Journal of Microbiology and Related Research
Volume 2 Number 1, January - June 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2116.7

Original Article

Molecular Typing of Bluetongue Virus 16 From Karnataka State of India

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Abstract

Bluetongue disease (BT) is a vector borne infectious but non-contagious disease of wild and domestic ruminants. The BTV isolate (7bp) of sheep origin was inoculated to 9-11 day old chicken embryo followed by BHK-21 cell culture. Upon appearance of 75% cytopathic effect in cell culture viral nucleic acid was extracted. The viral nucleic acid showed BTV specific migration pattern of 3:3:3:1 in RNA-PAGE. The group specific ns1 gene RT-PCR confirmed the sample as BTV. The vp2 gene based serotype specific RT-PCR revealed the isolate as BTV16. The nucleic acid sequence of vp2 gene PCR products showed a high degree of identity (>99.0%) with other BTV16 isolates from different regions of India. It also showed maximum nucleotide identity of 99.7-96.4% with several other eastern BTV16 viruses from India, Israel, Japan, Cyprus, Greece etc. Sequence identity study also revealed that 7bp isolate only showed 75.5% identity with western isolate of BTV16 from Nigeria. The phylogenetic study also showed a close relation between isolate in study and BTV16 isolates from India Japan, Israel and Greece which form a separate eastern cluster. Thus, molecular study showed that the isolate in study is of eastern origin and closer to BTV16 isolates from India, Greece, Japan, and Israel.

Key words: Bluetongue Virus 16; Topotype; vp2 Gene; RT-PCR.

Introduction

Bluetongue (BT) is non-contagious and infectious viral disease of domestic and wild ruminants in several parts of the world. BT is caused by Bluetongue virus (BTV) of genus *Orbivirus* under family *Reoviridae*. BT is a vector borne disease and transmitted by *Culicoides* vector (MacLachlan, 1994). BT is characterized by high morbidity, mortality, still birth, foetal abnormality, abortion, weight loss, wool break, reduced meat and milk yield which lead to huge economic loss to farming community and livestock industry. The clinical form of BT is characterized by pyrexia, swelling of tongue and lips, coronitis, cyanotic discoloration of tongue and muzzle which may lead to death. However, subclinical infection of BT may also cause reduced milk yield, loss of condition and abortion leading to infertility in animals (Osburn, 1994). Therefore, due to severe economic losses mandatory disease surveillance and trade barrier have been imposed on movement of ruminant animals, their products and germplasm from BT endemic countries to BT free countries (Velthuis et al., 2009). The disease is seen in more severe form in sheep and white-tailed deer (Howerth *et al.*, 1988; Darpel *et al.*, 2007). Several other domestic animals such as buffalo, cattle and goats

act as silent reservoirs and may remain viraemic for several months post infection (Maclachlan *et al*., 2009). BT may infect several species of domestic and wild ruminanats. Therefore, it is listed as multi species disease by Office International des Epizooties (OIE, 2013).

BTV is icosahedral virus having ten segmented linear double-stranded RNA (dsRNA) genome. The BT genome segments encode 7 structural (VP1 to VP7) and 4 non-structural proteins (NS1, NS2, NS3/ NS3a and NS4). The structural proteins play essential role in viral nucleic acid replication and viral capsid assembly. However, non-structural proteins have role in egression of viral particle from infected cell (Mertens et al., 1989; Ratinier et al., 2011). They are produced in infected host cells only. The viral inner capsid is composed of two major proteins (VP3 and VP7) and three minor proteins (VP1, VP4, and VP6) (Roy, 1989). Similarly, outer capsid is consists of major and minor serotype specific VP2 and VP5 proteins for individual BTV serotype (Ghiasi et al., 1987).

Because of segmented nature of BTV genome, reassortment is a common phenomenon. BT viruses exchange genome segments with other BT viruses which lead to evolution of newer serotypes. There are twenty seven distinct BTV serotypes (BTV1 to BTV27) have been reported worldwide (Hofmann et al., 2008; Maan et al., 2011; Jenckel et al., 2015). A large number of BTV serotypes have also been reported from India. The serum neutralization assay and virus isolation in cell culture showed the prevalence of 22 distinct BTV serotypes in different geographical regions of India (Prasad et al., 2009; Susmitha et al., 2012). Several serotypes of BTV have been isolated from Karnataka state. In this study vp2 gene based molecular characterization of BTV isolate of sheep origin from Karnataka state has been reported.

Materials and Methods

Sample Preparation

The Blood sample was collected from a sheep suspected for BTV infection from Karnataka state in 2009. The blood sample was designated as 7bp. The sample was processed by ultrasonication followed by filtration. The filtrate was inoculated to 9-11 day old chicken embryo through intravenous route. On 7 day post inoculation, embryo showing embryopathic effect was harvested. The embryonic fluid was inoculated to one day old monolayer of BHK-21 cell culture.

Viral Nucleic Acid Extraction and RNA-PAGE

BHK-21 cells were harvested after appearance of about 75% cytopathic effect (CPE). The harvested BHK-21 cells were centrifuged at 2000Xg for 10 minutes (Remi, India). The supernatant materials were discarded and pellet was used for viral dsRNA extraction using Guanidinium isothiocynate method (Chomoczynski and Sacchi, 1987). The viral nucleic acid was subjected to 8% RNA-poly acrylamide gel electrophoresis (RNA-PAGE). The BTV specific nucleic acid was visualized using silver staining (Svensson et al., 1986).

cDNA Preparation and PCR

The viral nucleic acid was used for cDNA preparation using moloney murine leukemia (Mo-MuLV-RT) virus reverse transcriptase enzyme (Sibzyme, Russia) and random decamer primer (Ambion, USA) in thermal cycler (Biorad i-Cycler, USA) as per manufacturer's protocol. The cDNA was allowed for group specific ns1 gene based PCR to confirm the samples as BTV. The group specific PCR was performed using primer pairs F: 5'GTTCTCTAGTTGGCAACCACC3' and R: 5' AAGCCAGACTGTTTCCCGAT3' which produced an amplicon of 274bp size in agarose gel electrophoresis (Prasad et al., 1999).

The serotype of virus isolate was confirmed by vp2 gene based serotype specific RT-PCR using primers specific to all the BTV serotypes. The cDNA was allowed to PCR using individual serotype specific primers in a 20 µl reaction mixture having 20 µM of serotype specific primers, 2 µl cDNA, 3% DMSO, 0.4 µl of 10mM dNTPs mix (Finnzyme, Finland), 4 µl 5X HF buffer and 0.4 U (2U/ µl) phusion high- fidelity DNA polymerase (Finnzyme, Finland) in thermal cycler (Biorad iCycler, USA). The PCR amplification cycle was set as initial denaturation at 98°C for 2 minute, followed by 32 cycles of denaturation at 98°C for 10 second, primer extension at 72°C for 20 second and annealing for 20 second at 55°C. The final PCR extension was allowed at 72°C for 10 minute. The PCR products were visualized using gel documentation system (Biovis, USA) in 1% agarose gel (Sigma, USA) electrophoresis.

Nucleic Acid Sequencing and Sequence Data Analysis

The vp2 gene specific PCR products were purified using QIA quick gel extraction kit (Qiagen, USA). The purified PCR products were allowed for nucleic acid sequencing using serotype specific forward and reverse primers for final serotype confirmation. The

nucleic acid sequencing reaction was performed in Genetic Analyser ABI PREISM ™ 3130 XL machine using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) as per the manufacturer's instruction in our departmental laboratory.

The nucleic acid sequence data obtained was allowed for online available BLASTN+ 2.3.1 search (Zhang *et al.,* 2000) for serotype confirmation. The forward and reverse sequences of virus were aligned to generate contig sequences using Bioedit v7.2.5 software (Hall, 1999), which were used for further analysis. The percent nucleotide identity with global isolates of BTV16 was calculated using Bioedit v7.2.5 software (Hall, 1999). The phylogenetic analysis of vp2 gene sequences of our isolate (7bp) along with other global sequences were done using Mega 6 programme (Tamura *et al.,* 2013).

Results and Discussion

India has several BTV serotypes reported from different geographical regions. In the present study, one of the BTV isolate (7bp) was isolated from sheep in Karnataka state, adapted in BHK21 cell line and used for vp2 gene based serotyping and molecular characterization. The 7bp isolate adapted to BHK-21 cell line produced BTV specific CPE such as vacuolation in cells, aggregation and rounding of cells, floating of dead cells in medium within 36 hours (Sekar *et al.* 2009) (Figure 1). The viral nucleic acid was extracted using Guanidinium isothiocynate method (Chomoczynski and Sacchi, 1987) from pelleted cell culture materials and screened by RNA-PAGE followed by silver staining. The RNA-PAGE analysis showed characteristics BTV specific migration pattern (3:3:3:1) of viral dsRNA (data not shown). The viral nucleic acid was subjected to group specific ns1 gene based RT-PCR. The ns1 gene PCR amplicon showed 274bp product size on agarose gel electrophoresis. This indicates the sample as BTV (Figure 2). The characteristic CPE in BHK-21 cell culture, specific migration pattern of viral nucleic acid (3:3:3:1) in RNA-PAGE and 274 bp amplicon of ns1gene group specific RT-PCR confirmed the samples as BTV.

Further, the cDNA of 7bp isolate was allowed for serotype specific RT-PCR using vp2 gene specific primers for all the BTV serotypes. The PCR amplicon showed 768bp amplification product on agarose gel electrophoresis, which is specific for BTV16 serotype (Figure 3). The remaining serotype specific primers did not show any amplification. Thus the 7bp isolate was serotyped as BTV16. For final confirmation of serotype of 7bp the vp2 gene PCR product was allowed for direct nucleic acid sequencing. The BLASTN+ 2.3.1 search of nucleotide sequence of vp2 gene of 7bp isolate showed the maximum identity only with several isolates of BTV16 from different regions of the world. Thus, vp2 gene specific RT-PCR followed by nucleotide sequencing confirmed the 7bp isolate as BTV16. The nucleotide sequence of 7bp isolate was deposited to GenBank database and accession number GU931316 was assigned. The contig sequence of 7bp isolate was generated and percent nucleotide sequence identity of 7bp isolate with several other BTV16 isolates from India and different regions of the world were calculated using Bioedit v7.2.5 (Hall, 1999) programme (Table 1).

The nucleotide sequence identity analysis showed that 7bp isolate (accession number GU931316) possessed 99.7% nucleotide identity with IND2010/cattle/16 (accession no JX007924), Sheep/2010/Ind/Hisar (accession no JQ904061) and IND/Goat/2010/16/HSR (Minakshi et al., 2012) isolate of BTV16 from India. However, 7bp isolate also showed nucleotide identity of 99.4-98.0% with several Indian BTV16 isolates such as G53/ABT/HSR, CU-NAU/IND/2010 (accession no JQ478482), INDAPMBNAP04/10 (accession no KC751423), G4/IND/2011 (Dadawala et al., 2013), VJW66/IND (Minakshi et al., 2015), GNT-27/IND (Minakshi et al., 2015), G53/IND/2011 (Dadawala et al., 2013) and MBN48/IND (Minakshi et al., 2015).

The 7bp isolate also showed nucleotide identity of 97.7-94.6% with several isolates from Israel (ISR2008/03, BTV16/ISR-2404/08 and BTV16/ISR-2228/08) (Nomikou et al., 2015), Greece (GRE2008/10, BTV-16/Gree1999/13(S-2) and GRE1999/13) (Nomikou et al., 2015), Cyprus (CYP2006/01) (Nomikou et al., 2015), Japan (MZ-1/C/01) (Shirafuji et al., 2012) and South Africa (RSArrrr/16) (Maan et al., 2004) (Table 1).

The BTV nucleotide sequence analysis from different geographical regions of globe broadly categorised BTV in to 'eastern' or 'western' topotypes (Maan et al., 2010). The sequence analysis of 7bp isolate showed an overall nucleotide identity of 99.7-94.6% with several eastern BTV16 viruses from India, Israel, Japan, South Africa, Greece and Cyprus. However, 7bp isolate showed only 75.5% nucleotide identity with Western isolate (NIG1982/10) of BTV16 from Nigeria (Mertens et al., 2013). Thus, it confirmed the eastern origin of 7bp isolate.

The phylogenetic study of BTV16 nucleotide sequences using Mega 6 software programme formed two separate major, western and eastern clusters. The isolate in study (7bp) formed a separate close cluster with several other Indian BTV16 viruses (IND2010/

cattle/16, Sheep/2010/Ind/Hisar, IND/Goat/2010/16/HSR, Cattle/2010/Ind/Hisar and INDAPMBNAP04/10) under eastern cluster. They were also found slightly distantly related to other eastern BTV16 isolates from India, Japan, Greece, Israel, South Africa and Cyprus. The BTV16 from Nigeria (Isolate NIG1982/10) was placed in western cluster (Mertens et al., 2013). Thus phylogenetic and sequence identity study revealed that 7bp isolate was found much closer to BTV16 isolates from Indian, Japanese, Israel or Greece.

India is a rainy tropical country which provides suitable environment for *Culicoides* vector growth and multiplication. Out of more than 1400 different *Culicoides* species globally reported, about 63 are identified from different geographical regions of India (Reddy et al., 2008; Halder et al., 2013; Archana et al., 2014). Thus, persistence of large numbers of *Culicoides* vectors explains the prevalence of 22 different BTV serotypes in India. The serological study revealed the presence of antibodies against BTV in several species of wild and domestic ruminants in India (Prasad et al., 1998).

Karnataka state is one of the major hubs of BT infection in India. Based on virus isolation and serum neutralization several BTV serotypes such as 1, 2, 4, 12, 16, 17, 18, 20 and 23 have been reported from Karnataka state (Prasad et al., 2009). Moreover, BTV16 was also reported from sheep population in adjoining states such as Tamil Nadu (isolate IND/Goat/2010/16/HSR) (Minakshi et al., 2012) and Andhra Pradesh (isolate, VJW66/IND, GNT-27/IND and MBN48/IND) (Minakshi et al., 2015).

The 7bp isolate showed a high degree of identity (99.7-98.0%) with several isolates of BTV16 from Tamil Nadu and Andhra Pradesh. Since Tamil Nadu and Andhra Pradesh states are neighbouring states to Karnataka and are also endemic for a known BTV vector (*Culicoides oxystoma*) in India (Minakshi, 2010). Thus, it may be assumed that BTV16 might be transmitted from neighbouring states to Karnataka either through vectors or migrating sheep population or through wind velocity. Moreover, due to serious BTV16 outbreaks in some states of India such as Andhra Pradesh, Karnataka, Gujarat and Tamil Nadu, it is included in inactivated Pentavalent vaccine formulation along with other serotypes such as 1, 2, 10 and 23 (Reddy et al., 2010). However, the knowledge about the molecular epidemiology of all the BTV serotypes is essential for a successful BTV control programme. The conventional serotyping methods along with molecular tests such as RT-PCR based typing and nucleic acid sequencing can be used for BTV surveillance in a particular geographical area. The surveillance information can be used for proper BT vaccine formulation.

Table 1: Percent nucleotide identity of vp2 gene of 7bp isolate with other bluetongue virus 16 from different regions of the world

S.N.	BTV16 vp2 gene sequences	7bp.GU931316.India Percent nucleotide identity
1	7bp.GU931316.India	100
2	IND2010/cattle/16.JX007924.India	99.7
3	Sheep/2010/Ind/Hisar.JQ904061.India	99.7
4	IND/Goat/2010/16/HSR.JQ924821.India	99.7
5	G53/ABT/HSR.KF664134.India	99.4
6	Cattle/2010/Ind/Hisar.JQ904063.India	99.2
7	Goat/2010/Ind/Hisar.JQ904062.India	98.9
8	CU-NAU/IND/2010.JQ478482.India	99.2
9	INDAPMBNAP04/10.KC751423.India	98.9
10	G4/IND/2011.JQ478483.India	98.6
11	VJW66/IND.JN106022.India	98.6
12	GNT-27/IND.JN106018.India	98.6
13	G53/IND/2011.JQ478486.India	98.0
14	MBN48/IND.JN106020.India	98.0
15	ISR2008/03.KP820992.Israel	97.9
16	CYP2006/01.KP820986.Cyprus	97.9
17	BTV16/ISR-2404/08.KP306785.Israel	97.9
18	BTV16/ISR-2228/08.KP306782.Isreal	97.9
19	GRE2008/10.KP820990.Greece	97.7
20	MZ-1/C/01.AB686220.Japan	97.6
21	GRE1999/13.KP820989.Greece	97.4
22	BTV-16/Gree1999/13(S-2).AM773709.Greece	97.4
23	GRE1999/13.AM773702.Greece	97.4
24	RSArrrr/16.AJ585137.South Africa	96.4
25	NIG1982/10.AJ585150.Nigeria	75.5

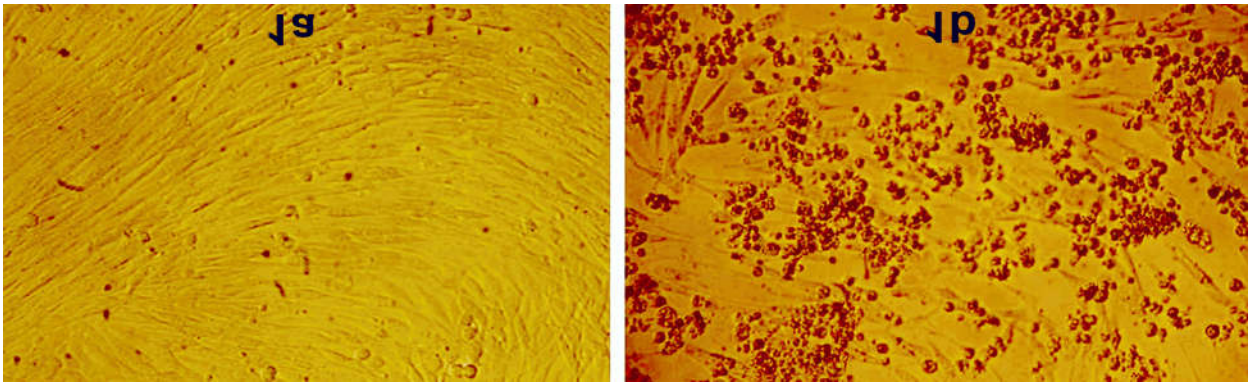

Fig. 1: Bluetongue virus isolation in BHK-21 cell line. (1a): Normal uninfected BHK-21 cell monolayer (48hours); (1b): BTV infected BHK-21 cells (48 hours) showing cytopathic effect characterized by degeneration and rounding of infected cells

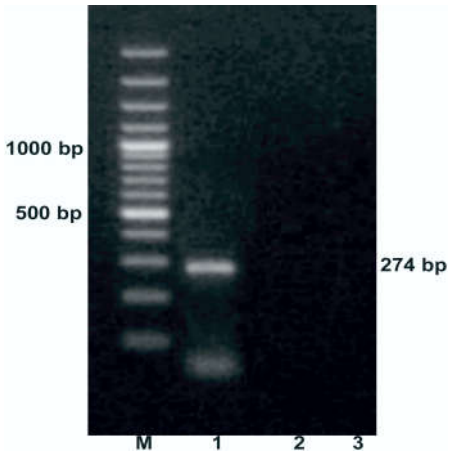

Fig. 2: 1% agarose gel electrophoresis of ns1 gene RT-PCR of Indian BTV16 isolate. Lane L: Ladder 100bp, 1: 7bp, 2: BHK21 cell control, 3: Nuclese free water control. The left side numbers indicate DNA marker and right side indicated size of PCR product.

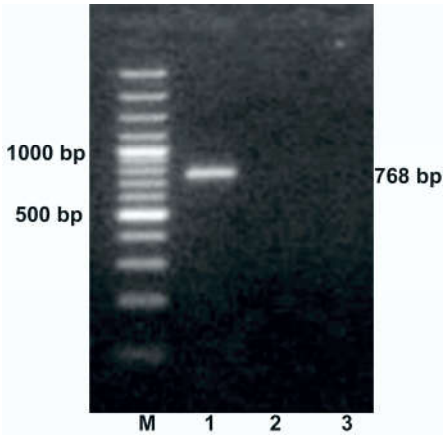

Fig. 3: 1% agarose gel electrophoresis of vp2 gene RT-PCR of Indian BTV16 isolate. Lane L: Ladder 100bp, 1: 7bp, 2: BHK21 cell control, 3: Nuclease free water control. The left side numbers indicate DNA marker and right side indicated size of PCR product.

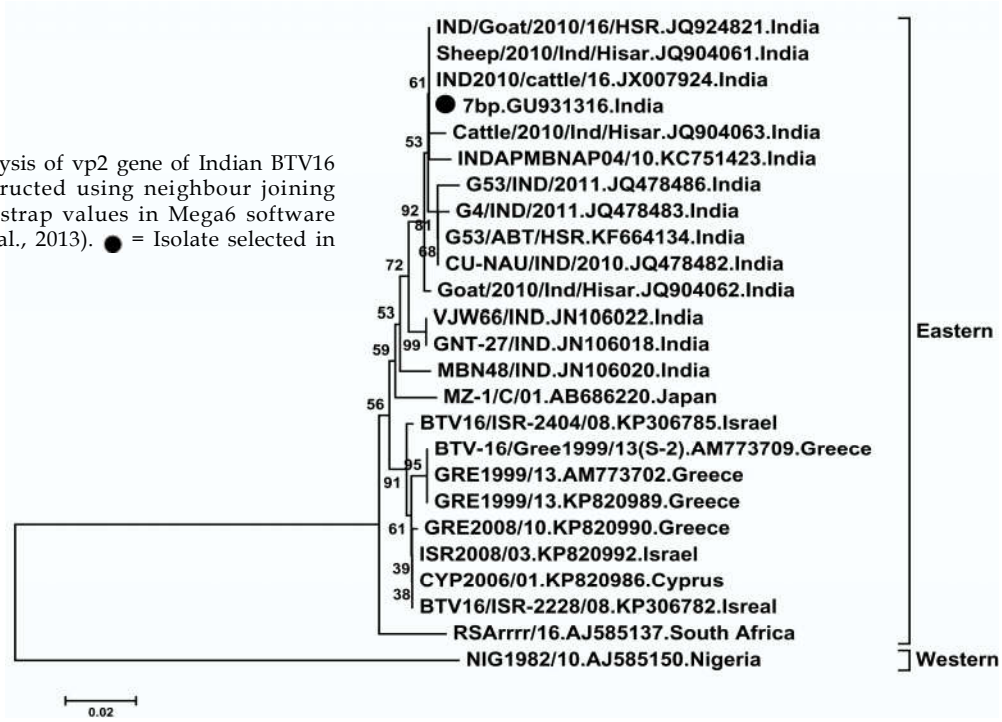

Fig. 4: Phylogenetic analysis of vp2 gene of Indian BTV16 isolate. Tree was constructed using neighbour joining method with 1000 bootstrap values in Mega6 software programme (Tamura et al., 2013). ● = Isolate selected in this study

Conclusion

BT is mostly a disease of sheep in India. However, several reports of BT outbreak in other ruminants such goat and cattle has also been reported from country. The BTV isolate (7bp) of sheep origin from Karnataka state was used for vp2 gene based serotyping and molecular characterization. The 7bp isolate was confirmed as BTV16 serotype based on RT-PCR, nucleic acid sequencing and vp2 gene sequence similarity search in GenBank data base. The nucleotide sequence identity and phylogenetic analysis revealed that 7bp isolate is much closer to other BTV16 isolates from India, Japan, Israel, Cyprus and Greece. Therefore, to control BT in India the close surveillance regarding import of live animal and its products should be initiated.

Competing Interest

All authors declare that they have no conflict of interest.

Acknowledgements

Authors are thankful to ICAR, New Delhi for providing financial support under 'All India network programme on Bluetongue' and Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana for providing infrastructural facility.

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