INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
Volume-4, Issue-2, April-June-2014
ISSN 2231-4490
Copyrights@2014
Coden : IJPAES www.ijpaes.com
*Received: 16th Feb-2014* *Revised: 10th March-2014* *Accepted: 13th March-2014*
***Research article***

# DETECTION AND CHARACTERIZATION BASED ON S7 AND S10 GENE SEGMENTS OF *BLUETONGUE VIRUS* ISOLATES FROM ANDHRA PRADESH, INDIA

P. Sangamithra[1,*], A.M. Anthony Johnson[1], D. Sreenivasulu[2] and D.V.R. Sai Gopal[1]

[1]Department of Virology, Sri Venkateswara University, Tirupati, A.P. India.
[2]Department of Microbiology, Sri Venkateswara Veterinary University, Tirupati, A.P. India.
*Corresponding author: psmithra2008@gmail.com mobile: +91 9849120359

**ABSTRACT :** The surveillance of Bluetongue disease (BT) during 2009-2010 outbreaks in the districts of Chittoor, Nellore, Nizamabad, Krishna, Guntur, Kadapa and Ongole, Andhra Pradesh, India revealed the occurrence of high morbidity, mortality, abortions, stillbirths, loss of fleece, reduced meat production in affected sheep leading to the vast economic loss to the poor section of Indian farming community. During 2009 and 2010 a total of 130 BT suspected clinical (blood) samples were collected from infected sheep. Analysis of the clinical samples in RT-PCR confirmed association of Bluetongue Virus (BTV) with the disease. RT-PCR was optimized for the amplification of S7 (VP7) and S10 (NS3) genes of field isolates with reference to BTV-2-Tirupati isolate. The amplicons were cloned, sequenced and submitted to Genbank. Phylogenetic characterization of these virus isolates based on S7 and S10 genes with all other reported isolates concluded that the present study isolates could be BTV-2 circulating in Andhra Pradesh, India.
**Keywords -** Bluetongue Virus, S7 gene, S10 gene, RT-PCR, Cloning, Phylogenetics

## INTRODUCTION

Bluetongue (BT) is an International office of Epizooties multiple species disease [6] and described as an economically devastating affliction of sheep. The vast economic losses of BT is due to high morbidity, mortality, abortions, stillbirths, loss of fleece, reduced meat production in affected animals and the restrictions placed on trading of animals and animal products from the endemic areas. Small ruminants like sheep and goats contribute significantly to the economy of developing country like India. The sheep are closely identified with poor people in pastoral system ranging from arid and semi-arid tropics to complex crop-livestock system in humid tropics. These animals are bestowed with sound innate capacity to thrive under extreme conditions with better economic returns than larger ruminants. Small ruminants are integral part of the poor section of Indian farming community.

Bluetongue Virus, the causative agent of BT is a member of the genus *Orbivirus* in the family *Reoviridae*. There are 24 serotypes of BTV, of which all 22 have been reported from India [11]. The epizootiology of virus is very complex in nature involving interaction of types of virus, vector, climate and the host. Bluetongue virus requires appropriate species of vector for its perpetuation in various geographic regions. The vectors are biting midges of genus *Culicoides*. The BTV genome is composed of 10 double stranded RNA segments, which encode 7 structural proteins (VP1-VP7) and four non-structural proteins (NS1, NS2, NS3 & NS3A). The outer capsid contains the serotype specific structural proteins VP2 and VP5 while the inner capsid contains the two major serogroup specific structural proteins VP3 and VP7 [5, 9]. VP7 gene has been demonstrated to have diagnostic capability for the detection of all serotypes of BTV due to the group specificity [2, 7]. The genome segments 7 and 10 are generally used for group – BTV detection. Serogroup specific RT-PCR, sequencing, Restriction enzyme analysis and phylogenetic analyses (targeting conserved genome segments) are used in many laboratories in India, for identification of BTV [1, 8, and 12]. The purpose of this study was to optimize the RT-PCR used for diagnosis of BTV infection and comparison of the two segments, S7 (VP7) and S10 (NS3), with the present study field isolates with each other for the identification of any differences at molecular level and to determine the serogroup.

## MATERIALS AND METHODS

### Sample collection

Bluetongue suspected blood samples were collected in EDTA (1-2 mg/ml of blood) from sheep during suspected outbreaks of bluetongue during the years of 2009-2010 from selected villages of Chittoor, Nellore, Nizamabad, Krishna, Guntur, Kadapa and Ongole districts of Andhra Pradesh.

### Detection of the Virus

The identification of the virus was confirmed by amplifying the conserved region of S10 (NS3) gene of the virus using reverse transcriptase polymerase chain reaction (RT-PCR). The viral genomic dsRNA from infected cell line (BHK21) was extracted using Trizole (Sigma, USA) method [3], followed by differential precipitation using lithium chloride. The Reverse Transcription was carried out at 42°C for 1 hour using ~ 100ng ds RNA, Random Hexamer primer, 012mM dNTPs and 5 Units of Molony Murine Leukemia Virus Reverse Transcriptase (MMuLV-RTase) (Fermentas, USA) followed by RTase inactivation at 75°C for 15 min. and Polymerase Chain Reaction was carried out using the NS3 gene specific primers, 20 picomoles each **(NS3F-GCGGGATCC ATGCTATCCGGCTGAT, NS3R- GCGTACGATGCGAATGCAGC,** available in BT lab, SVVU, Tiruapti) in the presence of 0.2mM dNTPs, 1.25mM $MgCl_2$ and 1 U of recombinant *Taq* DNA Polymerase (Fermentas, USA). Thermocycling conditions were Initial denaturation of 94° C for 3 min followed by 35 repeated cycles of denturation at 94° C for 30 sec, Annealing at 60° C for 30 sec, extension at 72° C for 30 sec and final extension at 72° C for 10 min.

### Amplification of S7 gene

RT-PCR was optimized for the amplification of S7 (VP7) gene for the BTV isolates viz., BTV-Krishna and BTV-Guntur with reference to BTV-2-Tirupati isolate (Personnel communication, D. Sreenivasulu, Department of Microbiology, SVVU, Tirupati). The Reverse transcription was carried out using two sets of gene specific primers, set-1 reported by Sangeeta Dalal *et al.*, 2009 [10] **(F - GTTAAAAATCTATAGAGATGGAC, R-GTAAGTGTAATCTAAGAGACG)** and set-2**(PSMBTVVP7F-AAGGATCCATGGACACTATCGCAGCAAGA,PSMBTVVP7R-AAGGTACCCTACACAT A AGCGGCGCG)** was designed and commercially obtained from Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad and 2.5 Units of Molony Murine Leukemia Virus Reverse Transcriptase (MMuLV-RTase) (Fermentas, USA) using ~300ng dsRNA as template in the presence of 6%DMSOand 0.12mM dNTPs. PCR amplification was performed using 25 pmoles of each primer, 5% DMSO, 200 µM of dNTPs. 1.25 mM of $MgCl_2$ and 1.25 U of recombinant *Taq* DNA Polymerase (Fermentas, USA). Thermo cycling conditions were same as reported by Sangeeta Dalal *et al.*, 2009 [10].

### Amplification of S10 gene

RT-PCR was also optimized for the amplification of S10 (NS3) gene of all three isolates and Reverse transcription was carried out using gene specific primers designed by us **(PSMBTVNS3F-GAAAGATCT ATGCTAT CCGGGCTGATCC, PSMBTVNS3R- AAGGTACCTCAGGTTAATGGCATTTCGAA)** 20 Pico moles each, 2.5 Units of Molony Murine Leukemia Virus Reverse Transcriptase (MMuLV-RTase) (Fermentas, USA) using ~300ng dsRNA as template in the presence of 6%DMSO and 0.12mM dNTPs. PCR amplification was performed using same concentrations of primers, 200 µM of dNTPs. 1.5 mM of $MgCl_2$ and 1.25 U of recombinant *Taq* DNA polymerase (Fermentas, USA). Thermo cycling conditions were Initial denaturation of 94° C for 3 min followed by 35 repeated cycles of denturation at 94° C for 30 sec, Annealing at 59° C for 30 sec, extension at 72° C for 1 minute and final extension at 72° C for 30 min.

### Cloning, Sequencing and Phylogenetic analysis

The RT-PCR products were cloned into pTZ57R/T vector (Fermentas, USA) as per the manufacturer's instructions of InsTA Clone-PCR Cloning Kit from Fermentas and were sequenced using M13 universal primers at Eurofins Genomics India Pvt.Ltd., Bangalore, India. The sequence homologs were searched by BLAST tool at NCBI server which revealed close similarity with BTV S7 and S10 genes. The percent identities of S7 and S10 genes of BTV - 2- Tirupati, BTV - Guntur and BTV - Krishna isolates with reported BTV sequences at nucleotide level and amino acid levels were calculated using BIOEDIT version 7.0.5.3 software [4]. Phylogenetic analysis was performed using Clustal X Package v. 2.1. The phylogenetic trees were visualized using Tree Explorer v.2.12.

## RESULTS AND DISCUSSION

A total of 130 BT suspected samples were collected in different outbreaks (Table.1) Out of 130 BT suspected blood samples 24 were found to be positive in RT-PCR.

**Table. 1: Details of sample Collection places and Detection of BTV in blood samples from BT suspected Sheep**

| S. No. | Date of sample collection | Place/village | District | Infected Host | No. of samples collected | No. of RT-PCR positive samples |
|---|---|---|---|---|---|---|
| 1. | 15.07.09 | Pedda Panduru | Chittoor | Sheep | 12 | 2 |
| 2. | 06.08.09 | V.R. Kandriga | -do- | -do- | 6 | 0 |
| 3. | 26.06.10 | Madamanur | Nellore | -do- | 11 | 1 |
| 4. | 23.08.10 | Mosra | Nizamabad | -do- | 7 | 0 |
| 5. | 20.09.10 & 21.09.10 | Kesarapalli | Krishna | -do- | 10 | 3 |
| | | Bhimavaram | -do- | -do- | 8 | 2 |
| | | Paritala | -do- | -do- | 5 | 1 |
| | | Maddirala | -do- | -do- | 4 | 0 |
| | | Kattubadivaripalem | -do- | -do- | 2 | 1 |
| 6. | 12.10.10 | Mangalagiri | Guntur | -do- | 9 | 1 |
| | | Boppudi | -do- | -do- | 6 | 2 |
| | | Macharla | -do- | -do- | 12 | 4 |
| | | Edavalli | -do- | -do- | 6 | 0 |
| 7. | 15.11.10 | Pendlimarri | Kadapa | -do- | 5 | 2 |
| 8. | 22.11.10 | Alluru | Ongole | -do- | 15 | 3 |
| 9. | 11.12.10 | Rapur | Nellore | -do- | 8 | 2 |
| 10. | 17.12.10 | Rompicherla | Chittoor | -do- | 4 | 0 |
| | | | | **Total** | **130** | **24** |

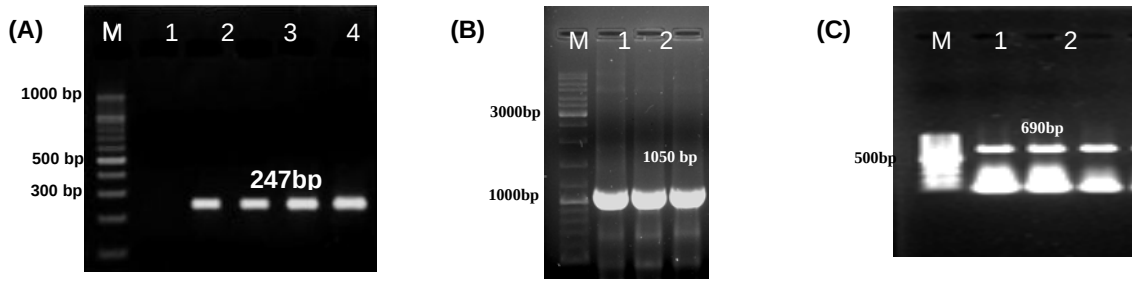


**Figure 1.** Gel photographs showing Detection of BTV and amplicons of S7 and S10 genes in RT-PCR

**A.** Detection of BTV in field isolates using RT-PCR
Lane M: 100bp DNA Ladder (Fermentas)
Lane 1: Negative control
Lane 2: Positive control
Lane 3: 247bp amplicon of S10 gene of BTV-2-TPT isolate
Lane 4: 247bp amplicon of S10 gene of BTV-Krishna isolate
Lane 5: 247bp amplicon of S10 gene of BTV-Guntur isolate

**B**. Gel picture showing the amplification of S7 (VP7) gene of BTV isolates in RT-PCR
Lane M: 1 kb GeneRuler™ DNA Ladder (Fermentas)
Lane 1: 1050bp amplicon of BTV-2-TPT isolate
Lane 2: 1050bp amplicon of BTV-Krishna isolate
Lane 3: 1050 bp amplicon of BTV-Guntur isolate

**C**. Gel picture showing the amplification of S10 (NS3) gene of BTV isolates in RT-PCR
Lane M: 100bp DNA Ladder (Fermentas)
Lane 1: 690 bp amplicon of BTV-2-TPT isolate
Lane 2: 690 bp amplicon of BTV-Krishna isolate
Lane 3: 690 bp amplicon BTV-Guntur isolate

Detection of virus in these samples was confirmed by the amplification of 247bp conserved region of S10 (NS3) gene of BTV isolates (Fig. 1.A). RT-PCR resulted in amplification of ~1.15kb (primer set 1), 1.05kb (primer set 2) of S7 gene and 0.69kp of S10 gene in all the three BTV isolates of the present study (Figure 1. B and C). A total of 1154 ntds and 1050 ntds were obtained comprising complete sequence and open reading frame of S7 gene and 690 ntds ORF of S10 gene respectively. The sequences were submitted to Genbank Database JQ080436, JQ080437 (BTV -2- Tirupati), KF460442, KF460444 (BTV - Guntur) and KF460443, KF469445 (BTV - Krishna). The sequence comparisons at amino acid level for S7 gene BTV-Guntur and BTV-2- Tirupati isolates shows 99.7% and 99.7% homology with JQ713561 isolate. BTV-Krishna shows 99.1% homology with AM261976 isolate. The sequence comparisons at amino acid level for S10 BTV2/Tirupati shares 100% of homology with BTV/Krishna, BTV6 isolates and also shares 99.5% with that of BTV-Guntur isolate. BTV-Guntur and BTV-Krishna isolates share 99.5% of homology with each other. BTV-Krishna shares 100% maximum homology with that of BTV-2-Tirupati and BTV6 isolates. Phylogenetic analysis based on complete sequence resulted in an un rooted bootstrapped NJ trees showing relationship of the present study BTV isolates with that of all BTV isolates reported from India (Figure 2. A and B).

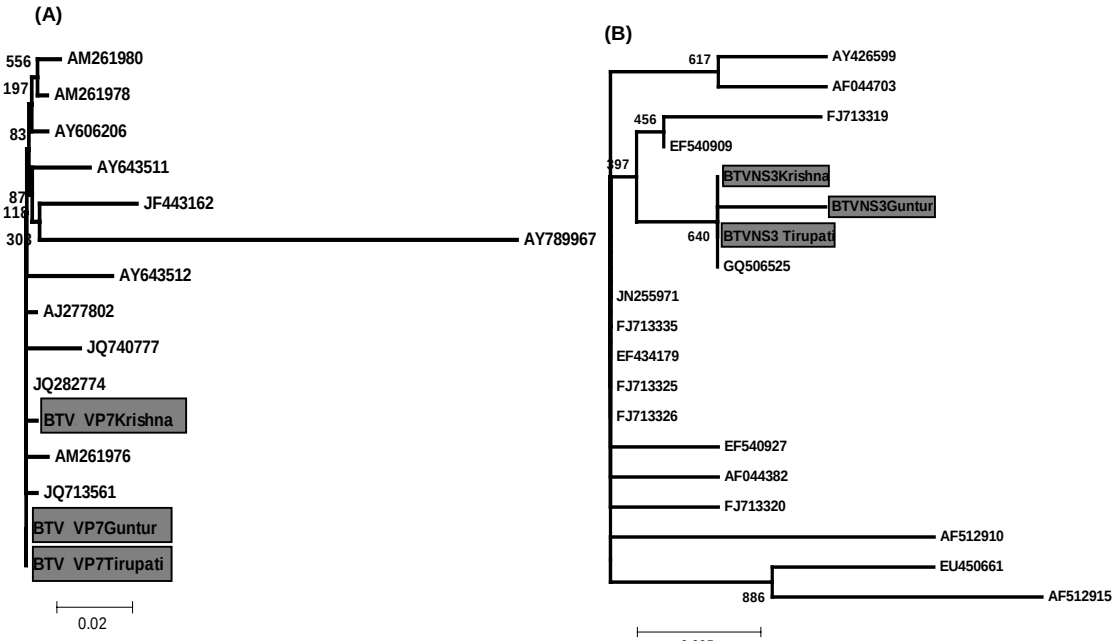


**Figure 2.A. Bootstrapped NJ Unrooted Phylogenetic tree constructed for Indian isolates of BTV VP7 gene amino acid sequences. BTV-2-Tirupati, BTV-Krishna and BTV-Guntur sequences generated from the present study are shade marked.**

**B. Bootstrapped NJ Unrooted Phylogenetic tree constructed for the representativeBTV serotypes NS3 gene aminoacid sequences. BTV-2-Tirupati, BTV-Krishna and BTV-Guntur sequences generated from the present study are shade marked.**

Therefore it can be concluded that the two field isolates of BTV (Untyped) viz., BTV-Krishna and BTV-Guntur belonging to two different regions fall in same cluster along with BTV-2-Tirupati. In addition, the sequence comparison studies also revealed that the two present study isolates could be BTV-2 circulating in Andhra Pradesh, India and the optimization of RT-PCR for the amplification of S7 and S10 genes and their characterization would be useful for the grouping of any BTV suspected isolate.

## REFERENCES

[1] A, L., Singh, K. P., Nomikou, K., Potgieter, A. C., Attoui, H., van Rooij, H., van Rijn, P., Clercq, K.D., Vandenbussche, F., Zientara, S., Breard, E., Sailleau. C., Beer. M., Hoffman, B., Mellor, P.S., Mertens, P.P.C. 2008. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains, Virology. 377: pp. 308-313.

[2] Anthony, S., Jones, H., Darpel, K.E., Elliott, H., Maan, S., Samuel, A., Mellor, P.S., Mertens, P.P.C. 2007. A duplex RT-PCR assay for detection of genome segment 7 (VP7gene) from 24 BTV serotypes. J Virol Meth 141: pp.188-197.

[3] Chomezynski, P., Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate – phenol chloroform extraction. Analytical Biochemistry 162: pp.156-159.
[4] Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp 41:pp. 95–98.
[5] Mertens, P.P.C., Brown, F., Sanger, D.V. 1984. Assignment of the genome segments of bluetongue virus type 1to the proteins which they encode. Virology 135: pp.207- 217.
[6] O.I.E. 2000. Bluetongue. In: O.I.E. manual of standards for diagnostic test and vaccine.
[7] Oldfield, S., Adachi, A., Urakawa, T., Hirasawa, T., Roy P. 1990. Purification and characterization of the major group specific core antigen VP7 of bluetongue virus synthesized by a recombinant baculovirus. J Gen Virol. 71: pp. 2649-2653.
[8] Prasad, G., Minakshi., Malik, Y., Maan, S. 1999. RT-PCR and its detection limit for cell culture grown bluetongue virus, using NSI gene specific primers. Indian Journal of Experimental Biology 37: pp.1255-1258.
[9] Roy, P. 1989. Bluetongue virus genetics and genome structure. Virus Res. 13:pp. 179-182.
[10] Sangeeta Dalal, Prasad, G., Prasad M., Maan, S. 2009. Restriction enzyme analysis of VP7 gene of Indian isolates of bluetongue virus. Indian J Exp Biol. 47(4): pp.245-249.
[11] Sreenivasulu, D., Subba Rao, M.V., Reddy, Y.N. Gard, G.P. 2004. Overview of bluetongue disease, viruses, vectors, surveillance and unique features: The Indian subcontinent and adjacent regions. Vet Italy 40: pp.73–77.
[12] Tiwari, A.K., Kataria, R.S., Desai, G., Butchaiah, G., Bandyopadhyay, S.K. 2000. Characterization of an Indian Bluetongue virus isolate by RT-PCR and restriction enzyme analysis of the VP-7 gene sequence. Vet Res Commun. 24: pp. 401-406.