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Phylogenetic analysis of bluetongue virus serotype 2, 9 and 15

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Abstract
The present study was under taken with a view to characterize BTV 2, 9 and 15 isolates employed for trial vaccine. BTV 2, 9 and 15 virus isolates were cultivated in BHK_{21}cell line and RNA was isolated. Further, RT-PCR was standardized for detection of NS1 gene of BTV 2, 9 and 15. Molecular characterization of these serotypes were taken up by the sequencing of NS1 gene. The sequences were compared to the available sequences in Genbank. Further analysis indicated that all NS1 nucleotide sequences segregated into 6 phylogenetic clades. The BTV-15 (N12) is closely related to BTV-15 (N15), BTV-15 (DQ399835). BTV-2 (M11) and BTV (N12) are clustered together with BTV-9 and BTVTPT, BTV-9 and BTV KMTAI. The identity of BTV-2 (M11) with USA and Taiwan isolates of BTV-2 was 95% and 90%, the identity of BTV -15 with USA isolates of BTV 2 and 17 was 93% and 98% and with BTV Taiwan isolate was 87%. Further analysis indicated that BTV serotype isolates were closely related to BTVTPT.

Keywords: Phylogenetic, analysis, bluetongue virus, serotype

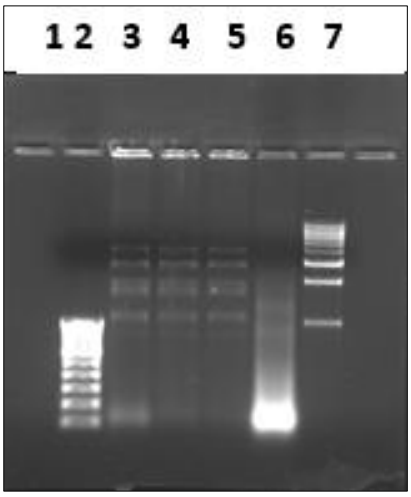
Introduction
Bluetongue (BT) is a major Office International Des Epizooties listed disease of sheep and goats and is endemic to India, causing significant economic losses to the sheep industry (OIE 2018) [17]. It is caused by bluetongue virus (BTV) that belongs to the genus Orbivirus of family Reoviridae and subfamily Sedoreovirinae (ICTV 2011) [11]. The transmission of BTV is mainly by the bite of a Culicoides midge (Mellor P S *et al.* 1995) [16]; Apart from this, Transmission through Direct contact (Batten C., 2014 and Bréard *et al.* 2018) [3, 7] and through transplacental (Saegerman C *et al.* 2011) [21] route were also reported.
Currently, 29 distinct serotypes of BTV are reported to be circulating geographically (Thota R *et al.* 2021) [23]. BT is endemic in India with a total of 24 serotypes reported and the majority of the outbreaks were reported from southern states in Peninsular India (Thota R *et al.* 2021) [23]. The BTV genome is composed of ten linear segments of double-stranded RNA (dsRNA). The 10 segments code for seven structural (VP1-VP7) and four non structural proteins (NS1, 2,3,3A) (Bommineni *et al.* 2008., Rao PP *et al.* 2012., Maan NS *et al.* 2012.) [5, 19, 13]. NS 1 gene is highly conserved among the different serotypes. In BTV infected cells three non structural proteins are made *i.e* NS1,NS2 and NS3(Huisman 1979) [9, 10]. NS1 is identified as a positive regulator of viral protein synthesis and it creates a positive feedback loop of expression, which rapidly increase the expression of all the viral proteins (Boyce *et al.* 2012) [6]. Two virus specified entities, tubules and granular inclusion bodies which are routinely observed in the BTV infected cells (Lecatsas 1968) [12]. The tubular structures are composed entirely of one type of polypeptide that is a gene product of BTV middle-size segment no. 6 (The 64 kDa NS1 protein) (Huismans and Els 1979) [9, 10]. Genetically engineered NS1 antigen was suitable for identifying all BTV serotypes in US (Urakawa and Roy 1988) [24]. Detailed information on this gene may useful for characterizing the strain, genotyping and development of diagnostic kit. In the present study, NS 1 gene based phylogenetic analysis of Bluetongue virus serotype 2, 9 and 15 was studied.

Materials and Methods
The BTV serotype 2, 9 and 15 were serotyped at serotyping Centre, All India Net Work Project on Bluetongue disease, Haryana Agricultural University, Hissar was used in this study. Bluetongue virus was passaged five times in BHK_{21} cell lines. The RNA was isolated from BTV infected cell cultures by Acid Phenol method (Chomczynski and Sacchi (1987) [8].

The quality of isolated RNA was checked by agarose gel (1% w/v) electrophoresis. NS 1 gene was amplified by using the set of primers described by OIE (2018) [17]. The primers were NS 1 F 5' GTTCTCTAGTTGGCAACCACC 3' and NS 1 R 5' AAGCCAGACTGTTTCCCGAT3'. The product size was 274 bp. For cDNA synthesis RNA mix was prepared by adding 1µl primer A (20 pmol/µl) and 1µl Primer B (20 pmol/µl) to 8 µl of isolated RNA. The mix was heated at 68^0C for 5 min. and snap cooled on ice. cDNA mix was prepared by adding 4.0µl of 5XRT buffer, 1 µl of DTT(0.1M), 0.5 µl of RNase out(40U/ µl), 1.0 µl of AMV RT(15U/ µl), 2.0µl of dNTPs and 1.5 µl of ultra pure water. cDNA mix was added to RNA mix, incubate at 25^0c for 10min and 42^0C for 60min then kept at 4^0c. cDNA of NS 1 gene was amplified by Polymerase chain reaction as per the protocol of OIE (2018) [17] with some modifications. The PCR mix consisted 5µl of 10X PCR buffer, 5µl of Magnesium chloride (25mM), 1µl of 10mM dNTPs, 1µl of taq DNA polymerase (5U/µl), 28 µl of Nuclease free water, 10 µl of cDNA. The PCR was carried out by thermal cycler machine (Thermo scientific). The cycling conditions were initial denaturation at 95^0Cfor 3min, 30 cycles of 95^0C/25sec,58^0C/20sec primer annealing and 72^0C/30min extension followed by final extension of 72 ^{0}C for 5min. After completion of the cycles the PCR product obtained was subjected to 1% agarose gel electrophoresis. PCR product was purified with QIA quick gel extraction kit (cat no. 28704) as per the manufacturer's instructions with suitable modifications. Purified PCR product was sequenced at M/s Bioserve Biotechnologies Ltd, Hyderabad, using PCR sequencing method in Beckman CEQTM 8000 Genetic Analysis System using NS 1 gene specific primers. The sequence of NS1 gene of BTV 2, 9 and 15 isolates obtained after sequencing were then aligned with database sequences using NCBI BLAST (www.ncbi.nlm.nih/gov/blast) to confirm their identity. Further, nucleotide sequence was multiple aligned using CLUSTAL W with 27 different BTV NS 1 gene sequences, obtained from GenBank. All available sequences were loaded in CLUSTAL W (www.ebi.ac.uk/clustalw*)* software to obtain the cladogram.

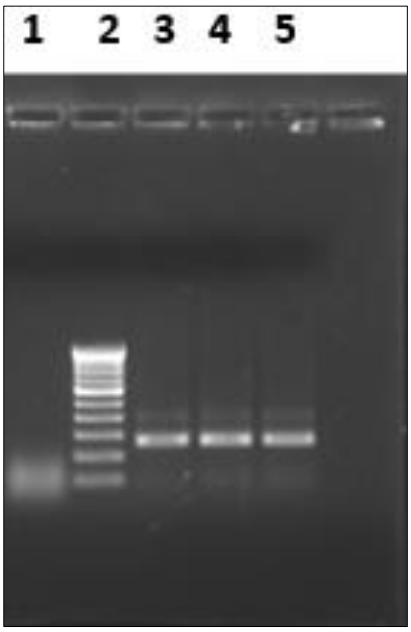
Results and Discussion

Double stranded RNA isolated from BHK-21 cell cultures infected with BTV-2, 9 and 15 serotypes were analyzed by agarose gel electrophoresis and revealed segmented genome pattern with 10 bands including two inseparable bands (Fig 1). Segmented pattern of genome is characteristic feature of BTV nucleic acid (Verwoerd *et al.* 1972; Squire *et al.* 1983 and Roy 1992) [25, 22, 20]. Resolving RNA bands was not clear for two segments. dsRNA needs to be denatured for annealing of primers before synthesis of cDNA. Different methods of denaturation were adopted by Earlier workers. *viz*: Formamide and heat (McColl and Gould, 1994) [15], DMSO and heat (Malik *et al.* 2001) [14] methyl mercuric hydroxide (Potgieter *et al.* 2002) [18]. However Abha (2004) [1] obtained better results using heat (95 ^{o}C) as denaturant instead of DMSO. In the present study RNA was denatured by heating at 68^0C for 5 min. It was found that the amplification of RT-PCR product was superior when denaturation of ds RNA was carried out at 68 ^{o}C for 5 min. For amplification the annealing temperature used was 58 ^{o}C and extension temperature was set to 72 ^{o}C. After 30 cycles of amplification a product of 274bp was observed on 1.8% of agarose gel. The annealing at 60 ^{o}C and extension at 72 ^{o}C was carried out by Aguero *et al.* (2002) [2] and they used 40 cycles for amplification. RT-PCR was standardized for NS1 gene of BTV serotype 2, 9 and 15. A specific band of 274bp was observed (Fig 2) as expected. The sequence of the isolate was compared with available sequences in the database (genbank) using clustalW software.



Lane: 2 100bp ladder
Lane: 3, 4 and 5: BTV 2,9 &15 ds RNA
Lane: 6 BHK21 Cell RNA
Lane 7: 1kb ladder

Fig 1: Migration patterns of the BTV 2, 9 & 15



Lane: 1negative control
Lane: 2 100 bp ladder
Lane: 3,4 & 5 BTV2,9, 15 isolates

Fig 2: NS 1 gene PCR product of BTV 2,9 & 15

The percentage identity between nucleotide and protein sequences of K8 and MBN isolates (BTV-9) was 99% and 100%. Phylogenetic analysis revealed that NS 1 gene sequence of available BTV serotypes can be distributed into 6 clades (Fig 3). The isolates of BTV-2, BTV-9 and BTV-15 are segregated into clade 1(Fig 3). Further analysis of NS 1 gene revealed that the BTV-9 showed relationship of 97% and 95% with nucleotide sequences of BTV-2 and BTV-15 (N12) isolate whereas its translated sequence showed 95% identity

with BTV-2 and 91% with BTV-15 and there was 73% homology between BTV-15 (N12) and BTV-2 (M11). BTV-15 (N12) was closely related to BTV-15 (N15), BTV-15 (DQ399835), BTV-15 China than to BTV-15 Australia and BTV-9 (MBN). Maan *et al.* 2012 [13] observed that Seg-5/NS1 of IND2003/08 showed up to 99% identity with western topotype viruses (prototype600565strain). Bitew *et al.* 2013 [4] revealed that NS1 gene of BTV-3 of USA clusters with the eastern lineage which indicating introduction of western BTV strains and reassortment between eastern and western field strains in India.

Phylogenetic analysis based on NS1 gene sequence of all three isolates revealed their identity of >98% with western topotypes and <90% with eastern topotypes of BTV. It clearly reflects that either the isolates are western topotypes circulating in India or they are reassortants of eastern strains with the segment-5 (ns1 gene) from western topotypes (Pawan Kumar thesis).

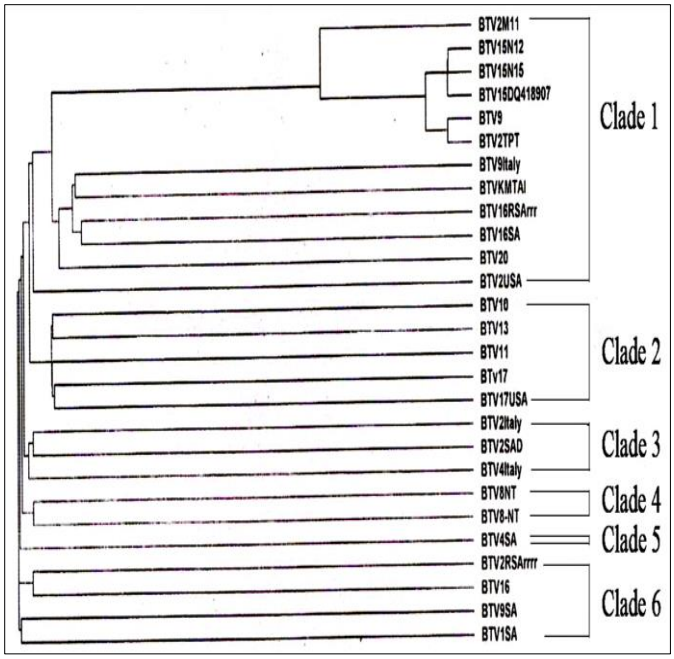


Fig 3: Phylogenetic analysis of NS 1 gene sequence of various BTV isolates

From this study it can be concluded that the BTV-15 (N12) Indian isolate might have been originated from China strain. The present available sequence data is not sufficient to give firm conclusions. Data of other conserved gene of BTV 15 might be more useful in topotying than the VP7 gene. Further the reassortment and genetic drift may be playing an important role in evolution of new BTV strains.

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