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Research Article

SEGMENT-3 BASED GENETIC DIVERSITY AMONG INDIAN ISOLATES OF BLUETONGUE VIRUS

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ABSTRACT

In this study seven sheep bluetongue virus (BTV) isolates adapted in BHK-21 cell line were taken. All the isolates were confirmed as BTV based on 380bp amplicon size with vp3 gene/segment-3 (seg-3) RT-PCR. For further study, the vp3 gene of all the seven isolates was amplified to a longer product size of 708bp which were sequenced and analysed. *In silico* restriction enzyme analysis (REA) of 708bp amplicon with *PvuII* revealed that three isolates namely IND/2007/04/BT1, IND/2007/04/BT2 and IND/2007/04/BTONG5 had a single restriction site at 1208 while the remaining five isolates lacked the same site. Whereas *in silico* REA with *BsgI* revealed that BTV isolates IND/2007/04 and IND/2007/04/BT4 lacked the restriction site. The remaining isolates had one restriction site at 1278bp. The vp3 gene is relatively conserved yet certain degree of genetic variation could be observed on the basis of REA, phylogenetic analysis and percent identity matrix within Indian isolates of BTV.

Key words: BTV, nucleotide sequences, restriction enzyme analysis, segment-3, topotyping

Bluetongue virus (BTV) is the type species of the genus *Orbivirus* and belongs to family *Reoviridae* (Mertens *et al.*, 2005). BTV infects most of the domestic and wild ruminant species and causes bluetongue (BT) disease primarily in sheep (Mertens and Mellor, 2003). The disease is characterised by fever, lameness (coronitis), swelling and cyanosis of lips and tongue leading to death. It is listed as a notifiable disease by the Office Internationale des Epizooties (OIE, 2006).

BTV genome consists of double-stranded RNA (dsRNA) ten linear segments which encodes seven structural proteins (VP1 to VP7) organised into two concrete shells i.e. the inner and outer capsid and four non-structural proteins (NS1, NS2, NS3 and NS3a). The size of the seg-3 is 2,772 bp. The VP3 protein interacts with both the minor protein complexes and the dsRNA of the viral genome, and consequently also helps to determine the internal structure of the virus particle (Gouet *et al.*, 1999). Despite high level of homology, both seg-3 and VP3 show significant level of variations that reflect the geographic origins of the virus, dividing individual isolates into eastern and western topotypes, and a number of further sub-groups (Pritchard *et al.*, 1995). The seg-3 nucleotide sequence based comparisons have been used as a way to identify the geographical origin (different topotypes) of BTV isolates.

Till 2008, twenty four distinct serotypes of BTV (BTV-1 to BTV-24) have been isolated and characterised worldwide which can be distinguished based on neutralisation assays (Mertens *et al.*, 2005). Recently, two more serotypes i.e BTV-25 from Switzerland (Hofmann *et al.*, 2008) and BTV-26 from Kuwait (P. Mertens, personal communication) have been reported. A total of 21 different BTV serotypes based upon serology and virus isolation have been reported from different parts of Indian subcontinent (Prasad *et al.*, 2007). In addition, BTV-21 has been isolated from West Bengal state of India (Jordar *et al.*, 2009); thus, a total of 22 different BTV serotypes have been reported from Indian subcontinent. The objective of the study was to find out vp3 gene based diversity among Indian isolates of BTV.

MATERIALS AND METHODS

Sample Preparation: A total of seven Indian isolates of BTV (year 2007) were propagated in BHK 21 cell line. After appearance of complete cytopathic effect (CPE) in infected BHK-21 cells, the virus was harvested and cells along with virus was pelleted at 3,000X g for 10 minutes in a table top centrifuge. The supernatant was decanted carefully and viral dsRNA was

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isolated from cell pellet using Tri reagent (Sigma). cDNA synthesis and amplification of vp3 gene:Viral genomic dsRNA was used as template for cDNA synthesis using random decamer primer (Ambion, USA) in a thermal cycler (BIO-RAD iCycler, USA). For 25 µl reaction mixture, 7 µg of viral dsRNA was subjected to heat denaturation along with 6% DMSO and 30 pM of random primer followed by addition of 400 µM each dNTPs and 500 U of Mo-MuLV reverse transcriptase (Sibzyme) for reverse transcription was added. The primer was subjected to anneal at 25^0C for 10 min, reverse transcription at 42^0C for 60 min and finally heat inactivation at 90^0C for 10 min.

The cDNA from all seven isolates were subjected to seg-3 specific PCR using published primer pairs and thermal cyclic condition (McCall and Gould, 1991). The forward primer (nt 1055) 5'CCTGATGTTTCCAGGAC AAATTATACTC3' and reverse primer (nt 1763) 5'CCGATTAAAGGCAAACCAAAGCGATATCC3' were used for first PCR amplification to yield 708bp product. The PCR was carried out in 20 µl reaction mixture containing 2 µl cDNA, 3% DMSO, 20 µM of primer along with 0.4 µl of 10mM dNTPs mix (Fermentas), 4 µl 5X HF buffer and 0.4 U (2U/ µl) phusion high-fidelity DNA polymerase (Finnzyme) in a thermal cycler (BIO-RAD iCycler, USA). The amplification programme consisted of initial denaturation for 3 min at 98^0C, followed by 35 cycles of denaturation at 98^0C for 10 sec, and primer extension at 72^0C for 20 sec. Annealing temperature was set at 54^0C for 20 sec. Final primer extension was carried out at 72^0C for 10 minutes. For further confirmation of isolates as BTV, the PCR products from earlier set of reactions of all the seven samples were subjected to nested PCR using one of the generic forward primers and another nested primer (nt1430) 5'TATGTAACGCTGAGCATGTACGTAG3' to generated 380bp amplicon using the thermal cyclic conditions as described by McCall and Gould (1994). The annealing temperature for this primer pair was kept at 56^0C and remaining conditions were kept unchanged.

In-silico Restriction Enzyme Analysis (REA): The in silico REA was carried out for all the seven Indian isolates of BTV along with two other isolates from GenBank database using web based algorithm (http://www.restrictionmapper.org/).

Nucleotide Sequence Analysis: PCR products were purified using QIA quick gel extraction kit (QIAGEN, USA) as per manufacturer's instructions. The partial length vp3 gene of all the isolates were sequenced with its respective primer pairs in both directions in the Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar using automated DNA sequencer (ABI PREISMTM 3130 Version 3.0). Sequence analysis was done using online software BLASTN+2.2.26 (http://blast.ncbi.nlm.nih.gov) (Zhang *et al.*, 2000). Phylogenetic tree was constructed based on nucleotide sequences for Indian BTV isolates using Mega 4.0 software (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

India is a vast country with tropical climate and wide prevalence of BTV as 22 of 24 serotypes of BTV have been reported from India (Prasad *et al.*, 2007). In the present study, seven BTV isolates were sequenced and phylogenetic analyses based on seg-3 was carried out. The dsRNA isolated from cell culture grown virus was subjected to vp3 gene specific RT-PCR with standard set of primers. All the samples showed an expected amplicon size of 708bp without any nonspecific amplification in 1% agarose gel electrophoresis (Fig. 1). For further confirmation of samples as BTV, the semi-nested PCR was carried out using another set of standard primers following the same protocol and condition of amplification as used for first PCR. All the samples showed an expected amplicon size of 380bp without any non-specific amplification (Fig. 2) thereby confirming the isolates as BTV.

The sequencing data obtained from amplicon size of 708bp was further analysed using online software such as Bioedit v7.0.8 (Hall, 1999). The sequences obtained were submitted to the NCBI GenBank database and the accession numbers are: EU880280.1, EU880281.1, EU880282.1, EU880283.1, EU880287.1 EU880291.1 and EU880292.1. The analysis of the sequencing data was carried out to find out the genomic diversity if present any. The pair wise nucleotide sequence identity of vp3 gene of Indian BTV isolates used in this study with similar region of vp3 gene from the GenBank database of Indian origin of BTV were determined using Bioedit v7.0.8 (Hall, 1999) software programme. It was observed that the sequences of Indian BTV isolates used in this study showed up to 91% identity within themselves while they showed up to 89% identity with previously published Indian

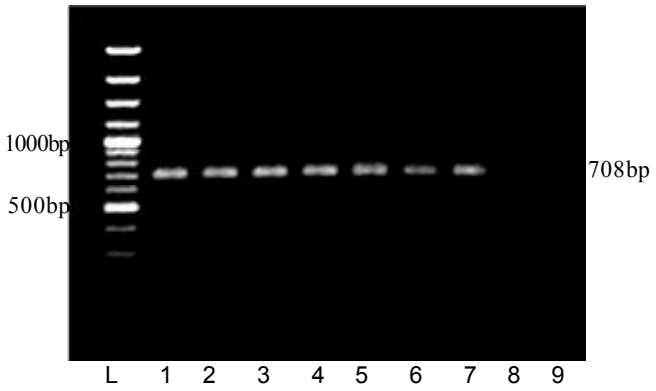

Fig 1. Vp3 gene specific RT-PCR showing amplicon size of 708bp. Lane L=Ladder 100bp; 1=IND/2007/04/BT1; 2= IND/2007/04/BT2; 3=IND/2007/04/BT3; 4=IND/2007/04/ BT4; 5=IND/2007/04/BT10; 6=IND/2007/04/BTONG5; 7= IND/2007/04/BT11H1; 8=BHK-21 cell negative control; 9=Nuclease free water (NFW) negative control.

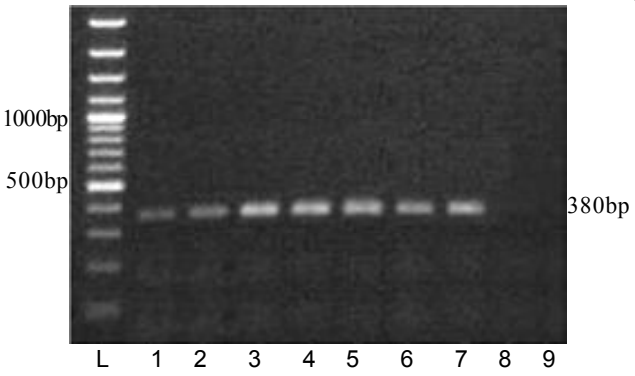

Fig 2. Vp3 gene specific RT-PCR showing amplicon size of 380bp. Lane L=Ladder 100bp; 1=IND/2007/04/BT1; 2= IND/2007/04/BT2; 3=IND/2007/04/BT3; 4=IND/2007/04/ BT4; 5=IND/2007/04/BT10; 6=IND/2007/04/BTONG5; 7= IND/2007/04/BT11H1; 8=BHK-21 cell negative control; 9=NFW negative control.

isolates DQ186810 and DQ186811 (Table 1).

In-silico REA with *PvuII* revealed that three BTV isolates IND/2007/04/BT1 (EU880280.1), IND/ 2007/04/BT2 (EU880281.1) and IND/2007/04/BTONG5 (EU880291.1) had a single site at 1208 while the remaining four isolates lacked the same site (Table 2). Similarly, in silico REA with *BsgI* revealed that BTV isolates IND/2007/04/BT3 (EU880282.1) and IND/ 2007/04/BT4 (EU880283.1) lacked the restriction site for this enzyme whereas the remaining five isolates had same restriction site at position 1278bp. However, in silico REA with *BtsI* enzyme all the BTV isolates showed a single restriction site at position 1126bp. REA with *PvuII* and *BsgI* showed maximum genetic diversity among Indian BTV isolates based on vp3 gene.

To observe more genetic diversity, the phylogenetic tree based on nucleotide sequencing data of all the 7 Indian BTV isolates (used in present study) as well as other Indian isolates available in GenBank was constructed (Fig. 3). The phylogenetic analysis indicated that of the seven Indian BTV isolates, five isolates were in the same cluster while the remaining two isolates (IND/2007/04/BT4 and IND/2007/04/BT11H1) formed a separate cluster indicating the presence of genetic diversity among BTV isolates.

The findings of this study suggested that vp3 gene based RT-PCR could be a reliable and rapid method for amplification of Indian isolates of BTV irrespective of the source of origin. The study also provided valuable information regarding seg-3 based genetic diversity among Indian isolates. In-silico REA carried out with three restriction enzymes (*PvuII, BsgI, BtsI*) indicated maximum genetic diversity among Indian BTV isolates with *PvuII* and *BsgI*. There is a difference in nucleotide

Table 1

Partial vp3 gene based nucleotide identity of Indian BTV isolates

Name of the isolate (Accession number)	1	2	3	4	5	6	7	8	9
IND/2007/04/BT1 (EU880280.1)	100								
IND/2007/04/BT2 (EU880281.1)	91.2	100							
IND/2007/04/BT3 (EU880282.1)	84.6	85.0	100						
IND/2007/04/BT4 (EU880283.1)	73.0	75.2	71.6	100					
IND/2007/04/BT10 (EU880287.1)	78.6	76.0	76.4	68.1	100				
IND/2007/04/BTONG5 (EU880291.1)	78.4	82.5	78.5	75.7	69.4	100			
IND/2007/04/BT11H1 (EU880292.1)	80.4	83.6	81.5	81.5	73.5	82.9	100		
BTV23 IND1998/01 (DQ186810)	85.5	89.6	86.5	80.0	80.7	82.8	89.3	100	
BTV 1 IND2001/01 (DQ186811)	85.2	88.5	85.9	80.8	80.5	82.3	89.9	98.3	100

Table 2
In-silico restriction enzyme analysis of partial vp3 gene of Indian BTV isolates

Name of the isolate (Accession number)	Restriction Enzymes		
	PvuII CAGCTG	*BsgI* GTGCAG	*BtsI* GCAGTG
IND/2007/04/BT1 (EU880280.1)	1208	1278	1126
IND/2007/04/BT2 (EU880281.1)	1208	1278	1126
IND/2007/04/BT3 (EU880282.1)	-	-	1126
IND/2007/04/BT4 (EU880283.1)	-	-	1126
IND/2007/04/BT10 (EU880287.1)	-	1278	1126
IND/2007/04/BTONG5 (EU880291.1)	1208	1278	1126
IND/2007/04/BT11H1 (EU880292.1)	-	1278	1126
BTV23 IND1998/01 (DQ186810)	-	1278	1126
BTV1 IND2001/01 (DQ186811)	-	1278	1126

sequences of BTV cognate genes based on geographical area of virus isolation which provide information on the potential geographical origin of virus isolates known as topotyping or genotyping. Thus, the determination of the nucleic acid sequence of portions of RNA may provide information on where the virus came from Gould (1987). The information generated in this study could be useful in tracking origin of Indian BTV isolates within India as well as their global origin in case of outbreaks. The seg-3 is less affected by the nature of the hosts' species than the outer capsid proteins with low evolution rates and the role of the VP3 protein is mainly structural. The sequencing of seg-3 may potentially facilitate future epidemiological studies of BTV as it provides a valuable analytical target to trace the origin of BTV strains during epizootics.

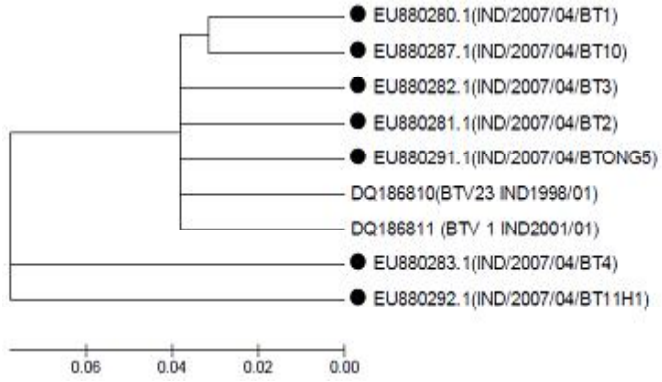

Fig 3. Phylogenetic tree based on partial vp3 gene nucleotide sequences (1055-1763) of BTV isolates. Tree was constructed from partial nucleotide sequences of vp3 gene using the Neighbour Joining method in Mega4 software programme with default parameters (Tamura *et al.*, 2007). Numbers at the major nodes indicate the bootstrap values. ● = BTV isolates used in present study.

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