



Research Article

Vp5 Gene Based Molecular Characterization of Bluetongue Virus 9 from South India

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ABSTRACT

Bluetongue virus (BTV) is a prototype species of the genus *Orbivirus* within the family *Reoviridae* that causes bluetongue disease (BT) in domestic as well as wild ruminants. The BTV isolate Ong5/06/Ind was isolated from sheep in Andhra Pradesh state in 2006. The isolate was confirmed as BTV based on characteristics CPE in BHK21 cell culture, 3:3:3:1 migration pattern of viral nucleic acid in RNA-PAGE and 366bp amplicon with group specific ns1 gene based PCR. VP5 gene based serotype specific primer produced specific PCR amplicon of 823bp corresponding to BTV serotype 9. Nucleotide sequence analysis revealed 97% identity with other Indian and European BTV-9 isolates, more than 90% with Asian and, only 68.8% with South African isolates. However, based on deduced amino acids, the identity was more than 97% with Indian, Asian and European and, only 73.4% with South African BTV-9 isolates. The *in-silico* restriction enzyme analysis (REA) with *AflIII*, *BtrI* and *HindII* showed a common pattern of restriction sites in Indian and European isolates at 1152, 1153 and 591, respectively. The lack of any of these restriction sites in African and other Asian isolates revealed the European origin of the study isolate. Similarly, the lack of *BsmAI* restriction site in Ong5/06/Ind isolate (Accession no. JF969307), differentiate it from European, Asian and South Africa isolates. The sequence analysis revealed that Ong5/06/Ind, Asian and European BTV-9 isolates were placed in nucleotide 'C' while South African isolates were placed in nucleotide 'B'.

Key Words: Bluetongue virus 9, RT-PCR, Vp5 gene, *In silico* RE analysis, Nucleotide.

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INTRODUCTION

Bluetongue (BT) is an insect-borne viral disease of domestic and wild ruminants caused by bluetongue virus (BTV), belonging to genus *Orbivirus* and family *Reoviridae*. BTV is a non enveloped dsRNA virus. The genome of BTV consists of 10 segments dsRNA encoding 7 structural (VP1 to VP7) and 4 non structural proteins (NS1, NS2, NS3/NS3a and NS4) (Ratinier et al., 2011; Belhouchet et al., 2011). The segment 2 (VP2 gene) is major serotype specific along with minor serotype specific segment 6 (VP5 gene). Genetic analysis of various BTV serotypes and strains has revealed the existence of distinct Eastern and Western topotypes associated with specific geographical origin of virus (Maan et al., 2010). In addition, VP2 and VP5 nucleotides have also been proposed based on nucleotide sequence relatedness, and this roughly correlates with closely related serotypes as well as cross-neutralization patterns (Maan et al., 2010). BTV is transmitted by biting midges (*Culicoides* spp.) and can infect all species of ruminant (Maclachlan and Guthrie, 2010) especially small ruminants such as sheep. Clinical signs of BTV infection are more severe and confined to sheep and white-tailed deer (Darpel et al., 2007).

Due to rapid evolutionary changes in genome through reassortment and mutations, BTVs are consistently evolving new serotypes globally. Twenty-four distinct serotypes of the BTV have been reported around the globe (Mertens et al.,

2004). However two more serotypes BTV25 from Switzerland (Hofmann et al., 2008) and BTV26 from Kuwait (Maan et al., 2011) have been isolated recently.

Since India is endemic for BTV, a total of 21 different serotypes have been reported based on virus isolation and serum neutralization test (Prasad et al., 2009). Recently, BTV serotype 21 has been reported from India (Jordar et al., 2009). The complete genome sequence of BTV16 of goat origin from India has been done recently (Minakshi et al., 2012). BTV is spreading in newer areas continuously; seroprevalence of BTV has been reported from Kerala state (Ravishankar et al., 2005). However, most of the studies were done on VP2 gene basis but in present study VP5 gene based serotype confirmation and molecular characterization of one of BTV isolate is done.

MATERIALS AND METHODS

Virus Sample Preparation

The virus was isolated during an outbreak in a sheep flock (of Nellore breed) in Andhra Pradesh state in 2006. The sample was designated as a new isolate Ong5/06/Ind since it was not reported earlier. The virus was propagated in BHK-21 cell line. After appearance of about 75% cytopathetic effect (CPE) at 6th Passage in infected BHK-21 cell line, the virus was harvested. The dsRNA viral nucleic acid was extracted from cell culture grown virus by Trizol® (Sigma, USA) method as per manufacturer's instructions.

RNA Poly Acrylamide Gel Electrophoresis (RNA-PAGE) of Viral Nucleic Acid

The viral nucleic acid was subjected for 8% RNA-PAGE to determine the specific migration pattern of BTV specific nucleic acid. To visualize the nucleic acid segments RNA-PAGE was allowed for silver staining (Svensson et al., 1986) (data not shown).

Oligonucleotide primers

To confirm the new isolate as BTV, a serogroup specific NS1 gene based primer of amplicon size 366 bp was used (Kovi et al., 2005). The primer pair P1 (443–462nt) – 5' CGGGAGGAGGCGGAACAGGA3' and P2 (1246 –1265nt) – 5'CGTGCCAGTGGGCAGCAAGA3' specific to VP5 gene of BTV–9 serotype was designed using sequences available in GenBank with the help of Primer Blast software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The specific amplicon size of this primer pair was 823bp. Specificity of the primer was tested by BLASTN similarity search in GenBank database.

Polymerase Chain Reaction (PCR)

The cDNA was prepared from viral nucleic acid using random primers (decamer) (Ambion, USA) and moloney murine leukemia virus–reverse transcriptase (Mo–MuLV–RT) enzyme (Promega) as per manufacturer's instruction. The cDNA was used for NS1 gene based PCR to confirm the isolate as BTV using standard protocol (Kovi et al., 2005). The cDNA was subsequently used for amplification of VP5 gene using primers specific to all the BTV serotypes including primers P1 and P2. The amplification was carried out in 20 µL reaction mixture containing 2 µL cDNA, 3% DMSO, 20 µM of primers along with 0.4 µL of 10mM dNTPs mix, 4 µL 5X HF buffer and 0.4 U (2 U/µL) phusion high– fidelity DNA polymerase (Finnzymes, Finland) in thermalcycler (Biorad iCycler, USA). The thermal condition for primer P1 and P2 was set as, initial denaturation for 3 min at 98°C, followed by 35 cycles for 10 sec denaturation

at 98°C, 20 sec primer annealing at 57°C and 30sec primer extension at 72°C. Final primer extension was carried out at 72°C for 10 min. The PCR products were analysed by agarose gel electrophoresis using 1% agarose (Sigma, USA).

Nucleotide Sequencing and Data Analysis

The VP5 gene specific PCR amplicon was sequenced using Genetic Analyser ABI PRISM™ 3130 XL. The sequence data obtained was analysed using online software BLASTN+ 2.2.28 (<http://blast.ncbi.nlm.nih.gov/>) (Zhang et al., 2000). The nucleotide sequence was translated to deduced amino acid sequence according to its open reading frame. BioEdit v7.2.3 software was used for calculation of percent nucleotide and amino acid sequence identity of VP5 gene of Ong5/06/Ind isolate (Accession no. JF969307) with BTV9 global isolates and all the 26 BTV serotypes. MEGA5 (Tamura et al., 2011) software was used for nucleotide as well as amino acid based phylogenetic analysis. The *in silico* REA of VP5 gene of virus isolate used in present study along with other global isolates were carried out using Restriction Mapper version 3.0 software available at <http://www.restrictionmapper.org/>.

RESULTS

The Ong5/06/Ind isolate (Accession no. JF969307) produced BTV specific CPE such as detachment, aggregation of the cells, foamy degeneration of the cells etc. in BHK–21 cell culture. The RNA–PAGE analysis showed BTV specific migration pattern (3:3:3:1) of viral dsRNA (data not shown). The Ong5/06/Ind isolate produced an expected amplicon size of 366bp with group specific NS1 gene based PCR (Figure 1). The VP5 gene of Ong5/06/Ind isolate was successfully amplified using primers P1 and P2 (specific to BTV9) as evidenced by expected PCR amplicon size of 823bp in agarose gel electrophoresis (Figure 2).

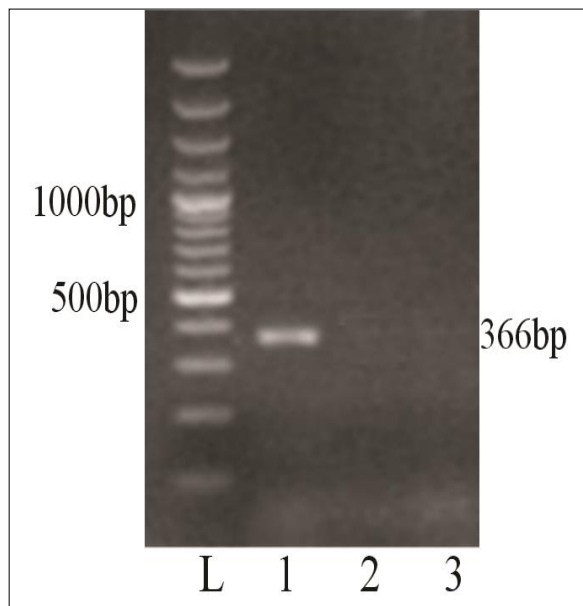


Figure 1: RT–PCR products of Ong5/06/Ind isolate using ns1 gene specific primer yielding 366bp amplicon; Lanes L: Ladder 100bp; 1: Ong5/06/Ind; 2: BHK21–cell; 3: Nuclease free water control.

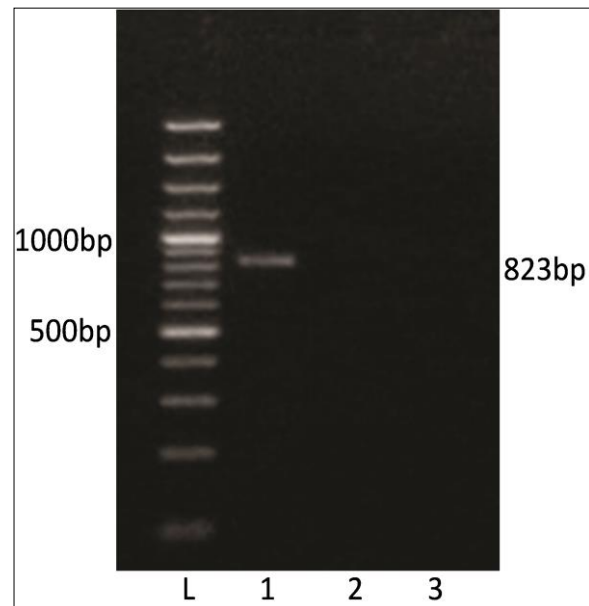


Figure 2 RT–PCR products of Ong5/06/Ind isolate using vp5 gene specific primer yielding 823bp amplicon. Lanes L: Ladder 100bp; 1: Ong5/06/Ind; 2:BHK21–cell; 3: Nuclease frees water control.

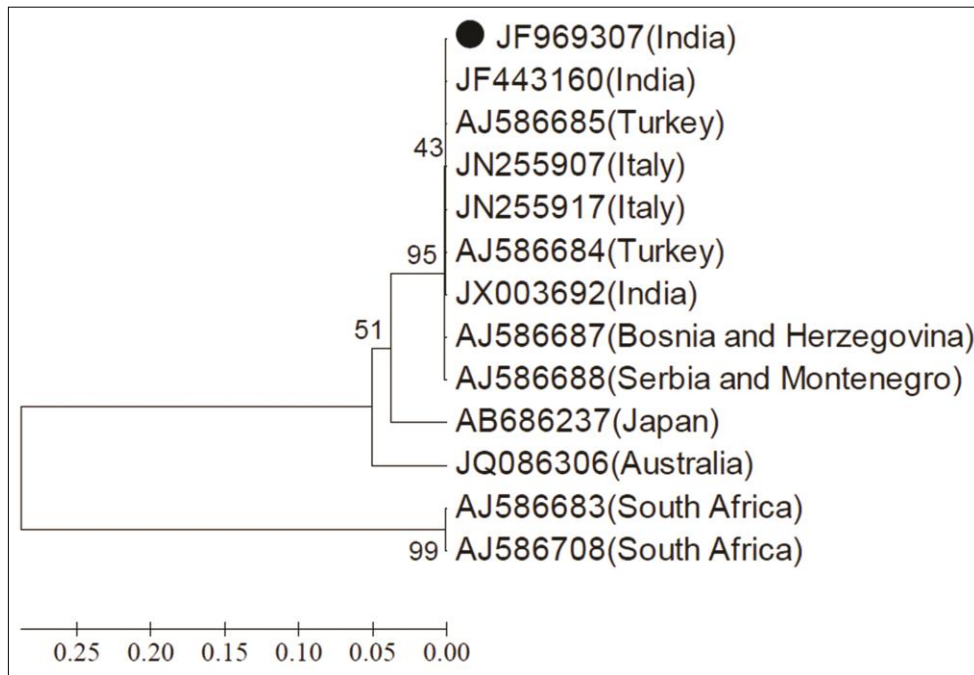
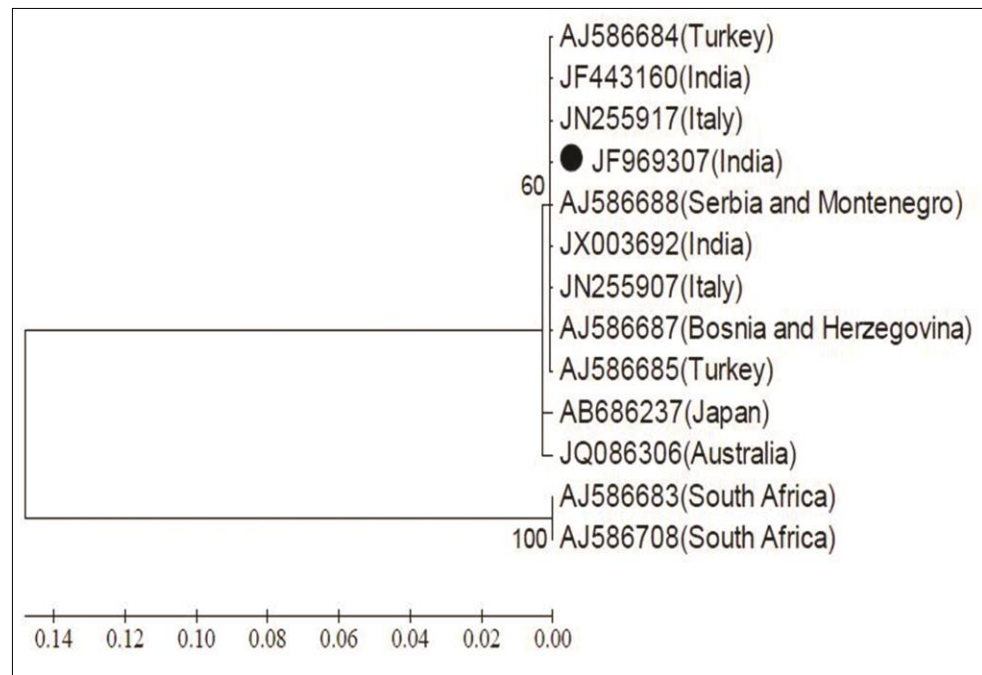


Figure 3: Vp5 gene nucleotide sequence based phylogenetic analysis of Ong5/06/Ind isolate along with other global isolates of BTV9. ●= isolate used in present study

Figure 4: Vp5 gene deduced amino acid sequence based phylogenetic analysis of Ong5/06/Ind isolate along with other global isolates of BTV9. ●= isolate used in present study



Similarly, Ong5/06/Ind isolate was also subjected to VP5 gene specific primers of remaining BTV serotypes. However, it did not give any amplification with VP5 gene specific primers of remaining serotypes (data known shown). The VP5 gene PCR amplicon was sequenced and sequence data was deposited to GenBank with accession no. JF969307. Ong5/06/Ind isolate showed maximum nucleotide (>97%) as well as deduced amino acid (>98%) sequence identity with European and other Indian isolates (Table 1). The phylogenetic analysis of Ong5/06/Ind isolate (Accession no. JF969307) along with representative BTV sequences from different parts of the world were done using

Mega5 software (Tamura et al., 2011). The nucleotide as well as deduced amino acid sequence based phylogenetic analysis of partial VP5 gene revealed that Ong5/06/Ind isolate (Accession no. JF969307) form a close cluster with European and other Indian isolates of BTV9 (Figure 3 and 4). The phylogenetic analysis of VP5 gene of all the 26 BTV serotypes revealed that Ong5/06/Ind isolate along with other Indian and European BTV9 isolates was placed in nucleotide 'B' whereas South African BTV9 isolates were placed in nucleotide 'C' (Figure 5)

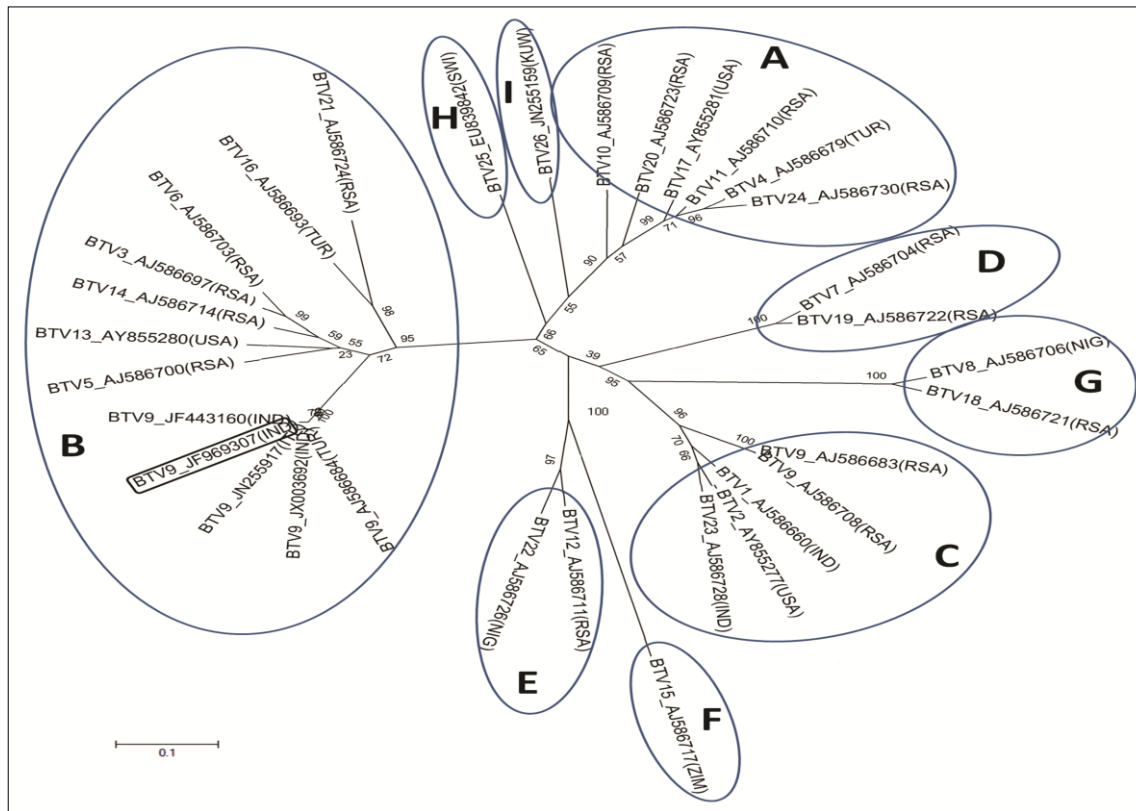


Figure 5: vp5 gene based nucleotide study of all the 26 BTV serotype from different parts of the world. The isolate written under rectangle is used in present study

Table 1: Percent identity of BTV9 isolates based on vp5 gene nucleotide and amino acid sequence

Sr. No.	BTV9 Isolates	Percent identity of JF969307 (India) isolate based on	
		Nucleotide	Amino Acid
1	JF969307(India)	100	100
2	JF443160(India)	99.2	97.8
3	AJ586684(Turkey)	97.8	98.5
4	JN25907(Italy)	97.8	98.5
5	JN255917(Italy)	97.8	98.5
6	AJ586685(Turkey)	97.6	98.5
7	JX003692(India)	97.6	98.5
8	AJ586687(Bosnia and Herzegovina)	97.5	98.5
9	AJ586688(Serbia and Montenegro)	97.5	98.5
10	AB686237(Japan)	92.1	98.1
11	JQ086306(Australia)	90.2	98.5
12	AJ586683(South Africa)	68.8	73.4
13	AJ586708(South Africa)	68.8	73.4

Sr. No.	BTV 9 isolates	BsmAI GTCTC	BtrI CACGTC	HindII GTYRAC	AflIII ACRYGT
1	JF969307(India)	-	1153	591	1152
2	JX003692(India)	-	1153	591	1152
3	JF443160(India)	-	1153	591	1152
4	JN25907(Italy)	575	1153	591	1152
5	JN255917(Italy)	575	1153	591	1152
6	AJ586684(Turkey)	575	1153	591	1152
7	AJ586685(Turkey)	575	1153	591	1152
8	AJ586687(Bosnia and Herzegovina)	575	1153	591	1152
9	AJ586688(Serbia and Montenegro)	575	1153	591	1152
10	AJ586683(South Africa)	581	-	-	-
11	AJ586708(South Africa)	581	-	-	-
12	JQ086306(Australia)	557	-	-	-
13	AB686237(Japan)	557	-	-	-

Table 2: *In silico* restriction enzyme analysis of BTV9 vp5 gene



Table 3: Percent nucleotide and amino acid identity of vp5 gene of Ong5/06/Ind isolate (Acc no. JF969307) with all the 26 BTV serotypes

Sr. No.	BTV26 serotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	BTV1 AJ586660(IND)	ID	92.3	74.1	74.1	75.1	73.6	72.2	84.2	73.2	66.0	71.7	72.7	72.7	88.4	88.4	73.6	74.1	51.8	75.1	71.7	51.8	74.1	73.2	84.2	72.2	73.6	74.1	52.8	87.5	73.6	63.1	70.3
2	BTV2 AY855277(USA)	77.8	ID	73.2	73.6	75.1	74.1	73.2	84.6	73.2	66.0	71.7	72.7	72.7	89.4	89.4	74.6	74.1	52.3	74.6	72.7	51.8	74.1	73.2	85.1	73.2	73.2	74.1	52.8	89.4	73.6	63.6	71.2
3	BTV3 AJ586697(RSA)	67.5	66.7	ID	76.4	92.7	99.0	70.6	75.0	91.8	86.6	91.3	92.3	92.3	73.2	73.2	77.2	76.4	54.6	91.3	93.7	50.9	90.3	75.9	74.5	70.6	78.4	90.8	54.2	74.1	76.4	69.3	70.8
4	BTV4 AJ586679(TUR)	68.6	69.8	68.7	ID	78.3	75.9	70.6	75.0	75.9	68.2	75.0	75.0	75.0	71.7	71.7	92.7	99	49.5	76.4	75.0	49.0	78.3	97.5	75.0	70.6	89.4	78.3	49.5	71.2	98.5	75.1	75.5
5	BTV5 AJ586700(RSA)	67.6	66.8	79.4	70.1	ID	92.7	70.1	74.5	93.2	86.0	91.8	92.3	92.3	73.6	73.6	75.9	78.3	52.8	89.9	92.7	50.0	91.3	77.4	75.0	70.1	77.9	91.8	52.3	73.2	77.4	71.2	70.3
6	BTV6 AJ586703(RSA)	68.2	66.5	96.2	68.6	78.6	ID	70.6	75.4	91.8	86.6	91.3	92.3	92.3	73.6	73.6	76.8	75.9	54.2	91.3	93.7	50.4	90.3	75.4	75.0	70.6	77.9	90.8	53.7	74.6	75.9	69.8	71.2
7	BTV7 AJ586704(RSA)	68.2	68.1	65.7	67.3	66.5	66.0	ID	73.0	70.1	63.4	69.2	69.7	69.7	72.2	72.2	71.1	71.1	52.8	71.6	69.2	52.1	70.6	71.1	72.5	100	71.7	70.1	53.7	71.2	70.6	63.1	68.7
8	BTV8 AJ586706(NIG)	72.3	72.3	68.4	69.2	70.0	69.0	67.6	ID	75.3	68.1	73.9	75.3	75.3	83.7	83.7	75.8	74.5	52.8	75.4	72.1	51.4	74.3	74.5	99.5	73.0	74.6	74.3	52.3	83.7	74.0	66.9	71.7
9	BTV9 JF969307(IND)	67.1	67.3	80.2	69.5	78.3	78.6	64.5	68.9	ID	98.5	97.8	98.5	98.5	73.4	73.4	75.8	75.9	53.2	91.3	91.8	50.9	88.8	76.4	75.3	70.1	77.0	89.3	53.2	73.6	75.0	70.8	69.3
10	BTV9 JX003692(IND)	66.5	66.7	79.4	68.7	77.3	78.0	63.5	67.9	97.6	ID	88.8	90.7	90.7	65.0	65.0	68.5	68.2	49.0	86.0	83.6	46.7	86.6	68.7	68.1	63.4	69.3	86.6	48.1	66.5	67.7	63.9	62.6
11	BTV9 JF443160(IND)	66.8	67.0	79.7	69.2	77.8	78.1	64.2	68.6	99.2	97.3	ID	96.6	96.6	70.8	70.8	74.8	75.0	52.8	89.3	91.3	50.0	87.9	75.4	73.9	69.2	76.0	88.4	52.3	72.2	74.0	69.3	68.4
12	BTV9 JN255917(ITL)	67.5	67.6	80.0	69.5	78.3	78.8	64.2	68.7	97.8	97.9	97.4	ID	100	72.2	72.2	75.8	75.0	52.8	91.3	91.8	50.4	88.8	75.4	75.3	69.7	76.0	89.3	52.8	73.6	75.0	70.3	69.3
13	BTV9 AJ586684(TUR)	67.5	67.6	80.0	69.5	78.3	78.8	64.2	68.7	97.8	97.9	97.4	100	ID	72.2	72.2	75.8	75.0	52.8	91.3	91.8	50.4	88.8	75.4	75.3	69.7	76.0	89.3	52.8	73.6	75.0	70.3	69.3
14	BTV9 AJ586683(RSA)	77.3	76.2	66.8	67.9	68.6	68.2	68.2	74.0	68.8	67.8	68.6	68.7	68.7	ID	100	71.7	71.7	51.8	74.1	70.3	51.4	71.2	70.8	83.7	72.2	72.2	71.7	52.3	87.5	71.2	64.5	69.8
15	BTV9 AJ586708(RSA)	77.5	76.8	67.0	67.8	68.6	68.4	68.1	73.9	68.8	67.8	68.6	68.7	68.7	99.8	ID	71.7	71.7	51.8	74.1	70.3	51.4	71.2	70.8	83.7	72.2	72.2	71.7	52.3	87.5	71.2	64.5	69.8
16	BTV10 AJ586709(RSA)	67.1	66.0	72.5	78.8	69.8	72.0	66.8	70.0	70.8	70.0	70.3	70.4	70.4	67.1	67.1	ID	93.7	51.4	78.3	75.0	49.0	78.2	92.2	75.8	71.1	87.0	78.2	50.0	72.2	93.2	73.2	74.1
17	BTV11 AJ586710(RSA)	69.0	68.2	68.6	91.2	70.0	67.8	67.5	67.3	70.4	69.3	70.1	70.1	70.1	67.5	67.6	79.4	ID	49.5	76.4	75.0	49.0	78.3	97.5	74.5	71.1	88.9	78.3	49.5	71.2	98.5	74.6	75.5
18	BTV12 AJ586711(RSA)	56.9	56.7	56.7	57.6	58.1	57.5	57.8	55.2	57.6	56.4	57.3	56.7	56.7	57.3	57.2	56.2	56.4	ID	53.2	53.2	68.6	52.3	49.5	52.3	52.8	51.4	52.3	91.9	54.2	49.0	51.4	48.1
19	BTV13 AY855280(USA)	66.8	68.2	78.9	70.6	79.4	78.8	66.4	68.6	76.2	76.5	76.0	76.1	76.2	67.9	67.8	69.5	70.4	56.7	ID	89.4	51.4	87.0	75.9	75.4	71.6	78.4	87.5	53.7	74.1	76.4	69.8	71.2
20	BTV14 AJ586714(RSA)	65.3	66.4	81.1	69.3	79.2	79.5	64.3	66.2	76.7	76.0	76.2	76.6	76.6	67.0	66.8	70.4	68.4	56.6	78.8	ID	50.0	89.0	75.0	73.0	69.0	76.0	90.0	53.0	73.0	75.0	67.0	69.0
21	BTV15 AJ586717(ZIM)	56.1	55.5	55.3	57.7	56.6	54.7	56.7	58.5	56.4	55.3	56.1	55.8	55.8	56.4	56.4	55.2	57.8	65.9	55.6	54.4	ID	50.4	49.0	50.9	52.1	49.0	50.4	69.1	52.3	49.5	50.0	51.1
22	BTV16 AJ586693(TUR)	67.6	67.5	77.8	72.0	79.5	77.5	66.2	70.4	78.6	78.1	78.1	79.2	79.2	66.2	66.0	72.3	71.8	56.2	78.0	76.1	58.6	ID	76.9	74.8	70.6	77.9	97.5	51.8	73.6	78.3	69.8	70.8
23	BTV17 AY855281(USA)	66.8	68.1	70.0	89.7	70.1	68.7	67.5	69.2	70.9	70.1	70.6	71.2	71.2	67.3	67.5	77.8	89.4	55.8	67.6	68.7	56.7	70.4	ID	75.0	71.0	89.0	77.0	50.0	70.0	97.0	74.0	74.0
24	BTV18 AJ586721(RSA)	73.6	72.9	68.7	69.7	69.7	69.8	67.6	96.0	68.4	67.6	68.1	68.6	68.6	74.7	74.8	69.3	67.8	53.8	69.0	65.9	57.4	69.7	70.4	ID	73.0	75.0	75.0	52.0	84.0	74.0	67.0	72.0
25	BTV19 AJ586722(RSA)	69.0	68.6	65.3	67.5	66.4	65.3	97.3	67.8	65.3	64.3	64.9	64.9	64.9	68.4	68.2	66.8	68.2	58.3	66.0	64.2	57.4	66.5	67.6	67.1	ID	72.0	70.0	54.0	71.0	71.0	63.0	69.0
26	BTV20 AJ586723(RSA)	68.2	68.9	69.3	80.6	68.9	70.1	67.5	67.3	67.6	66.8	67.3	67.8	67.8	68.7	68.6	77.2	79.5	56.1	71.4	69.8	55.0	71.4	78.0	66.7	67.1	ID	78.0	51.0	71.0	88.0	75.0	75.0
27	BTV21 AJ586724(RSA)	67.6	67.6	75.8	73.1	77.5	76.1	67.0	68.2	76.4	76.0	76.1	77.2	77.2	68.4	68.2	70.6	74.2	57.5	76.4	76.4	58.6	89.3	72.5	69.0	66.8	72.6	ID	51.8	73.6	78.3	69.3	70.8
28	BTV22 AJ586726(NIG)	55.9	53.8	58.3	54.7	59.0	58.0	55.3	55.0	58.4	57.0	58.1	57.6	57.6	55.6	55.5	57.5	55.2	78.0	57.8	57.8	63.4	58.7	56.4	54.8	55.3	56.1	59.2	ID	55.0	49.0	52.0	49.0
29	BTV23 AJ586728(IND)	76.1	79.1	66.0	67.9	66.4	66.7	67.0	72.9	69.5	68.4	69.0	69.3	69.3	77.0	76.9	68.1	67.8	56.2	68.9	66.2	56.3	67.5	67.5	73.1	67.3	66.7	67.8	55.0	ID	71.0	66.0	71.0
30	BTV24 AJ586730(RSA)	68.7	70.9	69.2	94.9	70.8	69.7	67.6	68.1	68.2	67.6	67.9	68.6	68.6	68.7	68.6	78.9	90.8	57.5	70.1	70.0	56.3	72.2	88.5	69.3	67.9	80.6	73.7	56.4	68.7	ID	75.1	76.5
31	BTV25 EU839842(SWI)	63.1	62.6	66.4	70.2	67.8	65.9	65.0	62.0	67.5	66.6	67.2	67.2	67.2	64.1	63.9	69.1	71.1	55.0	67.5	64.8	56.0	68.3	70.8	62.5	65.2	71.0	66.7	55.2	63.1	69.7	ID	69.3
32	BTV26 JN255159(KUW)	66.0	66.8	64.0	68.2	66.2	64.0	61.6	66.7	64.9	64.0	64.5	64.8	64.8	67.0	67.1	68.9	66.7	54.7	65.6	64.9	54.9	67.3	67.3	66.8	62.6	68.9	65.9	54.2	66.5	69.2	65.6	ID

Percent amino acid identity

Percent nucleotide identity

The *in silico* REA of VP5 gene with enzymes *BtrI*, *HindIII* and *AflIII* revealed that Ong5/06/Ind isolate share a common pattern of restriction sites with that of European and other Indian isolates (Table 2). However, restriction enzyme *BsmAI* showed genetic diversity of Indian isolates with other global isolates of BTV9. The VP5 gene based nucleotide and deduced amino acid sequence identity of all the 26 BTV serotypes was calculated using Bioedit v7.2.3 software programme. It was observed that Ong5/06/Ind isolate along with other Indian and European isolates had shown maximum identity with nucleotype 'B' serotypes and south African BTV9 isolate had shown maximum identity with Nucleotype 'C' serotypes (Table 3).

DISCUSSION

BT is an endemic disease in India for domestic and wild ruminants. A large number of BTV serotypes have been reported from India and BTV9 is one of the common serotype prevalent in country. BT is reported from various states of India such as Himachal Pradesh, Uttar Pradesh, Gujarat, Maharashtra and Andhra Pradesh (Prasad et al., 2009). VP5 gene based molecular characterization and phylogenetic relationship of BTV1 of Indian isolates (Manjunath et al., 2010) and reference strains of all 24 serotypes (Singh et al., 2004) have been reported earlier. The prevalence of BTV-9 was reported earlier from southern part of India (Rao et al., 2012). The present investigation was carried out for VP5 gene based serotype confirmation of an Indian BTV isolate and its comparison with other BTV isolates from India and global origin.

The BTV specific characteristics CPE in BHK-21 cell culture, migration pattern in 8% RNA-PAGE and an expected amplicon size with group specific *nsI* gene based RT-PCR confirmed the samples as BTV. The VP5 gene nucleotide based phylogenetic analysis using neighbour joining (NJ) method of MEGA5 software revealed that Ong5/06/Ind isolate formed a closer cluster with European and other Indian isolates. However it formed a minor cluster with Japan and Australia isolates. South Africa isolates were related distantly to it. The similar result was observed with deduced amino acid based phylogenetic analysis.

The REA of full or partial length gene is useful in differentiating between serotypes and within isolates of the same serotype. The *in-silico* REA was done with *BtrI*, *AflIII*, *HindIII* and *BsmAI* restriction enzymes. The *in-silico* REA revealed the single restriction site for restriction enzymes *BtrI*, *AflIII* and *HindIII* at nucleotide 1153, 1152 and 591 respectively in all the Indian and European isolates including isolates used in present study. However, these enzymes did not show any restriction sites in Japan, Australia and South Africa isolates. Similarly, REA with *BsmAI* revealed single restriction site at nucleotide 575 in all the European isolates. The same *BsmAI* restriction site was present in South Africa at 581 and in Australia and Japan isolates at nucleotide 557. The restriction enzymes *BtrI*, *AflIII* and *HindIII* showed a common restriction pattern in Indian and European BTV9 isolates. It indicated the European origin of our Indian isolates. However, *BsmAI* can be used for differentiation of Indian BTV9 isolates with European, South Africa, Japan and Australia isolates.

The phylogenetic analysis and *in-silico* REA indicated that Ong5/06/Ind isolate was of European origin. The analyses of partial vp5 gene sequence revealed that there is no significant divergence among Indian BTV9 isolates. They show only 0–0.4% nucleotide and 0–0.7% deduced amino acid based diversity. It indicates the common origin of these viruses. The Segment-6 sequences of the all the 26 different BTV serotypes cluster as 8 distinct evolutionary lineages, called as segment 6

nucleotypes (nucleotypes A–I) (Maan et al., 2011). The nucleotype 'B' includes serotype 3, 5, 6, 13, 14, 16 and 21 whereas nucleotype 'C' includes serotype 1, 2, 9 and 23 (Maan et al., 2010). The grouping of the different BTV serotypes within these 9 nucleotypes also reflects the serological relationships between them i.e. low-level cross-reactions between different BTV serotypes. The identity percentage for nucleotides and amino acids within a nucleotype was reported as 76–100% and 86–100% for nucleotides and amino acids respectively. However, the identity percentage for nucleotides and amino acids between different nucleotypes and serotypes was reported as 56.7–76% and 54.2–85.9% respectively (Maan et al., 2010).

The Ong5/06/Ind isolate along with other Indian and European BTV9 isolates showed maximum 76–80.2% nucleotide and 88–93.2% amino acids, identity with nucleotype 'B' serotypes (serotype 3, 5, 6, 13, 14, 16 and 21). This placed our Ong5/06/Ind isolate along with other Indian and European BTV9 isolates in nucleotype 'B' (Fig. 5). However, the BTV9 isolates from South Africa had shown maximum 76.2–77.5% nucleotide and 87.5–89.4% amino acid identity with nucleotype 'C' serotypes (serotypes 1, 2 and 23). This placed South African BTV9 in nucleotype 'C' as reported earlier (Maan et al., 2010). However, all the Indian and European BTV9 isolates had shown 66.5–69.3% nucleotide and 66.0–73.6% amino acid, identity with nucleotype 'C' serotypes. Similarly, South African BTV9 isolates had shown 66–68.6% nucleotide and 70.3–74.1% amino acid identity with the nucleotype 'B' serotypes (Table 3). The above discussion clearly indicated the existence of at least two segment-6 nucleotype in BTV9 globally.

CONCLUSION

A new BT virus isolated from Andhra Pradesh state had been confirmed as BTV serotype 9 based on vp5 gene specific PCR and nucleotide sequencing technique. Our vp5 gene based molecular analysis confirmed the virus isolate is of European origin. The segment-6 analysis of all the 26 serotypes revealed presence of at least two nucleotype in BTV9. The vp5 gene based phylogenetic analysis and REA tools could be effectively used for establishing phylogenetic relationship and differentiation of Indian isolates from rest of the global BTV9 isolates. The vp5 gene specific primer tested in this study can be used for identifying the BTV9 serotype prevalent in endemic region, and suitable vaccine candidate can be identified for controlling the disease.

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CONFLICT OF INTEREST

We declare no conflict of interest.

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