



## Genetic and antigenic characterization of bluetongue virus serotype-1 isolated from goat in India

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### ABSTRACT

Bluetongue virus (BTV) affects domestic and wild ruminants and is transmitted by *Culicoides* spp. In the present study, genetic and antigenic characterization of the BTV-1 serotype (MKD20/08/Ind isolate) isolated from goat was carried out to understand the phylogenetic relationship and molecular epidemiology of bluetongue. Full genome sequencing of BTV-1 isolate was carried out using next-generation sequencing (NGS). The sequence data was analyzed and the phylogenetic relationship of the goat isolate with other BTV-1 was established. Phylogenetic analysis grouped the MKD-20/08/Ind isolate within BTV serotype 1, with a high level of nucleotide sequence identity (>99%) to the previous BTV-1, confirming its serotype. The phylogenetic tree constructed from the outer capsid protein genes, seg-2 and seg-6, segregated MKD-20/08/Ind with other Indian BTV-1 isolates reported earlier. All BTV genome segments were found to be evolving under strong purifying (negative) selection. The mean substitution rate of different segments of BTV-1 isolates varied from  $1.663 \times 10^{-2}$  (seg-8) to  $3.949 \times 10^{-3}$  (seg-1) substitutions per site per year. The time to the most recent common ancestor (tMRCA) indicates all the genome segments of BTV-1 might have an older ancestor. The phenotypic antigenic relationship (r) of the MKD20/08/Ind isolate with other BTV-1 isolate-specific hyperimmune serum (HIS) determined by serum neutralization test (SNT) was 0.44 to 0.80. The neutralization behaviours of the MKD20/08/Ind with other BTV-1 isolate-specific HIS suggest probable minor subtype antigenic variation. Full-genome sequencing and phylogenetic analysis of BTV-1/MKD-20/08/Ind isolate indicate ancestral relationships amongst the Indian and Mediterranean BTV-1 isolates.

**Keywords:** Antigenic relationship, Bluetongue virus, BTV-1 serotype, Goat, Next generation sequencing, Phylogeny

Bluetongue is an arthropod-borne disease of domestic and wild ruminants that is caused by the bluetongue virus (BTV). Goats and cattle are the most common asymptomatic reservoir of the virus (MacLachlan 1994). Bluetongue virus is the prototype species of *Orbivirus* genus in the family Reoviridae. Currently, 29 distinct serotypes of BTV have been identified worldwide (Bumbarov *et al.* 2020). Bluetongue is endemic in India and at least 23 serotypes have been identified based on serology and/or virus isolation (Mahapatra *et al.* 2022). The BTV genome consists of ten linear segments (seg-1 to seg-10) of double-stranded (ds) RNA genome which codes for seven structural (VP1-VP7) and five non-structural proteins (NS1, NS2, NS3/NS3A, NS4 and NS5) (Stewart *et al.* 2015). The outer capsid protein of BTV is composed of VP2 and VP5 encoded by segments 2 and 6, respectively. The VP2 is a

highly variable protein that determines virus serotype and is the primary target of neutralizing antibodies (Hofmann *et al.* 2008). The evolution of the BTV in a confined geographical territory of Asia, Africa and America led to 'topotype' based diversity of the BTV genome (Reddy *et al.* 2018). The serotype and topotype variation along with the re-assortment of the segmented genome has a huge impact on the evolution and epidemiology of BTV (Nomikou *et al.* 2015). The full genome sequencing of BTV will be the basis for its characterization and molecular epidemiology and for understanding genetic re-assortments among different topotypes (Maan *et al.* 2015). The present study describes the full genome sequencing and phylogenetic analysis of BTV-1/MKD-20/08/Ind isolate from a goat and its relationship with other global isolates. Further, the neutralization behavior of the BTV-1 was studied *ex vivo* to find the phenotypic antigenic relationship of virus.

### MATERIALS AND METHODS

Bluetongue virus serotype-1 isolate MKD20/08/Ind obtained from the BTV repository of the Division of Virology, ICAR-Indian Veterinary Research Institute, Mukteswar, India, was propagated in the BHK-21 cell

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line in Glasgow minimum essential medium (Sigma-Aldrich USA) supplemented with fetal bovine serum (Gibco®, USA). The virus was isolated earlier in BHK-21 cell line from a blood sample of goat received from Mathura, Uttar Pradesh, India, in 2008. The isolate was used for the generation of full genome sequences and to study neutralization behaviour. Hyperimmune serum (HIS) against BTV-1 isolates, viz. MKD18/08/Ind, MKD25/08/Ind, SKN7/07/Ind, SKN8/07/Ind, Avikanagar/94/Ind, Hisar/85/Ind, Chennai/03/Ind, KDP15/07/Ind, NLG3/07/Ind, and NRT37/07/Ind available in the BTV serum repository, were used to study the neutralization behaviour of MKD20/08/Ind.

Viral dsRNA of MKD20/08/Ind, was extracted using Trizol reagent (Sigma-Aldrich USA) and subsequently purified by lithium chloride precipitation as per the method described earlier (Attoui *et al.* 2000). The purified dsRNA was used to generate full genome sequence data through next-generation sequencing (NGS) using the Ion Torrent platform. The full-length cDNA library was constructed from purified dsRNA using the Anlon Xpress Plus Fragment Library Kit according to the manufacturer's protocol (Life Technologies). After multiplexing with different bar-coded adaptors, each library was sequenced using an Ion PI v2 chip (Life Technologies). To establish the full genome, overlapping sequences were assembled with the Ion Torrent server by mapping closely related reference sequences. The mapped file was viewed with an Integrative Genomics Viewer. The average coverage depth was 127.7 and genome base coverage at 20× was 95.91%. The identity of the sequences was confirmed by NCBI BLASTN (<http://www.ncbi.nlm.nih.gov>) analysis. Segment length and the coding sequences (ORFs) of the genomic segments were analysed using the DNASTAR software package. The full genome nucleotide (nt) sequences (segments 1–10) of BTV-1, MKD-20/08/Ind were submitted to GenBank with accession numbers KU234257 to KU234266 corresponding to segments 1 through 10.

Reference sequences of BTV-1 were retrieved from BTV-GLUE and GenBank and multiple alignments were carried out using the CLUSTALW algorithm (Thompson *et al.* 1994). Percentage identity calculation and phylogenetic tree construction were done using MEGA software v. 11 (Tamura *et al.* 2013). The model selection module available in MEGA software was used to find the best-fit substitution model using the Bayesian Information Criterion (BIC). The evolutionary history was inferred using the Maximum Likelihood (ML) method and the bootstrap consensus tree inferred from 500 replicates. Bayesian phylogenetic analyses of different segments of BTV-1 were performed using BEAST v 1.10.4 with the BEAGLE library and different substitution clock and population evolution models were evaluated by estimating their marginal likelihoods using Akaike's Information Criterion for MCMC samples (AICM). A relaxed uncorrelated exponential distribution clock (UCED) and exponential demographic model were employed to infer the evolutionary rate and time to most recent common ancestors (TMRCA). MCMC sampling was run for 150 million generations, with trees and posteriors sampled every 10<sup>4</sup> steps. The convergence of all parameters was verified visually using Tracer 1.7. The posterior tree distributions were summarized using Tree Annotator. Statistical uncertainties in the data are reflected by the 95 % highest probability density (HPD) values.

The neutralization behaviour of MKD20/08/Ind was studied *ex-vivo* using the beta method of serum neutralization test (SNT) with ten BTV-1 isolate-specific HIS. The neutralization titre was calculated using the Reed and Muench method (Reed and Muench 1938) and mean anti-log values of titers were used for calculation of antigenic relationship (r) as described earlier (Biswas *et al.* 2015).

## RESULTS AND DISCUSSION

BTV serotypes are present throughout the world, and

Table 1. Segment size, lengths of coded proteins, dN/dS ratio estimated for different segments and other details of BTV-1/MKD-20/08/Ind

| Genome segment<br>(Protein coded) | Sequence<br>length (bp) | Gene bank<br>accession no. | Coding<br>sequence<br>Length | Protein<br>length | dN/dS  | Number of sites<br>under pervasive<br>selection | Number of sites<br>under episodic<br>selection | Number of<br>sites under<br>selection |
|-----------------------------------|-------------------------|----------------------------|------------------------------|-------------------|--------|-------------------------------------------------|------------------------------------------------|---------------------------------------|
| 1 (VP1)                           | 3944                    | KU234257                   | 3909                         | 1302              | 0.0260 | 0                                               | 12                                             | 241 (6.2%)                            |
| 2 (VP2)                           | 2940                    | KU234258                   | 2886                         | 961               | 0.0787 | 0                                               | 16                                             | 182 (6.3%)                            |
| 3 (VP3)                           | 2772                    | KU234259                   | 2706                         | 901               | 0.0064 | 0                                               | 3                                              | 211 (7.6%)                            |
| 4 (VP4)                           | 1981                    | KU234260                   | 1935                         | 644               | 0.0411 | 0                                               | 9                                              | 116 (5.9%)                            |
| 5 (NS1)                           | 1765                    | KU234261                   | 1659                         | 552               | 0.0414 | 0                                               | 6                                              | 136 (8.2%)                            |
| 6 (VP5)                           | 1635                    | KU234262                   | 1581                         | 526               | 0.0247 | 0                                               | 6                                              | 82 (5.2%)                             |
| 7 (VP7)                           | 1154                    | KU234263                   | 1050                         | 349               | 0.0055 | 0                                               | 2                                              | 79 (7.5%)                             |
| 8 (NS2)                           | 1125                    | KU234264                   | 1065                         | 354               | 0.0854 | 0                                               | 2                                              | 67 (6.3%)                             |
| 9 (VP6)                           | 1052                    | KU234265                   | 993                          | 330               | 0.263  | 0                                               | 7                                              | 40 (4.0%)                             |
| 9 (NS4)                           |                         |                            | 234                          | 77                |        |                                                 |                                                |                                       |
| 10 (NS3)                          | 822                     | KU234266                   | 690                          | 229               | 0.0212 | 0                                               | 1                                              | 33 (4.8%)                             |
| 10 (NS3a)                         |                         |                            | 651                          | 218               |        |                                                 |                                                |                                       |
| 10 (NS5)                          |                         |                            | 180                          | 59                |        |                                                 |                                                |                                       |

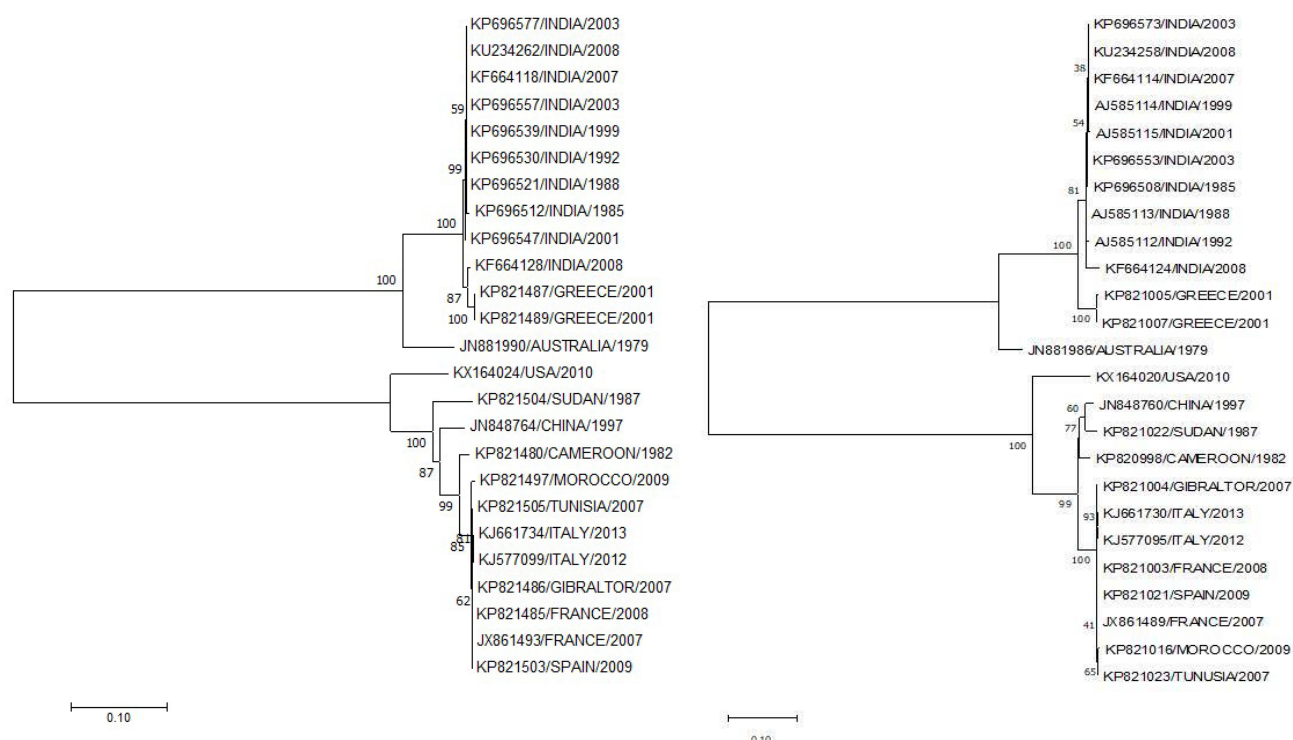


Fig. 1. Phylogenetic tree constructed by maximum likelihood (ML) analyses of BTv-1 MKD-20/08/Ind isolate using the complete coding region of the outer capsid protein genes segment-2 (GenBank Acc No/KU234258) and segment-6 (GenBank Acc No/KU234262). Isolate abbreviations: GenBank Acc No/country of origin/year of isolation; A. ML tree based on the seg-2 depicting the phylogenetic relationship with the global BTv-1; B. ML tree based on the seg-6 depicting the phylogenetic relationship with the global BTv-1.

new strains are generated through mutation and recombination. Therefore, full genome sequencing of BTv isolates is important to deduce relationships between serotypes and topotypes and to understand the virus's movement between different regions. Whole genome sequencing and phylogenetic analyses, along with neutralization behaviour, are key components of the characterization and molecular epidemiological studies of BTv. Phylogenetic studies give an idea about the topotype identity of each genome segment of BTv, which provides information regarding the evolution and stability of the virus in the ecosystem.

In the present study, author's report the genetic and antigenic characterization of BTv-1 (MKD-20/08/Ind) isolated from goat. The segment size, lengths of coded proteins, and other details of BTv-1/MKD-20/08/Ind are provided in Table 1. The isolate has the typical segment size reported for BTv-1 earlier (Boyle *et al.* 2014).

There was no evidence of intragenic recombination events in sequences as revealed from SimPlot, the RDP4 software package, or GARD analyses. Segments 2 and 6 code for serotype-specific proteins VP2 and VP5, respectively, and the serotype can be assigned based on the sequences of these segments, especially that of segment 2 (Maan *et al.* 2007). Conclusively, analyses of serotype-determining segments (Seg-2 and Seg-6) have grouped BTv-1/MKD-20/08/Ind within serotype 1, with a high level of nucleotide sequence identity (>99%) to the previous BTv-1, confirming its serotype. The phylogenetic tree constructed from the outer

capsid protein genes, seg-2 and seg-6, segregated BTv-1/MKD-20/08/Ind with other Indian BTv-1 isolates reported earlier. The cluster of Indian BTv-1 isolates was found in close proximity to the viruses reported from Greece. Viruses reported from Europe, Africa, China, Russia and America were found to be distantly related to Indian isolates. (Fig. 1 A-B). The percentage nucleotide identity of the VP2 gene (Seg-2) of MKD-20/08/Ind with other Indian isolates was 97.8–100%. In addition to it, the VP2 region shared high sequence homology (95.7–96.1%) with isolates from Greece collected in 2001. Similarly, MKD-20/08/Ind has a high sequence identity (99–100%) with other BTv-1 isolates of Indian origin.

Phylogenetic analysis showed that BTv-1/MKD-20/08/Ind contains genome segments derived mainly from Eastern lineages, as all the segments except Seg-5 have been grouped within the Eastern topotype along with viruses from Australia and Greece. The establishment of Segment 5 of Western origin in India, as evidenced by the closely related sequences of this segment with those of isolated viruses in the country, was also reported earlier (Rao *et al.* 2013). The phenomenon of re-assortment has repeatedly been seen in isolates collected post-1982, especially for Seg-5 (NS1). It has been postulated that the western NS1 contributes to enhanced transmission of the virus. BTv is classified into African, American, Australian, and Mediterranean topotypes based on geographical distribution (Rao *et al.* 2017). BTv-1 (MKD-20/08/Ind) isolate shares maximum nucleotide and amino acid sequence identity with viruses

Table 2. Estimates of the substitution rate and the tMRCA for the genome segments of BTV1 by Bayesian coalescent method

| Segment | No of sequences | Date range | Correlation coefficient | Substitution rate (95% HPD)                                                   | TMRCa (95% HPD)               |
|---------|-----------------|------------|-------------------------|-------------------------------------------------------------------------------|-------------------------------|
| 1       | 25              | 1979-2013  | 0.566                   | $2.005 \times 10^{-3}$ ( $4.903 \times 10^{-4}$ and $3.754 \times 10^{-3}$ )  | 1717.1 (1354.4 and 1939.8)    |
| 2       | 25              | 1979-2013  | 0.429                   | $3.749 \times 10^{-3}$ ( $1.399 \times 10^{-3}$ and $6.318 \times 10^{-3}$ )  | 1877.54 (1757.34 and 1960.45) |
| 3       | 25              | 1979-2013  | 0.406                   | $3.106 \times 10^{-3}$ ( $1.086 \times 10^{-3}$ and $5.107 \times 10^{-3}$ )  | 1895.86 (1804.61 and 1958.67) |
| 4       | 25              | 1979-2013  | 0.417                   | $2.605 \times 10^{-3}$ ( $8.655 \times 10^{-4}$ and $4.65 \times 10^{-3}$ )   | 1878.62 (1758.67 and 1964.68) |
| 5       | 25              | 1979-2013  | 0.704                   | $2.752 \times 10^{-3}$ ( $1.022 \times 10^{-3}$ and $5.128 \times 10^{-3}$ )  | 1928.09 (1865.44 and 1976.46) |
| 6       | 25              | 1979-2013  | 0.388                   | $1.907 \times 10^{-2}$ ( $7.161 \times 10^{-3}$ and $3.12 \times 10^{-2}$ )   | 1886.71 (1797.95 and 1954.40) |
| 7       | 25              | 1979-2013  | 0.358                   | $6.226 \times 10^{-2}$ ( $1.1720 \times 10^{-2}$ and $1.109 \times 10^{-1}$ ) | 1292.84 (553.61 and 1802.05)  |
| 8       | 25              | 1979-2013  | 0.424                   | $1.663 \times 10^{-2}$ ( $5.504 \times 10^{-3}$ and $2.87 \times 10^{-2}$ )   | 1881.99 (1755.95 and 1964.91) |
| 9       | 25              | 1979-2013  | 0.376                   | $4.748 \times 10^{-2}$ ( $7.521 \times 10^{-3}$ and $9.09 \times 10^{-2}$ )   | 1607.34 (1130.13 and 1920.77) |
| 10      | 25              | 1979-2013  | 0.446                   | $1.177 \times 10^{-3}$ ( $4.353 \times 10^{-4}$ and $1.797 \times 10^{-3}$ )  | 1914.94 (1841.89 and 1970.96) |

of Mediterranean topotypes, indicating the probable origin of the virus from the Mediterranean basin.

All BTV genome segments were found to be evolving under strong purifying (negative) selection, as none of the sites were found to be under positive selection pressure. On the other hand, 4–8% of the codon positions in different segments were found to be under negative selection pressure (Table 1). The dN/dS ratio estimated for different segments was also found to be less than 1, supporting the claim that negative selection plays a major role in shaping evolution. However, episodic positive selection appears to play a significant role in shaping evolution, as several codon positions were found to be under episodic selection pressure. Many codons experience purifying selection for the majority of their evolution, with bursts of strong positive selection within particular lineages, and mutations at such sites may experience transient positive selection, followed by purifying selection to maintain the change, and likely play a key role in adaptive evolution (Murrell *et al.* 2012). Among all segments, the dN/dS ratio for segment-9 coding for VP6 was found to be higher than for other segments.

The evolutionary rate and time to the most recent common ancestor (tMRCA) of BTV segments determined by using a relaxed clock model under the Bayesian coalescent framework are provided in Table 2. The mean substitution rate of different segments of BTV-1 isolates varied from  $1.663 \times 10^{-2}$  (seg-8) to  $3.949 \times 10^{-3}$  (seg-1) substitutions per site per year. Apparently, the substitution rate observed in this study for BTV-1 serotype alone is higher than those observed for BTV in general (Boyle *et al.* 2014). The estimates of tMRCA indicate all the genome segments of BTV-1 might have an older ancestor.

Phenotypic variations of BTV and neutralization resistance phenotypes within the serotype due to nt sequence differences in VP2 protein are reported (Bonneau *et al.* 2001). Neutralization-resistant phenotypes among the isolates of BTV serotypes 1 has been also reported earlier (Biswas *et al.* 2015). The phenotypic antigenic relationship (*r*) of BTV-1/MKD-20/08/Ind against a total of 10 isolate-specific HIS was studied. The mean *r* values ( $r_{\text{mean}}$ ) of the Indian MKD-20/08/Ind isolate ranged from 0.44 to 0.80. Comparatively moderate magnitudes

of neutralization resistance to most of the isolate-specific heterologous HIS were observed with Chennai/03/Ind (*r* = 0.44) and NRT37/07/Ind (*r* = 0.54). Except for these two isolates specific to HIS, all other Indian BTV-1 isolates specific to HIS showed strong antigenic relationships where the  $r_{\text{mean}}$  ranged from 0.61 to 0.80. The neutralization behaviours of the Indian BTV-1 isolate with the isolate-specific HIS suggest probable minor subtype antigenic variation. The HIS against BTV-1 isolates (Chennai/03/Ind and NRT37/07/Ind) recovered from the southern part of the country showed minor subtype antigenic variation.

In conclusion, genetic and antigenic characterization of BTV-1/ MKD-20/08/Ind isolate indicates ancestral relationships amongst the Indian and Mediterranean BTV-1 isolates. The time to the most recent common ancestor (tMRCA) points towards all the genome segments of the BTV-1 isolate might have an older ancestor. The antigenic characterization of goat isolate with the other BTV-1 isolate-specific HIS suggest minor subtype antigenic variation. Further studies targeting the vector biology and genetic characterization along with the phenotypic antigenic relationship will explain the molecular epidemiology of bluetongue in this northern region of the country and will help in effective control strategies.

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