoprotein (G) [3].

Since the exact vector of BEF has not been identified, prevention efforts are mainly aimed at efficient vaccination of susceptible animals. The earliest BEF vaccines were based on field isolates of BEFV which were attenuated by repeated passages in suckling mice and/or cell cultures [4]. These vaccines were prepared with various adjuvants such as Freund's complete or incomplete

adjuvant, aluminum hydroxide, dextran sulfate, or Quil [5] and [6]. Many of the live attenuated (LA) vaccines produced a long-lasting neutralizing antibody (NA) response which lasted more than 12 months after two vaccinations. These vaccines demonstrated variable protection from clinical disease after both experimental [5] and [7] and natural challenge [6].

The use of inactivated vaccines is considered a safer approach. In the process of inactivation, the pathogen's ability to propagate in the vaccinated host is destroyed but the viral capsid remains intact, such that it is still recognized by the immune system. Inactivation of BEFV has been achieved using a variety of agents such as formalin [8], β -propiolactone [9] and binary ethyleneimine [10].

Although BEFV appears to exist as a single serotype worldwide, an analysis of a number of Australian and Chinese isolates has revealed some antigenic variation [11]. Determining the nature of this variation will be important in understanding the epidemiology of BEFV infection and in the design of broadly protective vaccines.

In the last decade, Israel has been facing a dramatic increase in the number of BEF outbreaks [12] and [13].

As indicated by phylogenetic analysis, significant differences have been found between the field strains circulating in the Middle East and other isolates [14].

The need for a vaccine which is antigenically similar to the field strains circulating in the Middle

East led to the development of an experimental inactivated vaccine based on an Israeli strain mixed with the adjuvant MONTANIDE™ ISA 206 VG (w/o/w).

The present work aimed to determine if there is a genetic difference between the circulating field strains of BEFV and the vaccinal strain used in the production of the locally prepared BEF vaccine to ensure the efficacy of this vaccine to induce a protective immune response against the disease.

Materials and methods

1. Samples

1.1 Serum samples

One hundred and three serum samples were obtained from cattle showing clinical signs out of one thousand and six hundreds vaccinated animals from El-Salhia dairy farm, Alsharqia Governorate, Egypt during the period of July to August 2015.

1.2 Buffy coat and blood plasma samples

Three buffy coat samples and five blood plasma samples were obtained from diseased cattle.

2. Bovine ephemeral fever virus (BEFV)

Local bovine ephemeral fever virus (BEFV/Abbasia/2000) adapted on BHK₂₁ cell culture [15] was supplied by the Department of Pet Animal Vaccine Research (DPAVR); Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. With a titer of 10⁶TCID₅₀/ml and used in serum neutralization test and as a positive control in virus identification assays.

3. BEFV antisera

Anti-BEFV serum and locally prepared anti-BEFV serum conjugated with fluorescein isothiocyanate "FITC" were supplied by DPAVR and used in VNT and direct FAT.

4. Baby mice

Suckling Albino Swiss baby mice (3-4 days old) were supplied by DPAVR were used for trials of BEFV isolation.

5. Baby hamster kidney cell culture (BHK₂₁)

BHK₂₁ cell culture was supplied by DPAVR and used for virus isolation, virus neutralization (VNT) and serum neutralization (SNT) tests.

6. Serum neutralization test (SNT)

SNT was carried out using the microtiter technique according to [16] to determine the BEF antibodies in the collected serum samples and the antibody titer was expressed as the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 TCID₅₀ of the virus according to [17].

7. Virus isolation

7.1 In baby mice

Each buffy coat and blood plasma sample was inoculated intracerebrally in each of 5 baby mice with 0.3ml/mouse according to [18] Inoculated mice were kept under hygienic measures in separate cages with their dams subjecting for daily clinical observations. Healthy baby mice were kept as test control. On the 3rd to 4th day post inoculation, when affected mice showed specific signs of BEFV infection (nervous signs, limb paralysis and cyanosis followed by death), the brains of dead mice were collected and subjected to other 2 viral passages in baby mice brains. Brain smears were prepared from affected mice and subjected to fluorescent antibody technique for virus identification.

7.2 In cell culture

Buffy coat samples were inoculated in BHK₂₁ cell culture for three successive passages according to [15] where the obtained cytopathic effect was described.

8. Virus identification

8.1 Virus neutralization test

Samples showing specific signs of BEFV infection in baby mice and CPE in BHK cells were subjected to VNT according to [19].

8.2 Direct fluorescent antibody technique (FAT)

Direct FAT was carried out on BHK₂₁ cell culture infected with the obtained isolates of BEFV using specific anti-BEFV conjugated with FITC.

9. Polymerase chain reaction (PCR)

The primers were used according to [20].

Primers: The used primers for BEFV are demonstrated in table (1)

Table (1) BEFV primers

Primer of bovine ephemeral fever	Sequence
BEF (Forward primer) at position 19 of the G gene,	TTAATACGACTCACTATAGGGAGATTTACAAT
Reverse primer	TTCCGGTGAA GGTATCCATGTTCCGGTTAT

• Nucleic acid recognition of virus samples

RT-PCR was used to amplify genome fragment from the prepared samples followed by nucleotide sequencing using BEFV specific primers. These oligos were synthesized by BioBasic, Canada. were used to amplify the expected sequence at position 523 according to [21].

RNA Extraction was done using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) Cat.No 52904 according to the manufacturer's protocol.

RT-PCR was carried out using One-Step RT-PCR Kit (Qiagen, Germany). The cycling parameters of the reaction conditions were: 95 °C for 1 min; then 35 cycles of (94 °C for 45 sec, 56 °C for 45 sec, and 72 °C for 50 sec.) and then a final incubation at 4 °C overnight. Amplified products were analyzed on agarose gel. Positive and negative control samples and DNA ladder were involved in agarose gel electrophoresis.

• Nucleotide sequence and Phylogenetic analysis

Amplified viral RT-PCR products were sent to Macrogen Lab. (Korea) for DNA sequencing. The sequenced samples represented bovine ephemeral fever (BEFV) isolates. All the received sequence results were aligned with nucleotide sequences database at the National Centre for Biotechnology Information site (NCBI) using Basic Local Alignment Search Tool programs to assert the new sequences of BEFV.

Analysis of the sequences identity phylogenetic relationship was performed using the clustal W method.

3. Results and discussion

The total number of cows at Al-Salhia dairy farm was 1600 cows. Clinical examination of these cows revealed clinical signs suspected to be due to infection with BEFV. All diseased animals showed high fever more than 40°C, salivation, respiratory distress and stiffness and some animals showed different degrees of lameness Fig (1).

Disease complications appeared on six animals showing different forms of recumbency including sternal and lateral recumbences Fig (2,4) two cows revealed severe respiratory signs as much hurried respiration, stretching of neck and opening mouth Fig (3) and two cows revealed subcutaneous emphysema Fig (4) in addition, 12 cows were culled (7 cows were dead and 5 were slaughtered).

It was found that the morbidity rate was 6.4% while the mortality rate was 0.7% and case fatality rate was 11.7%

It was noticed that the disease clinical signs were more severe in heifers (lateral recumbancy and severe respiratory distress) than adult cows (lameness and sternal recumbancy) and no clinical signs were observed in calves.

The recorded clinical signs are in agreement with those reported by [22], [23] and [24].

Serum samples obtained from such animals and subjected to serum neutralization test revealed low antibody titers (≤ 2 to 8)

Regarding the level of BEF antibodies in the examined vaccinated and affected cattle, serum neutralization test revealed poor immune status with antibody titer of $\leq 2-8$ (table-3) reflecting poor immune status in 103 tested animals from 1600 vaccinated cattle with a total protection rate of 93.6%. The poor immune status increased the susceptibility for virus infection. In this respect, [25] and [26] concluded that when the titer of BEF neutralizing antibodies was equal to 5 or 6 animals became susceptible to virus infection showing typical symptoms of BEF and revaccination should be carried out. The protective level of BEF antibodies should not be less than 32 as demonstrated by [27], [28] and [29]. Also the infection in vaccinated cattle could be attributed to vaccination failure which may be due to the stress of high milk production (30 -40 liters of milk/ cow / day) on the general health of vaccinated cows. On the other side it was noticed that the used BEF vaccine is highly effective inducing 93.6% protection. Similar findings were obtained by [30] and [31] who stated that the use of live BEF vaccine which was inactivated on the time of administration induced high levels of specific BEF neutralizing antibodies.

Inoculation of buffy coat and blood plasma samples in the brain of suckling mice revealed that one buffy coat sample and one plasma sample from the same cow induced specific signs of BEF infection represented by paralysis of the limbs; cyanosis and death within 3 days post inoculation as recorded by [32]. The other 2 buffy coats and 4 plasma samples did not induce any symptoms in inoculated mice, with a positive percentage of 25%.

Successful isolation of BEF virus from the buffy coat and blood plasma in suckling mice inducing limb paralysis; cyanosis and death within 3-4 days post infection came in agreement with [18], [33] and [34]

The positive samples induced specific CPE of BEF virus Fig (6) when inoculated in BHK21 cell culture characterized by cell rounding and cell aggregation followed by detachment of the cell

sheet within 3-4 days post cell infection coming to be confirmed by what reported that BEF virus was isolated and propagated on different cell cultures as Vero cells [19] Vero, BHK and MDBK [15] and [35] and BHK [36].

Application of virus neutralization test and direct fluorescent antibody technique Fig (7) using specific anti-BEFV serum and fluorescence conjugate antibodies confirmed that the obtained isolate is BEF virus. On the other side, the results of direct FAT came to confirm the presence of BEF virus showing intracytoplasmic fluorescent reaction Fig (7) . The use of direct FAT for detection of BEF virus in cell culture was established by [33] and [34] obtaining similar results.

Reverse transcriptase polymerase chain reaction (RT-PCR) has been developed with many advantages as it is possible to detect as little as fragment of viral RNA from infected tissue by ethidium bromide staining after 35 cycle of PCR [21]. The application of RT-PCR on isolated field virus yielded a clear single specific band on agarose gel stained with ethidium bromide. The amplified

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DNA fragment corresponds to 523 bp. PCR confirms the diagnosis of BEF infection as a sensitive, specific and valuable rapid diagnosis of viral diseases.

Phylogenetic analysis of the sequences identity revealed that the obtained recent isolate of BEFV (Zagazig-2015) is closely related as 90% to BEFV/ isolate EGY- 2005 glycoprotein mRNA partial cds; BEFV/isolate TN-2004-124 glycoprotein mRNA, complete cds and BEFV/ isolate UL-1-2001 glycoprotein mRNA complete cds but as 88% with BEFV/ isolate EGY- 2012 glycoprotein G (G) gene partial cds.

4. Conclusion

Depending on the obtained results, it could be concluded that the present used BEF vaccine is a potent vaccine that is able to protect cattle against the virus infection (inducing 93.6% protection rate) under normal conditions. On the other side, there is no difference between the obtained isolate and that used in the vaccine preparation requiring no changes in the vaccine formula.



Fig (1) Cow showing stiffness and lameness



Fig (2) Cow showing lateral recumbancy with emphysema ysema



Fig (3) Cow showing lateral recumbancy with severe respiratory distress as a disease complication



Fig (4) Cow showing sternal recumbancy with emphysema

Table (2) Complications of the BEF

Animal number	Recorded signs			
	Recumbancy		Respiratory distress	Emphysema
	Sternal	Lateral		
1		+ve	+ve	+ve
2	+ve			
3		+ve	+ve	
4	+ve			+ve
5	+ve			
6	+ve			

Table (3) BEF immune status of tested cattle

Items	Mean serum neutralizing BEF antibody titer*						Non-valid
	0	≤2	2	4	8	16	
Number of samples	61	1	6	12	15	0	
Percentage	64.2	1.05	6.3	12.6	15.8	0	8
Total %**	3.8	0.06	0.38	0.75	0.94	0	

*Mean serum neutralizing BEF antibody titer= the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100TCID₅₀ of BEF virus

**Total percentage= percentage of tested sample from the total number of farm cattle (1600).

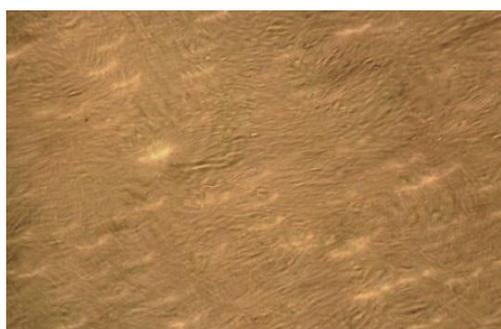


Fig (5) Normal BHK21 cell culture (H&E, 100X)

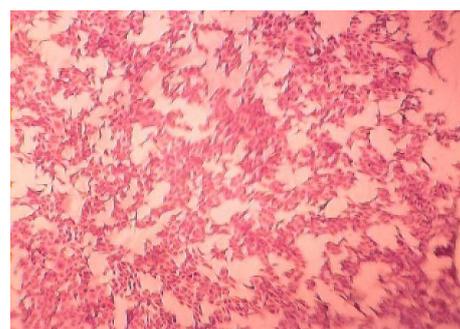


Fig (6) BHK21 cell culture infected with the isolated BEF virus showing cell rounding; cell aggregation and cell detachment (H&E, 100X)

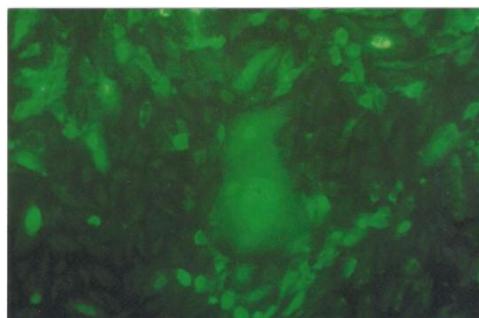


Fig (7) Positive FAT carried out on BHK21 cell culture infected with the isolated BEF virus showing intracytoplasmic apple green reaction

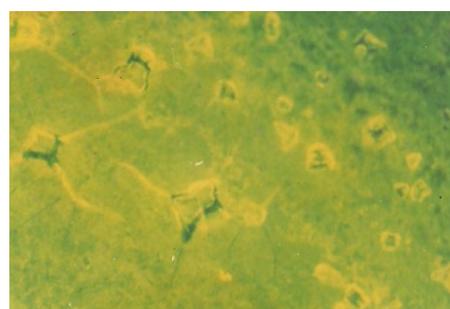


Fig (8) Negative FAT carried out on normal BHK21 cell culture (100X)

Lane (1): Buffy coat
 Lane (2): plasma sample
 Lane (3): tissue culture propagated virus
 Lane (4): positive control
 Lane (5- 6): negative samples
 Marker: 100 bp ladder.

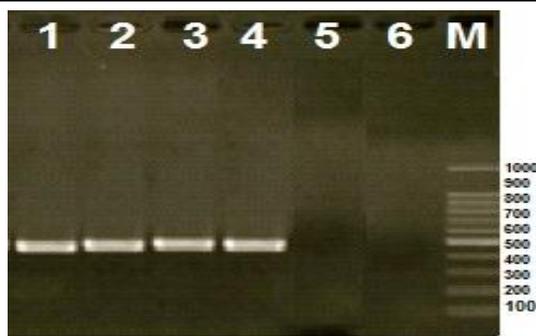


Fig (9) RT-PCR for detection of bovine ephemeral fever

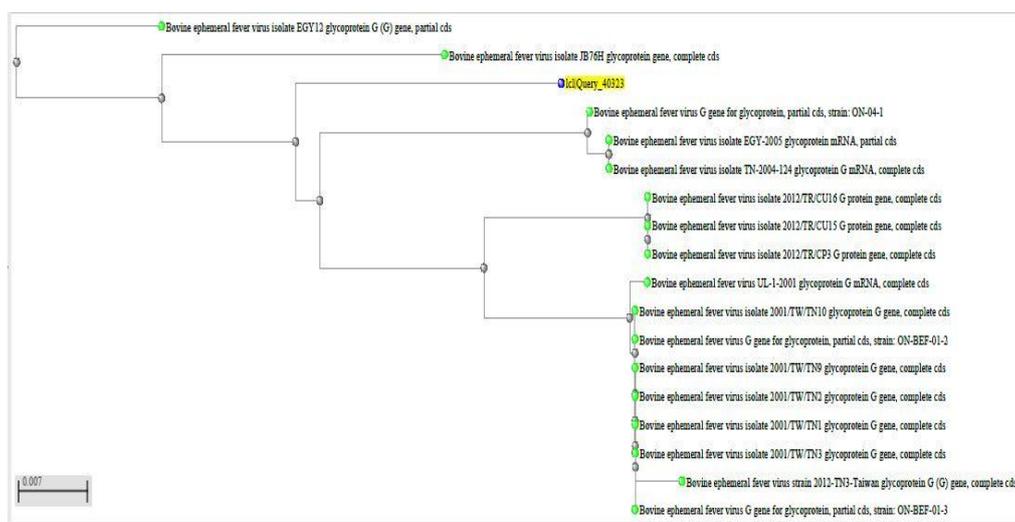


Fig (10) Phylogenetic tree showing relationship among BEFV depending on the virus partial cods gene sequence

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