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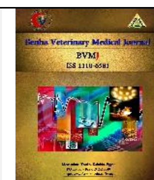
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Original Paper

Detection of bovine ephemeral fever virus (BEFV) in three Egyptian governorates during the summer of 2019

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ABSTRACT

Bovine ephemeral fever virus (BEFV), an economically important arthropod-borne viral pathogen, causes bovine ephemeral fever (BEF) in cattle and water buffalo. In the current study, we detected the BEFV in three Egyptian governorates (Giza, Menofia and Sharqiyah) through isolation, serological and molecular identification of the virus from naturally infected cattle. Fifty-five cattle showed symptoms suspected to be BEF represented by fever, lameness, subcutaneous emphysema, and recumbence. Serum samples were obtained for evaluation the immune status of affected cattle and buffy coat samples were subjected to trials of virus isolation on baby hamster kidney (BHK-21) cell line and also in baby mice where positive results were recorded among 10 samples only (4 samples form Giza, 3 samples from each Menofia and Sharqiyah). SNT results revealed that all the infected cattle had very low titers of BEFV serum neutralizing antibody which indicated that such were fully susceptible to the infection. Serological identification including direct fluorescent antibody technique (dFAT) and virus neutralization assay (VN) confirmed that the obtained viral isolates are BEFV. Further confirmation by reverse transcriptase polymerase chain reaction (RT-PCR) targeting the glycoprotein (G) gene showed positive amplification reaction of the target gene at the correct size of 500bp. Consequently, further future work for molecular analysis of such recent isolates will be conducted for evaluation of the circulating BEFV in these governorates in Egypt.

1. INTRODUCTION

The bovine ephemeral fever virus (BEFV) is classified as a member of the genus *Ephemerovirus* in the family *Rhabdoviridae*in order *Mononegavirales* and is known to cause Bovine ephemeral fever (BEF) also known as '3-day sickness', infecting both cattle and water buffalo. The disease is endemic in tropical and subtropical regions of Asia, Africa, Australia, and the Middle East and has major economic importance for both fattening and dairy industries due to reduced milk production at dairy farms and loss of conditioning of beef cattle (Kirkland, 2002; Walker et al., 2005). BEFV displays typical rhabdovirus bullet-shaped morphology, with negative-sense; single-stranded RNA genome tightly associated with the nucleoprotein (N) which, together with the phosphoprotein (P) and the large multi-functional enzyme (L) form a ribonucleoprotein complex (Walker et al., 1991). The virion surface glycoprotein (G) is responsible for cell attachment and entry; it is also a type-specific neutralizing antigen and induces protective immunity in cattle (Johal et al., 2008; Trinidad et al., 2014). BEVV causes disease which characterized by sudden onset of bi-phasic fever, depression, difficulty swallowing, serous nasal and ocular discharge, dyspnea, and lameness (Nandi and Negi, 1999). BEFV is considered to exist as a single serotype worldwide. Walker and Klement, (2015) reviewed that various neutralization assays performed using viral isolates from China, Australia, Japan, Kenya, Nigeria and South Africa have revealed strong antigenic cross-reactions. BEFV has been recorded in Egypt since 1909 and then, subsequent outbreaks affecting hundreds of cattle occurred in 1915. More recently, the disease was reported during the summers of 2010, 2014, 2017, 2018 and 2019 (Zaher and Ahmed, 2011; Kasem et al., 2014; El-Bagoury et al, 2014 and Albehwar et al, 2018; Nayel et al., 2019; El-Habbaa and Radwan, 2019). Despite the impact of BEFV in Egypt, little was known about disease epidemiology, the potential for vaccine management, and risks (Daoud et al., 2005; Younis et al, 2005). The current work aimed to detect the BEF virus in three Egyptian governorates (Giza; Menofia and Sharqiyah) through isolation of the virus from naturally infected cattle on baby mice and BHK-21 cell line and identification of the viral isolates by serological tests (VNT& FAT) as well as molecular identification by RT-PCR targeting the glycoprotein (G) gene.

2. MATERIAL AND METHODS

2.1. Ethical approval

The study protocol was approved by the Institutional Animals Care and Use Committee, Board of Research Ethics, Faculty of Veterinary Medicine, Benha University following animal welfare guidelines.

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2.2. Biological samples collected from clinically infected cattle

During summer 2019, a total number of cattle (n= 55) showing symptoms suspected to be bovine ephemeral fever were located in different three localities (20 in Giza; 15 in Menofia, and 20 in Sharqiyah Governorates) with different degrees of illness represented by fever (40-41°C); aggravated respiration, lameness, subcutaneous emphysema and recumbence (Fig. 1). Fifty-five samples of serum and buffy coat were obtained from cattle showing signs of what is clinically suspected to be bovine ephemeral fever.

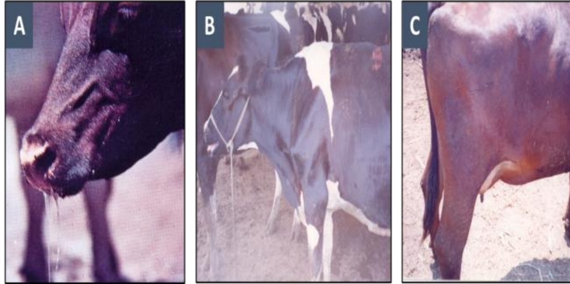

Fig (1): Suspected cattle with BEFV infection showed: Panel A, B &C; Salivation, difficult respiration, and Subcutaneous emphysema lesions especially on hindquarters

2.3. Cells and Viruses

The baby hamster kidney cell line (BHK-21) established by Macpherson and Stocker (1962) were used in serum neutralization test and trials of BEFV isolation and identification. BHK-21 cell culture adapted local strain BEFV (BEFV/Abbasia/2000) virus of a titer $10^{7.5}$ $TCID_{50}$/ml (Azab, et al., 2002) was supplied by Department of Pet Animal Vaccine Research (DPAVR),Veterinary Serum & Vaccine research Institute (VSVRI), Abbasia, Cairo for using in SNT and as positive control for molecular identification of BEFV isolates.

2.5. BEFV antisera

Locally prepared anti- BEFV serum alone or conjugated with fluorescein isothiocyanate "FITC" Florence et al, (1992), were supplied by DPAVR and used in VNT and direct FAT, respectively.

2.6. Serum neutralization test (SNT)

Serum neutralization test (SNT) was carried out on serum samples (n= 55) collected from diseased cattle for evaluation of the immune status of the clinically infectd cattle. SNT was carried out using the micro titer technique according to Yoneda et al (2008) to determine the BEFV 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 Singh et al. (1967)

2.7. Virus isolation

In baby mice: Each buffy coat sample (n= 55) was inoculated intracerebrally in each of 5 baby mice (3-4 days old) with 0.3ml/mouse according to Hamoda et al. (2002). Inoculated mice were kept in separate cages under hygienic measures with their dams subjecting to daily clinical observations. Healthy baby mice were kept as a 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 two viral passages in baby mice brains.

In cell culture: Buffy coat samples were inoculated in BHK-21 cell culture for three successive passages according to Azab et al. (2002) where the obtained cytopathic effect was described

2.8. Virus neutralization test (VNT)

Using micro titer technique according to Ferreira, (1976), tenfold dilution of the BHK-21 propagated virus at its third passages were prepared in HBSS, each virus dilution was mixed with equal volume of anti-bovine ephemeral fever virus serum (of a titer of 64), then inoculated in 6 wells of BHK-21 cell culture seeded 96 well tissue culture plate. This mixture was leaved for 1hr. at 37°C in incubator, and then the virus titer was calculated according to Reed and Muench (1938).

2.8. Direct fluorescent antibody technique (dFAT)

Direct fluorescent antibody technique was carried out according to Salheen et al., (2021) on the inoculated BHK-21 cells infected with suspected BEFV using specific anti-BEFV conjugated with FITC. The slides were kept in a humidified chamber for 45 minutes at 37 °C and then, were thoroughly washed with PBS for 15 min three times. The slides were then mounted with buffered glycerin, covered with a cover slip, and examined under a fluorescent microscope.

2.9. Extraction of viral nucleic acid and RT-PCR

RNA Extraction was carried out using QIAamp Viral RNA Mini Kit (Cat. No; 52904, QIAGEN, Germany) according to the manufacturer's protocol. RT-PCR was used to amplify genome fragments from the viral isolates on BHK-21 cells (selected three samples of high virus titer on BHK-21 cells) using BEFV specific primers. These oligos were synthesized by Bio Basic, Canada. The using primer was bef19T7-TTAATACGACTCACTATAGGGAGATTTAC-AATGTTCCGGTGAA at position 19 of the G gene. The reverse primer was GGTATCCATGTTCCGGTTAT-bef523R at position 523 according to Degheidy et al., (2011). RT-PCR was done according to Khalil et al., (2001) 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.). The PCR amplification products were analyzed on 1.5% agarose gel containing ethidium bromide gene ruler 100 bp ladder (Fermentas, Thermo, Germany) were used to determine the sizes of the amplicons

3. RESULTS

3.1. Immune status of diseased cattle

Serum neutralization test (SNT) was carried out on serum samples collected from diseased cattle showed that all of them had very low titers of BEF serum neutralizing antibody titers as tabulated in Table (1) indicated that such were fully susceptible to BEF virus infection.

Table (1): BEFV immune status in clinically suspected cattle

Animal locality	Number of samples	BEFV serum neutralizing antibody titer*		
		0 (Negative)	≤ 2	>2
Giza	20	15	3	2
Menofia	15	10	5	0
Sharqiyah	20	17	2	1
Total	55	42	10	3
%		42/55=76.63	10/55=18.18	3/55= 5.54

*Antibody titer= the reciprocal of the final serum dilution which neutralized 100 $TCID_{50}$ of BEF virus. *The protective BEFV-SNT titer is 32 (Albehwar et al, 2018)

3.2. Virus isolation

Inoculations of the buffy coat samples in the brain of suckling mice showed that 10 samples induced paralysis of the limbs, cyanosis and death within 3 days post-inoculation (Fig. 2A). The other samples (45 samples) did not induce any symptoms (Fig. 2 B) with a positive percentage of 18.18%. The positive buffy coat samples which showed characteristic symptoms on the inoculated baby mice were inoculated in BHK-21 cell culture for three successive passages inducing specific CPE of BEF virus (Fig. 3). The CPE on the inoculated BHK-21 cells was characterized by cell rounding and aggregation followed by a detachment of the cell sheet within 3-4 days post-inoculation with a gradual increase in the virus titer (2 log10; 3 log 10 and 5 log10 $TCID_{50}$/ml for each passage respectively.

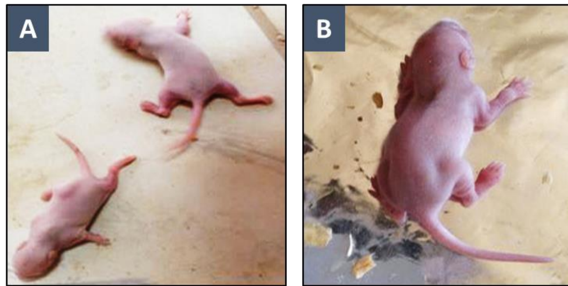

Fig (2): BEFV infected suckling mice showing limb paralysis (Panel A) and healthy control suckling mice (Panel B)

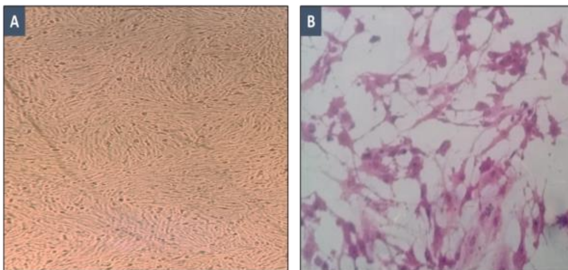

Fig (3): Representative picture of cytopathogenic effect of BEVF on inoculated BHK-21 cell culture showing cell rounding and cell aggregation (Panel B)and non-inoculated normal cells(Panel A) (Magnification; 100X)

3.3. Serological identification of the viral isolates by VNT and dFAT

Virus neutralization test (VNT) and direct immunoflourescence using specific anti-BEFV sera confirmed the BEFV isolates in 10 samples which have shown positive results on inoculated BHK-21 cell culture. The virus was found to be of 10^5 $TCID_{50}$/ml. The positive results of direct FAT came to confirm the presence of the BEF virus antigen showing apple-green fluorescence emission (Fig. 4)

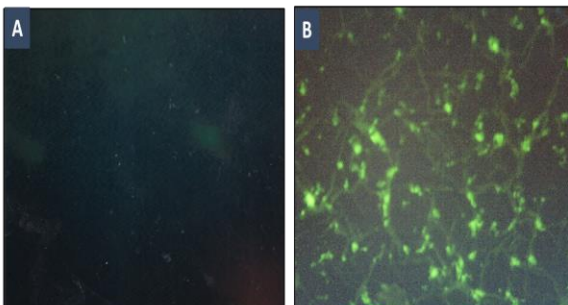

Figure (4): Direct fluorescent antibody technique (dFAT) detected the presence of BEFV in the inoculated BHK-21 cells showing apple green fluorescence (Panel B).Non-inoculated BHK-21 cells showing no emission (negative control)(Panel A).(Magnification; 100X)

3.4. Molecular detection of the viral isolates by RT-PCR

Three samples of the inoculated BHK-21 were selected to be subjected to molecular characterization by RT-PCR using specific BEFV primers set. Analysis of the RT-PCR amplicons obtained from the amplification reactions of extracted RNA by agarose gel electrophoresis. It showed the positive amplification result of the Glycoprotein (G) gene with expected correct size (500 bp) when compared with the positive control sample. (Fig.5) as expected, no amplicon product from the negative control reaction was shown

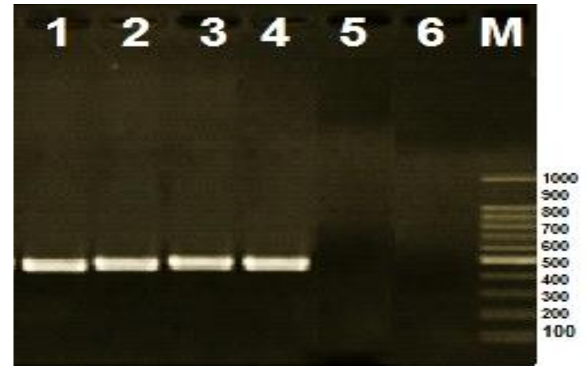

Fig (5): Agarose gel electrophoresis of RT-PCR products compared to 100bp marker, the electrophoretic pattern of the amplification products to the viral isolates (Lanes; 1, 2, and 3) on the gel electrophoresis (1.5%) at the expected correct size (500 bp). Positive control (Lane 4) and negative control (Lane 5 and 6) are included

4. DISCUSSION

Bovine ephemeral fever is one of the most important infectious diseases of cattle and water buffalo in Egypt. Differential diagnosis of BEFV based on clinical observation on the infected cattle is sometimes complicated. The diagnosis of ephemeral fever virus during epidemics is made on the presence of lameness, stiffness, rapid spread of the disease through herds and bi-phasic fever (Walker and Klement, 2015). The BEF virus can often be identified by viral isolation, serological and molecular tools conducted on a blood sample taken from animals in the acute febrile stage of the disease (OIE, 2008). Our obtained results of SNT which was carried out on serum samples collected from clinically infected cattle showed that all of them had very low titers of BEFV serum neutralizing antibody less than 32, which indicated that such were fully susceptible to the infection with BEF virus (Albehwar et al, 2018). The protective level of BEFV antibodies should not be less than 32 to achieve full protection of animals as demonstrated by (Daoud et al 2001; Salah, 2006; Aziz-Boaron et al., 2014). Isolation of the virus from blood of infected cattle can be successfully performed in baby hamster kidney (BHK-21) or monkey kidney (Vero) cell lines (Kasem et al., 2014; Azab et al., 2002Nayel et al., 2019) but not all BEFV strains can produce cytopathogenic effects on the inoculated cell culture (St. George, 1988). However, the short duration of viremia in the infected animal and inefficient replication of the virus before adaptation to mammalian cell cultures has limited the utility of this method. Suckling mice may also be used for primary isolation by intracerebral inoculation and the isolated viruses can be identified by neutralization assays which are considered a widely used serological method to diagnose BEFV infection using specific BEFV antisera (James et al, 2008; Bakhshesh and Abdollahi, 2015). Direct inoculation of the mosquito cell lines and detection by direct or indirect fluorescent antibody

techniques before passage in BHK-21 cells considered to be a more effective method (Uren et al., 1983). PCR-based detection of the virus during the acute stage of infection is commonly used for rapid laboratory diagnosis. Although virus isolation was the traditional "gold standard," real time RT-PCR assays are now routinely employed to achieve rapid confirmation of bovine ephemeral fever virus (Kirkland 2002; Zaher and Ahmed, 2011). It is evident from our obtained results that the RT-PCR targeting the glycoprotein (G) gene with the amplified product (500 bp) is a highly sensitive and specific tool for detection and identification of the BEFV isolates in cultured cells or mice inoculated with samples collected from the clinically suspected cattle (Wang et al., 2001; El-Bagouryet al., 2014; El-Habbaa and Radwan, 2019;Nayel et al., 2019).

5. CONCULOSION

Finally, further molecular analyses of our recent isolates of BEFV are recommended to confirm these findings to determine the molecular epidemiology of the BEFV strains circulating in Egypt and to update the nature of future vaccinal strains for successful preventive strategies.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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