

NUCLEOTIDE SEQUENCE COMPARISON OF VP5 GENE OF INDIAN ISOLATES OF BLUETONGUE VIRUS SEROTYPE 2

KOUSHLESH RANJAN, MINAKSHI PRASAD*, HARI MOHAN¹, RUPINDER, VISHWARADHAYA, T. M. and GAYA PRASAD

Department of Animal Biotechnology, College of Veterinary Sciences

Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar-125 004

¹Department of Medical Biotechnology, Maharshi Dayanand University, Rohtak

ABSTRACT

The present study was conducted to compare the nucleotide sequences of VP5 gene of Indian isolates of bluetongue virus serotype 2. A pair of VP5 gene specific primer (forward primer 755-774 bp and reverse primer 1589-1570 bp) giving an amplicon size of 835 bp was designed using VP5 gene sequences available in GenBank database. This primer set did not give any amplification with BTV serotypes 3, 9 and 16 showing serotype specificity of the primer. The nucleotide sequence of VP5 gene analysis revealed that Indian BTV2 serotype showed more than 99% identity with other Indian BTV2 isolate along with most of USA and South African isolates. However, it showed more than 86% identity with most of European BTV2 isolates. *In-silico* restriction enzyme analysis revealed that Indian BTV2 isolate can be differentiated from other BTV2 isolates from India, USA, South Africa and Europe using *Bgl*III, *Hae*III, *Bsm*AI, *Hap*II restriction enzyme.

Key words: Bluetongue virus 2, RT-PCR, sequencing, VP5 gene, *in-silico* RE analysis

Bluetongue (BT) is an infectious, non-contagious, insect vector borne disease of domestic and wild ruminants (MacLachlan, 1994). It is caused by BT virus (BTV), prototype species of the genus *Orbivirus* within the family *Reoviridae*. BT causes considerable economic loss to livestock industry which is mainly attributed to high morbidity, mortality, abortion, still birth, foetal abnormality, weight loss, wool break, reduced milk and meat yield. BT is listed as a 'notifiable disease' by the Office International des Epizooties (OIE) (OIE, 2006). It causes severe clinical signs as evidenced by fever, lameness, swelling of lips and tongue. Severe forms of the disease are frequently seen in sheep and in white-tailed deer (Howerth *et al.*, 1988; 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 (BTV1 to BTV24) of the virus have been identified worldwide (Mertens *et al.*, 2004). However, two more serotypes, BTV25 from Switzerland (Hofmann *et al.*, 2008) and BTV26 from Kuwait have been isolated recently (P. Mertens, personal information). Since India is endemic for

Culicoides vector, a total of 22 BTV serotypes have been reported from India (Prasad *et al.*, 2007; Jordar *et al.*, 2009). Based on nucleotide sequence analysis, most of BTV isolates can be broadly divided into two major groups 'Eastern' or 'Western' topotypes, and into a number of geographic subgroups (Balasuriya *et al.*, 2008).

BTV is a small icosahedral virus with a ten-segmented, double stranded RNA (dsRNA) genome. Each of the ten segments codes for at least one viral protein. Seven proteins (VP1 to VP7) are structural and form virus particle. In addition to structural proteins, there are four non-structural proteins NS1, NS2, NS3 and NS3a, expressed in virus infected host cells (Mertens *et al.*, 1989). 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 two major viral proteins, VP2 and VP5, which are serotype specific for individual BTV serotype (Ghiasi *et al.*, 1987). The VP5 protein participates in virus neutralization activity along with VP2 protein as it enhances the neutralization activity of VP2 protein (Roy *et al.*, 1990). VP5 also plays an important role in

*Corresponding author: minakshi.abt@gmail.com

membrane permeabilization and membrane fusion activity facilitating virus entry into the host cells (Forzan *et al.*, 2004). Genetic diversity between different BTV serotypes as well as various isolates within a single serotype has been reported by sequence analysis of genome segment encoding VP5 protein (Singh *et al.*, 2004). Although, isolation of BTV2 from India has been reported earlier, VP5 gene based molecular characterization of Indian isolate of BTV2 has not been reported so far. Therefore, the present study was carried out to determine the VP5 gene based genetic variation and phylogenetic relationship of Indian BTV2 isolate with isolates from different parts of the world.

MATERIALS AND METHODS

Sample Origin: The unknown Indian isolate suspected for BTV obtained from Andhra Pradesh state under 'All India Network Programme on Bluetongue' in 2004. The virus was propagated in BHK-21 cell line in the laboratory to raise the bulk virus stock.

Isolation of Viral dsRNA: After appearance of about 75% cytopathic effect (CPE) in infected BHK-21 cell culture, the virus was harvested. Viral RNA was extracted from cell culture supernatant by Trizol reagent (Sigma) as per manufacturer's instructions.

Oligonucleotide Primers: The VP5 gene specific primers P1-5'ACAGCCGTCGCAACGGGAAG3' (755-774 nt) and P2-5'AGAGGGGCACGTCCAACCGA3' (1589-1570 nt) were designed based on nucleotide sequences of serotype BTV2 available in GenBank with the help of Primer Blast software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). This primer pair P1 and P2 with expected amplicon size of 835 bp was selected for the present study. The primer sequences were got synthesized commercially (LifeTech, USA).

Polymerase Chain Reaction (PCR): Viral genomic RNA of BTV2 was used as a template for cDNA synthesis using random primers (decamer) (Ambion, USA) and moloney murine leukemia virus-reverse transcriptase (Mo-MuLV-RT) enzyme (Sibzyme, Russia). The reaction mixture consisted of 7 µg of heat denatured viral RNA, 6% DMSO and 30 pmol of random

decamer. Finally, 25 µl reaction mix was prepared using 400 µM each dNTPs and 500 U of Mo-MuLV-RT for reverse transcription. The primer was allowed to anneal at 25°C for 10 min followed by reverse transcription at 42°C for 60 min (Biorad i-Cycler).

The cDNA was subsequently amplified using vp5 gene specific primers. The amplification was carried out in 20 µl reaction mixture containing 2 µl cDNA, 3% DMSO, 20 µM of primers (P1 and P2) of BTV2 along with 0.4 µl of 10 mM dNTPs mix, 4 µl 5X HF buffer and 0.4 U (2U/ µl) phusion high-fidelity DNA polymerase (Finnzymes, Finland) in a thermalcycler (Biorad iCycler). The amplification programme consisted of initial denaturation for 3 min at 98°C, followed by 35 cycles of denaturation at 98°C for 10 sec, primer annealing at 57°C for 20 sec, primer extension at 72°C for 30 sec. Final primer extension was carried out at 72°C for 10 min. The amplified products were electrophoresed using 1% agarose gel (Sigma, USA) stained with ethidium bromide and visualized under UV transilluminator (Biovis, USA).

Serotype specificity of PCR primer was determined using RNA extracted from cell culture grown BTV serotypes viz. BTV2, BTV3, BTV9, BTV16. The uninfected BHK21 cells and nuclease free water served as negative controls.

Nucleotide Sequencing: PCR product was purified using QIA quick gel extraction kit (QIAGEN, USA). Purified PCR product was sequenced using Genetic Analyser ABI PRISM™ 3130 XL.

Nucleotide Sequence Analysis: Partial VP5 gene sequence of Indian BTV2 isolate was analysed using BLASTN+ 2.2.27 software (<http://blast.ncbi.nlm.nih.gov/>) (Zhang *et al.*, 2000). Bioedit v7.0.8 software was used for multiple sequence alignment and percent identity of partial vp5 gene of Indian BTV2 isolate with other global isolates. Similarly, deduced amino acid sequence of VP5 gene was generated and its percent identity with global isolates was calculated using Bioedit v7.0.8 software. The Mega 4.02 (Tamura *et al.*, 2007) software was used for phylogenetic analysis of the current isolate with other isolates of BTV2 as well as with all 25 BTV serotypes from different parts of the world.

To study the genomic diversity further, *in silico*

restriction analysis of partial vp5 gene of present Indian BTV2 isolate and other BTV2 isolates from different parts of world was carried out using Restriction Mapper version 3.0 software at <http://www.restrictionmapper.org/>.

RESULTS AND DISCUSSION

The VP5 gene of BTV2 was specifically amplified as evidenced by 835bp expected size of PCR product. No amplification was observed in serotypes BTV3, BTV9, BTV16 as well as in uninfected BHK21 cells and nuclease free water controls confirming the specificity of primers. The sequence data obtained was deposited to GenBank with accession no. GU213110.1 and the same has been used for comparison of our BTV2 VP5 gene sequence with global isolates. Indian isolate BTV2_GU213110.1 shared more than 99% nucleotide and deduced amino acid sequence identity with most of the Indian, South African and USA isolates of BTV2. Phylogenetic analysis of VP5 gene revealed that most of the USA, South Africa and Indian isolates formed a separate cluster and were closely related (Fig. 1). However, BTV2 isolates from Europe, Nigeria and Sudan formed a separate cluster. Comparison of partial VP5 gene sequence of Indian isolate with that of 25 BTV serotypes from all over the world grouped it closer to USA and South African isolates of BTV2 (Fig. 2).

In silico restriction analyses with *BsmAI* revealed that BTV2_GU213110.1 had single restriction site at nucleotide position 1514 whereas all other Indian, South African and USA isolates had *BsmAI* restriction site at nucleotide position 1513 except one of the Indian isolate BTV2_AJ783905.1 which had one more additional *BsmAI* site at 1432 suggesting mutation at that particular position (Table 1). Similarly, restriction analysis with *BglII* revealed that all the Indian, USA and South African BTV2 isolates have single restriction site at nucleotide position 829 whereas all the isolates from Europe, Sudan and Nigeria in addition had two more restriction sites at positions 993 and 1035. The restriction analysis with *HaeIII* revealed that all the European and Nigerian isolates, had two restriction sites at nucleotide positions

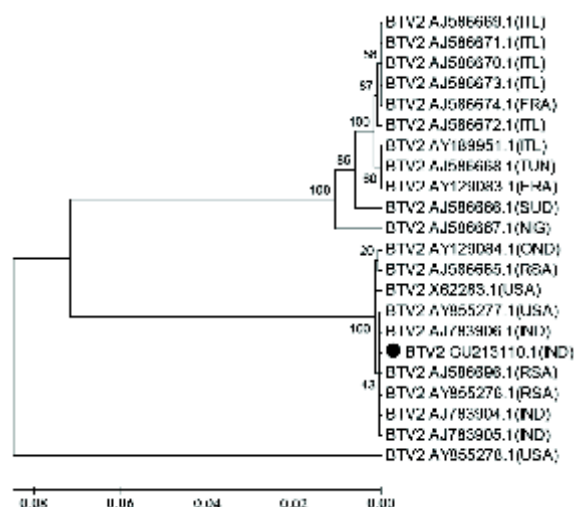


Fig1. Phylogenetic analysis of BTV2 isolates based on partial VP5 gene sequences. Tree was constructed from partial nucleotide sequences of VP5 gene using the neighbour joining method in Mega4 software (Tamura *et al.*, 2007). ●=Isolate of the present study; IND=India; RSA=South Africa; OND=Onderspoort; SUD=Sudan; TUN=Tunisia; ITL=Italy; FRA=France; NIG=Nigeria

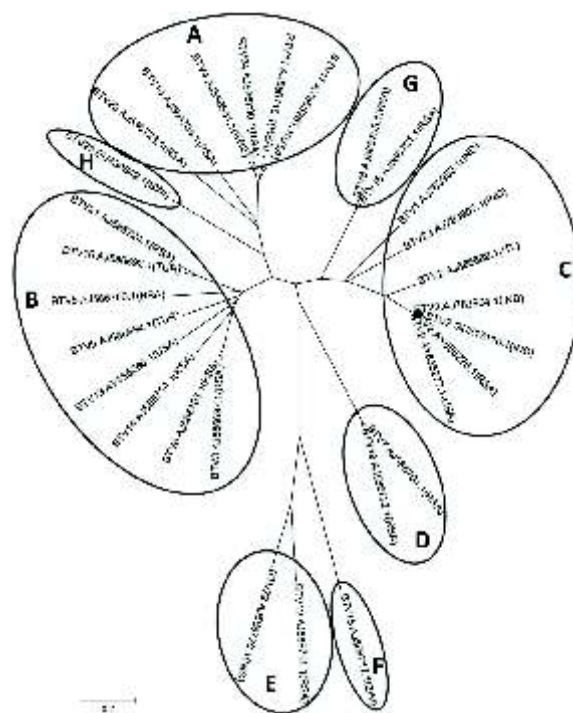


Fig 2. Phylogenetic comparison of Indian isolate of BTV2 with all 25 serotypes from different parts of the world based on partial VP5 gene sequences. Tree was constructed from partial nucleotide sequences of VP5 gene using the neighbour joining method using Mega4 software (Tamura *et al.*, 2007). ●=Isolate of the present study; IND=India; RSA=South Africa; ITL=Italy; TUR=Turkey; NIG=Nigeria; GRE=Greek; ZIM=Zimbabwe; SWI=Switzerland

1283 and 1331 whereas a single site at nucleotide position 1331 was observed in Sudan isolate. However, all BTV2 isolates from India, South Africa and USA did not have any restriction sites for *HaeIII*. All the European, Nigerian and Sudan isolate sequences used in the present study for comparison showed single *HapII* site at 908. Based upon *BglII*, *HaeIII* and *HapII* restriction sites, appreciable genomic diversity was observed between Indian BTV2 isolate (BTV2_GU213110.1) and the isolates from different parts of the world. These findings provide the basis for differentiation of Indian isolate from European, Nigerian and Sudanese BTV2 isolates.

BTV2 is one of the common BTV serotypes reported from Andhra Pradesh in India (Balumahendrian *et al.*, 2009). The total length of the VP5 gene is 1635 nucleotides, however, in some isolates it may be up to 1645 bp. Genetic diversity (3-43%) has been observed based on VP5 gene sequences of all the 24 serotypes of BTV across the world (Singh *et al.*, 2004). However, 73% nucleotide identity has been shown by recently evolved BTV25 serotype with that of BTV4 based on nucleotide sequence encoding VP5 protein (Hofmann *et al.*, 2008).

VP5 gene based molecular characterization and phylogenetic relationship of serotype BTV-1

Indian isolates (Manjunath *et al.*, 2010) and reference strains of all 24 serotypes (Singh *et al.*, 2004) have been reported earlier. The VP5 gene sequence based comparison of Indian BTV2 isolates (AJ783904.1, AJ783905.1 and AJ783906.1) other than GU213110.1 (used in the present study) has revealed 0-0.42% divergence among Indian isolates. The partial sequence of VP5 gene of Indian BTV2_GU213110.1 isolate showed more than 99% nucleotide and deduced amino acid sequence identity with most of the BTV2 isolates of India, USA and South Africa. However, the isolate used in present study had shown less than 86% nucleotide identity with European isolates. Based on deduced amino acid sequence, it had shown more than 97% identity with European isolates. This indicates that the isolate used in this study is closer to western topotype of BTV. The *in-silico* REA along with phylogenetic analysis clearly indicated that the isolate of this study is closely related to USA and South African BTV2 isolates as compared to European, Sudanese and Nigerian BTV2 isolates.

The segment-6/VP5 gene sequences of the reference isolates of all 25 different BTV serotypes cluster as eight distinct evolutionary lineages, called as segment 6 nucleotypes (nucleotypes A-H) (Maan *et al.*, 2010). The grouping of the different BTV serotypes within these eight nucleotypes also reflects the serological relationships between them i.e. low-level cross-reactions between different BTV serotypes. The comparative analysis of partial length VP5 gene sequences of Indian isolates of BTV2 (including the present isolate) with remaining 25 BTV serotypes from different parts of the world revealed the association of our isolate with nucleotype C along with BTV2 isolates of USA, South Africa and India. This nucleotype also include serotypes BTV1 and 23 of Indian origin indicating geographical closeness of BTV isolates of India and Western origin (Fig. 2). Thus, the analyses of partial VP5 gene sequence revealed that there is no significant divergence among Indian BTV2 isolates. However, based upon *in silico* restriction analysis Indian BTV2 isolates can be differentiated from European, Nigerian and Sudan isolates and are more closely related to USA and South Africa isolates.

Table1
***In-silico* restriction analysis of BTV 2 isolates from different parts of the world**

Isolates	<i>BglII</i>	<i>HaeIII</i>	<i>BsmAI</i>	<i>HapII</i>
BTV2_GU213110.1(IND)	829	-	1514	-
BTV2_AJ783904.1(IND)	829	-	1513	-
BTV2_AJ783905.1(IND)	829	-	1432, 1513	-
BTV2_AJ783906.1(IND)	829	-	1513	-
BTV2_AJ586665.1(RSA)	829	-	1513	-
BTV2_AJ586696.1(RSA)	829	-	1513	-
BTV2_AY855276.1(RSA)	829	-	1513	-
BTV2_AY855277.1(USA)	829	-	1513	-
BTV2_X62283.1(USA)829	-	1513	-	-
BTV2_AJ586666.1(SUD)	829, 993, 1035	1331	877	908
BTV2_AJ586667.1(NIG)	829, 993, 1035	1283, 1331	877	908
BTV2_AJ586668.1(TUN)	829, 993, 1035	1283, 1331	877	908
BTV2_AJ586669.1(ITA)	829, 993, 1035	1283, 1331	877	908
BTV2_AJ586670.1(ITA)	829, 993, 1035	1283, 1331	877	908
BTV2_AJ586671.1(ITA)	829, 993, 1035	1283, 1331	877	908
BTV2_AJ586672.1(ITA)	829, 993, 1035	1283, 1331	877	908
BTV2_AJ586673.1(ITA)	829, 993, 1035	1283, 1331	877	908
BTV2_AJ586674.1(FRA)	829, 993, 1035	1283, 1331	877	908

IND=India; RSA=South Africa; SUD=Sudan; TUN=Tunisia;
ITA=Italy; FRA=France; NIG=Nigeria

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