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The molecular signature & phylogenetic analysis of RNA binding and major essential inclusion body NS2 protein encoding S8 gene of field strain of *Bluetongue* *virus* from Central India

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Abstract

The *Bluetongue virus* is segmented genome encoding major 7 structural and 3 non structural proteins, out of which the S8 encoding non-structural NS2 is the only protein which is phosphorylated and has major component of virus encoded inclusion bodies. However, the functionality of this protein is still unknown and to decipher it, its characteristics need to be studied more by analysing its genome sequence. From the bluetongue suspected sheep, blood sample was analyzed for nonstructural genes using RT-PCR. Expected amplicons of 860 bp and 967 bp for the S5 (NS1) gene and 1087 bp and 822 bp for NS2 and NS3 genes, respectively were obtained, confirming BTV infection. The S8 gene was sequenced and sequence analysis of the S8 gene was carried out. The sequence analysis revealed that the amplified S8 gene fragment is 1027 bp in length, comprising 54.82% A+T content and 45.18% C+G content, indicating an AT-rich region. The ORF extended from nucleotide position 02 to 1023, with a coding sequence of 1023 bp encoding a protein of 340 amino acids, corresponding to the NS2 protein with an expected molecular weight of approximately 38.627 kDa. The homology search at nucleotide sequence showed 99.03% to 96.0% similarity & 99.03% to 64.95% identity at protein level with global BTV isolates, confirming the identity of the virus and validating the bluetongue outbreak. The phylogenetic analysis of S8 genes indicated clustering with Indian BTV 9 and other BTV 27 across different serotypes, at nucleotide level and placed in Clad IV and exhibits high commonality with the sheep-derived BTV-16, 9 and 4 serotypes suggesting regional evolutionary continuity and circulation of indigenous BTV lineage in Central India. The present study concluded that the RNA binding and major essential Inclusion body encoding NS2 gene of field isolate of *Bluetongue virus* from Central India is A+T rich, conserved in nature, can be used for microevolutionary studies.

Keywords: Bluetongue virus, RT-PCR, S8, NS2 gene, Central India, Phylogenetic analysis

1. Introduction

Blue tongue (BT) is an infectious, non-contagious, and arthropod-borne viral disease of domestic and wild ruminants including sheep, cattle, goats, and wild ungulates like deer caused by *Bluetongue virus* (BTV), which belongs to the genus Orbivirus, family *Reoviridae* and subfamily *Sedoreoviridae*. (Mertens *et al.*, 2009; Singh *et al.*, 2021; Haque *et al.*, 2025) [11, 14, 3]. There are 29 serotype of *Bluetongue virus* (BTV) (Yang *et al.*, 2021) [15]. The *Bluetongue virus* (BTV) is primarily transmitted by *Culicoides* midges, which act as biological vectors (Singh *et al.*, 2021) [14]. *Bluetongue virus* (BTV) possesses a segmented double-stranded RNA genome comprising 10 segments that encode seven structural (VP1–VP7) and four non-structural proteins (NS1, NS2, NS3/NS3a, and NS4) (Mertens *et al.*, 2004) [10].

Among Non structural genes, the non-structural protein NS2, encoded by genome segment 8, plays a crucial role in viral replication and assembly. NS2 is primarily involved in viral RNA packaging and the formation of viral inclusion bodies (VIBs), which serve as sites for viral replication and assembly within infected host cells (Hewat *et al.*, 1994) [4]. It acts as an RNA-

binding protein, selectively interacting with viral single-stranded RNA transcripts, thereby facilitating the assortment and packaging of the viral genome segments into progeny virions. Importantly, NS2 is one of the most highly conserved proteins among different BTV serotypes, including conserved regions such as the N-terminal domain. Due to this high level of conservation, NS2 is capable of inducing cross-reactive cellular immune responses across multiple serotypes (Jiménez-Cabello *et al.*, 2025) [5]. However, despite its immunogenicity, NS2 provides only limited protection against homologous BTV serotypes in natural hosts, indicating that it may not be sufficient alone for effective protective immunity. The conserved nature and binding to the RNA genome and a major component of formation of inclusion bodies make it a valuable target for molecular studies. So to understanding BTV biology and pathogenesis, as well functionality of this protein is still unknown hence the molecular signature of this NS2 protein encoding S8 gene need to be decipher by analysing its genome sequence. Hence the present study was carried out to understand its functionality by sequencing the gene encoding NS2 and analysing its phylogenetic relation with global isolates.

2. Materials and Methods

2.1 Collection of clinical samples

The blood samples were collected during an outbreak in sheep at "Umred" Tq. Umred in Dist. Nagpur, central India. Blood samples were collected from sheep via the jugular vein using 18-gauge needles. From suspected sheeps, 2 ml blood sample was collected in EDTA vials and brought to the laboratory on ice-packed container, with the temperature maintained at 4 °C. Blood samples were stored at 4 °C, while the separated serum was preserved at -20 °C.

2.2 RNA Isolation, cDNA synthesis

Total RNA was extracted from blood samples using TRI Reagent (Sigma-Aldrich, USA) following standard protocols. Briefly, blood samples were homogenized in TRI Reagent, followed by chloroform extraction and isopropanol precipitation. The RNA pellet was washed with 70% ethanol, air-dried, and resuspended in nuclease-free water. Extracted RNA was stored at -20 °C until further use. The cDNA synthesis was performed using the SuperScript™ IV First-Strand Synthesis System (Invitrogen, Cat. No. 18091050, USA), following the manufacturer's instructions. The reaction

was carried out in a total volume of 20 µl, using labeled microcentrifuge tubes and the reagents listed below. The cDNA reaction was comprise of template RNA around 11 µl (approximelty (500 ng), Random hexamer (50ng/µl) 1 µl, 1 µl 10mM dNTP mix (10mM each). The mixture was incubated at 65 °C for 5 minutes in a thermal cycler, followed by immediate chilling on ice for 2 minutes. Subsequently, 4 µl of 5X SSIV Buffer, 1 µl of 100mM DTT, 1 µl of Ribonuclease inhibitor (40U/ µl) and 1 µl of Superscript™ IV Reverse transcriptase(200U/µl)of were added. The cDNA synthesis reaction was carried out in cyclic condition of 23°C for 10 minutes, 50°C for 30 minutes and 80°C for 10 minutes. the reaction was cooled at 4°C and used for the PCR amplification.

2.3 PCR Amplification and confirmation and sequencing:

The PCR reaction was carried out in using the published primers S5, S8 and S10 gene specific forward primers and revers primers depicted in tables no.1. The PCR reaction was carried out in 50 ul PCR tune containing 5 µl of 10X PCR Buffer; 3 µl of MgCl₂ (50mM); 1 µl of dNTP (10mM); 2µl of forward and reverse primer (10 pmol/µl) each; 0.3 µl of Taq DNA polymerase (5U/µl); 3 µl of cDNA and Nuclease Free Water was added to make the volume 50 µl. For the NS10 gene the cyclic reaction was carried out in Prima-Trio thermal Cycler (Hi-Media, India) of cyclic conditions as initial denaturation 95°C for 2 min, followed by 3 step cycling for 35 cycles at denaturation for 95°C-20 sec; annealing- 52°C for 1 min, extension 72°C for 1 min and the final extension at 72°C for 10 min. whereas as for the BTV NS1 and NS2 gene amplification the initial denaturation 94°C for 2 min, followed by 3 step cycling for 10 cycles at Denaturation for 94°C-20 sec; Annealing- 45°C for 1 min, Extension 72°C for 1.30 minutes followed by 3 step cycling for 30 cycles of denaturation for 94°C-20 sec; Annealing at 50°C for 1 min, Extension 72°C for 1.30 minutes. the post cyclic final extension of 72°C for 10.00 minutes was carried out. The amplicons were analysed on 1.4% agarose containing the 0.5 ug/ml of ethidium bromide (SIGMA) in 0.5X Tris borate-EDTA buffer (TBE) at 12V/cm of gel in 0.5X TBE running buffer in submarine electrophoresis apparatus (Himedia, India) and power supply (LA960, Electrophoresis power supply, Himedia, India). Bands were visualized under a UV transilluminator and documented using a gel documentation system (BioZen Laboratories, India).

Table1: Primers sequences used in the present study

Primers	Primer sequence	Amplicon size	Reference
BTV-NS1-F1-13	TAG TTG GCA ACC ACC AAA CA	847bp	Gharat <i>et al.</i> , 2022 ^[2]
BTV-NS1-R1-860	TAT GGT GCA ACG GAA ACT CA		
BTV-NS1-F2-724	ATG ATT TCG CGA AGC ATT TT	967bp	
BTV-NS1-R2-1691	CAT CCA CAT CTG AGA CAT GC		
BTV-NS2-F-19	CAT GGA GCA AAA GCA ACG TA	1087bp	
BTV-NS2-R-1106	CTA ACC ACG CGG TCA CAA G		
BTV-S10-F-7	AAG TGT CGC TGC CAT GCT AT	725bp	
BTV- S10-F- 732	GGG CGC AAC TCT ACC TAC TG		

The PCR amplicon of the NS2 encoding gene S8 genes was subjected to bidirectional sequencing using commercial services (Eurofins Genomics, India). The obtained sequences were analyzed using bioinformatics tools. Sequence editing and trimming were performed using Chromas software, followed by homology searches using BLASTn. Consensus sequences were generated and analyzed for open reading

frames (ORFs). Phylogenetic analysis was carried out using MEGA software employing Neighbor-Joining and Maximum Likelihood methods with appropriate substitution models and bootstrap analysis. Global BTV sequences retrieved from GenBank were used for comparative analysis to determine genetic relationships and evolutionary patterns.

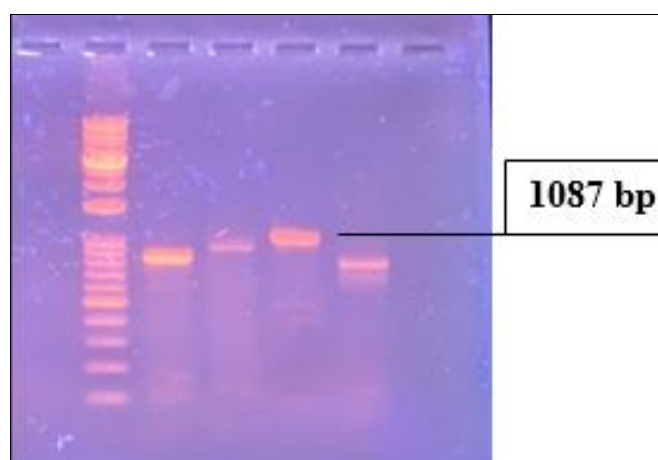
3. Results and Discussion

In the present study, amplification of the S5 (NS1) gene yielded bands of 860bp and 967bp. The S8 (NS2) and S10 (NS3) genes yielded distinct amplicons of 1087bp and 822bp, respectively. These results indicate that the all three non-structural coding gene of BTV viz S5, S8, and S10 genes were successfully amplified from blood sample, as confirmed by agarose gel electrophoresis (Fig. 1). The particular amplicon sizes of 1087bp which correspond to coding sections of the NS2 (S8) genes, demonstrated the high specificity and efficiency of the chosen primers for BTV genome identification.

Identification of BTV by S8 was performed through BLASTn analysis of the consensus sequence showed 99.03% identity with *Bluetongue virus* serotype 26 (Accession No. KP339211) and 99.03% to 96.0% similarity with global BTV sequences available in GenBank across 100 BLAST hits, confirming the identity of the virus and validating the bluetongue outbreak (Supplementary data). Similar high nucleotide identity ranges during BTV molecular characterization have been reported in earlier studies, demonstrating the reliability of segment-based molecular detection for confirmation of BTV infection (Maan *et al.*, 2007; Rao *et al.*, 2012) [9, 12]. The curated forward and reverse sequences were assembled to generate a contig, from which a consensus sequence was derived and designated as *BTV/S5/NAGPUR/MH/India/sheep/2025* (Accession No. PZ163108). Analysis of the sequence revealed that the amplified S8 gene fragment was 1027 bp in length, comprising 54.82% A+T content and 45.18% C+G content, indicating an AT-rich region. The ORF extended from nucleotide position 02 to 1023, with a coding sequence of 1023 bp encoding a protein of 340 amino acids, corresponding to the NS2 protein with an expected molecular weight of approximately 38.627 kDa. Similarly in another study, Seg-8 found to be 1121 bp long encoding the 353 aa of the viral inclusion body (VIB) matrix protein (Maan *et al.*, 2011) [8]. In another study, Segment 8 of BTV2/KM/2003 and BTV12/PT/2003 encodes a conserved 354 aa NS2 protein and shows higher genetic relatedness to eastern BTV strains than to western strains (Yin *et al.*, 2008) [16]. Protein BLASTp analysis demonstrated 97.94% identity with *Bluetongue virus* serotype 23 (Accession No. AGJ83628.1) and 99.03% to 64.95% identity with complete protein sequences of 100 global BTV isolates representing different serotypes.

The phylogenetic analysis of current isolate BTV/NS2/NGP-MS/2025/IND (Accession No. PZ163108) showed, it clustered into Clade IV with other previously reported *Bluetongue virus* isolates from India and other regions, based on the nucleotide phylogenetic analysis based on the S8 gene (Fig. no. 2). The isolate's S8 sequences of BTV serotypes reported from India, such as KP696658/NS2/BTV9/2009/INDIA and KX302641/NS2/BTV27/GOAT/2014/F, demonstrated close genetic relatedness. The clustering pattern shows that the S8 gene may be genetically conserved among circulating regional strains and that the current isolate has a common evolutionary origin with them. In another study, Phylogenetic analysis places these viruses in a distinct monophyletic group with Asian, Australian, and some Eurasian isolates, clearly separated from African, American, and European BTV strains, supporting their classification within the eastern topotype lineage (Yin *et al.*, 2008) [16]. The strong bootstrap support at this node makes the clustering more reliable (Fig. no. 3). Overall, the phylogenetic analysis confirms that the current research isolate belongs to the Clade IV branch of

BTV NS2 gene sequences and demonstrates its genetic relatedness to previously circulating Indian BTV strains. In another study, Seg-8 sequence analysis shows that Indian BTV isolates segregate into distinct eastern and western topotypes. Most Indian BTV serotypes cluster within the eastern topotype, while some isolates, particularly certain BTV-2 and BTV-10 strains, group within the western topotype, displaying very high nucleotide identity with vaccine strains from South Africa and prototype strains from the USA (Maan *et al.*, 2015) [7]. The current isolate BTV/NS2/NAGPUR/MH/2025/India showed a substantial clustering with worldwide reference serotypes, according to phylogenetic analysis based on the inferred amino acid sequences of the NS2 protein (Fig. 3). The investigation specifically showed that the isolate is located in Clade IV and exhibits high commonality with the sheep-derived BTV-16, 9 and 4 strains (namely, ANK78272/NS2/BTV16/SHEEP/2014 from Tamil Nadu, AKP24275/NS2/BTV9/2055/IND from Haryana, and AIK27566/NS2/BTV4/SHEEP/2008/I from Andhra Pradesh), particularly sheep origin isolates, suggesting regional evolutionary continuity and circulation of indigenous BTV lineage. Similar findings have been reported in earlier molecular epidemiological studies, where Indian BTV isolates showed geographical clustering and lineage-specific evolution due to endemic circulation and vector-host adaptation (Maan *et al.*, 2012) [6]. The close relationship of the present isolate with historical Indian strains indicates possible continuous viral evolution within the country rather than recent introduction from external geographical regions (Saminathan *et al.*, 2020) [13]. Previous molecular studies have also demonstrated that Indian BTV isolates often group according to topotype and regional circulation patterns (Maan *et al.*, 2015) [7]. The separation of the study isolate from other serotype clusters such as BTV12, BTV25, and BTV27 further supports serotype-specific genetic divergence within the S8 gene segment. NS2 is known to be relatively conserved but still provides sufficient resolution for studying evolutionary relationships and molecular epidemiology of BTV strains (Butan *et al.*, 2010) [1]. Overall, the findings of the present study are consistent with earlier reports suggesting continuous circulation and evolution of indigenous BTV strains in India, highlighting the importance of molecular surveillance for understanding virus evolution, outbreak tracing, and vaccine strategy planning



(Lane 1- DNA Ladder (1kbp Cat # MB011-R500, BIOCHEM, INDIA); Lane 2- Segment 5 (860bp); Lane 3- Segment 5 (967bp); Lane 4- Segment 8 (1087bp); Lane 5- Segment 10 (822bp))

Fig 1: Agarose gel electrophoresis showing S5, S8, S10 gene specific product by RT-PCR

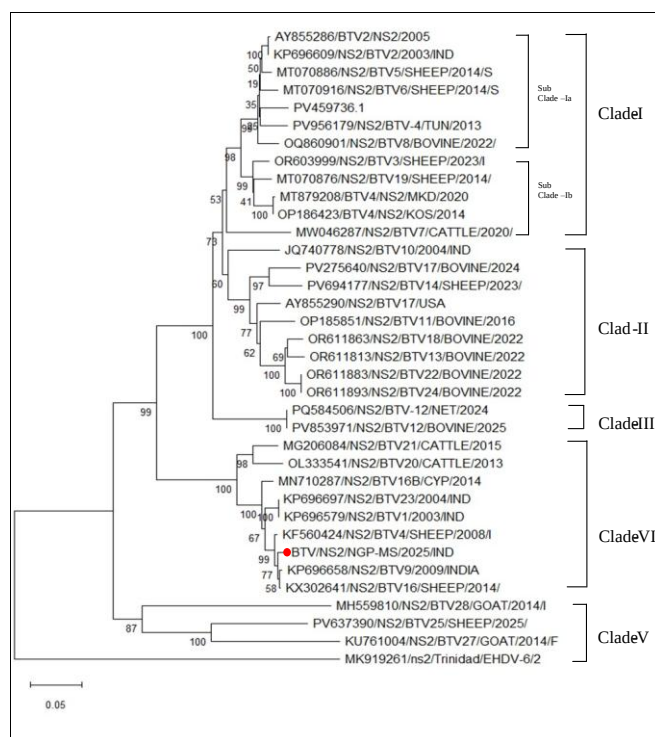


Fig 2: Molecular phylogenetic analysis of nucleotide sequences of S8 (NS2) genes by Neighbour-Joining method using MEGA 12

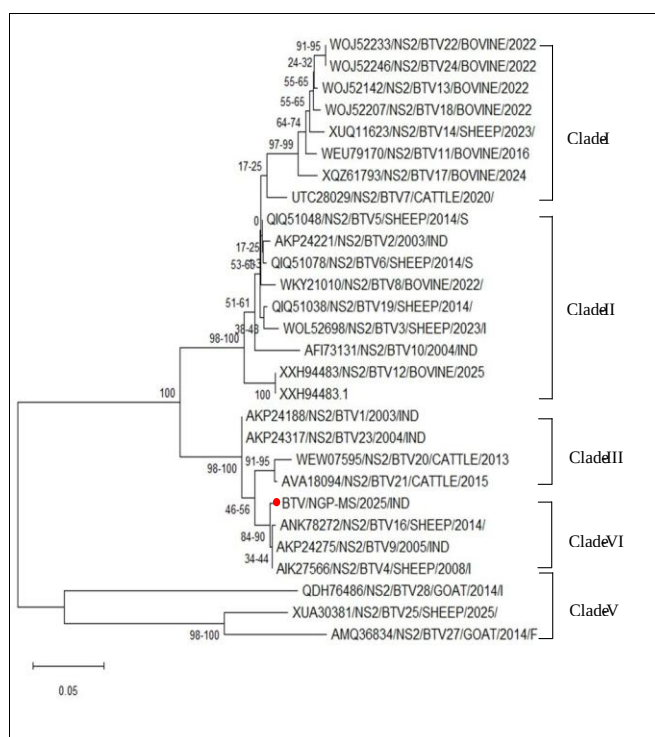


Fig 3: Molecular evolutionary relationship of BTV based on NS2 protein sequence using Maximum Likelihood method using MEGA 12

4. Conclusion

The present study concludes that RNA binding and major essential Inclusion body NS 2 protein encoding S8 gene of field strain of *Bluetongue virus* from Central India is A+T rich, due to its conserved nature and genetic stability, can be effectively utilized for molecular detection, genetic characterization, and AT rich, conserved in nature, can be used for microevolutionary studies.

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Conflict of Interest

Not available

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Not available

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