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Original Article

Molecular Characterization of Segment 6 of Indian Isolate of Bluetongue Virus 2

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Abstract

Bluetongue (BT) is a disease of domestic and wild ruminant caused by bluetongue virus (BTV) of genus *Orbivirus* and family *Reoviridae*. BTV is non-enveloped icosahedral virus having 10 segmented dsRNA genome. The VP5 protein encoded by segment 6 along with VP2 protein gives serotype specificity to the virus. The BTV serotype 2 (isolate M11) was isolated from an outbreak in sheep from Andhra Pradesh state. The virus sample was grown in BHK21 cell culture. The RNA-PAGE and NS1 gene based RT-PCR confirmed the sample as BTV. The viral cDNA was allowed for segment 6 (vp5 gene) specific RT-PCR using primers for all the serotypes. The BTV2 specific vp5 gene PCR amplicon was cloned and sequenced. Nucleotide sequence analysis revealed that Indian BTV2 serotype showed more than 99 / 97% nucleotide / amino acid sequence identity with western origin viruses from India, USA and South Africa. The study further indicated that western BTV2 in India may be originated from vaccine strains of BTV2 from South Africa.

Keywords: Bluetongue Virus 2; RT-PCR; Topotype; VP5 Gene.

Introduction

Bluetongue (BT) is an infectious, non-contagious, economically important, insect vector borne viral disease of domestic and wild ruminants (MacLachlan, 1994). It is caused by bluetongue virus (BTV), of genus *Orbivirus* of family *Reoviridae*. BT causes severe economic loss to small ruminant industry which is mainly due to its high morbidity, mortality, still birth, foetal abnormality, abortion, wool break, weight loss, reduced milk and meat yield. BT is listed under category of multiple species diseases by Office International des Epizooties, Paris (OIE, 2013). The clinical sign of BT is characterized by fever, swelling of lips and tongue, coronitis and lameness. The more severe forms of the disease are frequently seen in sheep and in white-tailed deer (Darpel *et al.,* 2007; Howerth *et al.,* 1988). Buffalo, cattle and goats act as silent reservoirs host, remain viraemic for several months and may transmit BT to other susceptible host (Maclachlan *et al*., 2009).

Due to rapid evolutionary changes in genome through reassortment, BTVs are consistently evolving new serotypes globally. Twenty-four distinct BTV serotypes have been identified worldwide (Mertens *et al*., 2004). Later on, two more serotypes BTV25 and BTV26 from Switzerland (Hofmann *et al.,* 2008) and Kuwait (Maan et al., 2011) respectively have been isolated. Recently, a novel virus from France has been characterized as BTV serotype 27 (Jenckel et al., 2015). The segmented nature of BTV genome favours the reassortment of genome segments especially when *Culicoides* vector or mammalian host is simultaneously infected by two or more different BTV

serotypes. This may led to evolution of new isolates or serotypes of BTV (Batten et al., 2008). The monsoon season in India is favourable to various insect vectors (including *Culicoides*) and infectious diseases (like BT) transmitted by them. Thus India became endemic for BT and a large number of BTV serotypes (total 22 BTV serotypes) have been reported from India. Based on nucleotide sequence analysis most of BTV isolates can be broadly divided into two major groups 'eastern' or 'western' topotypes, and into a number of geographic subgroups (Balasuriya *et al.,* 2008).

BTV is a small icosahedral virus with a capsid diameter of 70 nm. It has ten-segmented, double stranded RNA (dsRNA) genome, each of which codes for at least one viral protein. The genome segments code seven structural proteins (VP1 to VP7) to form virus particle. In addition to structural proteins, there are four non-structural proteins NS1, NS2, NS3/ NS3a and NS4 expressed in virus infected host cells (Mertens *et al.,* 1989; Belhouchet et al., 2011). The inner capsid of BTV is composed of two major (VP3 and VP7) and three minor proteins (VP1, VP4 and VP6) proteins (Roy, 1989). The outer capsid is composed of viral proteins, VP2 and VP5, which are serotype specific for individual BTV serotype (Ghiasi, 1987).

VP5 protein assists in virus neutralization along with major VP2 protein as it enhances the neutralization activity of VP2 protein (Roy *et al.,* 1990). The combination of VP2 and VP5 proteins can induce higher neutralizing antibody titre in comparison to VP2 protein alone (Huismans *et al.,* 1983). In addition VP5 can also play major role in membrane fusion and membrane permeabilization activity, facilitating virus entry into the host cells (Forzan *et al.,* 2004). The segment 6 (VP5 gene) of BTVs also showed genetic diversity between serotypes as well as within serotype (Singh *et al.,* 2004). In present paper, VP5 gene based genetic variation and phylogenetic study of Indian BTV2 isolate with global isolates has been reported.

Materials and Methods

Sample Origin

The BTV serotype 2 was originally obtained from Andhra Pradesh state under All India Network Program on Bluetongue in 2004. It was assigned as isolate M11. The Blood sample of Nellore breed of sheep was collected at height of temperature (40.5ºC). The sample was washed with sterile PBS of pH 7.4 and allowed for ultra-sonication and intravenous inoculation to 9-11 day old chicken embryo. The embryos showing characteristic embryopathic effect were harvested within 7 days and embryonic tissues were inoculated to day old confluent monolayer of BHK21 cell line. After showing characteristic cytopathic effect (CPE), it was passaged for 10 passages in BHK21 cell culture. After appearance of 75% CPE in infected cell culture, the virus was harvested along with BHK21 cells and cellular material was pelleted at 5000Xg using centrifuge machine (Remi, India). The viral dsRNA was extracted from pelleted cell culture material using Trizol reagent (Sigma, USA) as per manufacturer's instruction. The extracted viral nucleic acid was screened by RNA-PAGE followed by silver staining to visualize the 10 dsRNA segments to confirm the sample as BTV.

RT-PCR and PCR

The cDNA was synthesized using viral genomic dsRNA of M11 isolate as template, random decamer primers (Ambion, USA) and moloney murine leukemia virus-reverse transcriptase (Mo-MuLV-RT) enzyme (Sibzyme, Russia) in thermal cycler (Biorad i-Cycler, USA) as per manufacturer's instruction. The cDNA was subjected to group specific NS1 gene based PCR to confirm the sample as BTV using forward primer (11-31 nucleotide): 5'GTTCTCTAGTTGG CAACCACC3' and reverse primer (284-265 nucleotide): 5' AAGC CAGACTGTTTCCCGA 3' to generate an amplicon of 274bp size (Prasad et al., 1999). The cDNA was subsequently subjected to amplification of VP5 gene using published primer pairs specific to BTV2 (Ranjan et al., 2012) along with primers specific to all other BTV serotypes. The VP5 gene specific primers of BTV serotype 2, Forward (755-774 nucleotide) - 5'ACAGCCGTCGCAACGGG AAG3' and Reverse (1589-1570 nucleotide) - 5'AGAGGGGCACGTCCAACCGA 3' produces the PCR amplicon of 835 bp (Ranjan et al, 2012). The amplification reaction for BTV2 specific primer was carried out in 20 µl reaction mixture containing 2 µl cDNA, 3% DMSO, 20 µM of each primer along with 0.4 µl of 10mM dNTPs mix, 4 µl 5X HF buffer and 0.4 U (2U / µl) phusion high- fidelity DNA polymerase (Finnzymes, Finland) in thermal cycler (Biorad iCycler, USA). The amplification programme consisted of initial denaturation for 3 minute at 98°C, followed by 35 cycles for 15 second denaturation at 98°C, 20 second primer annealing at 57°C and 30 second primer extension at 72°C. The final primer extension was carried out at 72°C for 10 minute. Similarly cDNA was allowed for PCR using VP5 gene specific primers of all other serotypes. The amplified

PCR products were subjected to agarose gel electrophoresis in 1% agarose gel (Sigma, USA) followed by ethidium bromide staining and UV visualization under transilluminator (Biovis, USA).

Cloning and Sequencing of PCR Product

The PCR products were purified using commercially available QIA quick gel extraction kit (Qiagen, USA) to remove primer dimers and other PCR ingredients. The purified PCR products were cloned using commercially available cloning kit (Fermentas, USA) as per the manufacturer's instruction. The Pjet 1.2 plasmid was used as cloning vector and JM107 *E. coli* cell line as host system. The positive clones were selected by colony touch PCR using VP5 gene specific primer pairs. The positive clones were further grown in culture media and plasmids were extracted using commercially available kit Quiaprep (Quiagen, USA) as per the manufacturer's instruction. The plasmids from positive clones were allowed for nucleic acid sequencing using Genetic Analyser ABI PRESM ™ 3130 XL machine in our laboratory.

Nucleotide Sequence Analysis

The vector contamination sequence from nucleotide sequence obtained was removed using online software Vecscreen (http: //www. ncbi. nlm.nih. gov /tools /vecscreen/). The VP5 gene sequence of M11 isolate was analysed using online available software program BLASTN+ 2.2.31 (http: //blast.ncbi.nlm.nih.gov/) (Zhang *et al.,* 2000). Bioedit v7.2.1 software (Hall, 1999) was used for multiple sequence alignment and calculation of percent identity of nucleotide as well as deduced amino acid sequence of M11 isolate with other global isolates of BTV2. The phylogenetic analysis of M11 isolate with other isolates of BTV2 from different parts of the world was done using neighbour joining method with p-distance parameter and 1000 bootstrap value in Mega 5 software (Tamura *et al.,* 2011).

Result and Discussion

BTV 2 is one of the common BTV serotypes reported from Andhra Pradesh state of India. The segment 6 sequence based study showed significant genetic diversity (3-43%) among BTV isolates of all the 24 serotypes of BTV across the world (Singh *et al.,* 2004). The present study was carried out to investigate the vp5 gene based molecular variation of Indian isolate of BTV2 and its comparison with other BTV2 isolates from India and other countries. The dsRNA of cell culture grown BTV isolate (M11) was analysed using 8% RNA-PAGE. The RNA-PAGE analysis showed characteristic 3:3:3:1 migration pattern which is specific for BTV (Data not shown) (Minakshi et al., 2011). Furthermore, the group specific NS1 gene based PCR of viral cDNA revealed BTV specific amplicon of 274 bp in agarose gel electrophoresis (Figure 1). Thus, RNA-PAGE and NS1 gene specific PCR confirmed the sample as BTV. The VP5 gene based PCR of M11 isolate showed an amplicon of 835 bp with BTV2 specific primers as evidenced in 1% agarose gel electrophoresis (Figure 2). However, the cDNA sample did not show any amplification with primers specific to other serotypes. Subsequently, the BTV2 segment 6 (VP5 gene) specific PCR amplicon was cloned using PJet 1.2 cloning vector and sequenced to get complete nucleotide sequence of amplicon. After removal of terminal end vector sequences the VP5 gene nucleotide sequences showed identity with several isolates of BTV2 of GenBank database only. The nucleotide sequence of M11 isolate was deposited to GenBank database with accession number JF815523. The percent nucleotides (nt) as well as deduced amino acid (aa) sequence identity of isolate M11 with other BTV2 isolates from different regions of the world was calculated using Bioedit v7.2.1 (Table 1). The M11 isolate showed >86/95% nt/aa identity with western topotype isolates of BTV2 from different regions of the world. However, it showed maximum 99/97% nt/aa identity with several western BTV2 isolates from India (sheep/08/Ind/ABT/Hisar, IND2003/02, BTV-2/IND2003/01, IND2003/01, IND2003/03 and BTV-2/IND2003/03), USA (OnaA) and South Africa (557, RSArrrr/02 and RSAvvvv/02). Moreover, it showed only 75.6-78.4/91.0-91.7% nt/aa identity with eastern topotype isolates of BTV2 from India (IND1982/01 and BTV-2/IND1993/01), Japan (MZ-1/C/07) and Australia (Cooktown). It confirmed the western origin of segment 6 of M11 isolate.

Phylogenetic analysis of segment 6 classifies BTV2 into separate eastern and western topotypes (Fig. 3). The M11 isolate was placed within western topotype and it formed a separate close cluster with BTV2 from India (IND2003/02, sheep/08/Ind/ABT/Hisar, BTV-2/IND2003/01, IND2003/01, IND2003/03 and BTV-2/IND2003/03), USA (OnaA) and South Africa (557, RSArrrr/02 and RSAvvvv/02). However, it was slightly distant from other western topotype viruses from France, Italy, Tunisia, Nigeria, Sudan and USA, which formed separate cluster (Fig. 3). The eastern topotype was consists of BTV2 form India (IND1982/01 and BTV-2/IND1993/01), Australia (Cooktown) and Japan (MZ-1/C/07). The segment 6 (VP5 gene)

based molecular characterization and phylogenetic analysis of Indian isolate of BTV1, 2, 9 and 16 have been reported earlier (Manjunath *et al.,* 2010; Ranjan et al., 2013; Ranjan et al., 2014a; 2014b). The present study indicates that BTV2 serotype having both eastern as well western origin of segment 6 (VP5 gene) is circulating in India. Many of the western origin of BTV2 isolates in India including M11 isolate showed >99/97% nt/aa identities with South African live attenuated vaccine strain (RSAvvvv/02). Therefore, western origin of BTV2 might have entered to India either through import of live vaccinated animals or live vaccine itself. In South African BT control programme multivalent live attenuated vaccine containing BTV 2, 3, 4, 8-11, 16 had been used. Moreover, these vaccines were also imported by many other countries including Italy, Bulgaria, France, Spain, Portugal, Israel, eastern Mediterranean Islands (Savini et al., 2008; Coetzee et al., 2012). The several sheep breeds were imported to India during late 1970s and 1980s from these countries (Prasad et al., 2009; Jain et al., 1986). Furthermore, it was well established that live vaccines may cause significant levels of post vaccination viraemia leading to virus transmission by *Culicoides* vector during blood feeding. It may lead to either revert back of vaccine strain to virulent strain or reassortment of vaccine strain with other locally prevalent BTV isolate (Veronesi et al., 2010; Batten et al., 2008). Thus, new BTV isolate or serotype originated in an area. Moreover, the BTV2 was isolated from southern India especially from Andhra Pradesh and Tamil Nadu during 2003-04. All the western isolates of BTV2 were found to be sharing more than 99/97% nt/aa identity. It also indicates their common origin. Since, south India particularly Andhra Pradesh and Tamilnadu is endemic for a major species of *Culicoides* i.e. *Culicoides oxystoma* (Minakshi, 2010). Therefore, BTV2 serotype after entering to Indian subcontinent may be migrated either from Tamil Nadu to Andhra Pradesh or vice versa through migrating sheep population or vector.

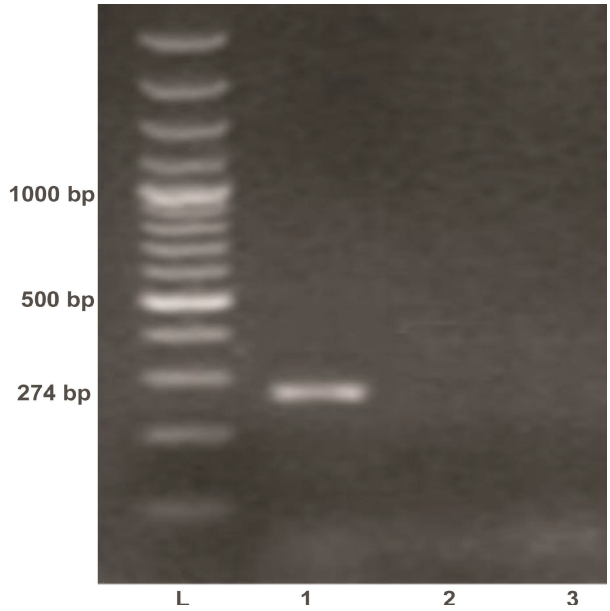

Fig. 1: Agarose gel electrophoresis of RT-PCR product of ns1 gene of M11 isolate. Lane L: Ladder 100bp, 1: M11, 2: BHK21 cell control, 3: NFW control.

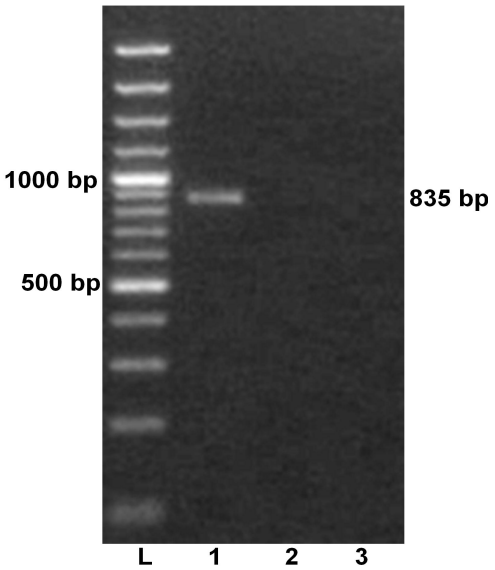

Fig. 2: Agarose gel electrophoresis of RT-PCR product of vp5 gene of M11 isolate. Lane L: Ladder 100bp, 1: M11, 2: BHK21 cell control, 3: NFW control

Table 1: Percent nucleotide and deduced amino acid sequence identity of segment 6 of bluetongue virus serotype 2

S.n.	BTV2 vp5 gene sequences	BTV2.M11.India.JF815523	
		Nucleotide	Amino acid
1	BTV2.M11.India.JF815523	100	100
2	BTV2.sheep/08/Ind/ABT/Hisar.India.JQ904068	99.1	97.4
3	BTV2.IND2003/02.India.AJ783905	99.1	97.8
4	BTV2.557.South Africa.AY855276	99.2	97.8
5	BTV2.OnaA.USA.AY855277	99.2	97.8
6	BTV2.BTV-2/IND2003/01.India.KP696597	99.2	98.2
7	BTV2.IND2003/01.India.AJ783904	99.2	98.2
8	BTV2.IND2003/03.India.AJ783906	99.4	98.2
9	BTV2.BTV-2/IND2003/03.India.KP696607	99.4	98.2

10	BTV2.RSArrrr/02.South Africa.AJ586696	99.2	97.8
11	BTV2.RSAvvvv/02.South Africa.AJ586665	99.0	97.1
12	BTV2.France.AY129083	87.4	96.7
13	BTV2.BTV-2IT2000.Italy.KM053273	87.4	97.1
14	BTV2.FRA2001/03.France.AJ586674	87.4	96.7
15	BTV2.SAD2001/01.Italy.AJ586672	87.4	96.7
16	BTV2.ITL2002/07.Italy.AJ586671	87.3	96.4
17	BTV2.TUN2000/01.Tunisia.AJ586668	87.5	97.1
18	BTV2.NIG1982/02.Nigeria.AJ586667	87.1	96.7
19	BTV2.SUD1985/01.Sudan.AJ586666	87.6	97.1
20	BTV2.FL99 13406-2.USA.AY855279	85.7	96.0
21	BTV2.OnaB.USA.AY855278	86.2	95.6
22	BTV2.MZ-1/C/07.Japan.AB686238	78.4	91.0
23	BTV2.Cooktown.Australia.JQ240326	79.5	91.0
24	BTV2.IND1982/01.india.AJ586675	78.2	91.7
25	BTV2.BTV-2/IND1993/01.India.KP696587	75.6	91.0

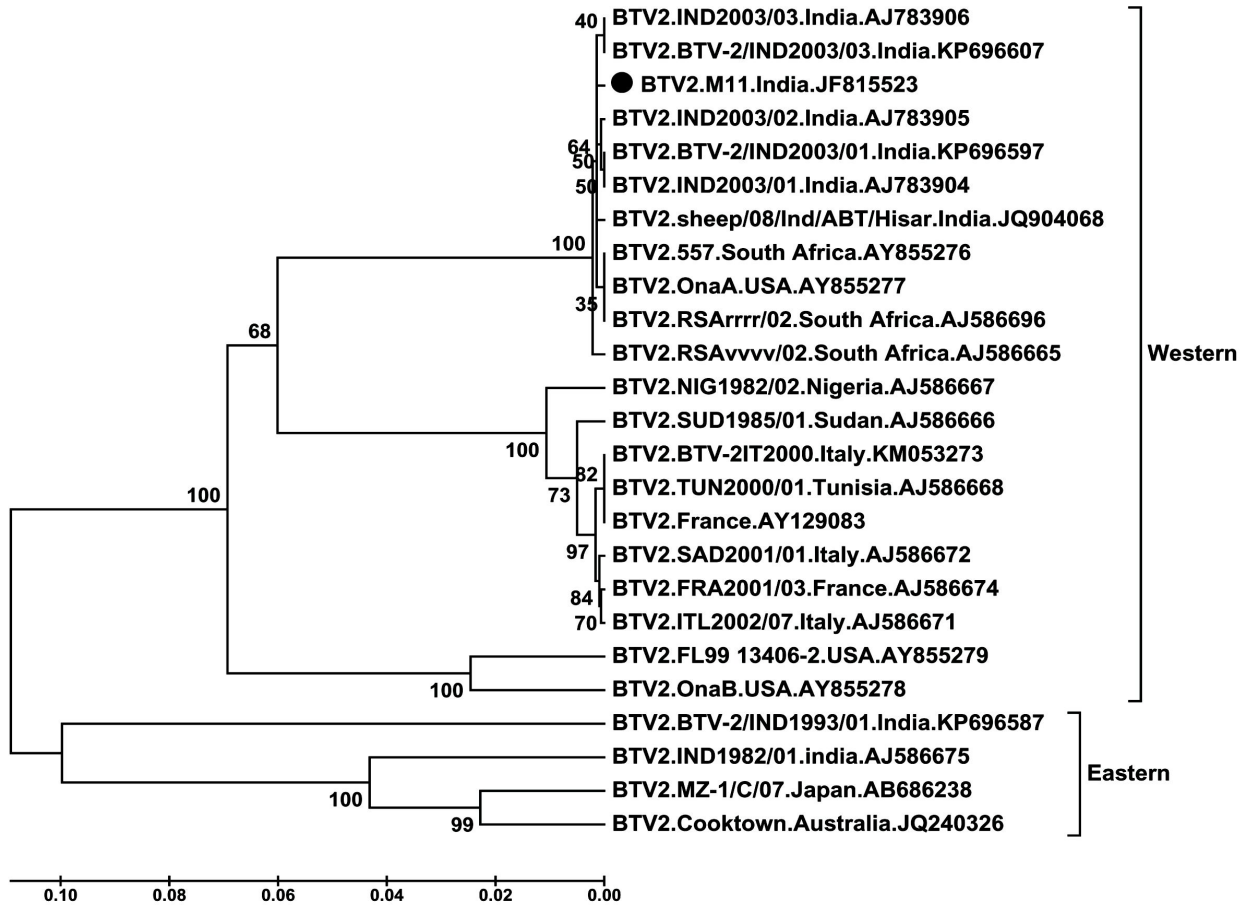

Fig. 3: The vp5 gene based phylogenetic tree of M11 isolate along with other BTV2 isolates from around the world. Tree was constructed using neighbour joining method in Mega5 software programme with default parameters and bootstrap value as 1000 replicate (Tamura *et al.,* 2011). ● = Isolate selected in this study

Conclusion

Based on percent nucleotide and deduced amino acid sequence identity of segment 6 of Indian isolate of BTV2 and phylogenetic analysis with global isolates, it can be concluded that M11 isolate is of western origin and more closely related to South African vaccine viruses. Moreover, the segment 6 sequence analyses also revealed little genetic divergence among western topotype of Indian BTV2 isolates. It indicates their common origin from South African Vaccine virus.

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