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Differentiation of Indian isolates of bluetongue virus serotype 1 from Australian and African isolates based on analysis of vp5 gene

B N Manjunatha[1], Minakshi Prasad, S Maan[1] and G Prasad*

Department of Animal Biotechnology, College of Veterinary Sciences
CCS Haryana Agricultural University, Hisar, India
[1]Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking
Surrey GU24 0NF, UK

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Bluetongue virus (BTV), prototype species of genus *Orbivirus,* belongs to the family *Reoviridae*. It is a non-enveloped, double shelled virus with ten segmented dsRNA genome. RNA segment 6 encodes an outer capsid serotype specific virus protein VP5. A pair of primers (forward 207-229 bp & reverse 1284-1304) was designed from the published BTV-1 segment 6 sequences to specifically amplify vp5 gene from Indian isolates of BTV. These primers specifically amplified PCR product of 1098 bp from cell culture adapted isolates of BTV-1 (Hisar isolate-BTV-1H, Avikanagar isolate-BTV-1A and Sirsa isolate-BTV-1S_3), but did not give any amplification with BTV-9 and BTV-23, indicating serotype specificity. vp5 coding sequences amplified from Indian BTV-1 isolates were cloned into pPCR Script™ Amp SK (+) vector and transformed into XL10-Gold® Kan ultracompetent *Escherichia coli* cells. The positive clones selected by blue–white screening and colony touch PCR were sequenced. The sequence analysis of the vp5 gene (253-1255 bp) revealed that Indian isolates of BTV-1 showed 89-91.1% nucleotide identity with Australian isolates of BTV-1, whereas it showed only 77-79.7% similarity with the BTV-1 African isolates. All three Indian isolates shared 99.4% nucleotide sequence similarity amongst themselves. Comparison of the deduced amino acid sequences revealed that the Indian BTV-1 isolates shared 96.7-98.8% and 94.9-95.8% amino acid similarity with Australian and African BTV-1 isolates, respectively. *In silico* restriction enzyme (RE) profile analysis of vp5 gene sequences showed that Indian isolates of BTV-1 can be differentiated from other BTV-1 isolates from South Africa and Australia using *Taq*I and *Bsm*I restriction endonucleases.

Keywords: Bluetongue, BTV-1, cloning, RE analysis, RT-PCR, sequencing, vp5 gene

Introduction

Bluetongue (BT) is an infectious, noncontagious, insect-borne viral disease of domestic and wild ruminants, mainly affecting sheep. BT virus (BTV) infection is widely prevalent throughout the tropical, subtropical and temperate regions of the world, between latitudes of approximately 40-50° N and 35° S, coincident with the distribution of haematophagous midges of genus *Culicoides* that are biological vectors of the virus[1,2]. Since 1998, the virus has spread northward in parts of the Mediterranean basin and appeared in North-western Europe in 2006 far beyond its prior known upper northern limits anywhere in the world[3,4]. Sheep and wild ruminants experience acute infection, often with high mortality. In cattle and goats the disease is generally described as inapparent or mild[5], although clinical disease have been seen during the initial stages of a new incursion of the virus into a serologically naive cattle population[6]. Due to the serious socio-economic consequences of BTV outbreaks on the international trade of animals and animal products, it has been included in the Office International des Epizooties (OIE) list of notifiable diseases (formerly List A).

BTV is the prototype member of the genus *Orbivirus,* family *Reoviridae.* BTV, a non-enveloped, icosahedral-shaped virus consists of a ten segmented, double-stranded (ds) RNA genome encapsidated in a double-layered protein coat. All the segments are monocistronic except segment 10, which codes for two related nonstructural proteins NS3 and NS3A[7,8]. The viral genome is surrounded by inner core composed of two major structural proteins VP3 and VP7 and three minor structural proteins [VP1 (Pol), VP4 (Cap) & VP6 (Hel)], which are the components of transcription complex. This in turn is surrounded by an outer capsid composed of two structural proteins (VP2 & VP5), which are serotype specific,

*Author for correspondence:
Tel: 91-1662-289132; Fax: 91-662-234613
E-mail: gprasad1986@gmail.com

highly variable, involved in cell attachment and penetration during initiation of infection and contain epitopes that bind neutralizing antibodies[8]. In addition, there are 4 nonstructural proteins NS1, NS2, NS3 and NS3A, which are expressed in the virus infected cells[9]. VP5 protein has been scribed an indirect role in augmenting neutralizing antibody response against VP2 (major neutralizing protein)[10]. In addition, VP5 protein has also been demonstrated to be involved in membrane fusion, cellular permeabilization and causation of apoptosis in the infected-mammalian cells[11,12].

To date, 24 distinct serotypes of BTV have been described in the world[1] with the evidence of prevalence of 21 serotypes in India[13,14]. The BTV strains isolated from different parts of world have shown high degree of variations in the nucleotide sequence of their dsRNA genome segments that reflect their geographic origins[15-17]. For many of the BTV genome segments, these variations divide these viruses into 'eastern' and 'western' groups/topotypes[18-20]. Previously, sequence analysis of genome segment 6 encoding VP5 protein also revealed genetic diversity between different BTV serotypes and between isolates within a single serotype[21]. Therefore, in the present study, analysis of the vp5 gene from Indian BTV-1 isolates was undertaken to find out the extent of genetic variability in this gene, which could be linked to trace the evolutionary origin of these isolates.

Materials and Methods

Viruses

Indian isolates of BTV-1 Hisar (BTV-1H)[22], BTV-1 Avikanagar (BTV-1A)[23], BTV-1 Sirsa 3 (BTV-1S_3)[24], BTV-9 Mehboobnagar (BTV-9MBN) and BTV-23 Izatnagar (BTV-23cIVRI) were propagated in baby hamster kidney (BHK-21) cell line. After 36-48 h of infection, 75% of the cells showed cytopathic effects, viruses were harvested and titrated as per the method of Reed and Muench[25].

Extraction of Viral RNA

The viral RNA was extracted by guanidinium isothiocyanate (GIT)-phenol-chloroform method[26] with minor modifications[27].

Preparation of vp5 Gene cDNA

BTV-1 specific primers [forward primer (P1) 5'-caggtgaatcgtatggcgaatcc-3' (nt. 207-229)] and [Reverse primer (P2) 5'-cggataacgtacgtcccatcg-3' (nt. 1304-1284)] were designed using FastPCR software from the sequences available in GenBank. A 100 ng viral RNA was heat denatured in the presence of 6% DMSO and 1 μ*M* concentration of each primer. Finally, 25 μL reaction mix was prepared using 400 μ*M* each dNTPs and 500 U of M-MuLV reverse transcriptase (SibEnzyme; 400,000 U/mL stock) for reverse transcription. The primers were allowed to anneal at 25°C for 10 min, and then reverse transcription at 42°C for 60 min, followed by heat inactivation at 90°C for 5 min in thermal cycler (Biorad iCycler).

Polymerase Chain Reaction (PCR)

The PCR was optimized with 12% of total cDNA generated, 5% DMSO, 0.8 μ*M* of each primer (Sigma), 200 μ*M* of each dNTPs, 1.5 m*M* $MgCl_2$ and 2.5 U of *Taq* DNA polymerase (MBI Fermentas 5 U/μL). The Cycling conditions for amplifying vp5 gene sequences were: initial denaturation of 95°C for 5 min, followed by 30 cycles of 45 sec denaturation at 95°C, 50 sec primer annealing at 63°C and 2 min primer extension at 72°C. Final extension was done at 72°C for 5 min. The PCR products were analyzed in ethidium bromide stained 1% agarose gel electrophoresis (AGE) and visualized under UV transilluminator (Biovis).

Restriction Digestion of PCR Product

The *in vitro* restriction analysis of 1098 bp PCR product of vp5 gene was done using *Bsm*I (New England Biolabs) and *Taq*I (New England Biolabs) as per the manufacturer's instructions.

Cloning of BTV-1 vp5 Gene

The 1098 bp PCR products of vp5 gene of all the three isolates of BTV-1 (BTV-1H, -1A & -1S3) were purified with StrataPrep® PCR purification kit (Stratagene). The A-overhangs of purified PCR products generated during PCR by *Taq* polymerase were polished using *Pfu* DNA polymerase to facilitate blunt end ligation in the subsequent steps. The pPCR-Script™ Amp SK(+) 2961 bp circular cloning vector (Acc. No. U46017), supplied with PCR-Script™ Amp cloning kit (Stratagene), was used for cloning. PCR products were ligated in the vector following the manufacture's instructions and kept on ice till further use. The XL10-Gold® Kan, ultracompetent cells of *Escherichia coli* supplied along with the PCR-script™ Amp cloning kit (Stratagene) were used as a host system for transformation. The transformed reaction mixture (150 μL per plate) was streaked on Luria

Bertani broth (LB) agar plates prepared with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (80 μg/mL), 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin (Amp) (50 μg/mL). The plates were incubated for 17 h at 37°C for blue–white selection of recombinant clones with further incubation for 2 h at 4°C for colour enhancement.

Touch PCR

The white and blue bacterial colonies grown in LB-Amp broth were subjected to touch PCR using P1 and P2 primers with cycling conditions standardized for 1098 bp product of BTV-1 vp5 gene. The amplicons were analyzed across 1% agarose gel stained with ethidium bromide.

Isolation of Plasmid by Alkaline Lysis Method

Mini preparation method described by Sambrook and Russel[28] was followed for isolation of plasmid DNA from white recombinant colonies which were found positive by touch PCR. The plasmid DNA (5 μL) was resolved in 0.7% agarose gel electrophoresis along with 1 kb DNA ladder.

Restriction Enzyme (RE) Digestion of Recombinant Plasmid

Plasmid DNA isolated by mini preparation method from the positive clones as well as from blue colonies was subjected to restriction digestion using *Xho*I RE (MBI Fermentas). The digested products were resolved in 0.7% AGE along with 1kb DNA ladder (MBI Fermentas).

Nucleotide Sequence Analysis

The recombinant plasmids pPCR-Script-Amp-BTV1-vp5-1098 having 1098 bp vp5 gene-insert of three Indian isolates of BTV-1 (BTV-1A, -1H & -1S3) were sequenced using automated DNA sequencer ABI PRISM™ 3130 Version 3.0. The sequence analysis was done after converting each sequence into FASTA format (http://www.ncbi.nlm.nih.gov). Clustal X version 1.83 programme was used for multiple sequence alignment. MEGA4 software[29] was used to determine the percentage nucleotide sequence identity, and phylogenic tree construction of 1003 bp coding region of vp5 gene sequences of three Indian isolates of BTV-1 sequenced in this study (BTV-1H, -A &-S3) and vp5 gene sequences of 23 BTV isolates belonging to different serotypes from other countries available in GenBank.

In silico RE Profile Analysis

The vp5 gene sequences of BTV-1H, -1A, -1S3 and selected isolates from other countries were subjected to *in silico* restriction analysis using software NEBcotter V2.0 at http://tools.neb.com/NEBcutter2/index.php

Results

All the three Indian isolates of BTV-1, viz., BTV-1H, -1A and -1S3, yielded an expected 1098 bp PCR product with BTV-1 vp5 gene specific P1 and P2 primer pair (Fig. 1). No amplification was observed with BTV-9, BTV-23 and nuclease free water controls, confirming that these primers were specific to vp5 gene of BTV-1. The *in vitro* restriction digestion of the 1098 bp vp5 gene PCR product of three Indian isolates of BTV-1 with *Bsm*I yielded two fragments of ~667 and 431 bp (Fig. 2), where as *Taq*I restriction enzyme yielded three fragments of ~433,

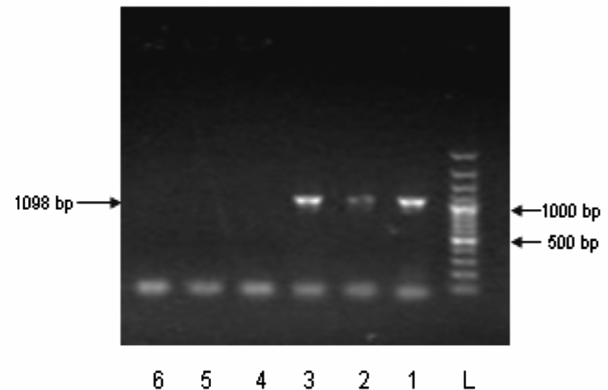

Fig. 1—Agarose gel electrophoresis of RT-PCR products of vp5 gene produced using the designed pair of primers. Lane L: 100 bp ladder, 1: BTV-1H, 2: BTV-1A, 3: BTV-1S3, 4: BTV-23 IVRI, 5: BTV-9MBN, 6: Nuclease free water control.

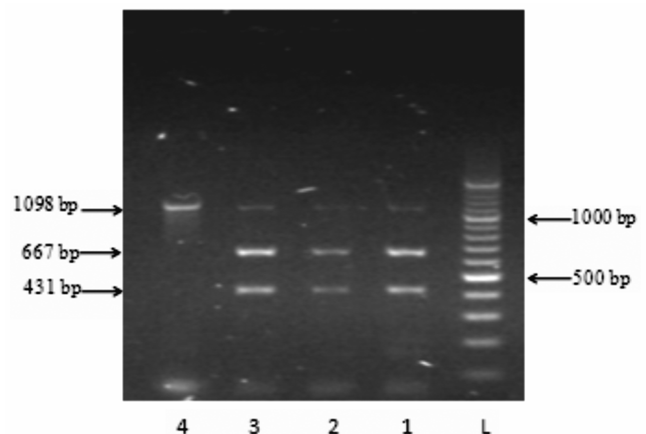

Fig. 2—Restriction profile of vp5 gene based 1098 bp RT-PCR product of BTV-1 Indian isolates with *Bsm*I. Lane L: 100 bp DNA ladder (Fermentas), 1: BTV-1H, 2: BTV-1A, 3: BTV-1S3, 4: BTV-1A uncut 1098 bp product.

366 and 299 bp (Fig. 3). After cloning of the 1098 bp PCR product, recombinant clones were selected on the basis of blue-white colony screening method. More than 90% of the colonies were white. The selected white colonies yielded expected 1098 bp amplicons on touch PCR using P1 and P2 primers. However, blue colonies, untransformed ultracompetent cells and LB-Amp broth did not yield any amplification. The plasmid DNA isolated from white and blue colonies yielded single band of 4059 (with 1098 bp insert) and 2961 bp (without insert), respectively in 0.7% agarose gel electrophoresis upon digestion with *Xho*I restriction enzyme, which has single target site in the vector and no site in the 1098 bp vp5 gene insert.

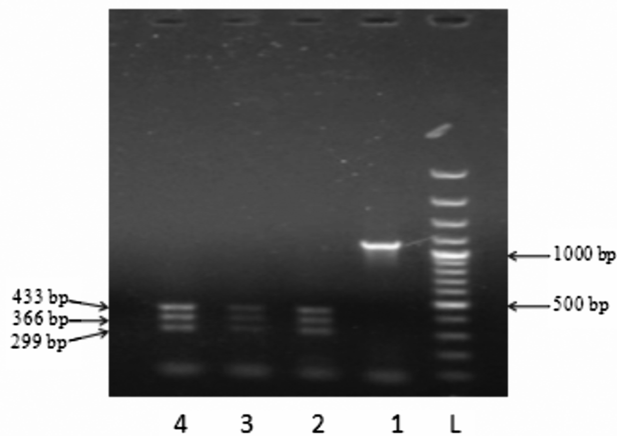

Fig. 3—Restriction profile of vp5 gene based 1098 bp RT-PCR product of BTV-1 Indian isolates with *Taq*I. Lane L: 100 bp DNA ladder (Fermentas), 1: BTV-1A uncut 1098 bp product, 2: BTV-1H, 3: BTV-1A, 4: BTV-1S3.

The recombinant vector having 1098 bp vp5 gene insert of each of the BTV-1 isolates (BTV-1H, -1A & -1S3) were sequenced from both directions. Sequences of vp5 gene obtained were corresponding to the region of 207 to 1304 bp for BTV-1A and -1S3 isolates; while for BTV-1H, it was from 253 to 1255 bp.

The sequences of 1003 nucleotides (corresponding to region 253 to 1255 bp) of vp5 gene of all the isolates were used for further *in silico* analysis after ignoring the terminal sequences. The sequences from vp5 gene of BTV-1H, -1A and -1S3 were submitted to NCBI GenBank and their accession numbers obtained were, respectively: EU443927, EU443928 and EU443929. The partial VP5 sequences (253-1255 bp) from BTV-1 Indian isolates were compared with that of 24 reference strains of BTV using Clustal X version 1.83.

The pair-wise nucleotide sequence identity of vp5 gene of Indian isolates of BTV-1 with other isolates and serotypes from different parts of the world were determined using MEGA 4 (Tables 1 & 2). The vp5 gene sequence analysis revealed that there was more than 99.6% sequence identity between three Indian isolates selected in this study. Indian isolate BTV-1H_EU443927 shared 100% nucleotide sequence homology with two (BTV-1IND_AJ586660 and BTV-1IND_AJ586662) Indian isolates of BTV-1, sequenced earlier[21].

The nucleotide sequences of three BTV-1 Indian isolates (BTV-1H, -1A & -1S3) were aligned with protein sequences available in protein database using BlastX. It was found that the nucleotide sequence corresponds to region from 77-410 amino acid sequence of BTV-1 VP5 protein. On multiple

Table 1—Per cent nucleotide sequence homology of vp5 gene of bluetongue virus serotype 1

No.	Serotype/isolate	BTV-1H_EU443927	BTV-1A_EU443928	BTV-1S3_EU443929
1	**BTV-1H_EU443927**	100	99.8	99.6
2	**BTV-1A_EU443928**	99.8	100	99.8
3	**BTV-1S3_EU443929**	99.6	99.4	100
4	BTV-1IND_AJ586660	100	99.8	99.6
5	BTV-1IND_AJ586659	99.9	99.7	99.5
6	BTV-1IND_AJ586662	100	99.8	99.6
7	BTV-1AUS_AJ631214	90.53	90.33	90.33
8	BTV-1AUS_M21845	89.23	89.03	89.03
9	BTV-1RSA_AJ586656	78.17	77.97	77.77
10	BTV-1RSA_AJ586695	79.06	78.86	78.66
11	BTV-1NIG_AJ586657	78.56	78.36	78.17
12	BTV-1SUD_AJ586658	78.36	78.17	78.17

IND= India, AUS=Australia, RSA=South Africa, NIG=Nigeria, SUD= Sudan.
The accession numbers in bold letters represent Indian isolated used for sequencing in the present study.

Table 2—Nucleotide sequence homology of vp5 gene of 24 serotypes of bluetongue virus

No.	Serotype/isolate	BTV-1H_EU443927	BTV-1A_EU443928	BTV-1S3_EU443929
1	**BTV-1H_U443927**	100	99.8	99.6
2	**BTV-1A_EU443928**	99.8	100	99.4
3	**BTV-1S3_EU443929**	99.6	99.4	100
4	BTV-1AUS_AJ631214	90.53	90.33	90.33
5	BTV-1RSA_AJ586656	78.17	77.97	77.77
6	BTV-2France_AY129083	77.74	77.54	77.54
7	BTV-2USA_AY855279	76.97	76.77	76.57
8	BTV-18IND_AY643510	75.77	75.57	75.57
9	BTV-23IND_AJ783907	76.57	76.37	76.37
10	BTV-8RSA_AJ586705	73.88	73.68	73.78
11	BTV-7RSA_AJ586704	68.49	68.3	68.39
12	BTV-19RSA_AJ586722	69.09	68.89	68.99
13	BTV-5RSA_AJ586700	66.9	66.7	66.8
14	BTV-9Serbia_AJ586688	68.2	68	68.1
15	BTV-16RSA_AJ586689	69.39	69.19	69.49
16	BTV-21RSA_AJ586724	69.59	69.39	69.69
17	BTV-3RSA_AJ586697	67	66.8	67.1
18	BTV-6RSA_AJ586703	67.1	66.9	67.2
19	BTV-14RSA_AJ586714	65.9	65.7	65.8
20	BTV-13RSA_AJ586713	68	67.8	68.1
21	BTV-4RSA_AJ586676	68.69	68.69	68.79
22	BTV-11RSA_AJ586710	69.09	69.09	69.19
23	BTV-17USA_X55359	68.1	68.1	68.2
24	BTV-24RSA_AJ586730	68.4	68.4	68.3
25	BTV-20RSA_AJ586723	67.6	67.6	67.4
26	BTV-10RSA_AJ586709	68	68	67.9
27	BTV-12RSA_AJ586711	58.42	58.33	58.42
28	BTV-22NIG_AJ586726	58.62	58.52	58.62
29	BTV-15RSA_AJ586716	57.98	57.88	58.38

IND= India, AUS=Australia, RSA=South Africa, NIG=Nigeria.
The accession numbers in bold letters represent Indian isolated used for sequencing in the present study.

alignment of these VP5 protein sequences using BioEdit software indicated four amino acid changes at 164, 198, 321 and 409 positions between the isolates within this region.

The *in silico* restriction analysis of 207-1304 bp region of BTV-1 isolates revealed that all the Indian and one Australian BTV-1 isolate (AJ631214-AUS) have only one *Bsm*I restriction site at 637 bp, whereas another Australian isolate (M21845-AUS) has restriction site at 636 bp. In contrast to Indian and Australian BTV-1 isolates, all African isolates of BTV-1 have restriction site at 445 bp, suggesting two mutations in these selected sequences (Table 3). However, one South African isolate of BTV-1 (AJ586656-RSA) did not have any restriction site for *Bsm*1 (Table 3).

In silico restriction analysis with *Taq*I revealed that all Indian isolates of BTV-1 have two restriction sites at 639 and 938 bp within the region from 207 to 1304 bp, whereas one Australian BTV-1 isolate (AJ631214 -AUS) has two extra restriction sites at 405 and 1221 positions, suggesting two mutations in these selected sequences (Table 3). Further, the RE analysis revealed that the another Australian BTV-1 isolate (M21845-AUS) has four *Taq*I sites at different locations (404, 638, 937 & 1220 bp) as shown in Table 3. On the other hand, all the African BTV-1 isolates, except AJ586656-RSA and M36713-RSA, have three restriction sites at entirely different locations (828, 858 and 1209 bp), indicating that there are 6 mutations in the selected sequence (Table 3). The South African isolate AJ586656-RSA exhibited *Taq*1 site at 858, 938 and 1209 bp, where as South African isolate M36713-RSA showed the RE sites at 828, 859 and 1209 bp (Table 3). Moreover, Algerian isolate EU422952-ALG exhibited an addition *Taq*1 site at 1214 bp (Table 3).

Phylogenetic anlaysis of vp5 gene of BTV-1 Indian isolates with those from around the world has revealed that BTV-1 Australian isolates forms a different cluster, which is closely related with three BTV-1 Indian isolates; whereas BTV-1 South African isolates form a different clade and distantly related to Indian isolates than Australian isolates (Fig. 4). Comparison of these partial vp5 sequences of Indian isolates of BTV-1 with those of isolates of 24 serotypes from all over the world group them with another type 1 eastern isolate from Australia. The sequences of Seg-6 from other serotypes group them as six distinct clades as shown in Fig. 5, which are described as segment 6 nucleotypes (nucleotypes A–F)[20]. Most of these groups correlate with the grouping of other outer capsid protein VP2[21,30].

Discussion

The vp5 gene is 1635 to 1645 bp long and has single open reading frame in one strand capable of coding a protein of 59.163 kDa (526 to 529 amino acids) across different BTV serotypes. The sequence analysis of genome segment encoding VP5 protein from representative isolates of all 24 BTV serotypes revealed significant level of genetic diversity between different BTV serotypes (3-43%) and as well as between isolates within a single serotype[21]. Therefore, the present investigation was carried out to find out the variation in vp5 gene within BTV-1 Indian isolates.

A 1003 bp product spanning a region of vp5 gene from 253-1255 bp from three Indian isolates of BTV-1 was used for *in silico* analysis. Nucleotide sequence alignment has revealed single nucleotide variation at six locations, viz., 454, 516, 617, 857, 986 and 1250 bp among the three isolates. This has shown two base pair changes between BTV-1H and -1A, 4 bp alterations between BTV-1H and -1S3, and 6 bp alterations between BTV-1A and -1S3. Among all BTV-1 Indian isolates from different geographical regions of the country, there was 0-0.6% divergence in vp5 gene.

The *in vitro* restriction digestion of the 1098 bp vp5 gene PCR product of three Indian isolates of BTV-1 with *Bsm*I yielded two fragments, suggesting one *Bsm*I restriction site, where as *Taq*I restriction enzyme yielded three fragments suggesting two *Taq*I restriction sites within this region. The results obtained by *in vitro* digestion were also confirmed with *in silico* restriction analysis. These data suggested that on the basis of restriction enzyme profile analysis (REPA) of vp5 gene, we can differentiate Indian isolates of BTV-1 from other isolates from different parts of the world. Similar studies on *in vitro* restriction digestion of vp2 and vp7 gene from Indian BTV-1 isolates has been used earlier to differentiate them from virus isolates from other parts of the world[31,32]. Hence, the restriction analysis of different genome segments with *Bsm*I or

Table 3—*In silico* restriction endonuclease profile analysis of vp5 gene sequences (253-1255 bp) of different BTV-1 isolates of the world

Acc. No.	*Bsm*I RE sites (bp)	*Taq*I RE sites (bp)
BTV-1H_EU443927	637	639, 938
BTV-1A_EU443928	637	639, 938
BTV-1S3_EU443929	637	639, 938
AJ586659 (IND)	637	639, 938
AJ586660 (IND)	637	639, 938
AJ586661 (IND)	637	639, 938
AJ586662 (IND)	637	639, 938
AJ586663 (IND)	637	639, 938
AJ783902 (IND)	637	639, 938
AJ783903 (IND)	637	639, 938
M21845 (AUS) (1634 bp)	636	404, 638, 937, 1220
AJ631214 (AUS)(1635 bp)	637	405, 639, 938, 1221
AJ586664 (GRE)	637	639, 938
AJ586695 (RSA)	445	828, 858, 1209
M36713 (RSA)	445	828, 859, 1209
AJ586656 (RSA)	No site	858, 938, 1209
AJ586657 (NIG)	445	828, 858, 1209
AJ586658 (SUD)	445	828, 858, 1209
EU422952 (ALG)	445	828, 858, 1209, 1214
EU422953 (MOR)	445	828, 858, 1209

IND= India, AUS=Australia, RSA=South Africa, ALG=Algeria, NIG=Nigeria, Mor=Morocco, SUD=Sudan, GRE=Greece.
The isolates in bold letters were used in the present study for cloning and sequencing.

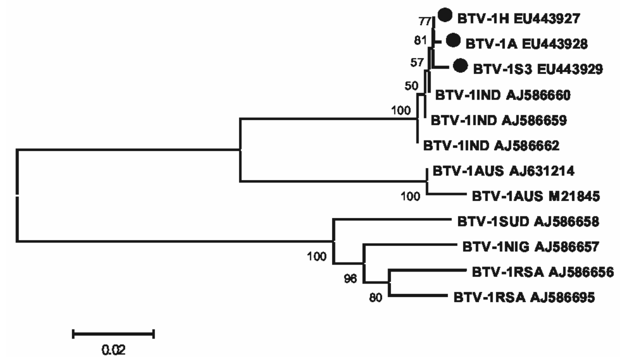

Fig. 4—Phylogenetic tree for BTV-1 isolates based on vp5 gene. The tree was constructed from nucleotide sequences of vp5 gene using the neighbor-joining method in Mega 4 (Tamura *et al*[29] and default parameters). Numbers at the major nodes indicate the bootstrap values. ● = Isolates selected in this study.

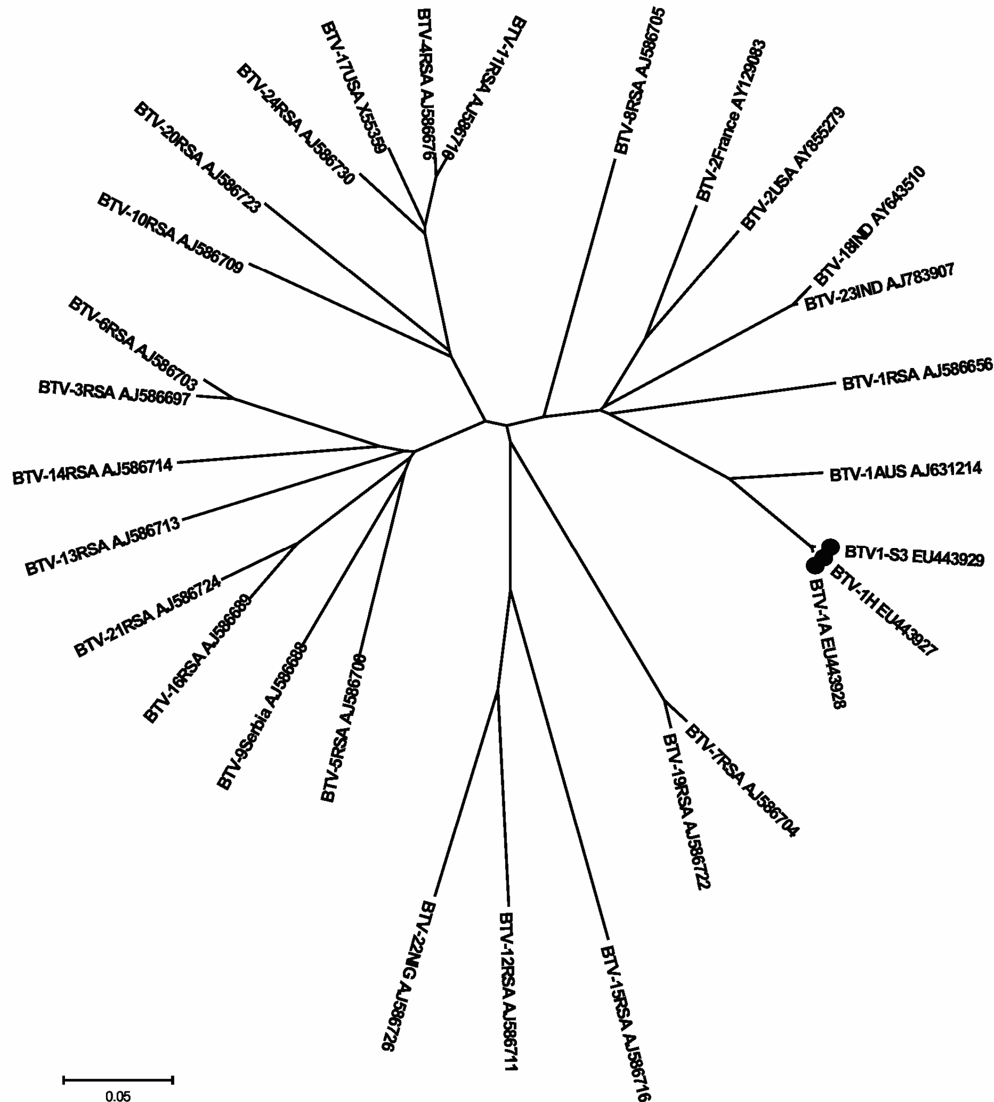

Fig. 5—Phylogenetic tree showing comparison of Indian BTV-1 isolates with those of 24 serotypes from around the world. The tree was constructed using partial nucleotide sequences of vp5 gene using the neighbor-joining method in Mega 4 (Tamura *et al*[29] and default parameters). ● = Isolates selected in this study.

*Taq*I enzymes would be helpful to determine the genetic relationship among BTV isolates in turn their evolutionary origin.

In genome segment 6, the Indian isolates of BTV-1 share 89-91.1% nucleotide sequence similarity with BTV-1 Australian isolates (eastern origin), whereas they only showed 77-79.7% similarity with the BTV-1 African isolates (western origin). This study supported the earlier studies on vp2 and vp7 genes indicating that Indian isolates share more similarity with Australian isolates than the African isolates[31,32]. This suggested that the Indian and the Australian isolates showed more close and recent common ancestry than the African isolates.

The *in silico* restriction analysis of vp5 gene of BTV-1 with *Taq*I revealed that all Indian isolates of BTV-1 have two restriction sites at 639 and 938 bp within the region from 207 to 1304 bp, whereas Australian isolates along with these two similar sites have two extra restriction sites. The South African isolates have three restriction sites at entirely different locations, except Algerian BTV-1 isolate EU422952-ALG (which has an additional site 1214 bp), indicating that there are 6 mutations in the selected sequence. The RE analysis also supported the phylogenetic analysis and confirmed that African BTV-1 isolates are distantly related to Indian isolates of BTV-1.

Comparison of these partial vp5 sequences of Indian isolates of BTV-1 with those of isolates of 24 serotypes from all over the world group them with another type 1 eastern isolate from Australia, which is more closely related to BTV-2. The sequences of Seg-6 from other serotypes group them as six distinct clades, which are described as segment 6 nucleotypes (nucleotypes A-F)[20]. Most of these groups correlate with the grouping based on the sequence of other outer capsid protein VP2[21,30]. In brief, BTV serotypes which are in segment 2 nucleotype 'A' are also grouped with segment 6 nucleotype A.

The region 253-1255 bp of vp5 gene selected for *in silico* analysis corresponding to region 77-410 amino acids of VP5 protein. This region of the protein has two important antigenic determinants. Yang *et al*[33] reported that the linear antigenic determinant site located between amino acid residues 175 and 189 aa was common and conserved. However, in our study at 164 aa position, there was a change of hydrophilic (glutamic acid) to hydrophobic aa (valine) in BTV-1S3. Whereas other two Indian isolates of BTV-1 did not show any change in this domain. Since there is a change from polar to non polar aa in BTV-1 S3 isolate, this mutation appears to have impact on the conformation of the epitope. However, biological significance of the mutation is not known. The other three variations observed in aa sequence of VP5 protein of BTV-1 Indian isolates are located at 198 (from isoleucine to valine) and 409 (alanine to tyrosine) in BTV-1A, and 321 (from lysine to glutamine) in BTV-1S3. Since these variations are located in the region which has not been reported to be associated with any significant biological function, these aa changes may not have significant impact in the virus pathogenicity.

Our study suggests that there is no significant variation in the regions (1003 bp regions) of vp5 gene of all the three Indian isolates used in the investigations. However, based on *in silico* RE analysis of *Taq*I and *Bsm*I, Indian isolates can be distinguished from Australian and African isolates.

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