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Restriction enzyme analysis of VP7 gene of Indian isolates of bluetongue virus

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The genome segment 7 of two Indian isolates of bluetongue virus (BTV) from Avikanagar (BTV-1-western India) and Hyderabad (BTV-Untyped Hyderabad-southern India) was amplified by RT-PCR using two sets of VP7 specific primers. A sequence of 1137 bp comprising the complete coding sequence of the VP7 gene from Avikanagar isolate and a 1154 bp full–length sequence from BTV-UT Hyderabad isolate were amplified. Further, restriction enzyme digestion of these full-length amplicons, using *Eco*RI, *Pst*I and *Taq*I revealed that genome segment 7 from both isolates were different from each other by absence of any *Eco*RI site in the BTV-UT Hyderabad isolate. There were also variations in the number and position of restriction sites for *Taq*I enzyme in these two isolates. *Taq*I has two sites in the Avikanagar isolate whereas four sites in BTV-UT Hyderabad. The restriction digestion fragments obtained after *Pst*I digestion were differentiated on the basis of their distinct sizes in both isolates. Comparison of their *in silico* restriction profiles with that of other isolates from different countries revealed that the two Indian isolates belonging to different parts of India had variations in their VP7 gene which was also distinguishable from at least some isolates from Australia and South Africa. Hence the restriction enzyme (RE) based analysis might be a useful tool in determining the genetic diversity in genome segment 7 and also for tracing their evolutionary relationships.

Keywords: Bluetongue virus, Restriction enzyme digestion, RT-PCR, Serogroup-specific

Bluetongue (BT) is an important disease of domestic and wild ruminants from animal health perspective and the economics of livestock and germplasm. In addition to production losses, the greatest economic impact is encountered due to trade restrictions on export of animals (or animal products) from regions, which harbour BT[1]. Such a restriction may cause huge financial loss for countries like India where BTV is endemic and the economy relies largely on livestock and livestock products.

Bluetongue virus (BTV), the causative agent of the disease, is a double-stranded RNA virus, a member of the genus *Orbivirus* in the family *Reoviridae*. There are 24 serotypes of BTV, of which at least 21 have been reported from different parts of India[2]. Genome of the virus consists of 10 segments, coding for seven structural (VP1–VP7) and four non-structural (NS1, NS2, NS3 and NS3a) proteins. 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[3,4]. VP7 gene has been demonstrated to have diagnostic capability for the detection of all serotypes of BTV due to the group specificity[5,6]. Molecular interactions based investigations of these viruses with their insect vector hosts have revealed that the group-specific viral capsid protein VP7 can mediate attachment of BTV to insect cells[7]. BTV genome segment 7 (S7), encoding the outer core protein VP7, also shows significant variations, which could relate to the insect populations that act as vectors for the different virus strains[8]. Cross-hybridisation and sequencing studies have shown that some of BTV genome segments can vary in a manner that reflects the geographic origin of the virus strain (topotype)[9,10].

Serogroup specific reverse transcription-polymerase chain reaction (RT-PCR), sequencing,

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restriction enzyme analysis (REA) and phylogenetic analyses (targeting conserved genome segments) are used in many laboratories in India, for identification of BTV[11-13]. In the present study we describe the sequence analysis and REA of Seg-7 from two Indian strains of BTV isolated from two different geographical regions of India (Avikanagar BTV-1-western India) and Hyderabad (BTV-Untyped-southern India) have been reported. Comparisons were also made to *in silico* REA from some selected strains of BTV from other parts of world in an attempt to determine the variability in this genome segment, which can also be used to define the geographic origin of a particular genome segment.

Materials and Methods

Bluetongue virus isolates—BTV serotype 1 Avikanagar (BTV-1A) was isolated from Central Sheep and Wool Research Institute, Avikanagar, Rajasthan, western India[14], whereas the another BTV untyped isolate from Hyderabad (BTV-UT Hyd) was isolated from Iskilla village, Nalgonda, Andra Pradesh, southern India (Reddy, 2003; Personal communication). These isolates were maintained in BT Lab, Department of Animal Biotechnology, CCS Haryana Agricultural University, Hisar, Haryana, India.

Cultivation of viruses—BHK-21 (baby hamster kidney-21) cells, grown in Glasgow modified minimum essential medium supplemented with L-glutamine, fetal calf serum (10%) and antibiotic, were used to propagate both isolates. One-day-old cell monolayer (~80% confluent) was used for infection with the two Indian isolates of BTV separately. Infected cultures were then incubated at 37°C and the cytopathic effects (CPE) were observed daily.

Processing of cell culture grown virus and extraction of viral RNA—The virus isolates were harvested when the infected BHK-21 monolayer showed about 75%-90% CPE (48-72 hr). The viral genomic RNA of BTV was extracted from cell pellets using Trizol method[15].

Primer used for RT-PCR—Two different sets of serogroup specific primers, set 1[16] and set 2[6], used for amplification of BTV Seg-7, were obtained comercially from Sigma. Primer set 1 was evaluated to amplify the coding sequence of 1137 bp and primer set 2 to amplify the complete sequence of 1154 bp from VP7 gene. Details of these primers, with nucleotide positions and sequence, are listed in Table 1.

Reverse transcription—Reverse transcription was carried out in a 25 µl reaction mixture containing approximately 100 ng of heat-denatured, purified BTV RNA, 25 pmol of each primers, 6% DMSO, 4 m*M* µmol of dNTPs and 2.5 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene). The primers were allowed to anneal at 25°C for 10 min and then reverse transcription was carried out at 42°C for 60 min in thermal cycler followed by heat inactivation of reverse transcriptase enzyme at 90°C for 5 min. The cDNA was stored at -20°C till further use.

Polymerase chain reaction and sequencing—Amplification of cDNA was carried out using 25 pmol of the same primers in 25 µl reaction mixture containing 5 µl heat-denatured cDNA, Taq buffer with 5% DMSO, 200 µ*M* of deoxyribonucleotide triphospahate (dNTPs), and 1.25 m*M* of $MgCl_2$ and 1.25 U Platinum *Taq* DNA polymerase (Invitrogen). The amplification program consisted of one cycle of initial denaturation at 95°C for 2 min and 30 cycles each having denaturation at 94°C for 45 sec, annealing at 56°C for 1 min, extension at 72°C for 1.30 min and final extension at 72°C for 10 min. The desired PCR products of 1154 bp (BTV-UT Hyderabad) and 1137 bp (BTV-1A) were then visualized by electrophoresis in agarose gel electrophoresis with ethidium bromide. The VP7 amplicons were sequenced using automated DNA sequencer ABI PRISMTM 3100 Version 3.0.

In vitro restriction endonuclease analysis—Full-length PCR products (~500 ng) of VP7 gene of the two Indian isolates of BTV were subjected to restriction digestion with a set of three restriction

Table 1—Details of oligonucleotide primers

Primer pair	Primer orientation and position	Primer sequence (5'-3')	Product size
Primer pair 1[(16)]	Forward (18-38)	ATGGACACTATCGCAGCAAGA	1137 bp
	Reverse (1156-1136)	GTAAGTGTAATCTAAGAGACG	
Primer pair 2[(6)]	Forward (1-23)	GTTAAAAATCTATAGAGATGGAC	1154 bp
	Reverse (1156-1136)	GTAAGTGTAATCTAAGAGACG	

enzymes; *Eco*R1, *Pst*1 and *Taq*1 (MBI Fermentas) according to manufacturers instructions.

In silico restriction endonuclease analysis—VP7 gene sequences of BTV-1A and BTV-UT Hyd were subjected to *in silico* restriction analysis using software 'Restriction Mapper version 3.0'. The *in silico* and *in vitro* restriction profile results were then compared. The GenBank accession numbers of VP7 gene sequence of all the isolates of BTV which were used for *in silico* restriction analysis is given in Table 2.

Table 2—*In silico* restriction endonuclease profile analysis of BTV-VP7 gene of Indian and other isolates from around the world

BTV isolates	Accession no.	*Eco*RI	*Pst*I	*Taq*I
BTV-1 Avikanagar	AM261976	1 (614)	1 (812)	2 (366,957)
BTV-UT Hyderabad	AM261975	Nil	1 (470)	4 (347,366, 589,926)
BTV-1SA	X53740	1 (614)	1 (812)	2 (771,926)
BTV-1AUS	M63417	1 (619)	1 (817)	2 (776,931)
BTV-1PRC	AF172825	1 (597)	1 (795)	2 (572,909)
BTV-2 France	AF346302	1 (993)	Nil	4
BTV-15AUS	L11724	Nil	Nil	3

Results

Bluetongue virus RNA extracted from infected BHK-21 cells by Trizol lysis and purified by differential lithium chloride precipitation method yielded high quality RNA. In the PCR, with the set 1 primers, a product of 1137 bp was amplified from BTV-1A isolate, whereas a 1154 bp product was obtained from BTV-UT Hyd isolate as observed in 1% agarose gel. The resultant PCR products were sequenced and sequences were submitted to EMBL database. The accession numbers of BTV-1A and BTV-UT Hyd are AM261976 and AM261975 respectively. The restriction digestion of PCR amplicons of VP7 gene of these two BTV isolates with *Pst*1 yielded two fragments of approximately 812 bp and 325 bp with BTV-1A whereas with BTV-UT Hyd two fragments of 470 and 684 bps were obtained (Fig. 1). This clearly indicated that there is only one site of *Pst*I within VP7 gene of these two isolates but the cutting site varies in terms of nucleotide position. The restriction digestion of PCR product of VP7 gene of two isolates of BTV with *Eco*RI yielded two fragments of approximately 523 and 614 bp with BTV-1A whereas BTV-UT Hyd remained undigested as indicated by only one band of 1154 bp (Fig. 1). This revealed that there was no restriction enzyme site for *Eco*RI in BTV-UT Hyd.

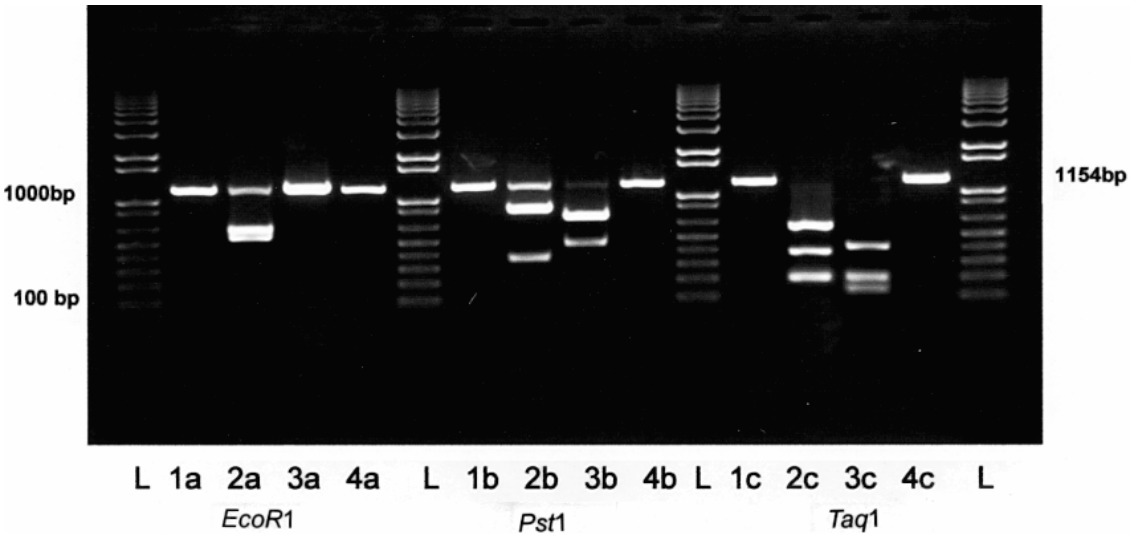

Fig. 1—Restriction endonuclease analysis of PCR amplicons from VP7 gene of Indian isolates of BTV using – *Eco*RI (Lanes 1a-4a); *Pst*I (Lanes 1b-4b); and *Taq*I (Lanes 1c-4c); [Lane (L) Marker 1 kb plus DNA ladder (Invitrogen); (1a) – Uncut 1137 bp PCR product; (2a)- BTV-1 (Avikanagar) showing products of 523 and 614 bp; (3a) - BTV-UT (Hyderabad) remained uncut with *Eco*R1; (4a) - Uncut 1154 bp PCR product; (1b) - Uncut 1137 bp PCR product; (2b) - BTV-1 (Avikanagar) showing products of 812 bp and 325 bp; (3b) - BTV-UT (Hyderabad) showing products of 684 and 470 bps; (4a) - Uncut 1154bp PCR product; (1c) - Uncut 1137 bp PCR product; (2c) - BTV-1 (Avikanagar) showing products of 591, 366 and 180 bp; (3c) - BTV-UT (Hyderabad) showing products of 347 and 335 (as single band), 228, 123, and 19 bp (not visible) and (4c) - Uncut 1154 bp PCR product].

The digestion of VP7 amplicons with *Taq*I yielded three visible fragments in each case, of approximately 123, 228, 350 bps with BTV-UT Hyd and 180, 366 and 591 bps with BTV-1A (Fig. 1). These results have indicated that there are two sites of *Taq*I in both the isolates. The VP7 gene sequences of Indian isolates and that of other isolates from different countries available in GenBank were subjected to *in silico* restriction analysis with a set of three restriction enzymes; *Eco*RI, *Pst*I and *Taq*I. The variations shown by these enzymes in terms of number of cuts and nucleotide positions in isolates of different serotypes from different geographical locations are presented in Table 2. *Eco*RI has one cutting site at nucleotide position 614 in BTV-1A where as no site in BTV-UT Hyd isolate. Therefore, the expected sizes of fragments in BTV-1A after *Eco*R1 digestion are 614 and 523 bp whereas 1154 bp in BTV-UT Hyd isolate. *Pst*I has one site in each isolate but cutting site varies in terms of nucleotide position. It cuts BTV-1A VP7 gene at position 812 whereas at position 470 in BTV-UT Hyd isolate. Therefore, the expected fragments size in these two isolates is 812, 325 bp and 684, 470 bps, respectively. *Taq*I has two sites in VP7 gene of BTV-1A (positions 366 and 957) isolate whereas four sites in BTV-UT Hyd isolate (positions 347, 366, 589 and 926). *Taq*I digestion therefore should produce three fragments of 591, 366 and 180 bp with Avikanagar isolate and five fragments of 19, 123, 228, 335 and 347 bp with Hyderabad-untyped isolate.

Discussion

Recent re-emergence of BTV in European countries, once again has indicated the need for development of new molecular tools for easy, quick and reliable methods of BTV detection worldwide. There are only a few reports of specific genome based molecular characterization of Indian isolates of BTV along with their restriction digestion profile analysis[12,17]. In this study, RT-PCR assays and primers were evaluated for the detection of BTV in cell culture. These assays successfully amplified an expected portion of the genome and no spurious products were observed with any of the BTV isolates proving the sequence specificity of these primers. The restriction digestion of full-length PCR amplicons of VP7 gene of two BTV Indian isolates with *Pst*I yielded two fragments for each of these isolates, yet they can be differentiated from each other due to difference in the size of restriction fragments obtained. The BTV-UT Hyd remained undigested when treated with *EcoR*1 while BTV-1A yielded two fragments. The *Taq*I restriction enzyme produced distinct restriction pattern for BTV-UT Hyd and BTV-1A with three visible fragments in each case. The *in vitro* restriction profiles of the nucleotide sequence of VP7 gene for the two Indian isolates were in perfect agreement with their *in silico* restriction profile results in most cases. However, the difference observed in the *in vitro* (3 visible fragments of ~123, 228, 350 bps) and *in silico* (4 cutting sites and 5 fragments of 19, 123, 228, 335 and 347) restriction profile generated by *Taq*I with BTV-UT Hyd isolate can be explained that a fragment of 19 bp is too short and not present on the agarose gel, whereas fragments of sizes 335 and 347 bp are visible as single band on the gel (Fig. 1). This study therefore indicated that all three restriction enzymes (*Eco*R1, *Pst*I and *Taq*I) could be used to differentiate these two isolates of BTV-1A and BTV-UT Hyd from each. The VP7 gene of these two isolates showed some degree of variability by having no *Eco*RI restriction site in one isolate (BTV-UT Hyd) and the distinct restriction profile pattern generated after digestion with *Pst*I and *Taq*I enzymes. Hence, it can be concluded that restriction profile analysis using *Pst*I, *Eco*RI and *Taq*I can be used to differentiate these two Indian isolates. The restriction analysis (*in silico* and *in vitro*) of VP7 gene sequences of Indian isolates with three restriction enzymes and their comparison with that of other isolates from different countries of the world revealed that both Indian isolates had distinct profiles which were also quite different to that of Australian and South African isolates that were compared. Other workers[6,8,10,16] have also reported that Seg-7 also shows some type of geographical grouping and variability. REA of sequences generated in these studies agrees with these earlier reports and separation of Seg-7 nucleotide sequences into distinct groups. Hence, the restriction analysis of genome segment 7 would be a helpful diagnostic and research tool in determining the genetic diversity in this gene and also in tracing their evolutionary and geographical origin.

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