VP2 Gne based Molecular Characterization of Blue tongue Virus Serotype 16 Isolated in 2017 from Andhra Pradesh, India

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ABSTRACT

Bluetongue virus (BTV) genome consists of 10 segmented double-stranded ribonucleic acid (dsRNA). Of these, genome segment 2 (Seg-2), which encodes outer capsid protein VP2 is the primary determinant of virus serotype. In the present study, partial VP2 gene of KRL2/2017 (BTV-16) isolate collected from Kurnool district of Andhra Pradesh was amplified to know the evolutionary relationship of this isolate with previously reported Indian and global BTV-16 isolates. Seg-2 sequence data of KRL2/2017, showed >98% nucleotide identity with other Indian isolates. Comparison of this isolate with BTV-16 isolates across the world, showed higher sequence homology to isolates of Japan followed by Greece and China than to those of isolates from South Africa, Italy, UK, Australia and Indonesia. Phylogenetic analysis suggest that the isolate of the current study belong to eastern topotype similar to the viruses recovered from Japan, Greece and China reflecting their common eastern origin.

Key Words: Bluetongue, serotype-16, VP2, topotype

INTRODUCTION

Bluetongue (BT) is an economically important, infectious, non-contagious, haemorrhagic disease caused by bluetongue virus (BTV), which is classified as type species of the genus *Orbivirus* in the family *Reoviridae* and subfamily *Sedoreovirinae.* BTV can infect both domestic and wild ruminants as well as some carnivores (MacLachlan, 1994 and Alexander, 1994). Currently, 27 distinct serotypes of BTV have been recognised worldwide and two additional putative novel serotypes were detected in a Capripox vaccine in the Middle East (BTV-28) and in an alpaca in South Africa (BTV-29) (Zientara *et al.,* 2014; Wright, 2014 and Maan *et al.,* 2015). BTV is primarily transmitted among susceptible hosts through the bite of a competent *Culicoides* midge (Du Toit, 1944). However, transplacental transmission is demonstrated in naturally and experimentally infected cattle and in experimentally-infected sheep with BTV-8 (Saegerman *et al.,* 2011).

The BTV genome is composed of ten linear segments of double stranded ribonucleic acid (dsRNA) and enclosed

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within a three layered icosahedral protein capsid, consisting a total of seven structural proteins (VP1-VP7) and four non-structural proteins (NS1, NS2, NS3/NS3a and NS4) (Verwoerd *et al.,* 1970; Mertens *et al.,* 1984 and Ratinier *et al.,* 2011). BTV outer capsid proteins VP2 and VP5are encoded by genome Seg-2 and -6, respectively. The VP2 gene particularly contains epitopes that bind to neutralizing antibodies generated during infection of the mammalian host and is main determinant of virus serotype (Mertens *et al.,* 2004). Topotypes were proposed for segments of BTV based on the high degree of nucleotide similarity among BTV isolates from the same geographical region (Gould and Pritchard, 1990). Sequence analysis of all segments of BTV revealed two broad topotypes. The BTV isolates from Africa, the Mediterranean and America were considered as 'western' types, and those from Australasia, the Middle East and the Mediterranean were considered as 'eastern' types (Maan *et al.,* 2010). Virus isolation and serum or virus neutralization tests (SNT or VNT) are slow, taking weeks whereas molecular methods like RT-PCR, sequence analysis, phylogenetic comparison etc. are time saving, in the detection of virus (Maan *et al.,* 2012a). Occurrence of multiple serotypes and presence of antigenic diversity within serotypes of BTV is a major constraint for developing an effective vaccine. Hence, there is a felt need to identify and characterize currently circulating BTV serotypes. In this study, we describe VP2 gene based molecular characterization of BTV-16 isolated from the samples collected during 2017 BT outbreak from Andhra Pradesh state. We report the evolutionary relationship of BTV-16 isolate from the current study with global BTV-16 isolates.

MATERIALS AND METHODS

Virus isolate

Blood sample was collected aseptically in Ethylene diamine tetraacetic acid (EDTA) vial from suspected field outbreak of BT disease in sheep during 2017 that occurred in Kurnool district of Andhra Pradesh and designated as KRL2/2017. Blood was collected from sheep exhibiting BT symptoms like pyrexia, oral lesions, swollen face, nasal discharges, frothy salivation and torticollis.

Virus dsRNA extraction and cDNA synthesis

Virus from blood sample was isolated by initially inoculating the washed erythrocyte lysate on to *Culicoides sonorensis* (KC) cell line and after 10 days, the KC cell culture adapted sample was passaged three times in BHK-21 cell line (Clavijo *et al.,* 2000). Viral dsRNA was isolated from infected BHK-21 cell line showing cytopathic effect (CPE) using TRIZOL method and then subjected to 1% agarose gel electrophoresis, for testing *Orbivirus* like pattern. cDNA was

synthesized using PrimeScript™ 1st strand cDNA Synthesis kit (TaKaRa) using random hexamers. The resulting cDNA was used as template in PCR.

Polymerase chain reaction

The PCR was carried out by using EmeraldAmp® GT PCR Master Mix (TaKaRa) with group specific primer pair; Seg10F-TTGGAYAAA GCRATGTCAAA;Seg10R-ACRTCATC ACGAAACGCTTC targeting Seg-10, and serotype-specific primer pairstargeting Seg-2 as described previously (OIE, 2012 and Reddy *et al.,* 2016). PCR was carried out with initial denaturation at 94^{0}C for 3 min followed by 35 cycles of 30 sec denaturation at 94^{0}C, 30 sec of primers annealing at 55^{0}C for all sets of group specific and serotype specific primers except for Seg-2 of BTV-16 where 50^{0}C was used, followed by extension at 72^{0}C for 1 min and then final extension at 72^{0}C for 10 min. PCR products were visualized by agarosegel electrophoresis. Amplified PCR product with VP2 gene primers of BTV-16(1196 bp) was purified using XcelGen® PCR Purification MiniKit as per the manufacturer's instructions.

Sequencing and Phylogenetic analysis

Sequencing was done from Xcelris Labs Ltd, Gujarat using VP2 gene specific primers as mentioned above for purified PCR products. The partial sequences of VP2 gene of KRL2/2017 isolate obtained after sequencing was compared with other available sequences in GenBank using NCBI BLAST (www.ncbi.nlm.nih/gov/blast). Phylogenetic trees were generated using the Neighbor-Joining (NJ) method with the Tamura 3-parameter model and bootstrapped on the set of 1,000 replicates with MEGA version 7.0 software to study the evolutionary relationship of isolate from the current study with other BTV-16 isolates from various geographical regions of the world.

RESULTS AND DISCUSSION

Virus BTV-16 (KRL2/2017) was isolated by initially inoculating the blood sample collected from sheep suspected with BT on to KC cells for 10 days followed by culturing in BHK-21 cell line. CPE was noticed after three passages in BHK-21 cell line. BTV nucleic acid was extracted from the infected cell culture fluid by TRIZOL method when the cells were showing above 75% CPE (Fig. 1A&B). The extracted RNA was subjected to 1% agarose gel electrophoresis and 10 segmented pattern of BTV RNA was observed (Fig. 1C). The isolate was confirmed as BTV by Seg-10 based RT-PCR (Fig. 1D). When subjected to Seg-2 based serotype specific RT-PCR, the isolate was confirmed as BTV-16. The size of the amplified product was1196 bp(Fig. 1E); it was purified and sequenced. No amplification of similar size was observed in negative control indicating that the amplified product was specific for VP2 gene ofBTV-16.

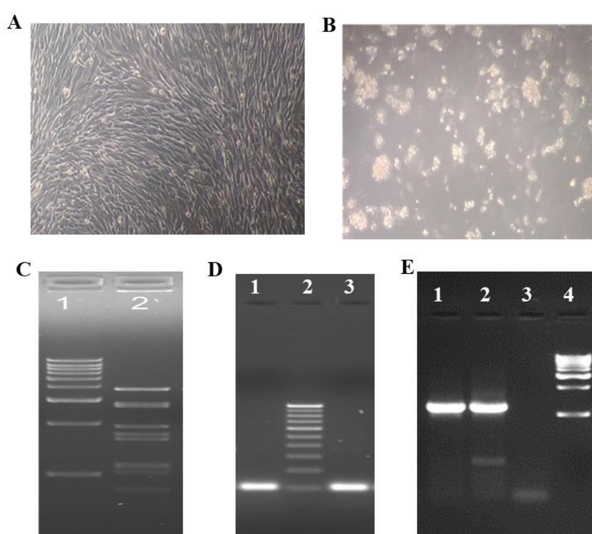

Fig 1:Molecular characterization of BTV-16 (KRL2/2017).*A-Uninfected BHK-21 cell line (10X); B-Infected BHK-21 cell line exhibiting CPE (10X) like cell rounding, clumping and detachment of cell bunches; C-Segmented pattern of BTV dsRNA, Lane 1- 1Kb Ladder, Lane 2- Segmented dsRNA of BTV-16 isolate (KRL2/2017); D-NS3group-specific RT-PCR (98 bp), confirming BTV, Lane 1- positive control, Lane 2-100 bp DNA ladder and Lane 3- BTV-16 isolate (KRL2/2017); E-VP2 gene specific RT-PCR (1196 bp) confirming BTV serotype-16E, Lane 1- BTV-16 isolate (KRL2/2017), Lane 2- Positive control, Lane 3- Negative cotrol and Lane 4-1 Kb ladder.*

PCR product of BTV-16 was sequenced and analysed. The BTV-16 Seg-2 sequence data (1196 bp; nucleotide421-1616) for KRL2/2017showed 98.5% nucleotide identity with Indian isolates (GeneBank Acc. No. KX302636; KC751423; KC751424 & KC751425), 98% nucleotide identity with isolates (GenBank Acc. No. JQ924821; JX007924; MG710531; KC751419; MG710529; JN572917; KF664134; KY934051; KF664104; KY934050 & MG7105530). Comparison of this isolate with previously reported isolates across the world showed 97.3%, 96.9%, 96.5%, 96.4%, and 96% nucleotide identity with isolates of Japan (GenBank Acc. No. AB686220; AB686226 & AB686225), Greece (Gene bank Acc. No.KP820989; KP820990 & AM773709), China (Gene bank Acc. No.KP195134 & KP195134), Israel(GenBank Acc. No.KP820992), UK (GenBank Acc. No.KP820985; KP820986 & KP820987) and Turkey (GenBank Acc. No.AJ585146; AJ585147 &AJ585148), respectively.BTV-16 isolates of UK (GenBank Acc. No. KP820988), Italy (GenBank Acc. No. KF387522; MH990434; MH990424 & MK014493) and South Africa reference strain (Gene bank Acc. No. AJ585137) showed 95.29% nucleotide identity with isolate from the current study whereas isolates of

Australia (GenBank Acc. No. MF384481 & MF384480) and Indonesia (GenBank Acc. No. AJ585151) showed 91.58% nucleotide identity. Maximum nucleotide identity of 96.5-97.3% was showed by Indian BTV-16 isolates with isolates of Japan followed by Greece and China than to those of isolates from South Africa, Italy, UK, Australia and Indonesia having 91.58-95.29% nucleotide similarity. Sequence homology and phylogenetic analysis of Seg-2 indicated that the BTV-16 isolate of the current study belonged to eastern topotype (Fig. 2).

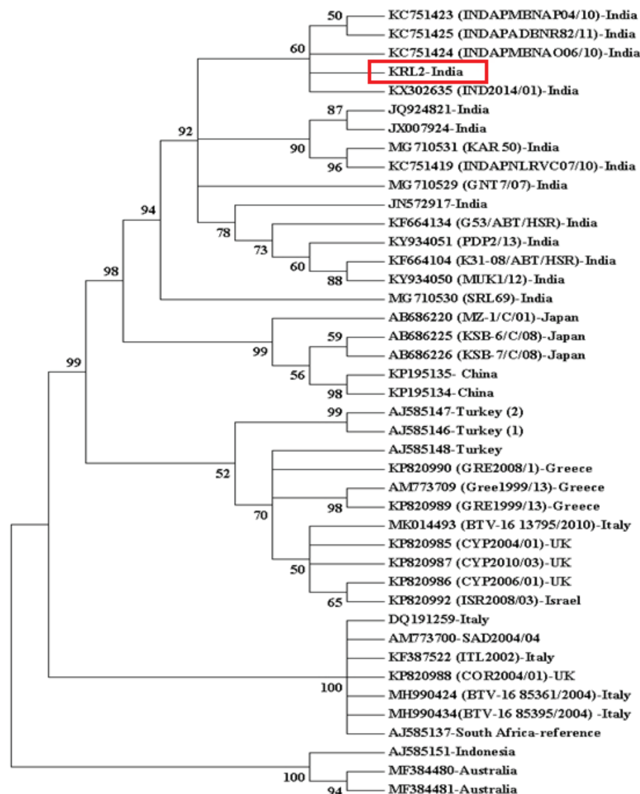

Fig 2: Phylogenetic analysis of BTV-16 (KRL2/2017) isolate against reference BTV-16 isolates isolated worldwide. Neighbor joining (NJ) tree showing relationship between KRL2/2017 (BTV-16) isolate sequenced in the current study and other 41 global isolates of BTV-16 based on nucleotide sequences of VP2 region of BTV using MEGA 7.0. The optimal tree with sum of branch length = 0.20209871 and the bootstrap test (1000 replicates) is shown. Based on the analysis the current isolate can be categorised as eastern topotype of BTV.

Sequence analysis of BTV segments have been extensively used for genetic characterization of isolates from various geographic regions of the world (Shirafuji *et al.,* 2012; Maan *et al.,* 2007 and Maan *et al.,* 2015). VP2 encoding genomic segments of *Orbivirus* are the most variable between serogroups as well as between serotypes of a serogroup (Gould and Pritchard, 1990). The sequence information on genome Seg-2 encoding outer capsid protein VP2 (the primary determinant of virus serotype), have been useful for understanding molecular epidemiology, phylogenetic relationship of BTV and are also capable of determining the geographic origin of a virustopotype (Gould and Hyatt, 1994, Maan *et al.,* 2015).

Most of the BT serotypes were isolated from Africa whereas BTV-16 was first isolated from the Indian subcontinent (Hazara, West Pakistan) in 1960 (Howell, 1970). The sequence analysis of BTV-16 (KRL2/2017) isolate from Andhra Pradesh state, presented here showed very high sequence homology of >98% nucleotide identity with previous BTV-16 isolates circulating in India and when compared with BTV-16 isolates from different geographical regions of the world showed an overall nucleotide identity of 91.58-97.3%.This similar observation was reported by Saxena *et al.* (2018) based on genetic and phylogenetic analysis of the outer capsid protein genes (VP2 and VP5) of Indian isolates ofBTV-16. Phylogenetic analysis based on VP2 gene segregated Indian BTV-16 isolates at proximity to the viruses recovered from Japan, Greece and China than to those of isolates from South Africa, Italy, UK, Australia and Indonesia. Supporting our data, Shafiq et al. (2013) previously reported the Indian isolates are at close proximity to Japan and Greece isolates based on phylogenetic analysis of Seg-2 of BTV-16. In this study a higher percentage of nucleotide similarity of VP2 gene of most of the globally isolated BTV-16 indicate that the eastern segment might have evolved from a common ancestor. Sequencing analysis and phylogenetic studies based on VP2 gene showed that isolate characterized from the current study belonged to eastern topotype. This observation was in accordance with Maan *et al.* (2012b) and Kumar *et al.* (2016), who reported the complete genome sequence analysis of BTV-16 isolates of India and identified as eastern topotype.

It may be concluded that BTV typing by molecular methods is considerably faster and cheaper than serological techniques. The VP2 gene based sequence analysis and phylogenetic approach will be useful for understanding molecular epidemiology, predicting BTV origin, patterns of distribution, variations within the serotypes, genotyping, and phylogenetic relationships. BTV-16 isolate from this study, could be grouped under eastern topotype. It will be possible to determine the origin of virus and genome segments with high degree of confidence only with complete genome sequence of BTV.

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