

Bluetongue Infection in Small Ruminants in Egypt

Kawther S. Zaher

Microbiology and Immunology Department,
National Research Center (NRC), Dokki, Cairo, Egypt

Abstract: The current study was undertaken to detect the presence of Bluetongue disease as a re-emerging disease also to establish the best (cheap, easy and accurate) method for detection of the disease. Two hundred serum samples, one hundred and fifty tissue samples of spleen and liver samples were taken from infected sheep and goats in different governorate Al Sharqiya, Al Daqhaliya and Al Qalyoubiya. Serum samples and tissue samples of tongue, spleen and liver were taken from sheep and goat showing clinical symptoms of Bluetongue such as lameness, coughing, mouth lesions, stillbirth, congenital abnormalities, pneumonia, enteritis, hepatitis and dead corpses of infected animals. The virus was isolated and propagated in Baby Hamster kidney cells (BHK-21) and identified by ELISA, Electron Microscopy and RT-PCR. The isolated virus showed various cytopathic effects on BHK-21 and the disease was clearly recognized by detection antibodies against the virus using ELISA, Electron Microscopy as well as RT-PCR. RT-PCR was more sensitive than ELISA in blood samples were 99, 98%, respectively. Also the ELISA results reveal that the virus is dominating in Al Sharqiya. The results also confirm the stability of the virus even in bad conditions and serum samples gave better results than tissue samples. The results confirm the presence of blue tongue infection in Egypt also it recommends the use of RT-PCR as fast, cheap, accurate and sensitive method for detection of the virus especially when samples are taken from tissue and to detect the virus as there is no ELIA for detection of the virus, the only ELISA found is to detect antibodies (Ab) against the virus.

Key words: Bluetongue • Virus RT-PCR • ELISA • Re-Emerging Disease • Vector-Born Disease

INTRODUCTION

In recent years vector-borne diseases (VBDs) are (re)-emerging and spreading across the world, having a profound impact on human and animal health, ecology, socio-economic and disease management [1]. Bluetongue virus (BTV) is an belongs to *Reoviridae family genus orbivirus*, arthropod-borne virus transmitted by *Culicoides* biting midges (*Diptera ceratopognidae*) that infects wild and domestic ruminants. Bluetongue (BT) virus infection is economically significant because of morbidity and mortality among clinically affected animals and restrictions on international movement of animals that have not been certified BTV free. The increase in emerging infectious diseases (EID) outbreaks in general and VBDs in particular, has been linked to global and/or local changes resulting from climate change, human-induced landscape changes and/or the changing activities

of human populations [2]. These ongoing changes and shifts have led to range shifts of endemic arthropod vectors, the establishment and spread of new arthropod vectors, the improved vector capacity of autochthonous arthropod vectors to established pathogens and the introduction, establishment and spread of new pathogens. Arguably the best documented example of veterinary importance in this field is the recent two-fold invasion of blue tongue (BT) worldwide [3-5].

The BTV particle is icosahedral, non-enveloped and has three concentric capsid layers [6]. The BTV core contains the 10 ds-RNA segments of the virus genome and the three minor protein components of the transcriptase complex, VP1 (Pol), VP4 (Cap) and VP6 (Hel) [7]. Cores also have two major protein components, VP3 (T2) (Which forms the innermost subcore shell) and VP7 (T13) (Forming the outer core-surface layer) [6]. In the intact virus particles the core is surrounded by an outer

Corresponding Author: Kawther S. Zaher, Department of Microbiology and Immunology, Veterinary Research Division, National Research Centre, Dokki, Giza, Egypt. Potal cod: 12622.

capsid layer, composed of two additional major proteins (VP2 and VP5). These proteins, which are more variable than the core proteins, or the three non-structural virus proteins (NS1, NS2 and NS3) [7], are involved in cell attachment and penetration during initiation of infection and contain epitopes that bind neutralizing antibodies [8]. VP5 is involved in the determination of virus serotype, possibly by influencing the conformation of the VP2 molecules in the outer capsid layer [9].

Although in 2008 Egypt declared free from BT, it has been notice the presence of BT infection in the last few years. No available data on disease history in Egypt. There have been some records of the disease in Kuwait [10] and Iran [11]. In Egypt the disease has become a threatening disease as it may attack animals in the form of massive outbreaks due to animal exportation during Eid Al Adha, which is a major feast in Egypt and due to the climate change as high temperature and humidity. This study is aiming for isolation and identification of the virus to notify the responsible authorities for the presence of the disease in Egypt and estimating a good and accurate diagnostic method for the disease to overcome it.

MATERIALS AND METHODS

This work was performed in Department of Microbiology and Immunology, National Research Center, between May and November 2012.

Samples Collection: Two hundred serum samples, one hundred and fifty tissue samples of tongue, spleen and liver samples were taken from sick sheep and goats in different governorate e.g Al Daqhaliya (Izbat al Malki, Izbat Bishlah and Izbat El Farayah), Al Qalyoubiya (Kafir El Gammal, Kafir Taha and Izbat El Ghafar) and Al Sharqiya

(Izbat Fitirah, Izbat El Hikr, Izbat Abu Sharkawi and Izbat Ash shanawani). The infected animals showed various clinical signs consistent with BT as lameness, coughing, mouth lesions, stillbirth, congenital abnormalities, pneumonia, enteritis, hepatitis and dead corpuses of infected animals as shown in Fig. 1.

Virus Isolation and Propagation: Blood samples were centrifugated at 3000 rpm for 5 min and the serum were collected and stored in-20°C till used. The tissues were crushed and centrifugated at 3000 rpm for 5 min and the supernatant were collected and stored in-20°C till used. The virus was propagated on Baby Hamster Kidney cells (BHK-21 clone 13), sub-clone of parent line derived from 51-day-old unsexed hamster kidneys which were obtained from Egyptian VACSERA Institute, Dokki, Giza. The cells was supplemented with Minimal essential Medium (Eagle's MEM) and 10% fetal calf serum and incubated in 37°C in CO₂ incubator as described by Zaher and Mohamed [12].

Bluetongue Competition Antibodies Test: Antibody to BT in sera was detected by a commercial ELISA (IDEXX[®] Laboratory Diagnostics, P00450-5) according to manufacturer's instructions. The IDEXX Bluetongue Competition antibodies (Ab) Test detects Ab specific to BTV in individual sheep, goat and cattle sera. It is based on competition between the serum to be tested and a monoclonal antibody.

Virus Purification: It was done according to Woolcocka and Shivaprasad using sucrose gradient ultracentrifugation [13]. Positive samples of culture and ELISA were centrifuged at 5000 rpm for 15 min and the supernatant was collected and stored at-20°C.



Fig. 1: Infected animal showing symptoms of BT such as mouth lesion, excessive salivation, swelling of the face and lips photo on the right. Ulceration on the lips and cyanosis of the tongue is very clear on the left photo.

The previously prepared supernatant was placed in Beckman TL-100 ultracentrifuge plastic centrifuge tube and exposed to high speed centrifugation at 45.000 X g (100.000 rpm) for 2 hours to pellet the virus on the bottom of the tube. The virus pellet was re-suspended in this buffer which contained 20 mM sodium chloride and different concentration of sucrose gradient 5 to 45% then centrifuged at 45.000xg (100.000 rpm) for two hours (Beckman L5-65 ultracentrifuge, SW50 rotor, Beckman, Palo Alto, California) the white band of the purified virus was sucked through sterile Pasteur pipette and stored in 2ml epindroff tube at -70°C till use.

Electron Microscopy: Pellets were re-suspended in 0.1 ml of distilled water and one drop of this suspension was placed on 200-mesh formvar carbon-coated grid. The material was stained with 2% solution of phosphotungstic acid, pH 6.5 [13]. The examination was performed at The Electron Microscope Unit, VACSERA, Dokki, Cairo, Egypt

Virus Diagnosis by RT-PCR: The viral ds-RNA was extracted by extraction kit (QIAamp Viral RNA mini kit QIAGEN®) according to the protocol of the manufacturer. RT-PCR was performed using one step RT-PCR QIAGEN kit and performed according to manufacturer's instructions. In this study, the genome segment coding for BTV-VP7 was amplified by RT-PCR using primers designed according to near perfect consensus sequences derived from published data [14, 15, 16]. The upstream primer PVP7-5 (5' GTTAAAAATCTMTAGAGATG 3', M: equal molar mixture A and C) anneals at the 5' end, whereas the downstream primer PVP7-3 (5' GTAAG TNTAATCNNAGAG 3', N: equal molar mixture of A, C, G

and T) anneals at the 3' end. Each primer has degenerate nucleotides incorporated to accommodate the sequence variations observed in published sequences. For c-DNA synthesis, total viral RNA was purified from purified BTV virus preparation. The first-strand c-DNA was synthesized using primer PVP7-3 [17] and subsequently amplified using primers PVP7-5 and PVP7-3 for 30 cycles at 94 °C for 1 min, 37 °C for 2 min and 72 °C for 2 min [14-16]. Amplified products were analyzed using a 100 bp DNA ladder (QIAGEN) (as a molecular marker on 1.5 % agarose gels. Gels were stained using Ethidium bromide 1 µg / ml) in Tris-EDTA (TE) and amplicons were visualized using an UV transilluminator at a wavelength of 590 nm. Positive reactions were confirmed according to size.

RESULTS AND DISCUSSION

Virus Propagation: Diagnostic tests currently used for the detection of BTV involve the isolation and growth of virus isolates in tissue culture cells (BHK-21). The cells showed rounding, piknosis and nuclear degeneration. Complete cell destruction was noticed after 73 hours after inoculation with the virus Fig. 2.

Antibody to BT detection by ELISA and Electron Microscopy: The virus is characterized using serological tests to detect reaction with reference antisera, such as the agar gel immunodiffusion test or serum neutralization test. These procedures are time-consuming and may fail to detect low levels of infectious virus or strains of BTV that do not replicate in eggs, mice or cell culture [10]. The use of the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to BTV in infected animals is faster. The IDEXX Bluetongue Competition Ab Test, which is called competitive ELISA (C-ELISA), detects

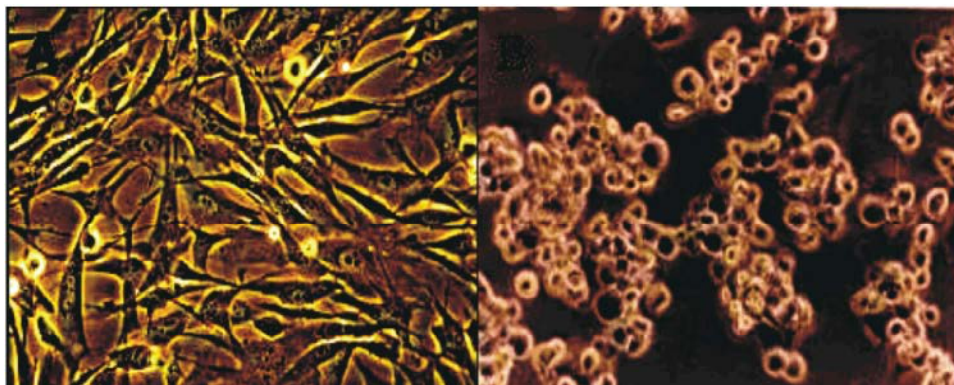


Fig. 2: Photo shows BHK-21. A: Normal BHK-21 cells. B: 36h post infection with the isolated virus. The cells are showing various cytopathic effects (CPE) such as rounding, piknosis and nuclear degeneration.

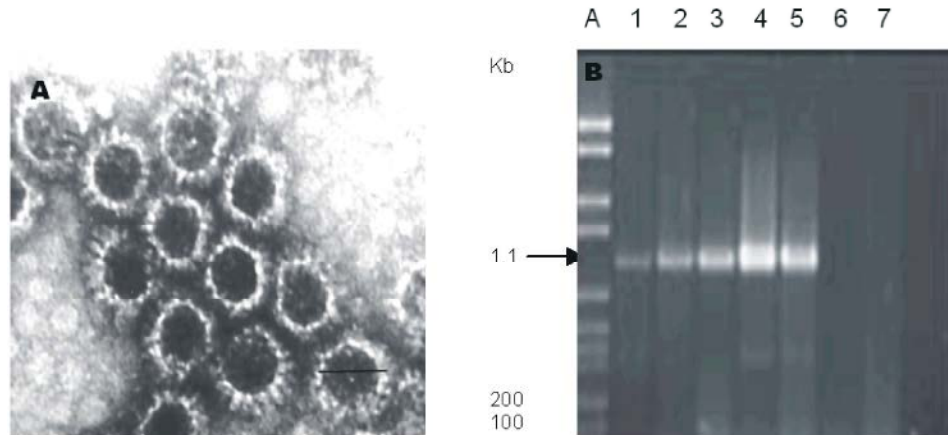


Fig. 3: A: Ultra-structure of BT. The virus is approximately 86 nm in diameter. B: Electrophoretic analysis of genomic double-stranded RNAs from Bluetongue virus. The double stranded RNA preparations were analyzed by electrophoresis in a 1% agarose gel which contains 0.5 µg/ml Ethidium bromide and visualized by exposure to ultraviolet light. In lane 1 DNA markers were run to enable estimation of molecular weights. In the rest of lanes (1-5) the tested BT samples were added with negative controls (Lanes 6 and 7) showing no band.

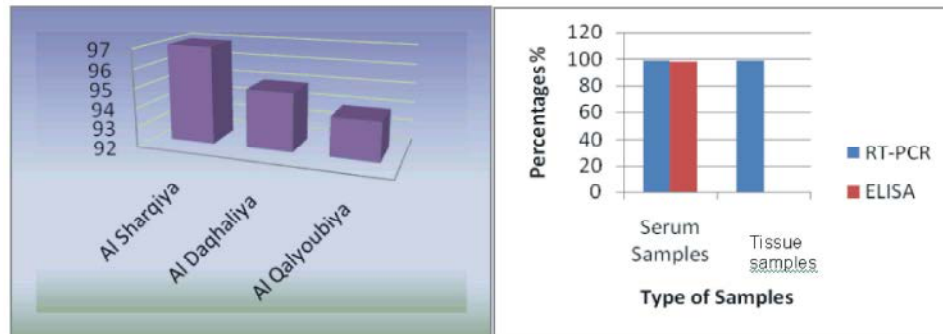


Fig. 4: A: Shows C-ELISA results in different governorate. B: Shows a Comparison between RT-PCR and ELISA

antibodies specific to the bluetongue virus (BTV) in individual sheep, goat and cattle sera. It is based on competition between the serum to be tested and a monoclonal antibody, which is coupled to the peroxidase and directed to the N-terminal part of the VP7 protein, a major core protein of the BTV (Specific for the BT serogroup). This method is easy to implement, rapid and reliable and it is particularly suited to analyzing a large number of samples. As no international standard for serum is available, the detectability has been settled on a French standard (J63) proposed by the CIRAD (Montpellier), which is the international reference laboratory for this disease. The test should give a positive reaction with dilution at 1:4, positive, doubtful or negative result with dilution at 1:8 and negative result with the dilution at 1:16 of this French standard. The test showed that 98% of clinically diseased animals showing clinical symptoms were positive to BTV, these finding matches

with Maan *et al.* [10]. Moreover, BTV show great stability against bad condition of sampling transportation as it persist high temperature. The virus extended in many villages in the three governorates where samples were taken from and these above mentioned villages were representative. All animals in this governorate were imported from Sinai. Further studies should be taken to investigate the portal of entry for this disease. Al Sharqiya showed more diseased animals with BTV as this governorate is rich with sheep and goats followed by Al Daqhaliya and Al Qalyoubiya (Fig 4A). Further more study should be done to estimate the serotype of the virus through sequencing

Electron microscopic examination revealed icosahedral virus containing two capsids. The virus particles were about 86 nm in diameter (Fig. 3A) the virus was easy to detect in blood as well as in tissue. This data matches the finding of Mertens *et al.* [7]

Virus Diagnosis by RT-PCR: The PCR is a method for *in vitro* amplification of DNA. It has substantially accelerated the pace of research in many fields of biology by reducing the time required to perform routine manipulations of DNA and by making new manipulations possible. This technique, referred to as RT-PCR, is used to as BT is a RNA virus. PCR amplification a major PCR band was observed at 1.1 kb which matched the predicted size (Fig. 3B) 99% of the samples were positives. Tissue samples gave clear results as well as blood samples.

Comparison Between ELISA and RT-PCR Results:

Unfortunately ELISA does not necessarily confirm recent infection also may be used to detect virus directly in tissue samples but the sensitivity is relatively low. A number of procedures have been developed to detect the presence of BTV antigens or nucleic acids. The polymerase chain reaction (PCR) technique has proved to be a powerful tool for BTV diagnosis [18, 19]. PCR techniques may be used not only to detect the presence of viral nucleic acid but also to ‘serogroup’ *Orbiviruses* and provide information on the serotype and possible geographic source (Topotype or genotype) of BTV isolates within a few days of receipt of clinical samples, such as infected sheep blood. Traditional approaches, which rely on virus isolation followed by virus identification, may require at least three to four weeks to generate information on serogroup and serotype and yield no data on the possible origin of the virus isolated. Moreover, PCR enables differentiation between field isolates and vaccine strains [19].

The PCR, first described in 1985, is a highly sensitive and specific technique used for the detection of nucleic acids [20]. The inventor of this technology earned a Nobel Prize for his achievement [21, 22] which has revolutionized research and diagnosis. As far as BTV diagnosis is concerned, the application of PCR technology has led to very rapid amplification of BTV RNA in clinical samples and PCR-based procedures are now available to provide information on virus serogroup and serotype. In PubMed more than 60 international publications deal with ‘bluetongue and PCR’ and more than 30 are related to the use of ‘PCR in BTV diagnoses.

In this study ELISA showed 98% sensitivity while RT-PCR showed 99% sensitivity in serum samples (Fig.4B) even though the target of both test are completely different, ELISA detects antibody while RT-PCR detects viral RNA.

CONCLUSION

This study confirms the presence of blue tongue infection in Egypt also it recommends the use of RT-PCR as fast, cheap, accurate and sensitive method for detection of the virus especially when samples are taken from tissue. The disease should be notifying its presence in Egypt since it was free from this disease and more studies should be attempts to estimate the serotype of BTV.

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