

ESTABLISHMENT OF DOT-BLOT HYBRIDIZATION FOR DIAGNOSIS OF *BOVINE EPHEMERAL FEVER VIRUS* IN EGYPT

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ABSTRACT

Bovine ephemeral fever (BEF) is an arthropode borne rhabdovirus, which causes a disabling febrile infection of cattle and water buffalo. A sever outbreak was recorded in Egypt during 2000 and 2001. Using RT-PCR, a primer designed to amplify 438 nucleotide fragments from the glycoprotein G gene allowed specific amplification of BEFV-cell culture isolates from Egypt (Menoufia and Alexandria Governorates) and Japan. The PCR product was sequenced and analyzed. PCR product of Alexandria isolate was cloned and labeled with digoxigenin and used as diagnostic probe for BEF virus infection using dot-blot hybridization. Thirty six samples were collected from Menoufia Governorate (Berket El-Sabaa, Tokh Tambesha and Salamon regions) and Demiate Governorate and were tested in dot-blot hybridization. BEFV was detected in 24 out of 36 samples (66.7%) as follow: 9/9 (100%) correspond to Berket El-Sabaa and Tokh Tambesha regions and 3/9 (33.3%) correspond to Demiate and Salamon regions. In conclusion, a local Egyptian probe was designed and applied in the dot blot hybridization technique for diagnosis of *bovine ephemeral fever virus*.

Key Words: BEFV, diagnosis, RT-PCR, Dot-blot hybridization

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INTRODUCTION

Bovine ephemeral fever virus (BEFV) is an arthropode borne rhabdovirus, which causes a disabling febrile infection of cattle and water buffalo. The disease is common in tropical and subtropical regions of Africa, Asia, Australia and the Middle East (St. George, 1997; Venter *et al.*, 2003; Wang *et al.*, 2001 and Yeruham *et al.*, 2003). The disease is caused by a virus belonging to the *Ephemerovirus*, which is a member of the *Rhabdoviridae* (Wunner *et al.*, 1995). Mosquitoes and *Culicoides* midges involved in transmitting the virus (Kirland, 1995; Mellor, 1996 and St. George *et al.*, 1977). The causative ephemerovirus is bullet shaped, consists of a negative-sense, single-stranded RNA genome and 5 nonstructural proteins, including a nucleoprotein (N), a polymerase-associated protein (P), a matrix protein (M), a large RNA-dependent RNA polymerase (L), and a surface glycoprotein (G), which induces the production of protective neutralizing antibody (Uren *et al.* 1994 and Walker and Cybinski, 1991).

Bovine ephemeral fever is characterized clinically by the

sudden onset of fever, stiffness, lameness, nasal and ocular discharges, depression, cessation of rumination, and constipation. A sever outbreak of BEF was observed in Egypt in summer 2000. Mortality rates ranged between 2-10% with fever, stiffness, lameness, dyspnea, abortion, subcutaneous emphysema and recumbency. Milk yield was reduced to about 25% during the peak of the outbreak. BEF virus was detected in leukocytes from animals showing clinical symptoms using immunofluorescence and immunoperoxidase techniques (Zaghawa *et al.*, 2000 and Hassan, 2000). PCR was established for diagnosis of BEF in Egypt by Khalil *et al.* (2001). The advantage of dot blot hybridization is to use crude samples without the need to RNA extraction step and avoiding RNA degradation.

There is an essential need for labeled DNA probe for improvement of the diagnosis of BEF. The introduction of these molecular techniques will provide a rapid, sensitive and specific diagnosis of the disease that will help us in the control of the disease and consequently reduce the economic losses.

MATERIALS & METHODS

Viruses

BEFV-Menoufia, isolated from an infected cattle (Zaghawa *et al.*, 2000), and BEFV a Japanese reference isolate were used. The following cell lines were used for isolation, propagation and titration of bovine ephemeral fever virus: (1) Baby hamster kidney cell line (BHK21) and (2) African green monkey kidney cell line (Vero). They were kindly supplied by Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. Minimum Essential Medium (MEM) with Hank's salts and L-glutamine without sodium bicarbonate was purchased from Sigma. It was used for the growth and maintenance of cell cultures. The growth medium was supplemented with 10% new born calf serum, while the maintenance medium was supplemented with 2% new born calf serum. The final pH of the growth and maintenance media were approximately adjusted to 7-7.3 by sodium bicarbonate solution (4.4%). Preparation of the media was done according to the instruction manual.

RNA extraction

RNA was extracted using one-step RNA reagent (Bio Basic

Inc.) as per the manufacturer's protocol. Homogenized tissue culture was mixed with 2 volumes of the reagent. The aqueous phase was separated by centrifugation for 15 min at 12 000 rpm (Eppendorf centrifuge). The aqueous phase was then washed with chloroform-isoamyl alcohol. RNAs were then precipitated with one volume of isopropanol at -20°C overnight. The precipitate of RNA was collected by centrifugation at 14 000 rpm for 30 min. The supernatant was discarded and the pellet was washed twice with 70% ethanol and by centrifugation at 14 000 rpm for 10 min. Pellet was air or vacuum dried and then dissolved in distilled water (RNase free). RNA extract was then kept at -70°C until use.

Reverse transcription-polymerase chain reaction (RT-PCR)

Attempts to reverse transcribe RNA were carried out with 18 nucleotide primer EG2 5' TACAACAGCAGATAAAAC 3' as a forward primer and 18 nucleotide primer EG3 5' CATTATGGGATAGGATCC 3' as a reverse primer derived from the glycoprotein gene sequence (10263-10700 nt) of the Australian isolate BB7721. Reverse transcription and PCR were carried

out according to the Slomka *et al.* (2009) using ONE STEP RT-PCR kit (QIAGEN). The RT-PCR conditions were 30 min at 50°C, 2 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C and 10 at 72°C. The amplified product was resolved by electrophoresis in 1% agarose gel.

Cloning of PCR Product

Cloning of BEFV glycoprotein region in pGEM-T easy Vector

CDNA-BEFV region obtained after PCR amplification of *Alexandria* isolate was directly cloned in the linearized and thymidylated pGEM-T easy plasmid. The ligation reactions were set up to insert BEFV-cDNA at 3'-T overhangs plasmid at the insertion site according to Promega Corporation standard protocol. Fifty ng of pGEM-T-easy vector (3018bp) were mixed with 25 ng of fresh PCR, 1 µl of T4 DNA ligase (3 Weiss units/µl), 5 µl of 2X T4 rapid DNA ligase buffer (300 mM Tris- HCl, pH7.8, 100 mM MgCl₂, 100 mM DTT, 10 mM ATP), and deionized water to a final volume of 10 µl. The ligation mixture was incubated overnight at 4°C. The construct was transformed in DH5α *E. coli* competent cells.

Transformation

The principals of the technique were followed according to Mahmoud, 2001. Five µl of the ligation reaction was carefully transferred into 100 µl competent *Escherichia coli* bacteria, isolate DH5α. The tube was gently flicked and placed on ice for 20 minutes. The mixture was heat-shocked for 45-50 seconds in a water bath at exactly 42°C and immediately returned to ice for 2 minutes. After adding 400 µl room temperature LB medium, the mixture was incubated for 1.5 h at 37°C with shaking (~150rpm). A 100 µl aliquot of the transformation culture were plated together onto duplicate LB-A plates. The plates were incubated overnight (16-24 hours) at 37°C. BEFV-cDNA insert was detected in the recombinant plasmid by PCR using BEFV primers. Picked colony was diluted in 40-80 µl distilled water then denatured at 90°C for 5 min. Amplification reaction was carried out in a volume of 25 µl of PCR reaction mixture using 8 µl of denatured culture as a template, 12.5 µl PCR Master Mix, 2X (Promega) and 1 µl of each BEFV-primer (10 µM). PCR conditions were 30 cycles of 1 min at 94°C, 1

min at 50°C, 1 min at 72°C and 10 at 72°C. The amplified product was resolved by electrophoresis in 1% agarose gel.

BEFV detection by Nucleic Acid Hybridization

Probe labeling

DIG-labeled DNA probe was generated according to Le Pogam *et al.* (2005) by using random primed labeling kit (Roche diagnostics Indianapolis, IN.), which is based on the hybridization of random oligonucleotides to the denatured DNA template (PCR product of Alexandria isolate clone n° 4).

Dot blot hybridization assays

Healthy or BEFV-crude blood samples were mixed with denaturing solution 8XSSC (1X SSC is 150 mM NaCl, 15mM Na acetate, pH 7.0 and 10% formaldehyde) then heated to 60°C for 15 min and kept on ice. The sample (2-10 µl) was spotted onto presaturated nylon membrane with 20X SSC. The membrane was then backed at 80°C for 30 min, followed by nucleic acid hybridization.

Hybridization technique

Hybridization technique according to the protocol provided by Roche inc. Briefly the nylon

membranes were prehybridized with at least 20 ml of hybridization solution (5X SSC, 0.1% N-lauroyl sarcosine Na salt, 0.02% SDS, 2% blocking reagent and 50% formamide) at 42°C for 1-3h. The hybridization solution then replaced with at least 25 ml of hybridization solution containing 10-25 ng of denatured labelled probe. The membranes then incubated overnight at 42°C. The membranes were then washed 2 times for 5min at room temperature with 50 ml of 2X SSC, 0.1% SDS (w/v) and 2 times for 15 min at 65°C with 0.1X SSC, 0.1% SDS (w/v).

Digoxigenin immunological detection

Digoxigenin immunological detection was performed according to the manufacturer's recommendations (Roche diagnostics Indianapolis, IN.) as follows. The membranes were equilibrated 2 min in buffer I (10 mM Tris-HCl, pH 7.5, 150 mM NaCl). The buffer was discarded then 100 ml of buffer II (1% blocking reagent dissolved in buffer I) was added. The membrane was then incubated for 30 min with gentle shaking. Anti-Dig alkaline Phosphatase was diluted 1: 15000 in buffer II, and membranes were

incubated in this buffer for 30 min. The membranes were washed twice for 15 min in buffer I. The buffer was discarded and the membranes were equilibrated in buffer III (10 mM Tris- HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 2 min. Finally, the color reaction was initiated at alkaline pH by the incubation of the membranes with 15 ml of color solution [200ul of Nitroblue tetrazolium (NBT) / 5-bromo- 4- chloro-3- indolye (BCIP)] in 15 ml Buffer III in the dark without shaking or mixing until the color developed. The reaction was stopped when desired spots were detected by washing the membrane with H₂O.

Sequence analysis

DNA sequencing was carried out with the ABI PRISM model 310 versions 3.4 semi-adaptive version 3.2 at gene link DNA Sequencing service, New York, USA. DNA sequencing was carried out using the PCR product specific primers. The sequence analysis was performed using the Chromas Pro Version 1.34 software. Sequences were compared to the sequences of GenBank database using BLAST applications (NCBI).

RESULTS

RT-PCR

Primers derived from the sequence of BEFV-Australian isolate BB7721 was used to amplify 438 bp correspond to a region in G glycoprotein. RT-PCR was used to detect BEFV from cell culture. The PCR-amplified product was about 400bp for BEFV Menoufia isolate 3rd passage and Japanese isolate. No product was detected when the BEFV Menoufia isolate 2nd passage or cell culture-virus free were tested. Agarose gel electrophoresis analysis of the PCR products is demonstrated in **Figure (1)**.

Cloning of the PCR products

Cloned BEFV-PCR product (Alexandria isolate) was tested. Five of the bacteria- containing white colonies were directly tested using PCR. The 5 samples yielded 5 recombinant plasmids containing cDNA inserts of about 400bp (**Figure 2**).

Hybridization analysis

Insert n° 4 was DIG-labeled and analysed by dot blot hybridization. The probe was shown to be virus-specific using dot-blot hybridization. The virus was detected in cell culture of BEFV-

Menoufia and Japanese isolates (Figure 3-E, N° 6 and 7) but not in PCR, RNA, RNA or DNA negative controls (Figure 3-E, N° 1-5). The probe was able to detect BEFV in blood samples collected from 2 different Governorates: Menoufia Governorate (Berket El-Sabaa, Tokh Tambesh and Salomon regions) and Demiate Governorate (Demiate region, Figure 3, A-D). BEFV was detected in 24 out of 36 samples (66.7%) as follow: 9/9 (100%) correspond to Berket El-Sabaa and Tokh Tambesha regions and 3/9 (33.3%) correspond to Demiate and Salomon regions. No

reaction was detected in blood samples collected from healthy cow (Figure 3-F, N° 1-4).

Sequence analysis

G gene of two BEFV Egyptian isolates (Eg-Alexandria and Eg-Menoufia) and a reference isolate from Japan was partially sequenced and analyzed. Nucleotide sequence identity of the 3 isolates was 100 %. Identity between our isolates and Genbank BEFV isolates was 80-100 % and 91.7-100 % for the nucleotide and the deduced amino acid sequences, respectively.

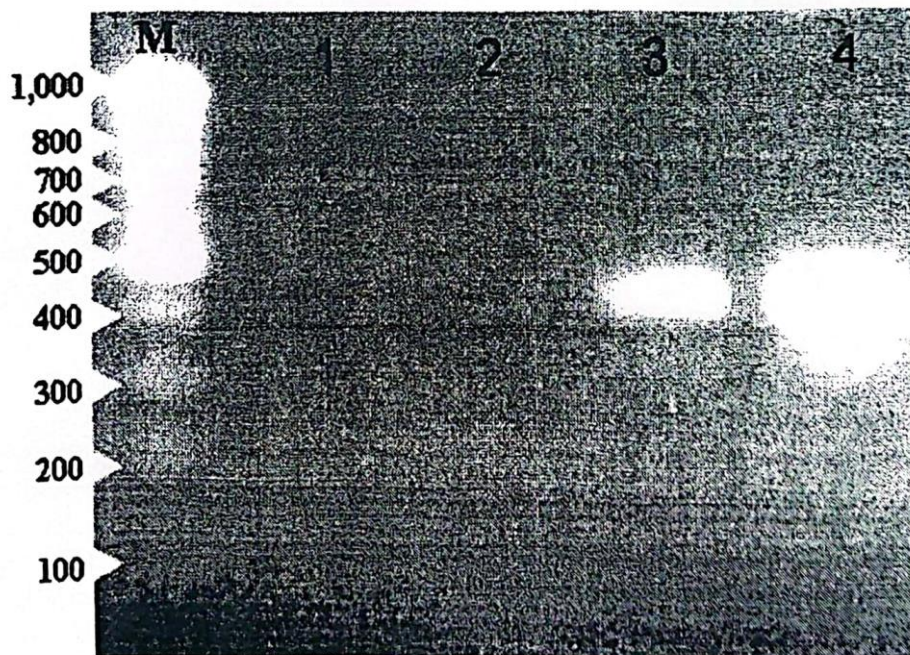


Figure 1. Electrophoresis of PCR products of both BEFV-Egyptian and Japanese reference isolates (1) cell culture-virus free, (2) and (3) are BEFV Menoufia isolate 2nd and 3rd passage and (4) Japanese isolate.

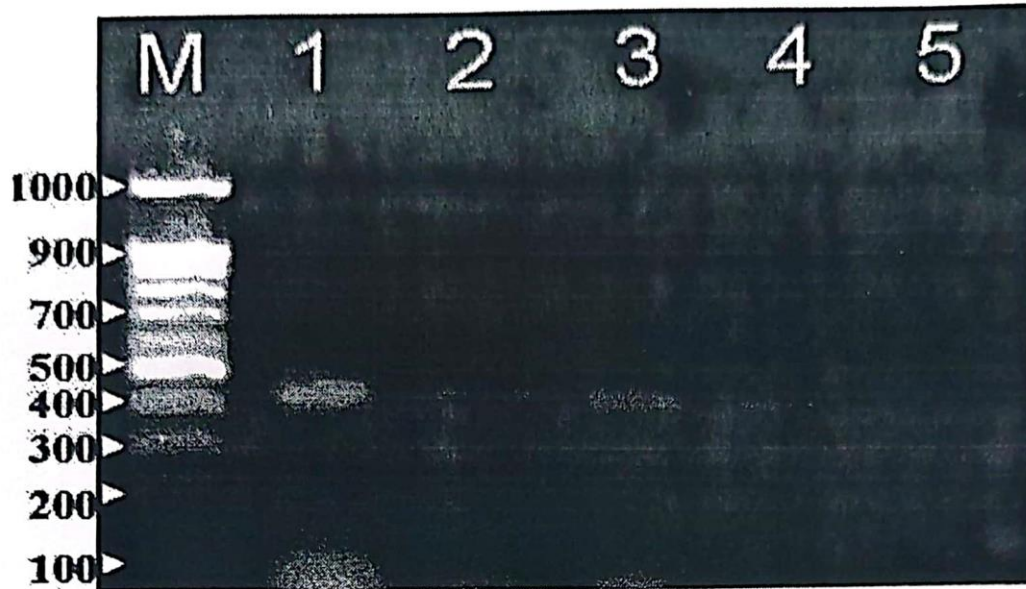


Figure 2. A 1% agarose gel showing PCR of DH5 α *E. coli* colonies-plasmid DNA containing BEFV inserts. Lane M is 1-1.5kb DNA ladder (0.075-12.2 kb, Life technologies).

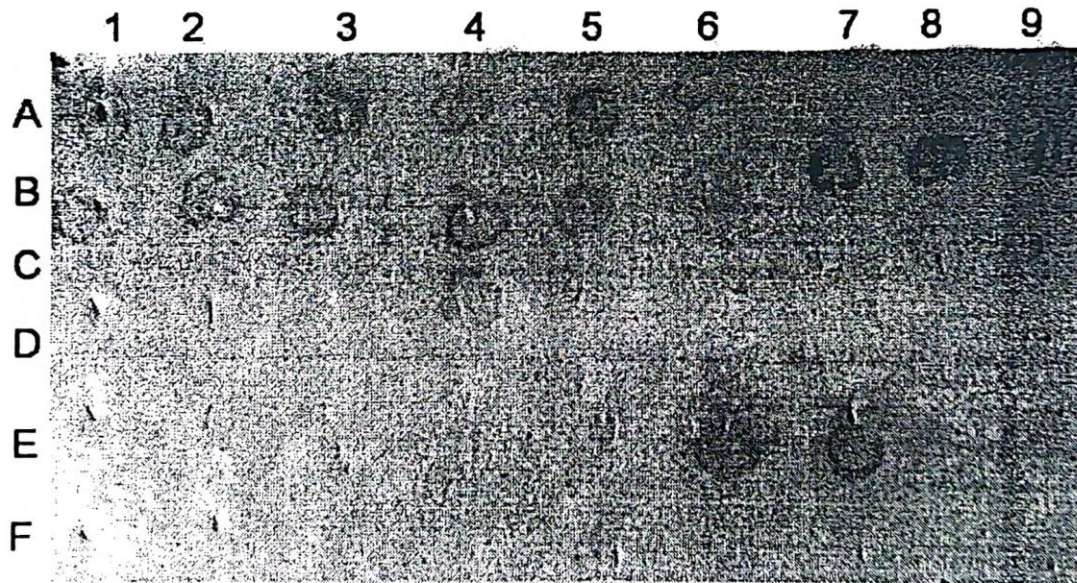


Figure 3. Dot-blot hybridization of crude blood samples of BEFV using BEFV DIG-labeled probes complementary to clone N° 4. (A-D) samples collected from regions Berket El-Sabaa, Tokh Tambesha, Demiate and Salamon respectively. (E), samples 1-5 PCR, RNA, RNA and DNA negative controls, samples 6-7 viral cell culture of BEFV-Menoufia and Japanese isolate respectively. (F) Samples 1-4 blood samples collected from healthy cow from the faculty farm Sadat city.

DISCUSSION

The serological identification of rhabdoviruses considered as field diagnostic problem due to the antigenic relationship between the members of this group (Calisher *et al.*, 1989). 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 (Wu *et al.*, 1992). There is no need for virus replication, moreover, RT-PCR is not time consuming since all procedures involved take about 6 hours to be completed (Davis and Boyle, 1990). The application of RT-PCR on leukocytes and brain tissue of infected mice with the suspected material of BEF virus yielded a clear single band on agarose gel stained with ethidium bromide. The amplified DNA fragment corresponds to 500bp. PCR confirms the diagnosis of BEF outbreak which is sensitive specific and of value for rapid diagnosis (Khalil *et al.*, 2001).

In the present work, RT-PCR was carried out with isolates from Egypt (Menoufia and Alexandria Governorate) and from Japan. The designed primers were derived from the glycoprotein gene

sequence of Australian isolate BB7721. BEFV isolates from Egypt and Japan were detected using RT-PCR (Figure 1). Virus was detected in the 3rd -cell culture passage but not in the 2nd passage, this could be explained as virus concentration was lower in the 2nd passage. The specificity of RT-PCR was confirmed as no product was detected when cell culture-virus free was used. RT-PCR was previously used for BEFV detection (Zheng *et al.*, 2009).

RT-PCR has become an important method for pathogen detection. However, RT-PCR requiring RNA extraction before amplification of each virus of interest are potentially expensive and resource intensive. The advantage of dot blot hybridization is to use crude samples without the need to RNA extraction step and avoiding RNA degradation.

In this research we develop a local Egyptian probe of BEF virus to be applied in the dot blot hybridization technique for diagnosis of bovine ephemeral fever virus. According to the hybridization data, Berket El-Sabaa and Tokh Tambesha were highly infected in a comparison to Demiate and Salamon (100% and 33.3% respectively). The specificity

of BEFV probe was confirmed using virus-cell culture from Egypt and Japan, no virus was detected from blood samples collected from healthy cow. The probe was not able to detect the virus from PCR product and RNA samples. The specificity of our probe was confirmed as no reaction was detected with blood samples from healthy cow or nucleic acid negative controls.

Analysis of the obtained partial G gene sequence of the 3 isolates (Menoufia, Alexandria and Japan) showed similar sequences for the 3 isolates (100 % similarity). The lowest sequence identity with genbank data was 80 and 91.7 % for nucleotide and amino acid, respectively. The entire G gene sequence of JT02L isolate from China was above 90% homology when compared to JB76H (Beijing strain), Taiwan strain and BB7721 (Australia's strain), indicating that BEFV was highly conservative between different epidemic periods and areas (Zheng *et al.*, 2009).

In conclusion a local Egyptian probe of the Egyptian isolate was designed and applied in the dot blot hybridization technique for diagnosis of bovine ephemeral fever virus. The application of the

dot blot hybridization technique offers the advantage of low costs and less precaution: (1) using crude blood of many samples on one Nylon membrane and (2), No need to RNA extraction step which avoid RNA degradation.

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