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Isolation and Characterization of Bovine Ephemeral Fever Virus in Delta Provinces, Egypt 2012

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Abstract: Bovine Ephemeral Fever is a non-contiguous arthropod born viral disease infecting cattle and water buffaloes. Suspected cases with Bovine Ephermeral Fever Virus (BEFV) were recorded in Delta provinces during summer season 2012. The present work aimed to isolate and identify the virus by RT-PCR. Blood samples were collected from infected animals in Kafrelsheikh, Al Gharbia, Al Dakahlyia, Al Behaira and Damietta. The virus was isolated in baby mice intracerebrally then passed to Baby Hamster Kidney (BHK-21) cell lines. Viral identification by indirect immunoflourcent technique showed intracytoplamic green dots. Molecular identification of the BEFV targeting glycoprotein G gene showed 420bp clear single band in agarose gel confirmed that the animals were infected with BEFV.

Key words: Bovine Ephemeral Fever · PCR · Egypt

INTRODUCTION

Bovine ephemeral fever virus (BEFV) is classified as the type species of the genus Ephemerovirus in the family Rhabdoviridae and is known to cause an acute febrile disease in cattle and water buffalo manifesting in anorexia, depression, ocular and nasal discharge, salivation, muscle stiffness, lameness, rumenal stasis, sternal recumbency and other inflammatory responses [1]. The disease is considered to cause major economic damage through reduced milk production at dairy farms and loss of conditioning of beef cattle at harvest time. BEFV is transmitted by hematophagous arthropod vectors (mosquitoes and Culicoides bitingmidges) and is widely distributed from tropical to temperate zones such as Australia, Asia, the Middle East and Africa [1- 3].

BEFV was Bullet or cone-shaped virion possesses a single stranded, negative-sense RNA genome (14,900 nucleotides) and five structural proteins comprising a nucleoprotein (N), a polymerase-associated protein (P), a matrix protein (M), a large RNA-dependent RNA polymerase (L) and a glycoprotein (G) spanning the viral envelope [4]. The G protein is a class I transmembrane glycoprotein that forms clear projections on the virion surface [5, 6]. It is a type-specific neutralizing antigen and induces protective immunity in cattle [7]. Four distinct neutralizing epitopes (G1, G2, G3 and G4) have been determined on the Australian BEFV strains by competitive binding assays using neutralizing monoclonal antibodies against prototype strainBB7721 and cross-reactivity analyses of neutralization resistant escape mutants [8]. A general structural model of the protein exhibiting variable antigenic sites has been proposed as a result of amino acid sequence analysis [9].

In Egypt, the disease was first described in 1895 by Piot and subsequent seasonal outbreaks have been occurred in summer of 1991, 2000, 2001 and 2004, 2010 [10 - 13]. 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 [14, 15].

The aim of this study to isolate and identify the causative agent of suspected cases of BEF appeared in Delta provinces Egypt in 2012.

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MATERIALS AND METHODS

Sampling: 353 Heparinized blood samples were collected from feverish animals during summer season in 2012 from 5 Egyptian governorates (Kafrelsheikh, Al Gharbia, Al Dakahlyia, Al Behaira and Damietta.), for separation of buffy coat for virus isolation [16]. The blood samples collected from infected cattle were centrifuged, the plasma was collected, washed in phosphate-buffer saline (PBS) for three times, stored at-70°C for virus isolation.

Virus Isolation: Isolation of BEFV was carried out in the brains of suckling mice and baby hamster kidney (BHK-21) cells [17]. The washed leucocytic fraction of the feverish animals was inoculated intracerebrally in baby mice (2-3 day age) and subjected to 3-4 blind passages in suckling mice. Thereafter, BHK-21 cells were inoculated with BEFV extracted from the brains of the 4th passage infected suckling mice. The virus underwent five passages in BHK-21 cells, until cytopathogenic effects (CPE) were observed.

Virus Titration: Virus titration was done in BHK-21 cells [18] and the titers were expressed as 50% tissue culture infectious doses (TCID 50). BEFV was also assayed and calculated by the method of Reed and Muench [19].

Production of Hyperimmune Sera and Virus Neutralization Test: Hyperimmune sera against BEFV were raised in rabbits. The supernatant of virus-infected BHK-21 cells or concentrated virus solution containing 10^7 TCID50/ml was used for immunization. Rabbits were inoculated intravenously with 2-3 ml of virus solution without any adjuvant. Three weeks later, the animals were immunized again in the same way. Serum was collected 10 days after the booster immunization and stored at -20°C until use [20].

Rabbit immune serum inactivated at 56°C for 30 min was serially diluted twofold with serum-free Eagle'sminimum essential medium using a double line of 96-well cell culture microplates. Each serum dilution was mixed with an equal volume of 200 $TCID_{50}$/0.05 ml of virus and incubated 37°C for 1 h. Then a 0.1 ml volume of BHK-21 cells suspended in MEM was added to each well. After incubation at 37°C for 3-5 days in humidified 5%carbon dioxide atmosphere, the antibody titer was expressed as a reciprocal of the highest serum dilution showing 50% inhibition of the cytopathic effect [20].

Immunofluroscent Antibody Assay: 100 $TCID_{50}$of BEFV was inoculated to 24-well Tissue culture plate contained confluent BHK-21 cell monolayer. The plate was incubated at 37°C with 5% C02. 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 [21].

Identification of the Virus by RT-PCR: The presence of BEFV was confirmed by reverse transcription polymerase chain reaction (RT-PCR) [22]. The BEFV RNAs were extracted from the infected BHK-21 cells using a Gene Jet viral RNA Purification kit (Thermo Scientific). For RT-PCR, the primers were 420F (5' AGAGCT TGG TGT GAA TAC 3') and 420R (5' CCA ACCTAC AAC AGC AGA TA 3'). The forward primer 420F was used to reverse-transcribe BEFV RNA to cDNA. A partial fragment of the BEFV G gene was amplified using the primers 420F and 420R. After the initial denaturation at 94°C for 5 min, the amplification proceeded through a total of 35 cycles consisting of denaturation at 94°C for 40s, annealing at 48°C for 1 min, primer extension at 72°C for 40s and a final extension for 10 min at 72°C. The expected DNA fragments were 420 base pairs (bp) in length.

RESULTS

Virus Isolation: BEFV was isolated by intracerebral inoculation of suckling mice and in BHK-21cells. The infected suckling mice showed convulsions, paralysis of hind limb, abnormal gait and death (Fig. 1A). The infected BHK-21 cells showed specific CPE which is characterized by cell rounding, granulation of the cytoplasm, cellular degeneration that end with detachment of cells from the surface of cell culture flask leaving empty spaces. These stages of cellular change take place in a period began from the 1st 24 hours post infection and end usually within 72 hours post cellular infection (Fig. 1C).

Neutralization Test: The titers of the isolate BEF viruses ranged from 10^5-10^7 $TCID_{50}$/ml. The standard positive serum to BEFV with 1:64 dilutions could neutralize completely the virus isolate.

Immunofluorescence Test: Infected BHK-21 cells showed greenish fluorescence granules in the cytoplasm of infected cells indicated positive result At the same time there was no fluorescent staining in the control non infected cells (Fig. 2A).

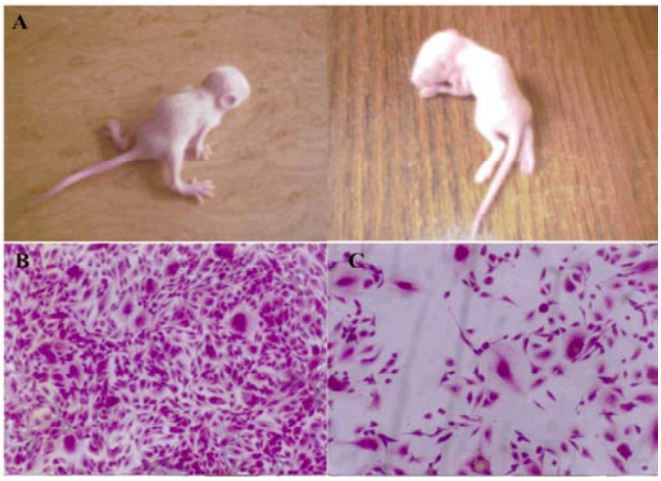

Fig. 1: Baby mice inoculated with BEF virus showing paralysis of the hind limbs (A). CPE in BHK-21 cells inoculated with BEF virus on the right (C) while, normal BHk-21 cells are on the left (B).

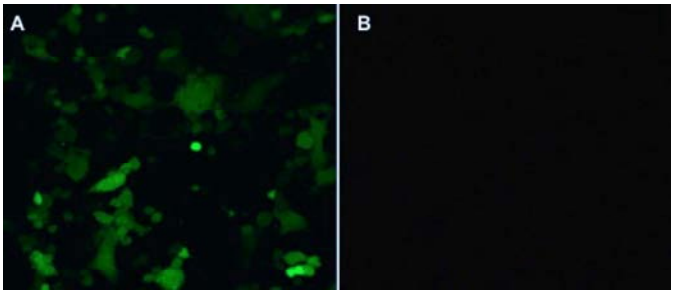

Fig. 2: IFA test where positive results were indicated by presence of specific green fluorescence in infected BHK-21 cells (A), while negative results were indicated by absence of green fluorescence (B).

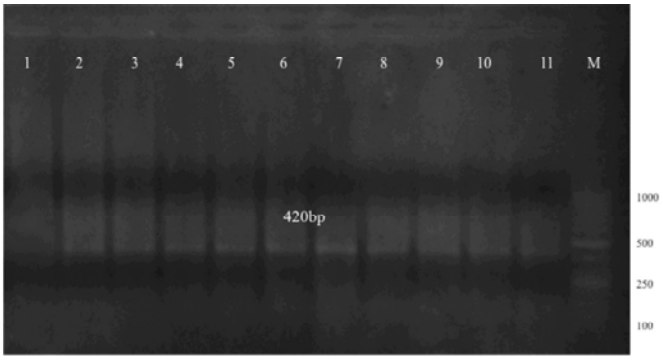

Fig. 3: Agarose gel electrophoresis of PCR product (420bp) using BEFV glycoprotein G specific primers. Lane M: DNA ladder molecular weight marker (100bp); lane 1: negative control; lanes 2: control positive, lane 3-11: positive samples, band at 420bp.

Identification of the Virus by RT-PCR: BEFVs were identified with RT-PCR using specific primers targeting the G gene. The cDNA were amplified producing a clear single band 420bp in length on agarose gel stained with ethidium bromide. All positive samples of Antibody neutralization and 1FT tests of BEFV gave band at 420bp (Fig. 3).

DISCUSSION

Diseases caused by various arthropod-borne viruses, such BEFV have been recognized as major threat to the production of beef and dairy cattle, mainly in Egypt. BEF is an acute febrile disease of cattle and water buffaloes caused by the bovine ephemeral fever virus a member of

the genus Ephemerovirus in the family Rhabdoviridae [23]. Affected animals are only sick for a few days; hence the alternative name Three Day Sickness but the clinical severity of the disease is inconsistent with the subsequent rapid recovery of most of the affected animals [1]. BEF causes significant economic losses through loss of condition, decreased milk production, lowered fertility of bulls and delays in marketing and restrictions on the export of live cattle [24]. In this study, the diagnosis of BEF was based on the characteristic clinical signs, which were typical for BEF and are in agreement with previous studies [25- 27]. These clinical signs were confirmed by virus isolation and RT-PCR.BEFV can be isolated in a number of common cell cultures including African green monkey kidney (Vero) and baby hamster kidney-21 (BHK-21). Cytopathogenic changes are visible on 2-5 days post inoculation including slight rounding of cell followed by destruction of the whole cell sheet within 24-48hours.In this study blood samples were collected from cattle showing clinical signs during summer season 2012 in Delta provinces (Kafrelsheikh, Al Gharbia and Al Dakahlyia, Al Behaira and Damietta governorates). BEF suspected virus was isolated by inoculation of both baby mice and BHK-21 cells showing convulsions, paralysis of hind limb and death of baby mice and specific CPE (cell rounding, granulation of the cytoplasm, cellular degeneration that end with detachment of cells from the surface of cell culture flask leaving empty spaces) in BHK-21 cells.

Reverse transcriptase polymerase chain reaction (RT-PCR) has been developed with many advantages as it is possible to detect as little as 2 fragments of viral RNA from infected tissue by ethidium bromide staining after 30 cycle of PCR [28]. The application of RT-PCR on isolated field virus yielded a clear single specific band on agarose gel stained with ethidium bromide. The amplified DNA fragment corresponds to 420 bp. PCR confirms the diagnosis of BEF outbreak, which is sensitive, specific and of value for rapid diagnosis of viral diseases.

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