

Electron Microscopic Characterization of Bovine Ephemeral Fever Virus

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Abstract: Bovine ephemeral fever virus (BEFV) isolates after their identification and confirmation by virus neutralization test (VNT) were subjected for characterization with negative staining electron microscopy (EM) before and after improving its performance with BEFV antibodies binded to *Staphylococcus aureus* protein A (SPA) using *Staphylococcus aureus* protein A-coagglutination ultrastructure analysis (SPA COA-EM). Negative staining EM could detect rhabdoviral particles, ranged from bullet to blunt cone shaped with different lengths. Aggregates of rhabdoviral particles coated with the specific antibody were observed in addition to their attachment to the surface of *Staphylococcus aureus*. It is concluded that negative staining EM and SPA COA-EM could characterize BEF viral particles following their isolation in green monkey kidney (vero) cells. Further studies are recommended to find relationship between forms of the BEF viral particles and cytopathic effect (CPE) in cell cultures of different passages using SPA COA-EM. These future studies may offer answer to why the antigenicity and pathogenicity of BEFV rapidly lost on passaging in suckling mice or cell cultures.

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1. Introduction

Bovine ephemeral fever (BEF) is a non contagious arthropode-borne disease of cattle and water buffaloes caused by the bovine ephemeral fever virus (**Nandi and Negi, 1999**).

Bovine ephemeral fever virus (BEFV) is a single stranded RNA of the genus Ephemerovirus in the family Rhabdoviridae. It mainly occurs in only one serotype (**ST.George, 1998**). There is no evidence of immunogenic diversity within the BEFV population, but antigenic variation has been demonstrated using panels of monoclonal antibodies and by epitope mapping (**Richmond, 2008**).

BEF is characterized by the sudden onset of fever, stiffness, lameness and depression with a high morbidity and 1% mortality (**Mackerras et al., 1940**). Recovery usually occurs within 3 to 4 days of the onset of clinical signs, hence the term "ephemeral" (**Uren, 1989**).

The disease is mostly severe in the more valuable classes of cattle such as bulls, pregnant and lactating cows, fat and well-conditioned cattle, hence significant economic losses can occur through loss of condition, decreased milk production, lowered fertility of bulls, miss-mothering of calves, delays in marketing and restrictions on the export of live cattle (**Young and Spradbrow, 1990**) as many countries require cattle and buffaloes free from BEF neutralizing antibodies to be imported from a country where the disease is prevalent. It is a closely affair to

keep the bulls whose semen is to be exported, in insect proof area and to monitor the evidence of BEFV infection continuously (**Nandi and Negi, 1999**).

BEF was firstly described among native cattle in central Africa in 1895 (**Buxton and Fraser, 1977**) and also was firstly described in Egypt (**Piot, 1896 and Rabagliati, 1924**). It mainly occurs in subtropical and temperate regions of Africa, Asia and Australia. The disease occurs in the Middle East (**Israel et al., 2010**) often in sweeping epizootics (**ST.George, 1998**).

Diagnosis is usually made on clinical grounds during major epidemics. Sporadic cases, or those occurring early in a possible epidemic can be confirmed by virus isolation or serology (**Bayer, 1998**). Serological diagnosis can be complicated by the previous infection of antigenically closely related ephemero-viruses fluorescent antibody (FA) and complement fixation (CF) tests.

These viruses such as Kimberley, Adelabde River and Berrimah (**Uren, 1989**). Although neutralization assay and a blocking enzyme linked immunosorbent assay (ELISA) can distinguish BEFV from these related viruses, a prior infection with Kimberley virus sensitize cattle so that a secondary instead of a primary antibody response occurs on first exposure to BEFV (**Nandi and Negi, 1999**).

Isolation of BEFV was achieved by inoculating

suckling mice intracerebrally (**ST.George, 1998**) and cell cultures (**Nandi and Negi, 1999**). The identity of the isolated virus is usually confirmed by immunofluorescence, virus neutralization test (VNT) or electron microscopy (EM). Non specific staining and background fluorescence make fluorescent antibody (FA) detection of antigens subjective and needs careful standardization before the results of the test can be interpreted (**Tuppurainen, 2004**). Reverse transcriptase-polymerase chain reaction (RT-PCR) are used in some countries for diagnosis of BEFV (**Fuying Zheng et al., 2011**) but mutation in the primer target region negate the effectiveness of primers. RT-PCR high cost and relative technical sophistication make it unsuitable for large scale testing. Moreover, RT-PCR will not identify subviral components such as empty virions, which may be produced late in an infection (**Hazelton and Gelderblom, 2003**).

The cell wall of *Staphylococcus aureus* containing protein A (SPA) binds Fc fragments of immunoglobulin G (IgG) of different mammalian species. When specific antibodies are added to a stabilized suspension of Staphylococci, the antibodies bind to the SPA located on the cell wall, thus orientating the Fab-located IgG-combining sites outwards and after mixing with homologous antigens, clumping can be detected with negative staining EM (**Bastawecy and Saad, 2007**).

There is a rapid loss of antigenicity and pathogenicity when BEFV is passaged in suckling mice or cell culture (**Tzipori and Spradbrow, 1973 and Uren, 1989**) because of presence of defective interfering particles (**Della-Porta and Snowdon, 1979**). The presence of interfering particles has posed considerable problems for the purification and characterization of the virus (**Uren, 1989**).

The aim of the present study is the characterization of BEF virions isolated in vero cells with their identification by VNT and negative staining EM before and after improving its performance with BEFV antibodies bind to SPA. Moreover, we will try to know why the antigenicity and pathogenicity of BEFV are attenuated during propagation in addition to benefit from this phenomenon for vaccine preparations.

2. Material and Methods:

Animals:

A total number of 13 cattle of both sex and age ranged between 6 months to 2 years. These animals were of Holstein breeds and belonged to private fattening farm in Sharkia Governorate (Egypt) during summer, 2011.

Samples:

Thirteen heparinized blood samples were collected from clinically diseased animals during febrile phase.

BEF Virus:

BEFV was obtained from Serum and Vaccine Research Institute, Abbasia, Cairo. The titer of the virus 10^6 TCID₅₀/ml.

Control Sera:

Positive and negative sera against BEFV were prepared in rabbits and supplied by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

Tissue Culture:

Green Monkey Kidney (Vero) cell culture was obtained from Virology Department, Animal Health Research Institute, Dokki, Giza.

Staphylococcus aureus suspension:

It was used to improve performance of negative staining EM. It is locally prepared according to **Kessler (1975)** and supplied by Animal reproduction Research Institute, El-Haram, Giza.

Diagnostic Methods:

Buffy coats were separated from the heparinized blood samples and subjected for inoculation in vero cell culture.

Virus isolation:

Vero cell cultures were inoculated with bovine leukocyte suspension according to **Wang et al. (2007)**. The cell cultures should be examined for cytopathic effect (CPE) for 5 days. If no CPE is detected, the cultures should be frozen and thawed 3 times and used for inoculation up to 3 blind passages.

Virus Neutralization Test:

The test was carried out with the virus isolates in vero cell cultures according to micro-neutralization test for BEF (**Wakeley et al., 2004**).

Negative staining electron microscopy:

Preparations of the supernatant from inoculated vero cell cultures showing CPE was mixed with a droplet of 3% phosphotungstic acid (PTA). A copper grid coated with carbon formvar was dipped into the mixture. After drying, the grid was examined by electron microscope (**Gard et al., 2007**).

BEFV coagglutination clumping ultrastructural analysis (SPA CoA-EM):

The test was performed according to **Bastawecy and Saad (2007)**. A drop obtained 5

minutes after mixing 20 µl of the 1:10 dilution of rabbit BEFV antiserum and SPA mixture with an equal amount of viral antigen (obtained from the supernatant of inoculated vero cell cultures showing CPE) was examined by electron microscopy using the same procedure used for negative staining EM.

3. Results:

The results of the present study illustrated that: The tested animals showed febrile reaction, listlessness and difficulty of standing, some of them showed emphysematous swelling in the shoulder, neck and back region in addition to lateral decubency (Fig. 1).

Results of isolation:

Out of 13 inoculated samples, 9 samples revealed CPE, characterized by rounding of cells, granular appearance of the cytoplasm (Fig. 2) followed by detachment from glass after 48 to 72 hours.

Results of negative staining-electron microscopy:

Supernatants of inoculated vero cell cultures detecting CPE and subjected for negative staining EM, showed bullet like appearance (Fig. 3), in addition to different forms of the virus which are blunt cone shaped of different lengths (Fig. 4).

Results of virus neutralization test:

The nine isolates were identified and confirmed to be BEFV isolates by VNT (Table 1).

Results of coagglutination clumping ultrastructural analysis:

Supernatants of inoculated vero cell cultures showing CPE when mixed with BEFV antiserum and SPA mixture, aggregates of viral particles coated with BEFV antiserum were shown to closely attached to surface of *Staphylococcus aureus* (Fig. 5). Viral particles are mainly bullet shaped in addition to considerable number of conical forms and short bullets.



Fig. (1): A cow shows lateral recumbancy and emphysematous swelling in the shoulder, neck and back region.

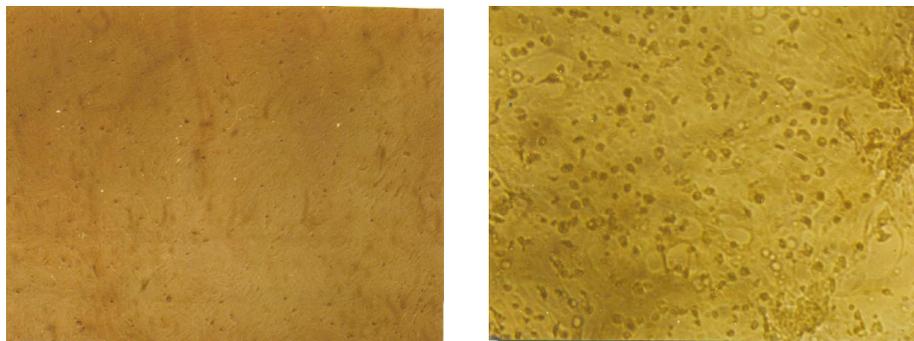


Fig. (2): Right: Vero cell line reveals a CPE characterized by rounding of cells, granular appearance of the cytoplasm followed by detachment from the glass (X 40). Left: Un-inoculated (control) vero cell line (X 40).

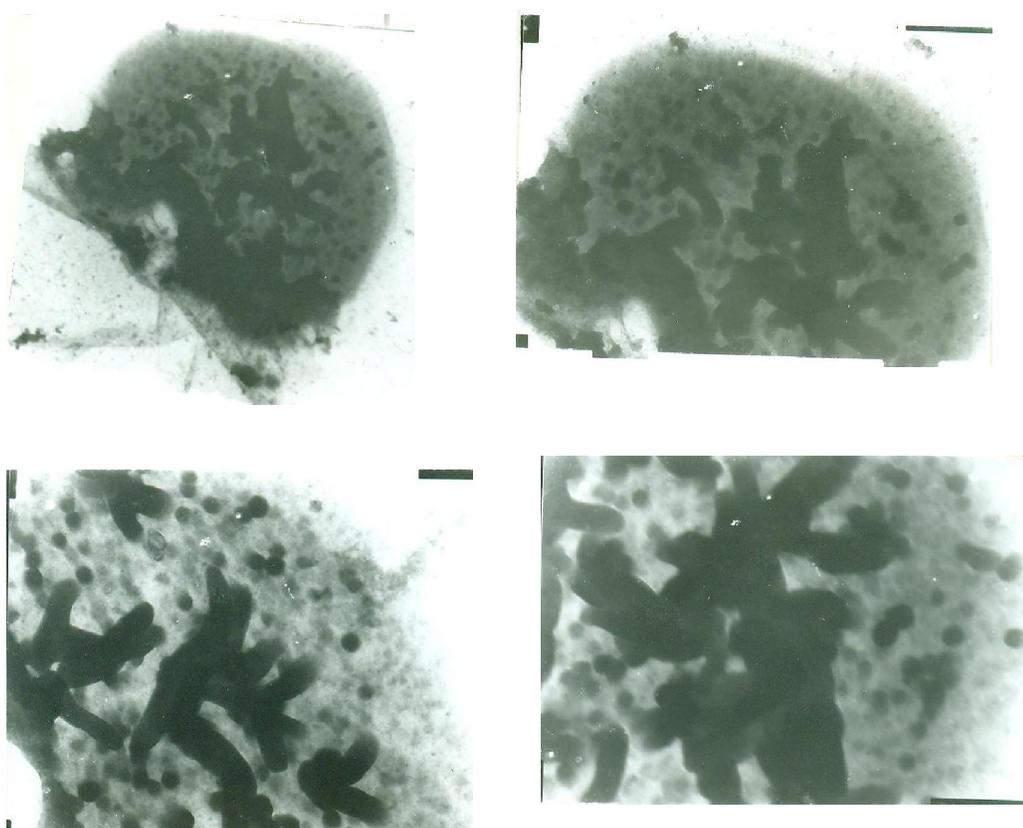
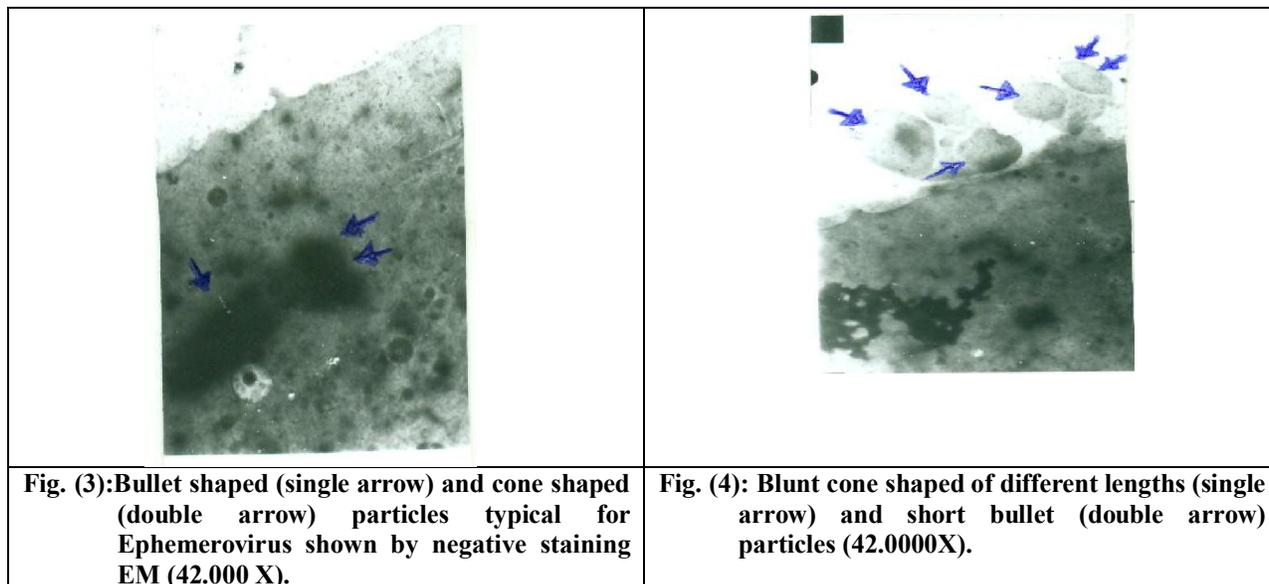


Fig. (5): Aggregates of Ephemerovirus like particles coated with BEFV antiserum were detected to be closely attached to surface of *Staphylococcus aureus*. A: (21.000 X) , B: (35.000 X), C: (56.000 X) & D: (56.000 X).

Table (1): Results of isolation and identification of the tested samples:

Number of samples	CPE in inoculated vero cell cultures	VNT	SPA CoA-EM
13	9	9	9

4. Discussion:

Bovine ephemeral fever is a disease of economic importance and its rapid diagnosis is the first step to plan a suitable control program (Nandi and Negi, 1999).

Diagnosis is made from clinical observations and the history of the outbreak, single cases are difficult to diagnose, but with a herd outbreak when cattle at various stages of disease will be observed, some with the characteristically rapid resolution of severe signs (Uren *et al.*, 1992).

In the present study samples were taken from the suspected animals to be infected with BEF in various stages of the disease for faster confirmation since the virus does not persist much beyond the 4th day after subsidence of the fever as stated by ST. George (1998).

Isolation is the most suitable method for BEF diagnosis where serology is too time consuming to be used as primary diagnostic method (Davies, 1991) and due to anamnestic responses to BEFV can be seen during the first exposure, if the animal was previously exposed to another member in the Ephemerovirus genus (ST. George, 1998).

Characterization of BEFV is very essential since isolation and vaccine preparations need viral propagation which rapidly loss its antigenicity and pathogenicity when it pasaged in suckling mice or cell culture (Snowdon, 1970 and Tzipori and Spradbrow, 1973).

BEFV could be isolated in a number of common cell cultures including African green monkey kidney (vero). This system is more practical than mice for producing vaccine (Nandi and Negi, 1999) despite the inherent loss of antigenicity (Uren, 1989).

In the current study, BEFV isolation was achieved by inoculation of vero cells and were identified as BEFV with VNT which can distinguish BEFV from their related Ephemeroviruses (Nandi and Negi, 1999).

Negative staining EM was attempted to identify BEF viral isolates. EM is the gold standard for identification and it is used as rapid and accurate diagnostic method due to after a simple and fast negative stain preparation, rapid morphologic identification are obtained (Davies *et al.*, 1971). BEFV could range from bullet (Fig. 3) to blunt cone shaped with different lengths (Fig. 4) and these results agreed with Richmond (2008) who stated

also that BEF viral particles have a diameter of approximately 37 nm but the length can vary from 70-183 nm. The shorter bullet and conical forms are considered to be defective particles that probably interfere with virus growth in cell culture. EM also can identify subviral components such as empty virions, which may be produced late in an infection (Hazelton and Gelderblom, 2003).

SPA CoA-EM was applied in the present study to improve the performance of negative staining EM (Bastawecy and Saad, 2007) where it is most instances, efficient but, it may give contradictory results in samples containing low levels of viral particles or masked by other viral particles (Bastawecy *et al.*, 2007). Aggregates of rabdoviral particles coated with the specific antibody were observed in isolates in addition to their attachment to the surface of *Staphylococcus aureus* (Fig. 5). Viral particles appeared to be bullet or cone shaped as previously described by Murphy *et al.* (1972). SPA CoA-EM could be helpful for diagnosis, as viral aggregation facilitates detection of BEF viral isolates with poor CPE and low multiplicity of infection due to defective interfering particles (Della-Porta and Snowdon, 1979) or specimens of infected animals with high titer of interferon α during the acute phase where peak titers of interferon appear 4 to 36 hour before the onset of pyrexia (Uren, 1989). This method also can roughly estimate percentage of bullet particles of BEFV versus other forms of the virus which may be defective interfering particles that may interfere with the replication of homologous virus (Brooks *et al.*, 1998). Moreover, SPA CoA-EM could be helpful for deciding, if the current passages of BEFV in cell lines used for vaccinal preparation or not, even it could be used for production of attenuated or killed vaccine.

In conclusion, our findings recommended the use of negative staining-EM and SPA CoA-EM for viral characterization when EM is available after isolation or after each passage of BEFV in cell cultures. Further studies are recommended to find relationship between forms of the BEF viral particles and CPE in cell cultures of different passages using SPA CoA-EM. These future studies may offer answer to why the antigenicity and pathogenicity of BEFV rapidly lost on passaging in suckling mice or cell culture.

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