Diagnosis, isolation and molecular identification of *Bovine ephemeral fever virus* in Dakahlyia province, Egypt 2015

Sahar Abd El Rahman[1]; Mohamed Eladl[2] and Mohamed M. El-Diasty[3]

[1] Department of Virology, Faculty of Veterinary Medicine, Mansoura University, Egypt
[2] Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Mansoura University, Egypt
[3] Animal Health Research Institute, Mansoura Provincial Laboratory, Egypt

ABSTRACT

Bovine ephemeral fever is a non-contiguous epizootic arthropod viral disease infecting cattle and water buffaloes. The present work aimed to isolate the causative agent and identify it by serological characterize of the viral antigens, then molecular characterization of its genome. Infection by the *bovine ephemeral fever virus* (BEFV) was noticed in Dakahlyia governorates during the summer season of 2015. Twelve samples of buffy coats were collected from dairy farms suspected from clinical investigations to be infected by *bovine ephemeral fever virus*. The virus was isolated intracerebrally in suckling mice then successfully identified by indirect immunofluorescence technique in the brain sections of positively inoculated mice. Reverse transcriptase polymerases chain (RT-PCR) reaction was performed directly on buffy coat samples for molecular identification of the virus showed 470 bp clear band in gel electrophoresis. The sensitivity of the utilized techniques in the identification and diagnosis of bovine ephemeral fever disease revealed that the virus isolation is a gold standard technique, as well, the immunofluorescence technique. RT-PCR proved to be a rapid, sensitive and specific tool for bovine ephemeral fever disease diagnosis.

Keywords: Bovine Ephemeral fever, Buffy coats, Suckling mice, Immunofluorescence technique, RT-PCR.

INTRODUCTION

Bovine ephemeral fever virus (BEFV) is one of the genus *Ephemeral viruses*, family *Rhabdoviridae* and the causative agent of an acute febrile disease in cattle and water buffalo called three day sickness. The disease is economically significant due to loss of milk production, mortality in dairy and beef animals and infertility problems. BEFV has been isolated from Culicoides brevitarsis, Culicoides coarctatus, and Anopheles bancroftii, however the arthropod vectors are yet to be defined (Walker *et al.,* 2012). BEFV is bullet shaped, negative sense, single stranded RNA virus and its genome consist of five structural protein genes; nucleoprotein (N), a polymerase-associated protein (P), a matrix protein (M), a surface glycoprotein (G) and a large RNA-dependent-RNA-polymerase (L). The G gene encodes glycoprotein (G) is responsible for cell attachment and entry, and also contains the type-specific and the neutralizing antigenic sites (Cybinski *et al.*,1990; Walker *et al.*, 1991; Uren *et al.,* 2004), the virus has only one serotype according to OIE, (2008). Experimental trials for controlling of the disease depend mainly on vaccination by live-attenuated, inactivated, subunit and recombinant vaccines (Radostitis *et al.*, 2007) referring that the live attenuated vaccine gives prolonged immunity comparable to others.

Lack of an accurate and rapid laboratory diagnosis of the disease, because of the short course of viremia in the affected animals, proper shipping requirements of blood samples to the laboratory and difficulties of isolation of BEFV, exaggerates the economic losses caused by the virus. Additionally, the international trade considerations may influence the selection of export animals, with the cost of laboratory testing being an added burden. Other

economic losses are the costs of treatment and costs of control program application as insect control and vaccination (Zaghawa *et al.,* 2002).

Diagnosis of the BEF disease depends mainly on clinical backgrounds and observations during major epidemics. Single cases are difficult to be diagnosed comparable to herd out-break, as cattle express the disease at various stages. Sporadic cases, or those occurring early in a possible epidemic can be confirmed by virus isolation and identification in suckling mice (Snowdon, 1970; Inaba, 1973), vero cell line (Burgess, 1974), serologically by detecting the specific antibodies in paired serum samples (Nandi & Negi, 1999) or detecting the viral antigen in the leukocytes of feverish animals by immunofluorescence (IFT) and immunoperoxidase techniques (Zaghawa *et al.,* 2002).

Advanced molecular technique as RT-PCR was firstly used for detection of BEFV in Egypt by Khalil *et al.,* (2001). As well, a labeled DNA probe was improved by Zaghloul *et al.,* (2012) for identification of the BEFV in the Egyptian farms without the need of RNA extraction by using dot-blot hybridization technique.

In the Middle East, the epidemic form of BEF disease has only been reported in Israel, Iran, Iraq and Saudi Arabia. The potential occurrence of ephemeral fever in the Eastern Mediterranean region was expected during the period 1989-1991. The ecoclimatic barrier of the disease has been investigated to be related to 38 ON latitude as the disease has not been reported north of this latitude (Tanaka & Inaba, 1986) except in China where it was reported at 44 ON (Zhang *et al.,* 1999). Clinical cases of ephemeral fever have not yet been reported in Europe, although antibody against BEFV has been detected in serological surveys in southern Russia. Many epidemic outbreaks of BEF disease occurred in Egypt and spread throughout the whole of the Nile Valley and Delta (Piot, 1896 & 1909; Rabagliati, 1924). The epidemic BEF disease appeared in most of Egyptian governments during 1990 and 1991as Aswan, Quena, Sohag and Assiut in Upper Egypt. Later to these years, more cases showed the signs the disease with a shorter duration, and lower production losses. Morbidity rates in individual herds of imported cattle varied from (20-90%) with a mortality rate from (1.5-3 %). The animals most likely to die were the largest, heaviest and highest producers. Thus the most valuable animals in the herd appeared to be the most susceptible to ephemeral fever.

Failure of the vaccine to protect animals against BEFV is probably due to the low average vaccination rate and/or amino acid alterations in antigen sites. Correct prediction of outbreak time or a booster vaccination done in late summer would be better for controlling the disease (Wang *et al.,* 2001). The aims of this study were isolation and adaptation of the viral agent in suckling mice, serological detection of the virus in the brain of suckling mice expressed the signs of the BVEF after three passages and molecular characterization of the circulating virus in Egypt during the outbreak of 2015. The results are required for the epidemiological study of the disease and useful for further research to manufacture a potent vaccine against the virus in Egypt.

MATERIALS AND METHODS

Blood samples collection

Twelve blood samples with anticoagulant, from sporadic cattle suspected to be infected by BEFV in Dakahlyia province during the summer of 2015, were collected during the febrile phase of the disease. Blood samples were centrifuged; buffy coats cells were separated and suspended in 4 volumes of sterile 0.2% NaCl to lyse erythrocytes then, after one minute, 7.2% NaCl was

added to reconstitute isotonicity. The cells were further washed in phosphate-buffered saline (PBS) and stored at -80^{o}C for virus isolation in suckling mice according to Van Der Westhuzen, (1967).

Virus isolation by intracerebral inoculation of suckling mice

Groups of suckling mice (3 day-ages) were divided for infection study of seven blood samples (5 mice per each group) and the control group (5 mice) (Figure 1A). The leukocytes cells were frozen, thawed three times for the liberation of the virus and centrifuged at 2000 x g for 5 min. 20 µl of the supernatant of blood samples were inoculated intracerebrally into the suckling mice (Figure 1B&C), while the control group was intracerebrally inoculated with PBS. Three passages of the inoculated brains of suckling mice were performed in suckling mice when clear clinical signs were observed.

Detection of the virus in the brain of mice by indirect immuno-fluorescence technique

Frozen sections, of the brains of the inoculated suckling mice showed clear clinical signs and control mice, were fixed by cold acetone for 10 min. and incubated with hyperimmune serum against BEFV raised in rabbit kindly provided by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt for one hour at room temperature. All slides were carefully washed by PBS and incubated with fluorescent isothiocyanate (FITC) conjugated anti-rabbit immunoglobulins for one hour in a dark humid chamber at room temperature according to Payment and Trudle, (1993). Carful washing of slides was performed and microscopically examined by fluorescent microscope.

Viral RNA isolation and reverse transcription to cDNA

Five blood samples of susceptible infected cattle by BEF disease were directly examined by molecular techniques. Viral RNA was extracted from the buffy coats using extraction kits (Qiagen, Hilden, Germany), according to manufacturer's protocol. The purified RNA was subjected to reverse transcription using RevertAid™ kits (Thermo) to cDNA synthesis as follows: 6 µl of extracting RNA, 1 µl Oligo (dT) primer and 5 µl DEPC-treated water heated at 65 °C for 10 minutes and cooled on ice. Then, 4 µl 5X Reaction buffer, 1 µl Ribolock™ Rnase inhibitor (20u/µl), 2 µl of 10 mM dNTP Mix, 1 µl RevertAid™ M-MulV Reverse Transcriptase (200 u/µl) were added to the solution to a final volume of 20 µl.

Polymerase Chain Reaction and gel electrophoresis

The resulting cDNA was amplified by polymerase chain reaction (PCR) using PCR PreMix (BIONEER), in which 2 µl of cDNA was mixed with 10 pmol of each forward: 5'-ATGTTCAAGGTCCTC ATAATTACC-3' (nt 1–24) and reverse: 5'-AATGATCAAAGAACCTATCATCAC-3' (nt 1874–1871) (Wang *et al.*, 2001) and dionized water to a final volume of 20 µl to amplify the G gene. Thermal cycling condition was initial denaturation at 95 °C for 5 min. then 35 cycles of 95 °C for 40 s, 52 °C for 40 s, 72 °C for 2 min and 30 s and final extension of 72 °C for 10 min. All PCR products were run on an agarose gel (1.5%) stained with ethidium bromide and amplicons were examined by ultraviolet transilluminator.

RESULTS

Virus isolation in suckling mice

The virus was isolated successfully in suckling mice for five buffy coats samples from the examined seven samples. Clinical signs including paralysis and stiffness in hind legs (Figure 2A) were detected at 6th day post inoculation with 21 dead mice from 35 inoculated mice in the first passage, increased to be 28 dead mice from 35

inoculated mice at the fifth day post inoculation in the second passage. In the third passage, all inoculated mice were dead in the fourth day post inoculation (Table 1). No clinical signs and death were observed in the control groups (Figure 2B).

Detection of the viral antigen of the brain of inoculated mice by indirect immunofluorescence technique

The viral inoculated brains of the third passages expressed fluorescent greenish coloration when examined under ultraviolet rays The application of indirect Fluorescent antibody technique (FAT) for BEFV antigen on smears from brain of positive mice appeared the form of greenish yellow fluorescence intracytoplasmic granules in the cytoplasm of the infected cell (Figure 3A). The brain sections of control mice showed no fluorescence staining (Figure 3B).

Molecular detection of BEFV in buffy coats by RT-PCR

Five field buffy coats were directly examined by RT-PCR reactions were successfully carried out. The reaction amplified a 470 bp product spanning part of G gene (G2). It produced a clear single band on agarose gel stained with ethidium bromide corresponded to 470 bp in length (Figure 4). Moreover, in the comparison study between Viral isolation in suckling mice, FAT and RT-PCR techniques for BEFV identification, it was obviously that PCR technique is more accurate, sensitive and rapid diagnostic method than other used techniques for detection of BEF disease.

Table 1: Isolation of BEFV in suckling mice

No. of passages	Mortality rate (%)	Average No. day of death
First	65	6^{th} day post inoculation
Second	80	5^{th} day post inoculation
Third	100	4^{th} day post inoculation

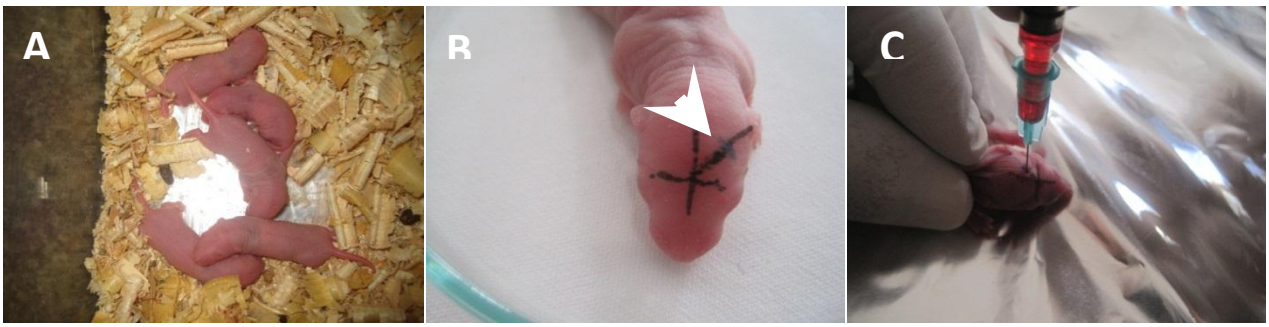

Figure 2: Intracerebral inoculation of suckling mice by BEFV**.** A: Suckling mice of 3 day-ages B: The site of intracerebral inoculation. C: Vertical inoculation of field samples in the brain of suckling mice

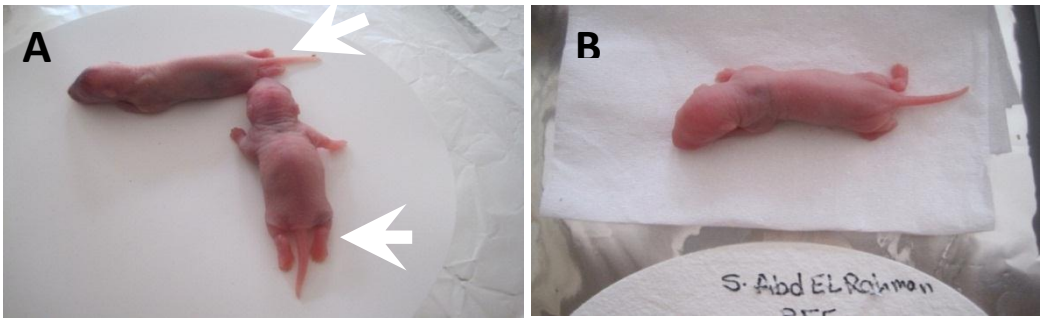

Figure 3: Clinical signs observed in inoculated mice. A: Paralysis of suckling mice (white arrow). B: Control mice

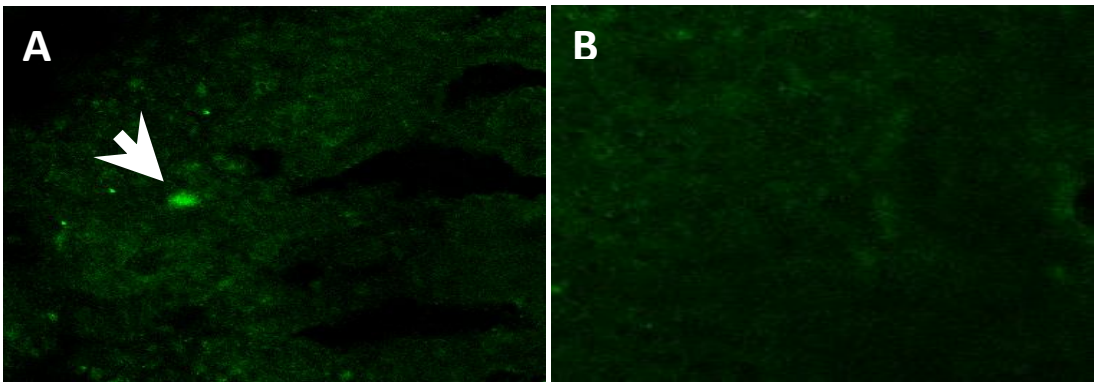

Figure 4: Detection of BEFV antigen in the brain of inoculated suckling mice by indirect immunofluorescence technique. A: Viral antigen in the brain of mice (white arrow). B: Control brain

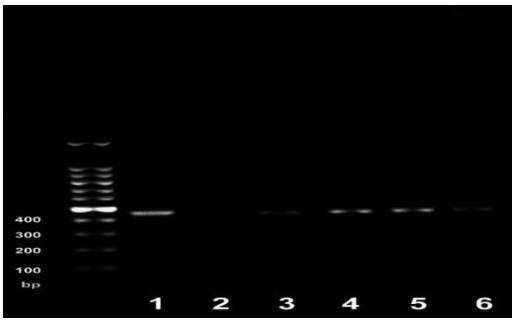

Figure 5: Gel electrophoresis for G gene of BEFV (amplicon of 470pb). Lane 1: Reference BEFV, Lane 2: Control negative, Lane (3-6): field isolates of BEFV.

DISCUSSION

Bovine ephemeral fever is one of the most important infectious diseases of live-stock in Egypt. Differential diagnosis of BEF based on clinical observation is sometimes difficult and complicated. There- fore, tentative diagnosis is essential and necessary to implement urgent and proper prophylactic action, evaluation of the extent of the disease and further epidemiological studies. The cases suspected of BEFV in the present study were sporadic in occurrence in Dakahlyia governorate. BEF virus was isolated from leukocytes of naturally infected cattle by intracerebral inoculation of baby mice and the obtained results were in agreement with those obtained with van der Westhuizen, (1967) who found that successful growth of BEF virus was achieved by inoculating leukocyte from a cow with clinical disease intracerebrally into suckling mice 1-3 days old. That isolation of BEFV in baby mice was more sensitive than isolation in vero cells (Bastawecy *et al.*, 2009), they observed that 16 samples were positive when they were inoculated intracerebrally in baby mice and identified with IFAT on their impression smears but they neither gave CPE in vero cells nor immunofluorescence technique, this could be explained due to presence of defective interfering BEFV particles in cell culture make autointerference (Tzipori, 1975). Isolation was confirmed by immunofluorescence staining in smears of mice brain inoculated by the field isolates because not all BEF strains produce CPE and the presence of virus is generally demonstrated by immunofluorescence (St. George *et al.*, 1977). The application of FAT on the positive isolates from the baby mice infected with the suspected material of BEFV revealed its ability to detect the virus in the tissues of suckling mice more than the field samples, this may be due to

the successful laboratory propagation of the virus in the brain cells and higher virus yield comparable to the field samples. As well, the processing of buffy coats preparation before FAT may cause cellular damage of the leukocytes and reduce the number of virus particles. Application of serological examinations for diagnosis of *rhabdoviruses* considered as a field problem because of the antigenic relationships between the members of this group (Calisher *et al.*, 1989). RT-PCR has become an important method for pathogen detection because of its advantages as no need for high virus particles titers, short time required for processing, specific and valuable test (Khalil *et al.*, 2001). The examined samples showed high percentages of diagnosis by RT-PCR, and so recommended for rapid field diagnosis of BEF disease.

This study represents an accurate and reliable diagnosis of BEFV in Dakahlia governorate in Egypt. The isolation of BEFV on suckling mice was successfully set up; using of immunofluorescence test is a suitable tool for viral antigen detection in the brain of positively infected suckling mice. Using of molecular approach for detection of the widely spread viral agent (RT-PCR) as a field diagnostic method is useful for rapid and fast identification of the virus. Further genomic sequencing and characterization of BEFV is required for raising our knowledge about the epidemiology of the virus in Egypt and the world. These viruses can also be applied for manufacturing an appropriate domestic vaccine against the virus and are also efficient for seroepidemiologic studies and controlling of the disease in Egypt.

REFERENCES

Bastaweesy, I.M.; Mahmoud, N.A.; Eweis, M. and Salem, S.A.H. (2009): Evaluation of different diagnostic techniques of *bovine ephemeral fever virus* in cattle Egypt. *J. Comparative Pathology and Clinical Pathology*, **23** (2): 7-17.

Burgess, G.W. (1974): A micro-titer serum neutralization test for *bovine ephemeral fever virus*. *Aust. J. Exp. Biol. Med. Sc.,* **52**: 851-855.

Calisher, C.H.; Karabatsos, N.; Thompson, L. and Smith, J.S. (1989): Protection against *rabies virus* conferred by immunizing mice with other *rhabdoviruses* and Preliminary evidence of molecular similarities between bovine ephemeral fever related viruses and rabies related viruses." In *Arbovirus* research in Australia; Preceding 5th Symposium

Cybinski, D.H.; Walker, P.J.; Byrne, K.A. and Zakrzewski, H. (1990): Mapping of antigenic sites on the *bovine ephemeral fever virus* glycoprotein using monoclonal antibodies. *J Gen Virol.*, **71**: 2065–2072.

Inaba, Y. (1973): Bovine ephemeral fever (three day sickness) stiff sickness. *Bull. Off. Int. Epiz.*, **79**: 627-673.

Khalil, S. A.; Khadr, A. M.; Zaghawa, A. and Akela, M. A. (2001): Application of PCR and immunoperoxidase for diagnosis of *bovine ephemeral fever virus* in Egypt during summer 2000. Proceeding of the 6th Sci. Cong., Egyptian Society for Cattle Diseases, Egypt. P: 135- 140.

Nandi, S. and Negi, B. S. (1999): Bovine ephemeral fever: a review. *Comparative Immunology, Microbiology and Infectious Diseases*, **22**: 81- 91.

OIE, (2008): Terrestrial Animal Health Code.

Payment, P. and Trudel, M. (1993): Methods and techniques in Virology. Marcel Dekker, New York.

Piot, J.B. (1896): Epizootic of dengue fever in cattle in Egypt. Prix National Academy of Medicine, France. Cited by Sahar, M.M. **– (1909)**: Department

of Public Health, Cairo, Annual Report, p: 101.

Radostitis, O.M.; Gay, C.G.; Hinchcliff, K.W. and Constable, P.D. (2007): Veterinary Medicine. 10th edition, Sounders/ Elsevier, Philadelphia.

Rabagliati, D.S. (1924): Three days fever of stiff sickness in cattle. *Vet. Rec*., **4** (23): 503-505

Snowdon, W.A. (1970): Bovine ephemeral fever: the reaction of cattle to different strains of *ephemeral fever virus* and the antigenic comparison of two strains of virus. *Aust. Vet. J*., **46**(6):258-66

St. George, T.D., Standfast, H.A.; Chrisie, D.G.; Knott, S.G. and Morgen, I.R. (1977): The epizootology of bovine ephemeral fever in Australia and Ppua New Guinea. *Aust. Vet. J.*, **53**: 17-28.

Tanaka, Y. and Inaba, Y. (1986): Epidemiology and economics of ephemeral fever in Japan. *Arbovirus* researchin Australia. Proceedings Fourth Symposium May 6-9, 1986, Brisbane, Australia, 276-279.

Tzipori, S. (1975): Plaque assay and characteristics of strains of bovine ephemeral fever virus in Vero cells. *Br. Vet. J.*, **131**: 720-726.

Uren, M.F.; George, M.F. and Murphy, G.M. (1992): Studies on the pathogenesis of bovine ephemeral fever in experimental cattle. III. Vi-rological biochemical data. *Vet. Microbiol.,* **30**: 297-307.

Van der Westhuizen, B. (1967): Studies on bovine ephemeral fever. 1. Isolation and preliminary characterization of a virus from natural and experimentally produced cases of bovine ephemeral fever. *Onderstepoort J. Vet. Res*., **34** (1): 29-40.

Walker, P.J.; Byrne, K.A.; Cybinski, D.H.; Doolan, D.L. and Wang, Y. (1991): Proteins of bovine ephemeral fever virus. *J Gen Virol.*, **80**: 1211–1220.

Walker, P.J.; Blasdell, K.R. and Joubert, D.A. (2012): *Ephemeroviruses*: arthropod-borne *rhabdoviruses* of ruminants, with large and complex genomes. In: Dietzgen RG, Kuzmin IV (Eds) *Rhabdoviruses*: Molecular Taxonomy, Evolution, Genomics, Ecology, Cytopathology and Control. Caister Academic Press, Norfolk, pp. 59–88.

Wang, F.I.; Hsu, A.M. and Huang, K.J. (2001): Bovine ephemeral fever in Taiwan. *J Vet Diagn Investi.*, **13**: 462–467.

Zaghawa, A.; Khalil, S.A.; Hassan, H.Y. and Al-Gabary, M.H. (2002): Diagnostic value of the antigenic relationship between b*ovine ephemeral fever and Rabies viruses*. 2nd Scientific Vet. Med. Congress. Fac. Vet. Med. Sadat City, Menufia University, 23-24 April, 2002.

Zaghloul, A.H.; Mahmoud, A.; Hassan, H.Y.; Hemeida, A.A; Nayel, M.A. and Zaghawa, A. A. (2012): Establishment of dot-blot hybridization for diagnosis of *bovine ephemeral fever virus* in Egypt. *International J. Virol.*, **8** (3): 271-278.

Zhang, Y.; Wang, X.; Yun, L.; Jiang, C. and Jin, R. (1999): Epidemiological investigations of bovine ephemeral fever in Jilin Province. The 1st International Symposium on *Bovine Ephenteral Fever and Related Rhabdoviruses*, 25-27 August, 1999, China, pp: 117-119.