DETECTION OF MULTIPLE GENETIC VARIANTS OF BTV SEROTYPE-1 ORIGINATED FROM DIFFERENT GEOGRAPHICAL REGIONS IN INDIA

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ABSTRACT

Bluetongue (BT) is an economically important viral disease of sheep and goat in India causing substantial morbidity and mortality. In the present study, four BTV-1 isolates of Andhra Pradesh and one from Utter Pradesh adapted in BHK-21 cell line were used to find the genetic variations. All the samples were confirmed as bluetongue virus (BTV) based on characteristics cytopathetic effect in BHK-21 cell line, visualization of ten segmented dsRNA genome in RNA-PAGE and 366 bp amplicon size with group specific ns1 gene RT-PCR. All the five isolates were confirmed as BTV serotype 1 based on segment 2 (vp2 gene) specific RT-PCR. Although, all the isolates were BTV serotype 1, significant genomic diversity in migration patterns of ten RNA segments was observed based on RNA-PAGE analysis. Five electropherotypic patterns were reported among BTV-1 isolates belonging to various geographical regions in India. These observations indicated occurrence of great genomic diversity among Indian BTV-1 isolates. These variations are significantly important and should be taken into account along with serotypes for developing future vaccination strategies.

KEY WORDS : Bluetongue virus, BTV serotptype 1, Cytopathetic effect, Genomic diversity, Electropherotypes and vaccine strategies.

INTRODUCTION

Bluetongue is an infectious non contagious economically important arthropod borne disease of domestic as well as wild ruminants (MacLachlan, 1994) The disease is caused by bluetongue virus (BTV) belonging to the genus Orbivirus of family Reoviridae. It is listed as a 'notifiable disease' by the Office International des Epizooties (OIE, 2006) Although disease is inapparent in many other ruminant species, particularly cattle that act as reservoir and spread virus to healthy animals through biting of blood sucking insect vector (Maclachlan et al., 2009) Therefore, mandatory export restrictions are imposed on movement of ruminants and their products of BT endemic countries to BT free countries (Velthuis et al., 2009).

BTV consists of linear 10 segmented double stranded RNA (dsRNA) genome which collectively encode seven structural (VP1-VP7) and four non-structural (NS1-NS3 and NS3A) proteins (Huismans and Erasmus, 1981; Huismans et al., 1983).

Twenty four distinct serotypes of BTV (BTV-1 to BTV-24) have been reported worldwide (Gibbs and Greiner, 1994). However, recently BTV-25 has been reported from Switzerland (Hofmann et al., 2008) and BTV-26 from Kuwait (P. Mertens, personal information). In India, 21 BTV serotypes have been reported from different states of the country based upon serology and virus isolation (Prasad et al., 2007; Hofmann et al., 2008). In the present study, a total of five Indian isolates of BTV were analysed in RNA-PAGE and agarose gel electrophoresis to see the migration patterns of the ten genomic RNA segments.

MATERIALS AND METHODS

A. Cultivation of viruses

A total of five BTV-1 isolates were grown in Baby Hamster kidney (BHK-21) cell line to raise the

stock of the virus. Out of these five isolates, one was, from Utter Pradesh (BTsamp1) and rest of the four (BTsamp2, BTsamp3, BTsamp4 and BTsamp5) were from Andhra Pradesh. All the virus isolates and cell line used for present study were maintained in Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar.

B. Extraction and purification of viral dsRNA

After appearance of complete cytopathic effect (CPE) in infected BHK-21 cell line, cells along with virus were kept at 4ºC for 12 hours and pelleted down at 5,000x g for 5 minutes in table top centrifuge (REMI, India). Supernatant was decanted carefully and viral dsRNA was extracted from pelleted material using Trizol (Sigma, USA) following the manufacturer's instructions. The single stranded RNAs were selectively precipitated using 4M Lithium Chloride and 7.5 M Ammonium acetate. The dsRNA pellet was washed with prechilled 70% ethanol and finally the air dried pellet was dissolved in nuclease free water.

C. RNA -PAGE of dsRNA

The dsRNA extracted from all five BTV isolates were analyzed by 8% RNA-PAGE followed by silver staining (Biometra, USA) using the discontinuous buffer system without SDS (Laemmli, 1970; Svensson et al. (1986). The characteristic migration pattern of ten segments of BTV genome was visualized after silver staining and recorded by photography.

D. Synthesis of cDNA and amplification

Viral genomic dsRNA of all five BTV isolates were subjected to cDNA synthesis and amplification by Reverse Transcription and Polymerase Chain Reaction (RT-PCR) using random decamer (Ambion, USA) in thermal cycler (Biorad i Cycler). Briefly, for 25 µl reaction mixture, 6µg of viral dsRNA was subjected to heat denaturation at 95^0C for 5 minutes along with 6% DMSO and 30 pM of random decamer primer. Finally 400µM each dNTPs and 500 U of Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase (Sibzyme) for reverse transcription were added. Thermal conditions were set as primer annealing at 25°C for 10 min, reverse transcription at 42°C for 60 min and finally heat inactivation at 90°C for 10 min.

The cDNA from all five BTV isolates were allowed to non-structural (ns1) gene specific PCR to confirm them as BTV, using primers S1 and S2 generating an amplicon of 366 bp size (Kovi et.al, 2005). For confirmation of their serotype, the cDNA from all the five isolates were further subjected to BTV serotype-1 specific PCR with vp2 gene specific primers S3 and S4 to generate an amplicon size of 604 bp (Table 1) (Dahiya et.al, 2004).

TABLE 1: Primers Used for RT-PCR of BTV 1 Indian Isolates

S.N.	Primers	Sequences(5'? 3')	Product size
1.	S1 (19-39bp)	GTT GGC AAC CAC CAA ACA TGG	366 bp
	S2 (384-361bp)	TCC CAC TTT TGC GCT AAT CCT CAA	
2	S3(1240-1271bp)	ATG GTC GAG TTA ACC TGT TTG ATT ATG TC	604 bp
	S4 (1844-1813bp)	AAT TCC ACG CCG TTG CAA GAT	

PCR was carried out in 20 µl reaction mixture containing 2µl cDNA, 3% DMSO, 20 µM of primers S1-S2 and S3-S4 separately 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 thermalcycler (Biorad iCycler). The amplification programme consisted of initial denaturation for 3 min at 98°C, followed

by 35 cycles for 10 sec denaturation at 98°C, 20 sec primer extension at 72°C. Annealing temperature for primers S1and S2 primer was kept at 54°C for 20 sec and for S3 and S4 at 57°C for 20 sec. Final primer extension was carried out at 72°C for 10 minute. The amplified products were analysed by agarose gel electrophoresis.

III. RESULTS

The segmented genome of all the five Indian isolates of BTV migrated in a pattern of 3:3:3:1 in RNA-PAGE that is characteristic of BTV genome (Fig. 1).

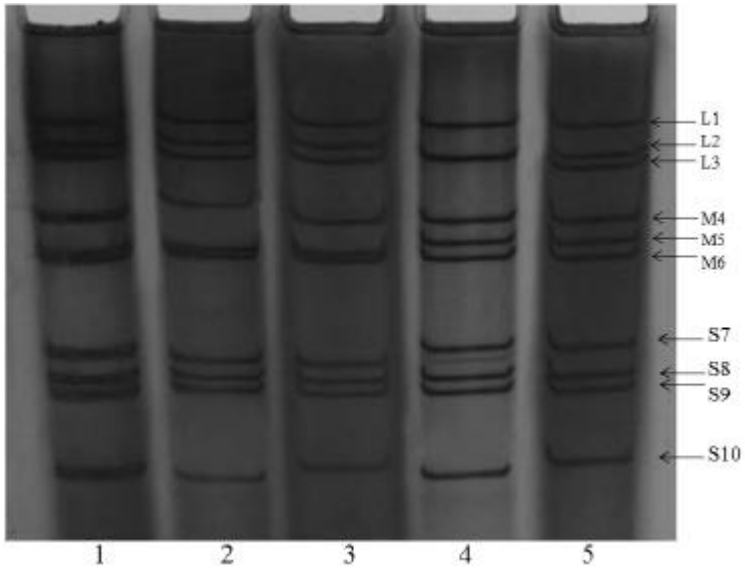

Figure 1: 1% Agarose gel electrophoresis of five BTV isolates showing characteristics ten segmented genome. Lanes: 1: BTsamp1, 2: BTsamp2, 3: BTsamp3, 4: BTsamp4 and 5: BTsamp5. The numbers on the right side indicate large (L1-L3), medium (M3-M6),

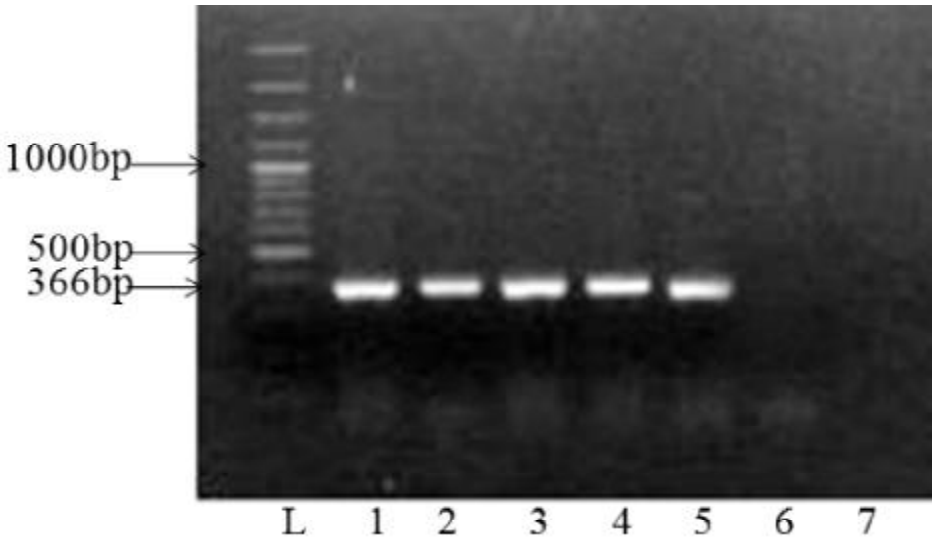

Figure 2: Ns1 gene specific RT-PCR of different BTV isolates showing characteristic 366 bp amplicon size in 1% agarose gel electrophoresis. Lanes: L: 100bp DNA ladder, 1: BTsamp1, 2: BTsamp2, 3: BTsamp3, 4: BTsamp4, 5: BTsamp5, 6: BHK-21 cell negative control and 7: Nuclease free water negative control

Surprisingly, the migration patterns of all the ten segments of dsRNA of BTV-1 were though typical of BTV genome segments, a great diversity was observed within the same serotypes as indicated by variation in the migration length of segments. A total of five different electropherotypic migration patterns namely E1-E5 were observed (Table 2).

TABLE 2: Migration Pattern of Different Bluetongue Virus Isolates in RNA-PAGE

S.No.	Electrop-revalance	Pattern	Isolates	Variations in migration of segments
1	E1	3:3:3:1	BTsamp1	Segments L2 and L3 close to each other, segments M5 and M6 co-migrating, segment S7 and S8 distantly migrating, segment S8 and S9 were closely migrating and segment S10 was fast migrating
2	E2	3:3:3:1	BTsamp2	Segments L2 and L3 were distantly migrating in comparison to E1, segment M4 was slow migrating in comparison to all other isolates, segments M5 and M6 co-migrating, segments S7 and S8 were closely migrating, segments S8 and S9 were closely migrating and S10 was fast migrating.
3	E3	3:3:3:1	BTsamp3	Migration of segments L2 and L3 was similar as in E2, segment M4 was fast migrating, M5 and M6 co-migrating, segments S7 and S8 were closely migrating, segments S8 and S9 closely migrating where as segment S10 was slow migrating
4	E4	3:3:3:1	BTsamp4	Segment L2 and L3 were co-migrating, segment M4 and M5 were closely migrating and M5 and M6 were distantly migrating in comparison to patterns E1, E2 and E3.Segments S7 and S8 were distantly migrating and having an extra segment in between. Segment S10 was fast migrating.
5	E5	3:3:3:1	BTsamp5	Very similar to E3 pattern, segments M5 and M6 were distantly migrating, segments S7 and S8 were also distant migrating, where as segment S10 slow migrating.

Segment M4 is slow migrating in comparison to all other isolates in electropherotype BTsamp2 (E2) and an extra segment was observed in between segment S7 and S8 in BTsamp4 (E4). These finding strongly suggested intratypic genetic diversity among different BTV isolates of the same serotype. The segment 2 of all the 9 isolates was similar as all of them belonged to serotype BTV-1 but the rest of the segments need to be explored on genome sequencing basis to find out the diversity in these isolates.

cDNA prepared from all the five BTV isolates were subjected to group specific ns1 gene PCR (with primers S1 and S2) and a single 366 bp amplicon was observed in all isolates. Characteristics ten segmented dsRNA in 8% RNA-PAGE and 366 bp amplicon with ns1 gene specific primer confirmed

the isolates as BTV (Fig. 2).

Serotype identification of these isolates was done by serotype specific segment 2 (vp2 gene) based RT-PCR assays. All five BTV isolates (BTsamp1, BTsamp2, BTsamp3, BTsamp4 and BTsamp5) were subjected to vp2 gene specific RT-PCR for all twenty four BTV serotypes (data not shown). However, all the five Indian isolates showed expected amplicon size of 604 bp only with BTV 1 specific primers (primer pairs S3 and S4) without any non specific amplification (Fig 3). These isolates did not show any amplification with vp2 gene specific primers for remaining 23 serotypes, thus confirmed the isolates as BTV serotype 1.

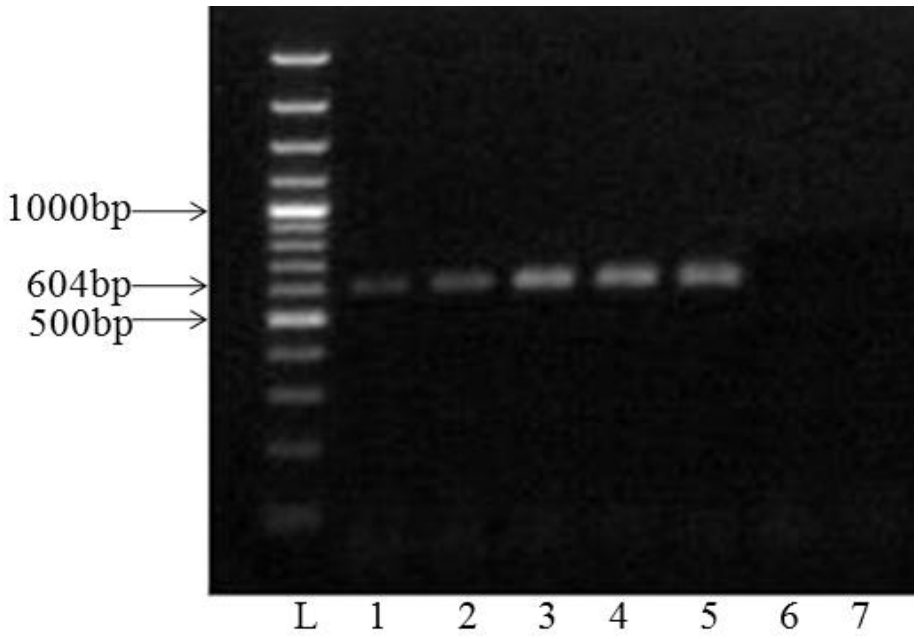

Figure 3: Vp2 gene specific RT-PCR based serotyping of different BTV 1 isolates showing 604 bp amplicon size in 1% Agarose gel electrophoresis. Lanes: L: 100 bp DNA ladder, 1: BTsamp1, 2: BTsamp2, 3: BTsamp3, 4: BTsamp4, 5: BTsamp5, 6: BHK-21 cell negative control and 7: Nuclease free water negative control

IV. DISCUSSION

Since India is a vast country with different climatic zones, provides suitable conditions for Culicoides breeding all over India, the vector responsible for transmission of the disease. BTV is endemic in India particularly to Tamil Nadu, Andhra Pradesh, Karnataka, Maharasthra, Gujarat, Rajasthan, Haryana, Himachal Pradesh, Utter Pradesh and Jammu and Kashmir states. BTV isolates used for this study were isolated from two different geographical regions of Andhra Pradesh and Utter Pradesh state. BTV serotype 1 is one of the common BTV serotypes prevalent in south as well as north India. All the five BTV isolates yielded a single 604 bp product without giving any non-specific amplification, confirmed the BTV serotype 1. Though all the isolates belonged to serotype -1 of bluetongue virus but exhibited diversity in their electropherotypic migration patterns, indicative of genomic diversity due to change in the length/ number of nucleotides because of mutations. The change in the size of segment could be due to addition or deletions in the nucleotides of the genome which could be determined by sequencing of the full genome. Our findings indicated that multiple genetic variants of a single serotype can be present that originate from different geographical regions.

Multiple genetic variants of a single serotype have been reported earlier also in Indian isolates of BTV serotype-16 (Ranjan et. al., 2011) The evidence for multiple variants based on RNA patterns have already reported in Indian isolates of avian, bovine, human and swine rotaviruses (Savita et. al., 2008), (Minakshi et. al., 2009). These polymorphisms have provided useful biochemical markers for the analysis of genetic reassortment of BTV. In order to understand the genetics and epidemiology of BTV and, further, to produce a vaccine for it, it is necessary to characterize BTV field isolates not only by serotyping but also by electropherotyping.

V. CONCLUSIONS

The variation in the migration patterns is suggestive of occurrence of high frequency reassortment in the animal host as well as in the vector responsible for spread of infection. The vector species could be considered as highly permissive host for reassortment mediated evolution of arboviruses. It has been suggested that reassortment must be considered an important factor for developing future vaccination strategies.

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