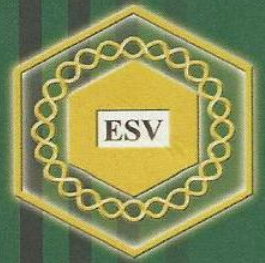


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## Serological and molecular identification of *Bovine ephemeral fever virus* isolates from cattle and buffaloes in Qaluobia province, Egypt 2014

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### ABSTRACT

In the present study, we report the isolation of *Bovine ephemeral fever virus* (BEFV) field strains from clinically infected cattle and buffaloes. Twenty Buffy coat samples were collected from each clinically infected cattle and buffaloes in Qaluobia province during the year 2014. The virus was isolated on BHK-21 cell line. Infected cells with BEFV showed 80% cytopathic effect (CPE) after 48 h by the 3<sup>rd</sup> virus passage. The isolates were serologically identified by neutralization and immunofluorescence techniques, and then molecularly identified using reverse transcription polymerase chain reaction (RT-PCR). Both cattle and buffalo isolates behaved the same pattern for serological and molecular identification in correlation with BEFV Webster reference strain and showed an amplicon size 473 bp for G2 gene. In conclusion, BEFV field strains from cattle and buffaloes seem like but further molecular characterization was required to study their homology.

**Keywords:** BEFV, Neutralization test, Immunofluorescence techniques, RT-PCR

### INTRODUCTION

Ephemeroviruses are arthropod-borne rhabdoviruses of ungulates, primarily infecting cattle and other ruminants. *Bovine ephemeral fever virus* (BEFV) causes a debilitating febrile illness in cattle and water buffaloes characterized by stiffness in gait; lameness; high morbidity and low mortality with a rapid spontaneous recovery of affected animals (Walker et al., 2012) and has been isolated on many occasions from biting midges (*Culicoides spp.*) and mosquitoes (*Anopheles bancrofti*), and from cattle in Africa, Asia and Australia (van der Westhuizen, 1967; Walker et al., 2012).

BEFV has a negative 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) (Walker et al., 1991). The 81 kDa BEFV G protein is a class I transmembrane glycoprotein which contains type specific and neutralizing

antigenic sites (Cybinski et al., 1990) and induces protective immunity in cattle (Uren et al., 1994; Hertig et al., 1996). BEFV and other ephemeroviruses also encode a second non-structural glycoprotein (GNS) and several other small polypeptides (Wang et al., 1994; Mc William et al., 1997). The GNS glycoprotein is also a class I transmembrane glycoprotein which is structurally related to the G protein but has not been detected in virions and does not induce a neutralizing or protective immune response (Walker et al., 1992; Hertig et al., 1996).

Four distinct neutralizing antigenic sites (G1, G2, G3 and G4) have been identified on BEFV G protein by competition binding studies using neutralizing monoclonal antibodies (MAbs) and cross-reactivity analysis of neutralization-resistant escape mutants (Cybinski et al., 1992)

First reports of the disease probably go back to the 19th century in the South

Africa, and then this infection was also described in Rhodesia, Kenya, Indonesia, India, Egypt, Palestine, Australia and Japan (St George, 1988). In Egypt, several outbreaks of BEFV have occurred in summer of 1991, 2000, 2001, and 2004 and 2010 affecting cattle and buffaloes (Kawther and Ahmed, 2011). Nowadays during October 2014, an outbreak of suspected BEFV was observed in a farm of faculty of veterinary medicine, Moshtohor, Qaluobia province. The sever clinical manifestations and economic losses during this time created substantial awareness for our aim to isolate and identify the causative agent of suspected cases of BEFV appeared in Egypt in 2014 using serological and molecular techniques.

## MATERIAL AND METHODS

### Specimen collection:

On October 2014, a total of 40 blood samples on EDTA were collected from BEF suspected cattle and buffaloes (20 samples from each) during the febrile period. These animals were located in the farm of faculty of veterinary medicine, Moshtohor, Qaluobia. Buffy coats were separated for a trial of virus isolation according to Van Der Westhuizen., 1967.

### Virus and cell culture:

The 3<sup>rd</sup> passage of BHK-21 cell culture adapted Webster strain of BEFV (Azab *et al.*, 2002) of a titer  $10^6$  TCID<sub>50</sub>/ml and BHK-21 cell line were supplied by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo and used in the present search.

### Antiserum and conjugate:

Antiserum of BEFV was supplied by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo and used in virus neutralization test and IFAT. Goat anti-bovine immunoglobulins conjugated with fluorescein isothiocyanate (FITC) are commercially available (ICN, U.S.A.). The conjugate was used for IFAT.

### Virus isolation on BHK21 cell line:

Confluent BHK21 cell monolayers in 25 cm<sup>2</sup> flasks were infected with 10% Buffy coats (2ml/flask) and incubated at 37°C for 1h, then cell maintenance medium was added into the flasks (10ml/flask). The cultures were maintained at 37°C for 5 days and observed every 24h interval. If no CPE within 5 days, the cultures were frozen and thawed 3 times and inoculated on BHK21 cells again till 3 blind passages (Van Der Westhuizen., 1967).

### Serological identification of BEFV isolate:

#### Virus neutralization (VN) test:

VN test was carried out with the virus isolate on the BHK 21 cell culture according to micro-neutralization test for BEFV as previously described (Wakeley *et al.*, 2004). Reference BEFV strain was a positive control.

#### Immunofluorescent antibody (IFA) test:

At the third passage on BHK21cell monolayer. When CPE was observed, the supernatant was removed and the cell sheet was fixed in cold acetone for 20 min at -20°C and then stained by an IFA technique (Payment and Trudle., 1993). Antiserum to BEFV was used as the primary antibody, then a fluorescein isothiocyanate-labelled goat anti-bovine IgG was used to stain the preparations. Also, uninfected cells treated as described above, were used as an indicators. The specific immunofluorescence reaction was examined with a fluorescence microscope.

### Molecular identification of BEFV isolate by RT-PCR amplification:

The total RNAs were prepared from infected BHK21cells using QIAamp Viral RNA Mini Kit (QIAGEN, Germany). Then the cDNA was synthesized using 1µg of the total RNA with primer (5' CCT CAC AAT GTT CAA GGT CCT C 3') and Avian Myeloblastosis reverse transcriptase enzyme (Promega). Reverse transcription was conducted at 50°C for 30

minutes. This mixture was then heated at 94°C for 2 minutes to stop the reaction. The cDNA was amplified by Taq polymerase with primers upstream (5' GGA ATA CGG AGA TGA ATC AA 3') start from nucleotide number 407 and end at nucleotide number 426 and downstream (5' ATT CTG TTC TAT CTG TGT GC 3') start from nucleotide number 861 and end at nucleotide number 880 in order to obtain BEFV G2 gene. After the initial denaturation at 94 °C for 5 min, the amplification proceeded through a total of 35 cycles of 94 °C for 45 s for denaturation; 56 °C for 45 s for annealing; and 72 °C for 50 s for primer extension and the final extension of 10 min at 72°C. The PCR products were identified by 1.5 %

agarose gel electrophoresis containing 0.5 µg/ml ethidium bromide and then visualized under UV trans-illuminator. A 100 bp DNA ladder (Gibco) was used as DNA base pair marker.

## RESULTS

### Isolation of BEFV on BHK-21 cell line:

Inoculation of the prepared samples from Buffy coats onto BHK-21 cell line were positive in 15/20 (75%) samples from cattle and 7/20 (35%) samples from buffaloes by the 3<sup>rd</sup> passage (Table 1). The CPE were cell rounding, cell aggregation and detachment of the cell sheet between the 3<sup>rd</sup> and 5<sup>th</sup> days post inoculation (photo1).

Table (1): isolation of BEFV suspected samples on BHK-21 cell line

Species	Total number of samples	Number of positive samples		
		1 <sup>st</sup> passage	2 <sup>nd</sup> passage	3 <sup>rd</sup> passage
Cattle	20	2	6	15
Buffaloes	20	0	4	7
Total	40	2	10	22

Positive samples showed rounding, cell aggregation and detachment of the cell sheet.

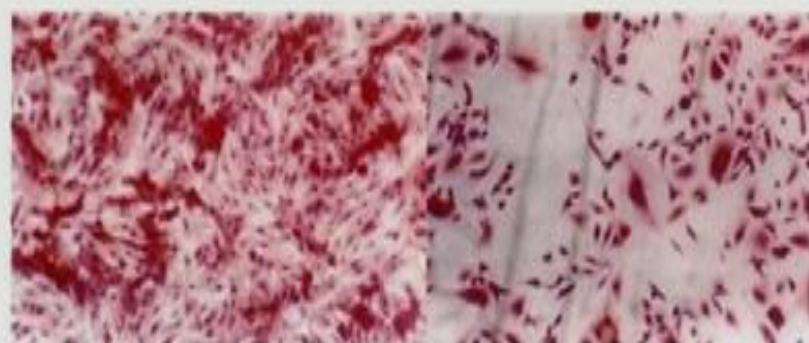


photo (1) CPE of suspected BEFV samples inoculated onto BHK cell line showing, cell rounding and cell lyses by the 3<sup>rd</sup> passage (right) compared with normal BHK cell line BHK cell line (left). Both were stained with Hematoxyline and Eosin, (Magnification power 100X)

### Serological identification of suspected BEFV isolates:

Suspected BEFV isolates were serologically identified by virus neutralization and fluorescent antibody techniques (table 2). Neutralization assay revealed that 14 out of 20 (70%) samples from cattle and 6 out of 20 (30%) samples from buffaloes showed the standard positive serum to BEFV with 1:64 dilution could neutralize completely the virus isolate being same as Webster strain. Direct FAT identified 15 out of 20 (75%) samples from cattle and 8 out of 20 (40%) samples from buffaloes with characteristic apple green fluorescent (photo.2)



Photo (2): Apple green fluorescent from BHK cell line inoculated with suspected BEFV isolates.

### Molecular identification of BEFV isolates from cattle and buffaloes:

The expected size of PCR product as 473 bp of the G2 protein encoding gene of BEFV was successfully detected in 17 out of 20 (85%) samples from cattle and 9 out of 20 (45%) samples from buffaloes (table 2). The size of the resulted fragments were analyzed by gel documentation system and showed that the BEF virus reference strain and the cattle and buffalo isolates had the same size of G2 gene fragment without significant differences between the strains as represented in (fig.1).



Fig. (1): RT-PCR detection of BEFV, 473bp amplified product of G2 gene for different strains. Lane M: DNA ladder of 100 bp, Lane 1: Reference BEFV strain, Lane 2: BEFV Buffalo isolate, Lane 3: BEFV Cattle isolate.

Table (2): Identification of BEF virus in inoculated BHK cell cultures by virus neutralization, fluorescent antibody techniques and RT-PCR.

Animal species	Number of inoculated samples	CPE positive samples	Serological assays			
			VN positive samples	FAT positive samples	RT-PCR positive samples	
Cattle	20	15 (75%)	14 (70%)	15 (75%)	17 (85%)	
Buffaloes	20	7 (35%)	6 (30%)	8 (40%)	9 (45%)	
Total	40	22 (55%)	20 (50%)	23 (57.5%)	26 (65%)	

## DISCUSSION

An outbreak of BEFV was recorded from dairy herd in the farm of faculty of veterinary medicine, Moshtohor during October 2014 in Qalubia province, one of the Nile Delta. The Nile Delta offers favorable conditions for breeding large population of biting flies, mainly mosquitoes, and it is known to be a permanent focus of BEF infection (Al-Busaidy and Mellor., 1991). The carriage of flies over long distance by air currents has also been reported (Alba et al., 2004).

Although infection of cattle with BEFV is associated with neutralizing antibody responses but an antibody rise does not necessarily indicate recent infection, and it should be considered of diagnostic value only when taken in conjunction with clinical signs of disease (Cybinski, 1987). In addition, an antigenic relationship between Rhabdoviruses is considered another problem (Calisher et al, 1989). So, diagnosis of BEFV was based on isolation, serological identification of viral antigen or molecular identification of viral genome (OIE, 2008).

BEFV was successfully isolated on BHK-21 cells inoculated with cattle and buffaloes Buffy coats by the 3rd passage showed characteristic CPE (table 1 and photo 1). This indicates the susceptibility and role of this cell for BEFV growth (Nandi and Nagi, 1999; Azab et al. 2002; El-Shamy, 2003).

Serological identification of BEF virus field isolates (cattle and buffalo isolates) using VNT and IFAT revealed higher sensitivity IFAT than VNT for identification of buffaloes isolate as 6 samples were detected by VNT while 8 were detected by IFAT. This confirmed the theory that not all BEFV strains produce CPE and the presence of virus is generally demonstrated by immunofluorescence (St. George 1988; Sayed-Ahmed, 2005). Our results might refer to delayed CPE produced by buffalo isolates.

Molecular identification of BEFV strains using RT-PCR assay revealed the presence of specific PCR product at the correct expected size of the G2 gene (473 bp). The size of the resulted fragments, analyzed by gel documentation system, showed that cattle and buffalo isolates and the reference Webster strain had the same size of G2 gene fragment 473 bp, without significant differences between them as represented in fig (1). The results indicated that RT-PCR is a very sensitive, specific and rapid confirmatory assay for rapid identification of BEFV as agreed and demonstrated by Wang et al (2001), and Zaghawa et al (2006) who found that RT-PCR is a very sensitive, specific and rapid confirmatory assay for rapid identification of BEFV. Comparison between the sensitivity of the utilized techniques in identification and diagnosis of infections revealed 55%, 50%, 57.5%, and 65% for virus isolation, neutralization, IFAT, and RT-PCR, respectively. These findings come in complete agreement also with those of Davis and Boyle, (1990) and Wu et al, (1992) who found that RT-PCR can detect as little as two fragments of viral RNA from infected tissue after 30 cycles of PCR which can be completed within 6 hours with no need for virus propagation or purification. Despite that virus isolation is the gold standard technique but IFAT and RT-PCR proved to be rapid, sensitive and specific for BEFV identification. A further study is still needed for studying homology between viral isolates using nucleotide and amino acid sequencing.

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