



## Serogrouping of United States and some African serotypes of bluetongue virus using RT-PCR

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### Abstract

The diagnostic potential of RT-PCR for detection of bluetongue virus (BTV) ribonucleic acid (RNA) sequence in cell culture and tissue samples from infected ruminants from United States, Sudan, South Africa and Senegal, was evaluated. The non structural protein 1 (NS1) gene of North American BTV serotype 11 was targeted for PCR amplification. The United States BTV serotypes 2, 10, 11, 13 and 17 and the Sudanese BTV serotypes 1, 2, 4 and 16 and BTV serotype 4 from South Africa and BTV serotype 2 from Senegal were studied. RNAs from all BTV field isolates used in this study, propagated in cell cultures, were detected by the described RT-PCR-based assay. The first specific 790 bp BTV PCR products were amplified using a pair of outer primers (BTV1 and BTV2). Specificity of the PCR products was confirmed by a nested amplification of a 520 bp PCR product using a pair of internal (nested) primers (BTV3 and BTV4). The BTV PCR products were visualized on ethidium bromide-stained agarose gels.

Amplification products were not detected when the RT-PCR-based assay was applied to RNAs from closely related orbiviruses including: epizootic hemorrhagic disease virus (EHDV) prototypes serotypes 1, 2, 4; RNA from Sudanese isolate of palyam orbiviruses serogroup and total nucleic acid extracts from uninfected Vero cells.

Application of the nested BTV RT-PCR to clinical samples resulted in amplification of BTV RNA from blood and serum samples from goats experimentally infected with BTV4 and from naturally infected sheep, goats, cattle and deer.

The results of this study indicated that this RT-PCR assay could be applied for rapid detection of BTV, in cell culture and clinical samples from susceptible ruminants during an outbreak of the disease, in the United States and African.

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## 1. Introduction

Bluetongue virus (BTV) is a double stranded (ds) RNA orbivirus of the family Reoviridae (Borden et al., 1971; Fenner et al., 1974; Gould et al., 1992). The virus has a worldwide distribution and exists in at least 25 distinct serotypes (Davies et al., 1992). In Sudan, previous studies have shown that the seasonal incidence of BTV is a predictable event related to the rainy season (Mohammed and Mellor, 1990). BTV serotypes 1, 2, 4 and 16 were recovered from a sentinel calf herd at the Khartoum University farm, Shambat (Mohammed and Mellor, 1990; Mohammed and Taylor, 1987). BTV serotypes 2, 10, 11, 13 and 17, which are known to be enzootic in the United States, cause febrile disease in sheep and clinical hemorrhagic disease in North American white-tailed deer (Shope et al., 1960; Hoff and Trainor, 1974; Aradaib et al., 1995). In cattle and goats, the infections are usually inapparent and evidence of clinical disease is seldomly observed. However, indirect losses associated with loss of body weight and condition, drop in milk production and poor subsequent reproductive performance were thought to have greater economic effect than occasional overt disease (Mohammed and Taylor, 1987; Gorman, 1992).

In addition, there is restriction on the international movement of livestock and associated germplasm from countries suspected to harbor the disease to BTV-free countries, unless the animals are certified free of infection by conventional virus isolation or serology (Gorman, 1992). Such a restriction could lead to economic losses for BTV-endemic countries, like Sudan, which rely on the sale of livestock for foreign exchange. Conventional virus isolation and serotyping are the most accurate methods for definitive diagnosis. However, they are time consuming, labor intensive and expensive (Maclachlan et al., 1990; Hammami and Osburn, 1992; Gould et al., 1992; Pearson et al., 1992; Work et al., 1992; Aradaib et al., 1995). In previous studies, hybridization assays using complementary cDNA probes and RT-PCR-based detection assays were developed and evaluated for detection of BTV. These RT-PCR assays have greatly improved the detection of BTV infection. In the present study, a nested BTV RT-PCR assay was evaluated for rapid detection of United States and African isolates of BTV serogroup.

## 2. Materials and methods

### 2.1. Cell culture and virus propagation

The four BTV serotypes 1, 2, 4 and 16 present in the Sudan (Faculty of Veterinary Medicine, University of Khartoum, Sudan) and the five BTV prototypes serotypes 2, 10, 11, 13, 16 and 17 present in United States (Arthropod-Borne Animal Disease Research Laboratory, Laramie, WY) were used in this study. Two isolates of BTV serotype 2 recovered from naturally infected sheep in Senegal (Veterinary Research Institute, Dakar, Senegal) and BTV serotype 4 from South Africa (Veterinary Research Institute, Onderstepoort, South Africa) were also included in the study. EHDV prototypes serotypes 1, 2 (National Veterinary Services Laboratory, USDA, APHIS, Ames, IA and Washington Animal Disease Diagnostic Laboratory, Pullman, WA) were used. The Sudanese isolate of EHDV-4 and palyam viruses serogroup (Faculty of Veterinary Medicine, University of Khartoum, Sudan) were employed as negative controls. The viruses were isolated and processed as described previously (Aradaib et al., 1995). All viruses were propagated on confluent monolayers of Vero cells. Minimal essential medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) was used for virus propagation. The infectious material was harvested and centrifuged at  $1500 \times g$  for 30 min and the cell-free supernatant was used for RNA extraction using QIAamp extraction kit (QIAamp, Hamburg, Germany).

### 2.2. Extraction of viral nucleic acid from infected cell culture and clinical samples

The BTV, EHDV and the palyam orbiviruses serogroup dsRNAs were extracted from the infected cell cultures and supernatant from infected tissues using QIAamp viral RNA kit (QIAamp, Hamburg, Germany) as per manufacturer's instructions. Briefly, 140  $\mu$ l of virus suspension were added to 560  $\mu$ l AVL buffer containing carrier RNA into a 1.5 ml microcentrifuge tube and mixed by pulse-vortexing for 15 s. The mixture was incubated at room temperature for 10 min. An amount of 560  $\mu$ l of absolute ethanol were added and mixed by pulse-vortexing for 15 s. An amount of 630  $\mu$ l of the mixture were transferred to

QIAamp spin column mounted on 2 ml collection tube and centrifuged at 8000 rpm for 1 min. The column was then transferred to another collection tube and the remaining 630  $\mu$ l of the mixture was again spin at the same speed. The column was then washed twice by 500  $\mu$ l of washing buffers WB1 and WB2, respectively. Finally, dsRNAs were carefully eluted by 60  $\mu$ l of buffer AVE equilibrated to room temperature. Total nucleic acid was quantified using a spectrophotometer at 260 nm wavelength.

### 2.3. Design of primers

BTV primers were designed from genome segment 6 of BTV11. Details for design of primers including primers number, nucleotide positions, nucleic acid sequences and expected PCR products were summarized in (Table 1).

### 2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR protocol used in this study was basically as previously described (Aradaib et al., 1998). Briefly, for the reverse transcription step, the thermal cycling profiles were performed at 42 °C for 30 min followed by incubation at 99 °C. For PCR amplification, the thermal cycling profiles at 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 45 s, and a final incubation at 72 °C for 10 min. An amount of 5 units/ $\mu$ l of the Taq DNA polymerase (Perkin-Elmer Cetus, The Perkin-Elmer Corporation, Norwalk, CT) was used per reaction. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ).

For nested amplification, 2  $\mu$ l of the first amplified 790 bp PCR product were transferred to a PCR tube containing amplification buffer. For each PCR

amplification, the amplification buffer consisted of 10  $\mu$ l of 10X PCR buffer; 10  $\mu$ l  $MgCl_2$  of 1.5 mM concentration; 2  $\mu$ l of primers (BTV3 and BTV4) at a concentration of 20 pg; 8  $\mu$ l of dNTPs including ATP, TTP, GTP, CTP; 1  $\mu$ l of Taq DNA polymerase (Perkin-Elmer Corporation, Norwalk, CT) at a concentration of 5.0 units/ $\mu$ l. Double distilled water was added to each PCR tube to obtain a total volume of 100  $\mu$ l. The PCR tubes were replaced in the thermal cycler for another 40 cycles at the same temperature per cycles described above.

Following amplification, 20  $\mu$ l from each nested PCR reaction containing amplified product were loaded onto gels of 1.5% SeaKem agarose (FMC Bioproduct, Rockland, ME) and electrophoresed. The gels were stained with ethidium bromide, and the nested PCR products were visualized under UV light.

## 3. Results

The described BTV RT-PCR-based assay afforded sensitive and specific detection of all BTV isolates used in this study. The first 790 bp PCR product were detected from 1.0 pg RNA of United States BTV serotypes 2, 10, 11, 13 and 17; Sudanese BTV serotypes 1, 2, 4 and 16; South African BTV serotype 4 and Senegali isolates of BTV serotypes 2; propagated in Vero cell culture (Fig. 1). The nested 520 bp PCR product was successfully amplified from the first 790 bp PCR product (Fig. 2).

The amount of 1.0 pg RNA from EHDV serotypes 1, 2, 4 and RNA from palyam virus and total nucleic acid extracts from uninfected Vero cells failed to demonstrate the primary or the nested BTV PCR products (Fig. 3).

Application of this nRT-PCR-based assay to clinical samples resulted in direct detection of BTV

Table 1

Primer	Position	Nucleic acid sequence	PCR product (bp)
External (outer primers)			
BTV1	71–90	5'-GATTACGCAAATGCCACGAG-3'	790
BTV2	841–860	5'-GGTGTGAATGGAAAATTCACCT-3'	
Internal (nested primers)			
BTV3	311–330	5'-TACGAGGAGGATGTCGAAGG-3'	520
BTV4	811–830	5'-TTCTGAAGAGCTGTGTGACA-3'	

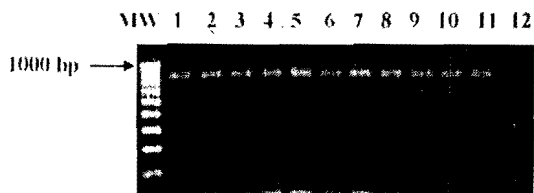


Fig. 1. Visualization of the 790 bp specific-BTV PCR product on ethidium bromide-stained agarose gel from 1.0 µg of RNA of eight different BTV field isolates. Lane MW: molecular weight marker; lanes 1–5: North American BTV serotypes 2, 10, 11, 13 and 17; lanes 6–9: Sudanese BTV serotype 1, 2, 4 and 16 field isolate; lane 10: BTV4 from Senegal; lane 11: BTV4 from South Africa; lane 12: non-infected Vero cells total nucleic acid extract.

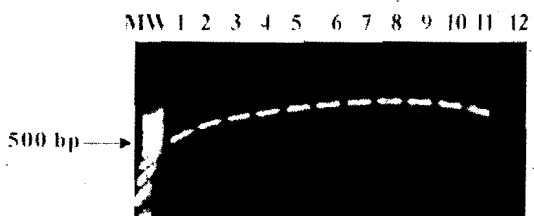


Fig. 2. Nested amplification of the nested 520 bp specific-BTV PCR product on ethidium bromide-stained agarose gel from 1.0 µg of RNA of eight different BTV field isolates. Lane MW: molecular weight marker (100 bp ladder); lanes 1–5: North American BTV serotypes 2, 10, 11, 13 and 17; lanes 6–9: Sudanese BTV serotype 1, 2, 4 and 16 field isolate; lane 10: BTV4 from Senegal; lane 11: BTV4 from South Africa; lane 12: non-infected Vero cells total nucleic acid extract.

RNA from blood and serum samples collected from Nubian goats experimentally infected with Sudanese BTV serotype 4 (Fig. 4). The nested 520 bp PCR product was also detected in tissue samples from

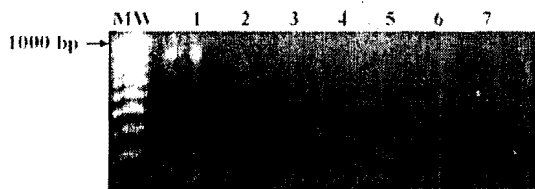


Fig. 3. Specificity of the polymerase chain reaction for BTV RNA. The 790 bp PCR amplification product was not detected from 1.0 µg RNA from EHDV serotypes 1, 2, 4; palyam virus and total nucleic acid extracts from non infected Vero cells. Lane MW: molecular weight marker (100 bp ladder); lane 1: 1 µg BTV4; lanes 2–4: EHDV prototypes serotypes 1, 2 and 4, respectively; lanes 5–6: dsRNAs from palyam virus; lane 7: non infected Vero cells total nucleic acid extract.

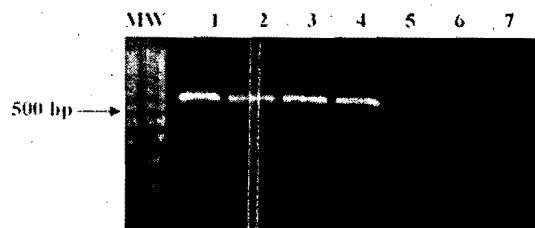


Fig. 4. Detection of the 520 bp specific-BTV PCR product by nested amplification from clinical samples. Lane MW: molecular weight marker (100 bp ladder); lanes 1–4: blood samples from BTIV4 infected goats; lanes 5–6: blood samples from non infected goats; lane 7: non infected Vero cell controls.

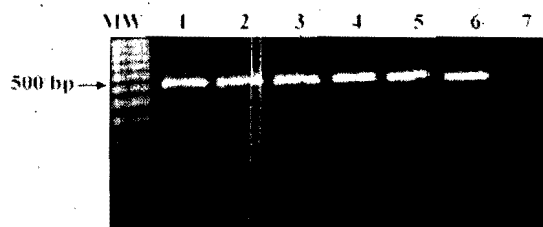


Fig. 5. Detection of the 520 bp specific-BTV PCR product by nested amplification from clinical samples. Lane MW: molecular weight marker; lanes 1 and 2: blood samples from BTIV11 infected calves; lanes 3 and 4: blood sample from BTIV infected deer; lanes 5 and 6: blood sample from BTIV infected sheep; lane 7: blood sample from non infected calf.

white-tailed deer, sheep and cattle (Fig. 5). All BTV which were PCR positive were also positive by conventional virus isolation and serotyping.

#### 4. Discussion

Bluetongue infection is a serious veterinary problem in sheep and North American white-tailed deer (Shope et al., 1960). However, in focal areas of endemicity, goats and cattle developed subclinical infection (Mohammed and Taylor, 1987; Gorman, 1992). At least four serotypes of BTV designated BTV1, BTV2, BTV4 and BTV16 are known to be enzootic in the Sudan (Mohammed and Mellor, 1990). Very little information is available about orbiviruses originally isolated in Sudan and Senegal. Further studies on these BTV serogroup are necessary to determine their biology, ecology and molecular epidemiology.

The BTV field isolates used in this study represented a range of topotype viruses, isolated from a diverse geographic location in North America and Africa, including Sudan (Central Africa), Senegal (West Africa) and South Africa. The isolates were recovered from different animal species including cattle, sheep, goats and deer. The described BTV RT-PCR assay using serogroup primers derived from segment 6 of BTV11 reproducibly and specifically detected BTV RNA in infected cell cultures and clinical samples. Selection of the primers was based on the observation that the NS1 gene of BTV is the most conserved among cognates of BTV serogroup (Aradaib et al., 1998). The specific 790 bp PCR products, visualized on ethidium bromide-stained agarose gel, were obtained from all BTV RNA samples tested. The BTV RT-PCR assay was a simple procedure that efficiently detected all BTV isolates under the stringency condition used in this study. It is well documented that nested amplification increases the sensitivity of the PCR assay and confirms the identity of the first PCR product (Aradaib et al., 1998, 2003). In the present study, the use of nested amplification removes the hazardous and cumbersome hybridization assay with radio-labeled cDNA probes. In addition, hybridization confirmation assay is tedious, laborious and usually takes overnight.

In a previous report, the sensitivity studies of the described BTV RT-PCR protocol indicated that the PCR assay was capable of detecting the amount of 0.1 fg of total BTV17 genomic dsRNA. The total molecular weight of the BTV genome is  $11.44 \times 10^6$  Da, and 0.1 fg of BTV RNA corresponds to five viral particles (Aradaib et al., 1998). The BTV RT-PCR amplification technology with nested amplification was more sensitive than the dot-blot hybridization using BTV cDNA probe, where at least a few nanograms of the dsRNA are required to produce a positive hybridization signal (Venter et al., 1991).

The specificity studies indicated that the specific 520 bp PCR product was not amplified from 1.0 pg of RNA from EHDV serotypes 1, 2, 4 and RNA from palyam virus or total nucleic acid extracts from Vero cell controls under the same stringency condition described in this study. Temperature and time for denaturation, primer annealing and extension, enzyme and  $MgCl_2$  concentration, and number of cycles of the three temperature per time segments were very important for

maintaining sensitivity and specificity of the PCR reaction. The nested amplification is necessary to confirm the identity of the first amplified product and to increase the sensitivity of the RT-nPCR assay.

The sample size used in this study represents all BTV serotypes recognized to be enzootic in United States and some African countries, including Senegal, Sudan, South Africa. We believe that, the samples were enough to validate the described BTV RT-PCR assay for detection of BTV serogroup from different geographical origin in North America and Africa. This finding also confirmed that BTV NS1 genome is highly conserved among cognates of BTV serogroup and could be used for serogrouping and topotyping of BTV serogroup from different continents.

The BTV RT-PCR assays provide supportive diagnostic techniques to the lengthy cumbersome conventional virus isolation procedures. The QIAamp kit provides a simple procedure that takes only 1 h for viral dsRNA extraction. The thermal cycling profiles for reverse transcription and RT-PCR assay, including the primary and nested amplifications, required 6 h. The time required from sample submission to interpretation of the final results was consistently 7 h. This means that confirmatory diagnosis of submitted samples, from BTV suspected animals, could be made within the same working day. The rapidity, sensitivity and specificity of the RT-PCR assay would greatly facilitate detection of BTV infection in an outbreak among susceptible ruminants in United States and Africa. In the present study, of the 25 serotypes of BTV serogroup, we validated the detection of BTV serotypes 1, 2, 4, 10, 11, 13, 16 and 17 by the described BTV RT-PCR-based detection assay. The described RT-PCR assay could also detect the remaining serotypes of BTV serogroup. This RT-PCR-based assay for detection of African and United States field isolates of BTV provides the basis for future diagnosis of BTV in the African Continent. Further studies are in progress to determine the capability of the described BTV RT-nPCR assay to detect additional serotypes of BTV serogroup, and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with current diagnostic techniques used for detection of BTV infection.

In conclusion, the described serogroup-specific BTV RT-PCR assay, using primers derived from genome segment 6 of BTV11, should provides rapid

detection of BTV infection during an epizootic of the disease among susceptible ruminants from United States, Sudan, Senegal and South Africa.

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