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Original Article

Phylogenetic Analysis of BTV1 from Northern and Southern States of India

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Abstract

Bluetongue (BT) is an economically important viral disease for sheep and goat causing high morbidity and mortality. In the present study, eight Bluetongue virus (BTV) isolates of goat origin from Utter Pradesh (UP) and four BTV isolates of sheep origin from Andhra Pradesh (AP) adapted in BHK-21 cell line were used. After appearance of 75% of cytopathic effect in BHK-21 cells the virus along with cells were pelleted down and nucleic acid (dsRNA) was extracted using Tri Reagent. All the isolates were confirmed as BTV based on characteristic cytopathic effect in BHK-21 cell culture, RNA-PAGE study (3:3:3:1 pattern) and 366bp amplicon size with group specific ns1 gene based RT-PCR. All the isolates were confirmed as BTV 1 based on segment 2 based serotype specific RT-PCR showing specific amplicon of 605bp size. The nucleotide sequencing of vp2 gene followed by BLAST search also confirmed the isolates as BTV1. The phylogenetic study revealed the eastern topotype origin of these isolates. The phylogenetic analysis also revealed that BTV1 isolates from north India form more closely related sub cluster with other Indian isolates of BTV1. However, BTV1 from south India form separate sub cluster with BTV1 from Greece with in same eastern cluster. The presence of a common *culicoides* vector species *C. oxystoma* has been reported in all these states and could be the possible cause for spread of virus in these parts of the India.

Keywords: Bluetongue virus serotptype-1, cytopathic effect, Phylogenetic analysis, vp2 gene

Introduction

Bluetongue (BT) is an economically important viral disease of domestic and wild ruminants. BT is non-contagious, infectious and insect borne (*Culicoides*) disease (Maclachlan, 1994). The BT disease is caused by Bluetongue virus (BTV) which belongs to genus *Orbivirus* and family *Reoviridae*. BT disease has high economic impact on livestock industry. It leads to high morbidity, mortality, abortion, foetal abnormality, still birth, weight loss, reduced milk and meat yield, wool break etc. in affected animals. It causes severe clinical signs such as lameness, fever, swelling and cyanosis of lips and tongue. The more severe forms of the disease are primarily seen in sheep and in white-tailed deer (Howerth et al., 1988; Darpel et al., 2007). However, Buffalo, cattle and goats act as silent reservoirs and remain viraemic for several months (Maclachlan et al., 2009). The disease has high potency to spread among large and small ruminants, which may cause socioeconomic problems in view of mandatory trade barrier on movement of animals, their embryos, germplasm and other animal products from BT endemic countries to BT free countries. BT affects

many ruminant animals. Therefore it is listed as a multiple species diseases by Office International des Epizooties (OIE, 2013).

There are twenty-four distinct serotypes (BTV1 to BTV24) of BTV have been identified worldwide (Mertens et al., 2004). However, due to rapid reassortment and mutations in genome, BTVs are consistently evolving new serotypes globally. Recently two more serotypes i.e., BTV25 from Switzerland (Hofmann et al., 2008) and BTV26 from Kuwait have been isolated (Maan et al., 2011).

In India, 21 different BTV serotypes have been reported from different states of the country based upon serology and virus isolation (Prasad et al., 2009). Recently, the 22nd BTV serotype i.e. BTV21 have been reported from Andhra Pradesh state (Susmitha et al., 2012). Similarly, the BTV12 has been isolated from Andhra Pradesh state of India (Rao et al., 2013). The nucleotide sequence analysis revealed that most of the BT viruses can be classified into two major groups i.e., 'eastern' or 'western' topotypes, and also into a number of geographic subgroups based on its geographical distribution (Balasuriya et al., 2008).

BTV is an icosahedral virus. The genome of virus consists of ten-segmented, double stranded RNA (dsRNA) molecule. Each of the ten segments codes for at least one viral protein. Seven proteins (VP1 to VP7) are structural and form virus particle. The virus also encodes four non-structural proteins NS1, NS2, NS3 and NS3a which are expressed in virus infected host cells (Mertens et al., 1989). However, recently segment 9 encoded another non-structural protein (i.e., NS4) has been reported (Ratinier et al., 2011; Belhouchet et al., 2011). The inner capsid of BTV is composed of five polypeptides: three minor proteins (VP1, VP4, and VP6) and two major proteins (VP3 and VP7) (Roy, 1989). The outer capsid is composed of serotype specific two viral proteins, VP2 and VP5 (Ghiasi et al., 1987).

The vp2 gene sequences are serotype specific and can be used for determination of respective serotypes. The vp2 gene based serotype–specific RT–PCR is a rapid assay that has shown perfect agreement with the serotyping by conventional virus neutralization methods (Mertens et al., 2007). The BTV1 is one of the most prevalent serotype in India. Recently, an outbreak of BTV1 was reported in a flock of goats in Mathura district of Uttar Pradesh (Biswas et al., 2010). The present study was carried out for vp2 gene based serotype confirmation and determining the phylogenetic relationship of Indian isolate of BTV1 with global isolates.

Materials and methods

Viral sample origin

A total of twelve BTV isolates collected in 2008 from various geographical regions of India were obtained under All India Network Programme on Bluetongue. The virus samples were propagated in BHK-21 cell line in our lab by passaging in BHK21 cell line. Out of these twelve, four samples were of sheep origin collected from Andhra Pradesh (NRT35/IND, NRT39/IND, CHT1/IND and BT1/IND) and remaining eight samples (MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND and MKD25/IND) were of goat origin from Mathura district, Uttar Pradesh.

Isolation of viral nucleic acid

The virus samples produced BTV specific cytopathic effect (CPE) in infected BHK-21 cell culture within 48 hours. After appearance of 75% CPE, the BHK-21 cell culture along with virus was harvested and pelleted down at 5,000x g for 5 minutes in table top refrigerated centrifuge (REMI, India). Supernatant was decanted carefully and BTV dsRNA was isolated from cell pellet using Tri Reagent method (Sigma, USA) (Chomoczynski and Sacchi, 1987). Using 4M Lithium Chloride and 7.5 M Ammonium acetate, single stranded RNA was selectively precipitated and removed. The dsRNA pellet was washed with prechilled 70% ethanol, air dried and dissolved in nuclease free water.

RNA Poly acrylamide Gel Electrophoresis (RNA–PAGE) of viral dsRNA

The viral dsRNA was subjected to RNA-PAGE using 8% poly acrylamide gel electrophoresis. The RNA–PAGE was allowed for silver staining to visualize the BTV specific nucleic acid segment (Svensson et al., 1986) (data not shown).

Preparation of cDNA and PCR

Viral genomic dsRNA of all the BTV isolates were subjected to cDNA synthesis by Reverse Transcription using Mo-MuLV reverse transcriptase enzyme (Promega, USA) and random decamer (Ambion, USA) in thermal cycler (Biorad i Cycler) as per manufacturer's instruction. The cDNA from all the twelve isolates were subjected to group specific (ns1 gene specific) PCR for confirmation of samples as BTV (Kovi et al., 2005). To confirm the serotype of

virus isolates the cDNA were further subjected to serotype specific PCR using vp2 genes specific primers of all the BTV serotypes.

Cloning of vp2 gene PCR product

The vp2 gene PCR products were purified using QIA quick gel extraction kit (Qiagen, USA) to remove primer dimmers and other PCR ingredients. The purified PCR products were cloned using Pjet 1.2 cloning vector and JM107 cell as host system (Fermentas, USA) as per the manufacturer's instruction. The positive clones were selected by colony touch PCR using vp2 gene specific primer pair. For nucleotide sequencing, the plasmids from positive clones were extracted using Quiaprep kit (Quiagen, USA) as per the manufacturer's instruction.

Nucleic acid sequencing and sequence data analysis

The plasmids from positive clones of all the twelve isolates were allowed to nucleic acid sequencing using vector specific primer by Genetic Analyser ABI PRISM TM 3130 XL machine in our laboratory. The vector sequence from nucleotide sequence obtained was trimmed using online available Vecscreen software (http: //www. ncbi. nlm.nih. gov /tools / vecscreen/). The nucleotide sequences of vp2 gene of all the isolates were subjected to BLASTN+ 2.2.30 (Zhang et al., 2000) analysis for serotype confirmation. The percent nucleotide as well as its deduced amino acid sequence identity matrix with global isolates was calculated using BioEdit 7.2.5 (Hall, 1999). The phylogenetic analysis of vp2 gene nucleotide as well as its deduced amino acid sequence in study along with other global sequences of same serotype was done using MEGA6 software (Tamura et al., 2013).

Result and Discussion

India is endemic for BTV infection. A large number of BTV serotypes were reported from different states of India. Conventionally, diagnosis of BT includes serological tests, virus isolation and serotype identification by virus neutralization, polymerase chain reaction (PCR), plaque inhibition and fluorescence inhibition tests. The vp2 gene sequences are serotype specific and can be used for determination of respective serotypes.

In present study 12 BTV samples from Uttar Pradesh and Andhra Pradesh states were used. These viruses were grown in BHK-21 cell line. All the viruses showed the characteristic cytopathic effect of BTV such as rounding off and increase in number of floating cells in medium followed by formation of empty areas in BHK-21 monolayers (Sekar et al., 2009). The viral dsRNA was prepared from cell culture pelleted material.

In RNA–PAGE analysis, all the 12 isolates showed the characteristics 10 segmented migration pattern (3:3:3:1) of dsRNA which is specific to BTV (data not

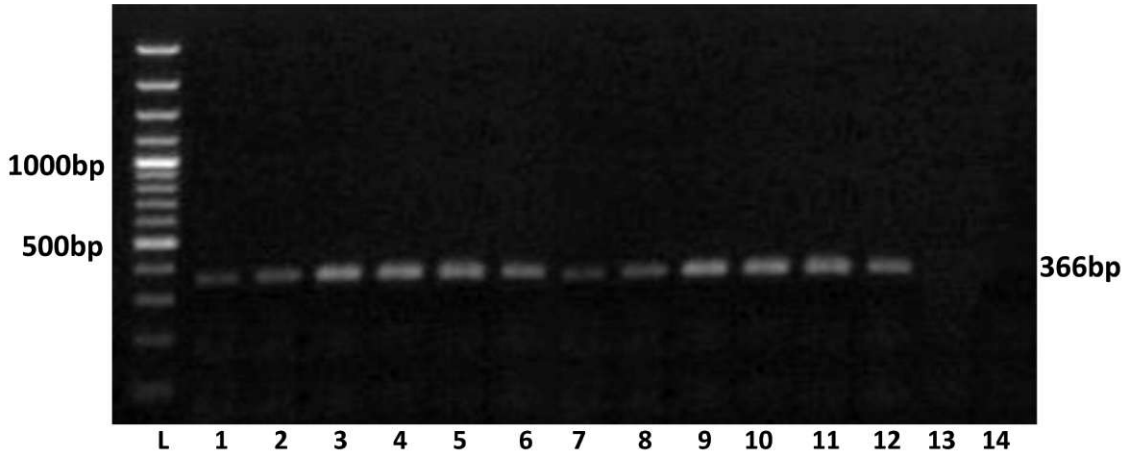

Fig. 1: Ns1 gene specific RT-PCR of twelve BTV isolates showing characteristic 366bp amplicon size in 1% agarose gel electrophoresis. Lanes: L: 100bp DNA ladder, 1: MKD18/IND, 2: MKD19/IND, 3: MKD20/IND, 4: MKD21/IND, 5: MKD22/IND, 6: MKD23/IND, 7: MKD24/IND, 8: MKD25/IND, 9:NRT35/IND, 10: NRT39/IND, 11:CHT1/IND, 12: BT1/IND, 13: BHK-21 cell negative control and 14: Nuclease free water negative control.

Table 1: BTV1 nucleotide and amino acid identity

Percent amino acid identity

BTV1 isolates	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
1 India.MKD18/IND.JQ037800	ID	100	99.5	100	99.5	100	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
2 India.MKD19/IND.JQ037801	100	ID	99.5	100	99.5	100	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
3 India.MKD20/IND.JQ037802	99.8	99.8	ID	99.5	99	99.5	97.5	97	97.5	98.5	98.5	98	99.5	92	88	89	99.5	80.5	80.5	81	98.5	81	94.5	94.5	94.5	94	94.5
4 India.MKD21/IND.JQ037803	100	100	99.8	ID	99.5	100	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
5 India.MKD22/IND.JQ037804	99.6	99.6	99.5	99.6	ID	99.5	98	98	97.5	98.5	98.5	98	99.5	92	88	89	99.5	80.5	80.5	81	98.5	81	94.5	94.5	94.5	94	94.5
6 India.MKD23/IND.JQ037805	100	100	99.8	100	99.6	ID	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
7 India.MKD24/IND.JQ037806	99.1	99.1	99	99.1	99	99.1	ID	99	96	97	97	96.5	98	90.5	86.5	87.5	98	79.1	79.1	79.6	97	79.6	93	93	93	92.5	93
8 India.MKD25/IND.JQ037807	98.8	98.8	98.6	98.8	98.8	98.8	99.5	ID	95.5	96.5	96.5	96	97.5	90	86.5	87.5	97.5	78.6	78.6	79.1	96.5	79.1	92.5	92.5	92.5	92	92.5
9 India.BT1/IND.JQ037811	98.6	98.6	98.5	98.6	98.3	98.6	97.8	97.5	ID	97	97	96.5	98	90.5	86.5	87.5	98	81	81	81.5	97	81.5	93	93	93	92.5	93
10 India.CHT1/IND.JQ037812	98.8	98.8	98.6	98.8	98.5	98.8	98	97.6	97.5	ID	99	97.5	99	91.5	87.5	88.5	99	80.5	80.5	81	98	81	94	94	94	93.5	94
11 India.NRT35/IND.JQ037810	97.6	97.6	97.5	97.6	97.3	97.6	96.8	96.5	96.3	97.8	ID	98.5	99	91.5	87.5	88.5	99	80.5	80.5	81	98	81	94	94	94	93.5	94
12 India.NRT39/IND.JQ037809	98.5	98.5	98.3	98.5	98.1	98.5	97.6	97.3	97.3	97.3	97.8	ID	98.5	91	87	88	98.5	79.6	79.6	80	97.5	80	93.5	93.5	93.5	93	93.5
13 India/Meerut01/India/2010/KC954625	100	100	99.8	100	99.6	100	99.1	98.8	98.6	98.8	97.6	98.5	ID	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
14 India/Chennai/AY559061	91.7	91.7	91.5	91.7	91.4	91.7	90.9	90.7	91.2	90.5	89.7	90.2	91.7	ID	86.1	86.1	92.5	75.6	75.6	75.6	91.5	76.1	87.5	87.5	87.5	87.5	87.5
15 India/Sirsa3/Y559060	88.4	88.4	88.2	88.4	88.2	88.4	87.6	87.4	87.7	87.2	86.4	86.9	88.4	81.4	ID	98.8	88.5	72.6	71.6	72.6	87.5	72.6	86.2	86.1	86.3	86.3	86.3
16 India/Avikanagar/AY559058	88.7	88.7	88.5	88.7	88.5	88.7	87.9	87.7	88	87.6	86.7	87.2	88.7	81.8	99.6	ID	89.5	72.6	72.6	72.6	88.5	72.6	86.1	86.2	86.2	86.2	86.3
17 India/India A/AJ585111	100	100	99.8	100	99.6	100	99.1	98.8	98.6	98.8	97.6	98.5	100	91.7	88.4	88.7	ID	81	81	81.5	99	81.5	95	95	95	94.5	95
18 Morocco/MOR2006/06/EU625362	72.8	72.8	72.7	72.8	72.8	72.8	72	71.7	73	73	72.8	72.5	72.8	68.4	68.4	68.6	72.8	ID	100	97.5	82	99.5	79.1	79.1	79.1	78.6	79.1
19 Algeria/ALG2006/01/EU625361	72.8	72.8	72.7	72.8	72.8	72.8	72	71.7	73	73	72.8	72.5	72.8	68.4	68.4	68.6	72.8	100	ID	97.5	82	99.5	79.1	79.1	79.1	78.6	79.1
20 South Africa-ref/South Africa/AJ585122	73.8	73.8	73.7	73.8	73.7	73.8	73	72.7	74	73.7	73.5	73.5	73.8	68.7	68.4	68.4	73.8	94.5	94.5	ID	82.5	98	80	80	80	79.6	80
21 Greece/GRE2001/07/JN635334	95.8	95.8	95.7	95.8	95.5	95.8	95	94.8	95	94.7	93.5	94.7	95.8	88	85.2	85.6	95.8	72.8	72.8	73.8	ID	82.5	96	96	96	95.5	96
22 Portugal/BTV1/PT/29058/07/ EU498674	73.2	73.2	73	73.2	73.2	73.2	72.3	72	73.3	73.3	73.2	72.8	73.2	68.5	65.6	65.9	73.2	99.6	99.6	94.7	73.2	ID	79.6	79.6	79.6	79.1	79.6
23 Australia/DPP8304/KM099539	87.7	87.7	87.6	87.7	87.4	87.7	86.9	86.6	87.2	87.6	87.2	87.6	87.7	80.8	78.8	79.1	87.7	74.3	74.3	74.3	87.9	74.3	ID	100	100	99.5	100
24 Australia/DPP8086/KM099538	87.1	87.1	86.9	87.1	86.7	87.1	86.2	85.9	86.6	86.9	86.6	86.9	87.1	80.1	78.2	78.5	87.1	74.2	74.2	74	87.2	74.5	98.1	ID	100	99.5	100
25 Australia/DPP7137/KM099537	87.4	87.4	87.2	87.4	87.1	87.4	86.6	86.2	86.9	87.2	86.9	87.2	87.4	80.6	78.3	78.6	87.4	74.2	74.2	73.8	87.6	74.5	98.5	98.3	ID	99.5	100
26 Australia/DPP6504/KM099536	87.6	87.6	87.4	87.6	87.2	87.6	86.7	86.4	87.1	87.4	87.1	87.4	87.6	80.8	78.5	78.8	87.6	74	74	74	87.7	74.3	98.6	98.5	99.5	ID	99.5
27 Australia/DPP6112/KM099535	87.6	87.6	87.4	87.6	87.2	87.6	86.7	86.4	87.1	87.4	87.1	87.4	87.6	80.6	78.5	78.8	87.6	74	74	74	87.7	74.3	98.6	98.5	99.5	99.6	ID

Percent nucleotide identity

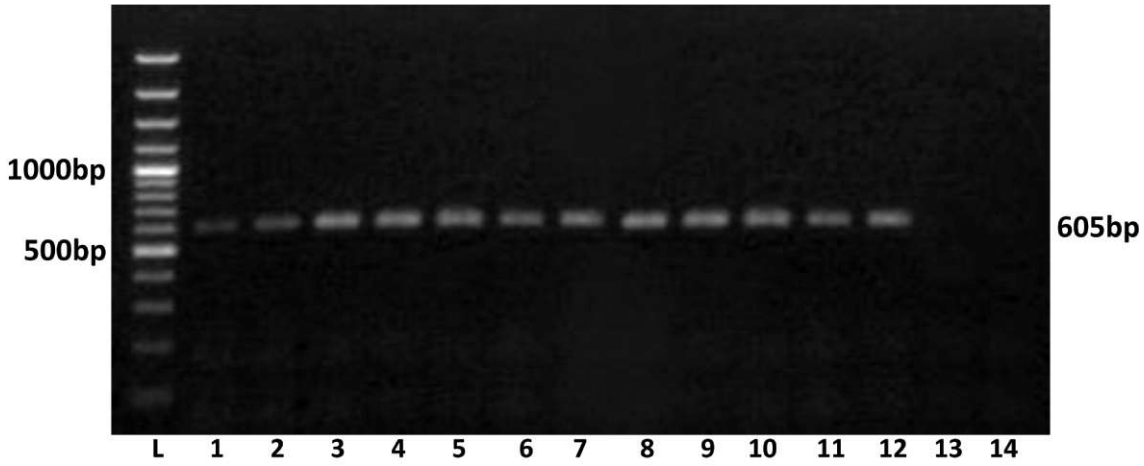

Fig. 2: Vp2 gene specific RT-PCR based serotyping of different BTV 1 isolates showing 605bp amplicon size in 1% Agarose gel electrophoresis. Lanes: L: 100bp DNA ladder, 1: MKD18/IND, 2: MKD19/IND, 3: MKD20/IND, 4: MKD21/IND, 5: MKD22/IND, 6: MKD23/IND, 7: MKD24/IND, 8: MKD25/IND, 9:NRT35/IND, 10: NRT39/IND, 11:CHT1/IND, 12: BT1/IND, 13: BHK-21 cell negative control and 14: Nuclease free water negative control.

shown). Upon group specific ns1 gene based RT-PCR all the isolates yielded an expected size of 366 bp of amplicon on 1% agarose gel electrophoresis (Figure 1). The characteristics cytopathic effect in BHK–21 cell culture, migration pattern in RNA–PAGE and group specific ns1 gene based RT-PCR further confirmed the samples as BTV. The BTV isolates grown in BHK–21 cells were evaluated by different RT–PCR assays by several other workers earlier (Aradabib et al., 1998; Biswas et al., 2010).

For serotype confirmation, all the isolates were subjected to vp2 gene specific RT-PCR using primers specific to all the BTV serotypes. All the isolates in study had shown specific PCR amplicon of 605bp with BTV1 vp2 gene primer only (Figure 2) without showing any amplification with remaining serotype specific primers. Thus, all the isolates were serotyped as BTV1. The cloned product of vp2 gene of all the isolates was allowed for nucleic acid sequencing.

BLASTN+ 2.2.30 analysis of all the isolates showed that vp2 gene sequences of all the isolates align only with BTV1 isolates from India and different parts of the world. It confirmed the serotype of all the BTV isolates as BTV1. The sequence data obtained was submitted to NCBI and accession numbers assigned to different isolates MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND, MKD25/IND, NRT39/IND, NRT35/IND, BT1/IND and CHT1/IND were JQ037800, JQ037801, JQ037802, JQ037803, JQ037804, JQ037805, JQ037806, JQ037807, JQ037809, JQ037810, JQ037811 and JQ037812 respectively.

The percent nucleotide and its deduced amino acid sequences identity of the isolates in study along with other BTV1 isolates from different parts of the world were calculated using Bioedit 7.2.5 (Hall, 1999). The nucleotide and its deduced amino acid sequence identity study revealed that BTV1 isolates from north India (MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND, and MKD25/IND) showed 98.8-100%/97-100% nucleotide/amino acid (nt/aa) identity among themselves (Table 1). Similarly, BTV1 isolates from south India (NRT39/IND, NRT35/IND, BT1/IND, and CHT1/IND) showed 96.3-97.8%/96.5-99% nt/aa identity among themselves. However, overall nt/aa identity of BTV1 isolates in study was found to be 96.5-100%/95.5-100%.

The BTV1 isolates in study showed 78.2-100%/86.1-100% nt/aa identity with eastern topotype BTV1 isolates from India (Meerut 01, India A, Chennai, Sirsa 3and Avikanagar isolates), Australia (DPP8304, DPP8086, DPP7137, DPP6504 and DPP6112) and Greece (GRE2001). However, they showed only 68.4-74.5%/72.6-81.5% nt/aa identity with western topotype of BTV1 viruses from Morocco (isolate MOR2006/06), Algeria (ALG2006/01), South Africa (South Africa-ref) and Portugal (BTV1/PT/29058/07). These topotype results agree with previous study made (Maan et al., 2010). It confirmed the eastern origin of segment 2 of BTV1 isolates in study.

The nucleotide sequence based phylogenetic analysis of these isolates along with other BTV1

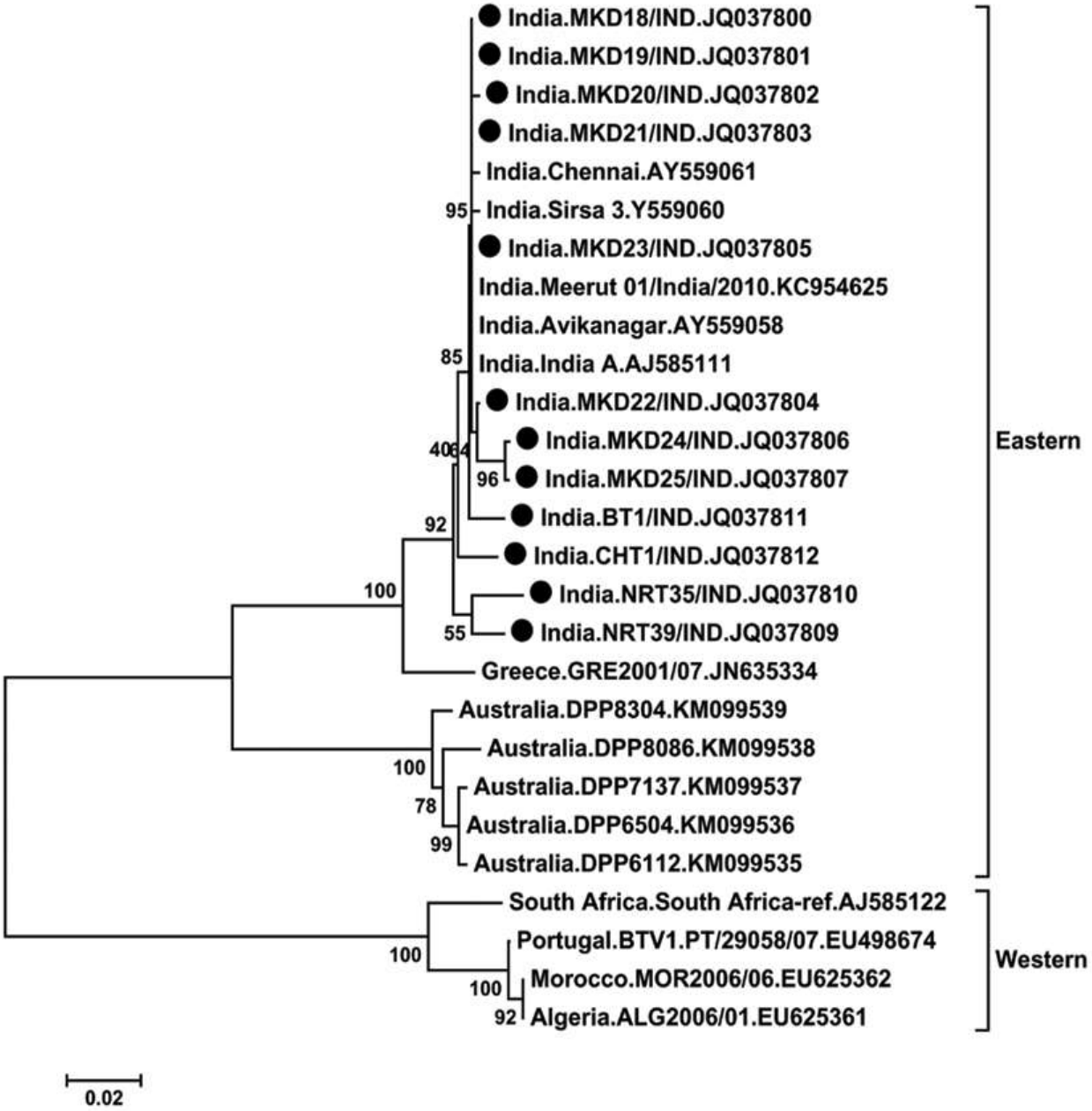

Fig. 3: Vp2 gene nucleotide sequence based phylogenetic analysis of Indian isolates of BTV1 with other global isolates of BTV1. ● Indian isolates used in this study

isolates using Mega 6 software programme revealed that BTV1 isolates in study form a separate eastern cluster with eastern topotype BTV1 isolates from India, Australia and Greece (Figure 3). The western cluster was consists of BTV1 viruses of western topotype from Morocco, Algeria, South Africa and Portugal. However, within eastern cluster BTV1 isolates from north India (MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND, and MKD25/IND) formed a more closely related sub cluster along with other Indian isolates. Similarly, south Indian BTV1 isolates (NRT39/IND, NRT35/IND, BT1/IND, and CHT1/IND) formed a separate more closely related sub cluster with BTV1 from Greece. The similar result was observed with deduced amino acid sequence based phylogenetic analysis (Figure 4).

The phylogenetic analysis study and sequence identity revealed that the segment 2 of BTV1 isolates in study might be originated most probably from India or Greece. However, to know the origin of individual segments of the BTV genome, sequencing of the all

Fig. 4: Vp2 gene deduced amino acid sequence based phylogenetic analysis of Indian isolates of BTV1 with other global isolates of BTV1. ● Indian isolates used in this study

the segments is required. Recently, reassortment in the segment 6 of BTV16 and BTV21 has been reported in the Indian BTV16 isolate (Shafiq et al., 2013). The transmission of BTV takes place along with live animals or its product transport and its *Culicoides* vector. Previous studies have suggested prevalence of BTV serotypes 2, 4, 6, 9, 12, 13, 14, 17, 18 and 19 in Andhra Pradesh whereas BTV 23 in Uttrakhand state. The circulation of BTV1 has been reported from many states of India including Rajasthan, Gujarat Maharashtra, Tamil Nadu and Karnataka (Prasad et al., 2009). Therefore, migration of BTV 1 within adjoining states is quite possible due to movement of animal from one state to another. The activity of the *Culicoides* vector may also play role in transmission and spread of the infection in adjoining areas. BT viruses undergo continuous genetic reassortment and are transmitted by Culicoides vector, posing the disease control a challenge. The knowledge of prevalence of various circulating serotypes could be used for development of sensitive diagnostics to study epidemiology and effective vaccination to control the disease in field. Rapid molecular based diagnosis of BTV isolates is required to improve surveillance of BTV serotypes prevalent and to facilitate the choice of an appropriate serotype–specific vaccine in a disease outbreak.

Conclusions

BT is one of the major infectious diseases of small ruminants in India. BT is primarily a disease of sheep. In this study sequence based serotyping and phylogenetic study of twelve isolates of BTV from north and south India has been reported. All the BTV isolates were serotyped as BTV1. Phylogenetic study confirmed the eastern origin of these isolates. The sequence and phylogenetic study revealed that all the isolates might be originated either from BTV1 isolates from India or Greece. The segment 2 based RT–PCR used here can be used for diagnosis of BTV from variety of biological samples such as blood, tissue samples and *Culicoides* vectors. The present study suggested that RT-PCR could be a reliable and rapid method for detection and determination of serotypes of Indian isolates of BTV. The information thus generated regarding circulation of BTV1 in various part of the India could be very useful for development of rapid and sensitive diagnostics and suitable vaccine candidates for control BT disease.

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