Global Veterinaria 6 (5): 447-452, 2011
ISSN 1992-6197
© IDOSI Publications, 2011

# Investigations on Bovine Ephemeral Fever Virus in Egyptian Cows and Buffaloes with Emphasis on Isolation and Identification of a Field Strain

[1]Kawther S. Zaher and [2]Wahid M. Ahmed

[1]Department of Microbiology and Immunology, National Research Centre, Giza, Egypt
[2]Department of Animal Reproduction and AI, National Research Centre, Giza, Egypt

**Abstract:** A total number of 160 local Egyptian cows (N=60) and buffalo cows (N=100) raised at Al Sharkia governorate was clinically examined during May-October 2010. Samples from blood and nasal swabs were collected from healthy animals as well as from animals showing clinical signs similar to those caused by Bovine Ephemeral fever (BEF) infection. These animals showed fever, reduced appetite, decrease milk production, ceased ovarian activity and abortion. The signs were more obvious in younger than older animals and in cows than in buffaloes. The virus was isolated on both Vero and BHK-21 cells. Infected cells with Bovine Ephemeral fever Virus (BEFV) showed 80% CPE after 24 h from the $6^{th}$ virus passage. The isolated virus was proved to be BEFV by neutralization test, electron microscopy, immunofluorescence antibody test and RT-PCR. All positive samples of Antibody neutralization and IFT tests of BEFV gave band at 1965bp during amplification of G gene.

**Key words:** Bovine Ephemeral fever · BEF · RT-PCR · IFT · NT · Electron microscopy

## INTRODUCTION

Bovine ephemeral fever (BEF) also known as Three Day Sickness is an acute and disabling arthropod vector-borne viral infection of cattle and water buffalo caused by the *Ephemerovirus* virus of the *Rhabdoviridae* family. The BEF is a bullet or cone shaped virion which consists of a negative, single stranded RNA genome with a lipid envelope and 5 structural proteins. The envelope glycoprotein G contains type-specific and neutralizing antigenic sites. The virus is transmitted by an insect vector. The particular species linked to the virus are the biting midges *Culicoides oxystoma* and *C. nipponensis* [1].

The characteristics of the disease are the sudden onset of fever, stiffness, lameness, nasal and ocular discharges, depression, cessation of rumination and constipation. It usually caused the bulls to be temporarily infertile. During fever, milk production almost ceased and the milk quality became poor. Lactation usually resumed on recovery, but the loss of milk production in a natural epizootic can be as high as 12% of the lactation in dairy cows [2].

In Egypt, the disease was first described in 1895 by Piot, 1924 by Rabagliati and subsequent outbreaks have been occurred in summer of 1991, 2000, 2001 and 2004. In summer 1991, a typical form of the disease has been recorded in different governorates in Lower Egypt .A second outbreak of BEF occurred in summer 2000, whereas it included several governorates in Lower and Upper Egypt and characterized by 50% morbidity and 2.5% mortality [3,5]. In the Near East, the disease has been reported in Jordan, Israel, Syria, Iran and Iraq. Two suspected outbreaks of bovine ephemeral fever have occurred in the Kingdom of Saudi Arabia [6,8].

The aim of this study was to investigate the effect of infection with BEFV in cows and buffaloes in Egypt. Special emphasis were given for carrying out accurate procedures for isolation and identification of the virus also to express a protein that is specifically for BEFV and suitable for our local strain using RT PCR.

## MATERIALS AND METHODS

The present study was carried out during the period from May to October 2010 on a total number of 160 heads of Egyptian cows (N=60) and buffalo-cows (N=100). Some of these animals showing clinical symptoms suggesting infection with BEF. These animals raised at Al Sharkia governorate (Lower Egypt) in the form of small holders

**Corresponding Author:** Kawther S.A. Zaher, Department of Microbiology and Immunology, Veterinary Research Division, National Research Centre (NRC), Postal code: 12622,Giza, Egypt. E-mail: zaherkus@yahoo.com.

farms, during different reproductive parities, received non sufficient nutrition and subjected to no regular health caring programs. Case history was recorded and animals were clinically inspected. Gynecological examination was carried out aided by ultrasonography (Pia Medical Falcs e`Saote, the Netherlands) with an endorectal linear array 6-8 MHz transducer.

**Sampling:** Samples of blood and nasal swabs were collected from healthy animals as well as from cows and buffaloes showing clinical symptoms similar to those caused by BEFV infection, mostly fever, anorexic and depression during a natural outbreak which occurred during the summer months (May to October 2010).

**Reference BEFV:** Reference BEFV and sera was kindly supplied from Abbasya Vaccine and Sera institute, Cairo, Egypt.

**Virus Isolation:** Swabs were prepared in Eagle's MEM (Gibco Co) containing streptomycine (0.1mg/ml) and gentamycine (0.1mg/ml). After centrifugation at 4°C for 20 minutes, the supernatants were collected and stored at-70°C for virus isolation. Heparinized blood samples were centrifuged at 3000 rpm for 10 minutes and the plasma was collected. The blood cell layer was then resuspended and washed in phosphate-buffer saline (PBS) for three times as previously described. After washing, the blood cell layer was again resuspended in PBS and stored at-70°C for virus isolation. After freezing and thawing, the blood samples and the supernatants of the homogenates were inoculated into cell cultures for virus isolation. The BHK-21 (baby hamster kidney continuous cell line) and Vero (green monkey kidney continuous cell line) were used for virus propagation. The monolayer cell culture, freshly prepared in 25cm$^2$ flasks, was individually inoculated with the samples. After incubation at 37° C for 5-7 days, each culture fluid was inoculated into another freshly prepared cell culture. This culture passages were repeated at least three times. Virus isolation was determined by the appearance of a cytopathic effect (CPE) in the culture [9, 11].

**Virus Assay:** Virus assay was conducted in Vero cells and BHK-21 cells as described by Snowdon [12] and the titers were expressed as 50% tissue culture infectious doses (TCID $_{50}$). BEF virus was also assayed and calculated by the method of Reed and Muench [13].

**Neutralization Tests:** Neutralization tests were performed by a technique similar to that previously described [14, 15]. Serial two-fold dilutions of heat-inactivated serum (56°C for 30 min) were mixed with an equal volume of cell culture fluid containing 100 TCID $_{50}$ of BEF virus. After incubation for 60 min at 37°C each mixture of virus and serum was used to inoculate at least four tube cultures of Vero cells. The highest serum dilution preventing cytopathic change in at least half the culture was taken as the endpoint. A titration of the test virus was included in each assay.

**Virus Purification:** Infected cell culture fluid was clarified [9] by centrifugation at 5000 r.p.m, for 30 min .Virus was pelleted from the supernatant fraction at 30000 r.p.m, for 90 min and resuspended in a small volume of 120 ml NaC1, 1.2ml EDTA, 0.1% BSA, 12 ml Tris-HC1 pH7.6 (NTE/BSA). The pelleted virus was applied to a 15% to 45% (w/v) sucrose gradient in NTE/BSA and centrifuged at 40000 r.p.m, for 30 min (Beckman SW41 Ti rotor). Two visible bands were detected in gradients; the upper band contained non-infectious truncated particles. The lower band, containing infectious virions, was diluted in two volumes of NTE/BSA and centrifuged at 70000 r.p.m, for 10min (Beckman TLA 100-3 rotor). The virus pellet was resuspended in a small volume of NTE/BSA, applied to a 15% to 60% (w/w) sucrose gradient in NTE/BSA and centrifuged to equilibrium density at 40000 r.p.m, for 16 h. The virus band was then diluted in two volumes of NTE/BSA and the virus was pelleted at 70000 r.p.m. for 10 min (Beckman TLA 100.3 rotor).

**Observation of the Virus by Electron Microscopy:** The purified virus was mounted on grids and negatively stained in 2% phosphotungstic acid and observed under electron microscope [16].

**Immunofluorescent Antibody (IFA) Test:** 100 TCID $_{50}$ of BEF virus was inoculated to 16-well Tissue culture plate contained confluent BHK-21 cell monolayer. The plate was incubated at 37°C with 5% $CO_2$. When CPE was observed in these wells, the supernatant was removed and the cell sheet was fixed in cold acetone for 20 min at-20°C and stained by an IFA Technique [17].

**Identification of the Virus by RT-PCR:** Commercial kit was used for extraction of BEFV RNA, namely RNA isolation kit (Qiagen Co., Valencia, CA, USA). RNA was extracted from BEFV-infected cultured cells according to manufacturer's protocol. A series of 10-fold dilutions were

made, by diluting the RNA with diethylpyrocarbonate (DEPC)-treated water. Then the cDNA was synthesized using approximately 1 μg of the total RNA with primer F (5`CCT CAC AAT GTT CAA GGT CCT C 3`). The cDNA was amplified by *Taq* polymerase with primers F (5` GCA GGA ACA TGA TTG CCC TGT T 3`) in order to obtain BEFV G gene [18]. One negative control containing the PCR mixture without the template was included. After completion of the PCR, 5 μl of the reaction mixture were loaded onto a 1.5% agarose gel, containing 5μg/ml ethidium bromide, for electrophoresis and subsequent visualization by UV transillumination [19].

## RESULTS

The clinical signs of BEF were identical in all the examined cases showing symptoms (40 out of 60 cows, 66.67% and 39 out of buffaloes; 39%). The duration of the disease extended from 3 to 6 days after which the animal may be returned to a normal state of health. Affected animals displayed transient anorexia, hyperpnoea, a sudden severe reduction in milk yield with pink discoloration of milk in some cases, fever (40-41°C) lasting for 3 days, seromucoid nasal discharge and occasionally lacrimation was observed and the nostrils were hyperemic and widely opened during respiration with labored respiration. The clinical signs were very clear in infected cows rather than buffaloes, which showed mild to moderate fever, anorexia, labored respiration and sudden reduction of milk yield (Fig. 1).

In general, the clinical signs of the disease were more obvious in younger than older animals and in cows than in buffaloes. Rectal examination aided by ultrasonography of affected cases revealed complete cessation of ovarian activity in 100% of cows (40) and 76.92% of buffaloes( 39) showing clinical symptoms of BEF. Some cases (3) of abortion occurred in pregnant cows only.

**Virus Isolation:** CPE showed after the 3rd passage on both cells Vero cells was very sensitive to BEFV while CPE were clearer in BHK-21 cells. In the 4th passage and the 5th passage CPE was seen 48 h after infection and approximately 50-60%. From the 6th passage to the tenth passage, CPE was observed 24 h post-infection and 80% (Fig. 2-3).

**Neutralization Test:** The titers of the virus isolate were $10^6$ $TCID_{50}$ $ml^{-1}$. The standard positive serum to BEFV with 1:64 dilution could neutralize completely the virus isolate.

Fig. 1: Show perfuse lacrimation in the left photo and depression and slight lameness in the right photo

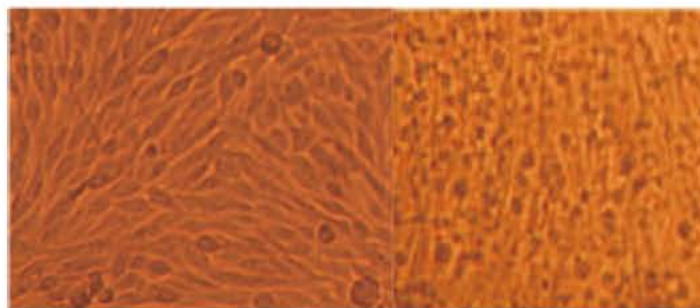

Fig. 2: CPE in BHK-21 cells inoculated with suspected virus. Normal BHK-21 cells is on the left, while BHK-21 cells showing CPE on the left

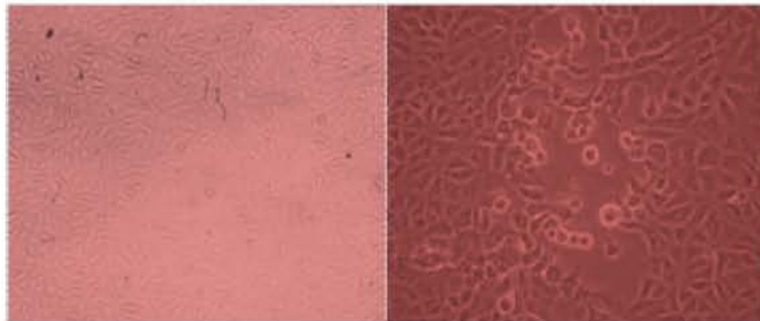

Fig. 3: CPE in Vero cells inoculated with suspected virus. Normal Vero cells is on the left, while Vero cells showing CPE on the left

Fig. 4: Electron micrograph of the virus isolate. Original magnification X 100000.

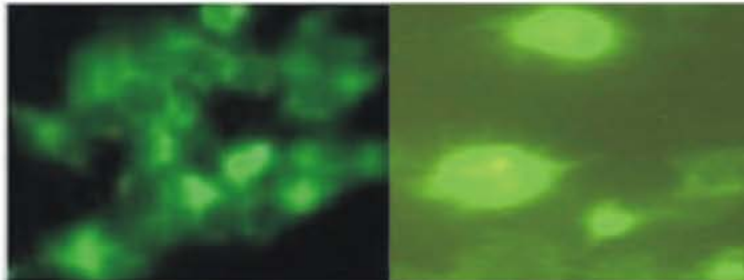

Fig. 5: Positive results of IFA test where specific fluorescence was produced in infected BHK-21 cells

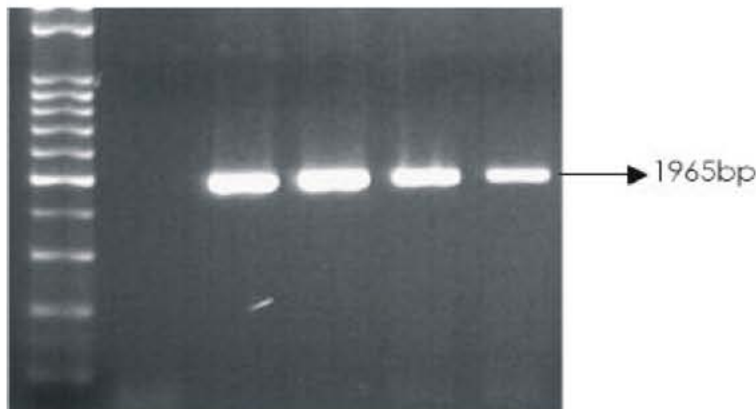


Fig. 6: Agarose gel electrophoresis of PCR-amplified cDNA fragment (1965 bp) using BEFV genomic extracted RNA. Lane 1at the left: DNA ladder molecular weight marker; lane 2: negative control; lanes 3-6 possitive band at 1965bp.

**Electron Microscopic Examination:** The virus particles were observed from purified virus particles from infected cell cultures, which showed bullet-like shape and had spikes on the surface of the envelope protein and were identical to BEFV`s shaped (Fig. 4).

**IFT Test:** Infected cells showed very intense fluorescence throughout the cell sheet. At the same time there was no fluorescent staining in the control non infected cells (Fig. 5).

**Identification of the Virus by RT-PCR:** The aim gene obtained by RT-PCR was G gene of BEFV which was 1965bp in size. All positive samples of Antibody neutralization and IFT tests of BEFV gave band at 1965bp (Fig. 6).

## DISCUSSION

Ephemeral fever is usually diagnosed from history and clinical signs. A diagnosis can be made from the sudden onset of febrile reactions lasting for 2-5days with spontaneous recovery. The seasonal occurrence and symptoms of oropharyngeal secretions, joint pains and stiffness are of value. However, a confirmatory diagnosis can be obtained by isolation of BEF virus from blood taken into heparin or EDTA anti-coagulant during fever and demonstration of rising titer of neutralizing antibodies sera collected during illness and two or three weeks later. The virus can be isolated by inoculating the virus particles in blood from clinically affected cattle to suitable tissue cultured cells. Serological diagnosis can be complicated by the previous infection of antigenically related virus such as Kimberley virus. Kimberley virus infection is subclinical and causes the development of low titter of serum neutralizing antibodies to BEF virus without conferring any protection against BEF virus. A prior infection with Kimberley virus sensitizes the cattle so that a secondary instead of primary antibody response occurs on first exposure to BEFV [20].

In this study blood samples were collected from cattle showing clinical signs during summer season 2010 in Al Sharkia governorate. Then the field strain of the virus was isolated by inoculation in both Vero and BHK-21 cells. The results of NT, IFA, electron microscopic examination and RT-PCRtests all showed the virus is BEFV. On the other hand, other members of Rhabdoviridae such as Kimberley virus has not been reported in Egypt. This indicates that NT is very suitable test for BEFV diagnosis. This conclusion meets with othr investigators [21, 22]. Since 1924 and the virus attacks Egpytian cattle in summer season. Most of the time the disease passes without noticing, but the disease exsist more than 80 years and vaccination program seams to be inadequate to illiminate the disease.

In conclusion The study reported that BEFV negatively afecting the productivity and reproduct6ivity of large farm animals . NT, IFA, electon microscopic examination and RT-PCR are the most effective tests for virus diagnosis.Also, ampliefication of G gene is effective for BEFV diagnosis.

## REFERENCES

1. Dhillon, J., J.A. Cowley, Y. Wang and P.J. Walker, 2000. RNA polymerase (L) gene andgenome terminal sequences of ephemeroviruses bovine ephemeral fever virusand Adelaide River virus indicate a close relationship to vesiculovirus. Virus Research, 70: 87-95.
2. Wang, J.Y., L.N. Lee, C.S. Chou, C.Y. Huang, S.K. Wang, H.C. Lai, P.R. Hsueh and K.T. Luh, 2004. Performance assessment of a nested PCR assay (the RAPID BAP-MTB) and the BD ProbeTec ET system for detection of Mycobacterium tuberculosis in clinical specimens. J. Clin. Microbiol., 42: 4599-4603.
3. Abuelyazeed, A. EL-Sheikh, 2006. Studies on Bovine Ephemeral Fever virus, PhD thesis, Department of Virology, Faculty of Veterinary Medicine Zagazig University.
4. El-Sawalhy, A.A., 1999. Bovine ephemeral fever In: Veterinary infectious diseases 2nd ed. Al-Ahram Dist. Agency, Egypt.
5. Rabagliati, D.S., 1924. Three days' fever or sickness in cattle. Vet. Rec., 4: 503-5.
6. Boorman, J., 1989. Culicoides (Diptera: Ceratopogonidae) of the Arabia peninsula with notes on their medical and veterinary importance. Fauna Saudi Arabia, 10: 160-224.
7. Burgess, G.W., 1971. Bovine ephemeral fever: a review. Vet. Bull., 41: 887-895.
8. Yeruham, I., M. Van Ham, Y. Stram, O. Friedgut, H. Yadin, K.Y. Mumcuoglu and Y. Braverman, 2010. Epidemiological investigation of bovine ephemeral Fever outbreaks in Israel. Vet. Med. Int., 7: 290541-52.

9. Walker, P.J., K.A. Byrne, D.H. Cybinski, D.L. Doolan and Y. Wang, 1991. Proteins of ephemeral fever virus. J. General Virol., 72: 67-74.
10. Wang, Y.S., Z.C. Wang, Y.D. Tang, Z.L. Shi, K.W. He, Y. Li, J.B. Hou, H.c. Yao, H.J. Fan and C.P. Lu, 2007. Comparison of 4 infectious bursal disease virus isolated from different bird species. Arch. Virol., 152: 1787-1797.
11. Tan, J.M., J. Cook, N. Clarke and Tannock, 2008. Optimization of methods for isolation of Merek`s disease virus in primary chicken cell cultures. J. Virol. Methods, 147: 312-318.
12. Snowdon, W.A., 1970. The reaction of cattle to different strains of ephemeral fever virus and the antigenic comparison of two strains. Australian Veterinary Journal, 46: 258-66.
13. Reed, L.J. and H. Muench, 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene, 27: 493-7.
14. Wakeley, P.R., J.L.E. Turner, G. Ibata, D.P. King, T. Sandvik, P. Howard and W.T. Drew, 2004. Characterization of a type 2 bovine viral diarrhoea virus isolated from cattle in the UK. Vet. Microbiol., 19: 19-24.
15. Xu, C.T., W.X. Fan, R. Wei and H. Zhao, 2004. Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003 (H9N2) virus. Microbes Infection, 6: 919-925.
16. Gard, J.A., M.D. Givens, K.P. Riddell, P.K. Galik, Y. Zhang, D.A. Stringfellow and M.S.D. Marley, 2007. Detection of Bovine viral Diarrhea Virus (BVDV) in single or small groups of preimplantation bovine embryos. Theriogenology, 67: 1415-1423.
17. Chua, K.B., C.L. Koh, P.S. Hooi, K.F. Wee, J.H. Khong, B.H. Chuna, Y.P. Chan M.E. lim and S.K. Lam, 2002. Isolation of Nipah virus from Malaysian Island Flying-foxes. Microbes Infect., 4: 145-151.
18. Guan, Y., B.J. Zheng, Y.Q. He, X.L. Liu, Z.X. Zhuang, C.L. Cheung, S.W. Luo, P.H. Li, L.J. Zhang, Y.J. Guan, K.M. Butt, K.L. Wong, K.Y. Yuen, J.S.M. Peiris and L.L.M. Poonl, 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science, 302: 275-278.
19. Wang Fun-In, A., M. Hsu and K.J. Huang, 2001. Bovine ephemeral fever in Taiwan. J. Vet. Diagn. Invest., 13: 462-467.
20. Tomori, O., A. Fagbarni and G. Kemp, 1974. Kotonkan virus: Experimental infection of white Fulani calves.Bull. Epiz. Dis. Africa., 22: 195-200.
21. Zheng, F.Y., G. Lin, C.Q. QIU, J.Z. ZHOU, X.A. CAO and X.W. Gong, 2009. Isolation and charactrization of field strain of Bovine Ephemoral Fever in China. J. Anim. Vet. Advances, 8: 1478-1483.
22. Zheng, F.Y., G. Lin, C.Q. QIU, J.Z. ZHOU, X.A. CAO and X.W. Gong, 2010. Serological detection of bovine ephemeral fever virus using an indirect ELISA based on antigenic site G1 expressed in Pichia pastoris. Vet J., 185:211-5.