ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (12) 635 - 641
doi: 10.30466/vrf.2023.1990376.3796
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Co-infection of bluetongue virus serotypes 12 and 16 in sheep from Haryana, India

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Article Info

Article history:

Received: 25 February 2023
Accepted: 17 June 2023
Available online: 15 December 2023

Keywords:

Bluetongue virus
Dual infection
Goat
Molecular diagnosis
Real time RT-PCR

Abstract

World Organization for Animal Health has listed bluetongue (BT) under notifiable diseases. The BT is an arboviral infectious disease of domestic and wild ruminants caused by the bluetongue virus (BTV). Southern states of India had remained the point of attention for BT since first presence in 1964 in Maharashtra. Recently, northern states of India have also been reported positive for BTV in small ruminants. The present study reported the dual infection of BTV serotypes, BTV-12 and -16 in sheep population from Sirsa district of Haryana in the year 2016. After detection and serotyping with Seg-2 specific real time polymerase chain reaction (PCR), the Seg-2 and Seg-6 of BTV were PCR amplified and sequenced. On phylogenetic analysis it was detected to be clustered in nucleotype G and nucleotype B specific for BTV-12 and BTV-16, respectively. This was the first report of BTV-16 from Haryana. The results signified the co-infection of two different serotypes in an animal from a single outbreak.

Introduction

Small ruminants like sheep and goats are domesticated by less affluent farmers for milk, wool, meat etc. as their main source of livelihood. Bluetongue (BT) is a notifiable disease listed by World Organization for Animal Health.[1] It is an economically significant infectious arboviral disease of small domestic and wild ruminants caused by the bluetongue virus (BTV). Prominently sheep and white-tailed deer (particularly of North America) are severely affected by the disease.[2] The BTV segmented double-stranded RNA viruses (dsRNA viruses) belongs to the genus *Orbivirus* of family *Reoviridae*. This virus has immense potential to alter its genome by various biological means like point mutations, recombination and reassortment. This has led to extremely diverse genetic pool that has resulted 28 distinct BTV serotypes and recently three novel putative BTV serotypes were identified.[2-6] Furthermore, BTV has multiple strains, nucleotypes, topotypes, ressortants within each serotypes depending on the sequences of individual segments.[4,7,8] By virtue of so much complexity, BTV easily befools host immune system, as immunity is not cross protective among different serotypes.[9] The great diversity has spread the BTV initially only in African countries but subsequently all parts of the globe except Antarctica.[10]

Bluetongue outbreaks have been more commonly seen in southern states (Andhra Pradesh, Tamil Nadu, Karnataka etc.) of peninsular India after the first incidence of BT in Maharashtra in 1964.[10,11] Different serotypes and exotic BTV strains have been found circulating in host animal population in the peninsular region.[11-18] Northern states of India like Haryana, Rajasthan, Himachal Pradesh etc. were found to be serologically positive along with very few isolations of BTV.[19-21] In Haryana, two BTV serotypes -1 and -4 were isolated in the past.[22,23] However, there were serological evidences of BTV-2, -8, -12 and -16.[24] Recently, BTV-12 and Peste des petits ruminants virus (PPRV) have been detected as a co-infection in sheep and goats in Haryana, thereby, concerning the need to investigate BTV epidemiology in the state.[1] The present study reported the detection of BTV-12 and BTV-16 in same sample from sheep in Sirsa district of Haryana by conventional and real time reverse transcription polymerase chain reaction (RT-PCR) along with phylogenetic sequence analysis. This was the first report of BTV-16 isolation from sheep in Haryana.

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Materials and Methods

A total number of 33 blood samples were collected from BT suspected sheep and goats from Sirsa district of Haryana in January 2016. Blood samples were taken in vacutainers from jugular vein of animals and transported on ice maintaining cold chain to laboratory. Blood samples (500 µL) were used for viral RNA extraction by QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The extracted viral RNAs were used for real time PCR for Seg-1, Seg-9 and Seg-10 for initial detection of BTV.[22,25,26]

The BTV positive samples were passaged in KC (*Culicoides sonorensis*, insect) and baby hamster kidney-21 (BHK-21 clone 13 mammalian) cell lines to increase the titer of virus. Briefly, the blood samples containing red blood cell, collected from BT suspected sheep were washed thrice with phosphate-buffered saline (PBS; pH= 7.20) and lysed in sterile distilled water. The blood cell lysate was pelleted, resuspended in PBS, passed through 0.22 µm millipore filter and 500 µL suspension was inoculated in KC cell line. The cells were kept in incubation for a period of 10 days at 26.00 ˚C. Supernatant along with infected KC cells (0.50 mL) were inoculated in BHK-21 70.00 - 80.00 % confluent cell sheet in 25.00 cm^2 tissue culture flask or 1.00 mL inoculum/ 75.00 cm^2 flask after decanting the growth medium and one wash with sterile PBS. Maintenance medium of 6.00 mL per 25.00 cm^2 or 15.00 mL per 75.00 cm^2 tissue culture flask was added after 1 hr of incubation at 37.00 ˚C. The cells were incubated at 37.00 ˚C and 5.00 % CO_2 until appearance of characteristic cytopathic effects (CPE) as ballooning of cells, cells aggregation or clustering. Cultures were grown till the CPE produced by virus covered 70.00 - 80.00% surface tissue culture flasks or complete detachment of cell sheet. The virus was harvested after appearance of > 75.00% cytopathic effects in BHK-21 cells and viral RNA was Trizol extracted with $LiCl_2$ precipitation method.[26] Briefly, 0.50 mL of cell culture grown virus was taken in a 2.00 mL Eppendorf tube and treated with equal volume of TRIzol followed by immediate vigorous vortexing to avoid formation of insoluble aggregates. Then, 0.10 mL of chloroform was added and vortexed vigorously. The mixture was kept for 15 min on ice for proper lysis of cells and centrifuged (2-16KL; Sigma, Osterode am Harz, Germany) at 13,201 *g* for 15 min. The aqueous phase was then separated in a 1.50 mL eppendorf tube without disturbing the inter phase and organic phase. Equal volume of isopropanol was added to the tube, inverted 4 - 5 times and kept at – 20.00 ˚C overnight. Next day, the RNA was pelleted by centrifugation at 13,201 *g* for 20 min and supernatant discarded. After washing two times with 1.00 mL of 70.00% chilled ethanol (to remove excess salts), RNA was pelleted by centrifugation at 13,201 *g* for 10 min and air dried. Pellet was directly dissolved in nuclease free water (NFW) by gentle tapping followed by quick spinning.

The resuspended RNA was further treated for Lithium chloride precipitation. For that, 100 µL of 4.00 M Lithium chloride was added and it was incubated at 4.00 ˚C overnight. Next day, it was centrifuged at 13,201 *g* for 5 min to pellet single-stranded RNA (ssRNA). Supernatant having dsRNA was collected in another centrifuge tube. To this, 200 µL of isopropanol and 50.00 µL of 7.50 M ammonium acetate were added and incubated at – 20.00 ˚C for 2 hr. The viral dsRNA was pelleted by centrifugation at 13,201 *g* for 10 min. The pellet was washed with 1.00 mL 70.00% ethanol and dsRNA was repelleted by centrifugation at 13,201 *g* for 10 min. The supernatant was discarded, pellet air dried, dissolved in 50.00 µL NFW and stored at – 20.00 ˚C. The extracted viral RNA was treated with S-1 nuclease for 45 min at 25 ˚C and DNAse for 15 min at room temperature to remove all the contaminating ssRNA or DNA.

Extracted viral dsRNAs were used in real time RT-PCR for Seg-2 of Indian panel of BTV serotypes to detect the BTV serotype involved[7,25,27,28] and further confirmed by PCR amplification in four overlapping products of Seg-2 and Seg-6 of the BTV serotypes with published primer sequences.[1,25,29] The amplified products were analyzed in 1.00% agarose gel, PCR purified (Qiagen PCR purification kit) and sequenced by Applied Biosystems ABI capillary sequencer 3130 using a BigDye cycle sequencing kit (Thermo Fisher Scientific, San Jose, USA).

Results

Out of 33 samples, 15 were found positive by real time RT-PCR with cycle threshold value ranging 19.30 to 31.00. On serotyping by real time PCR using Seg-2 specific primers of Indian panel of BTV serotypes, out of 15 samples, one sample IND2016/98 from sheep was positive for BTV-12 and BTV-16. The serotypes BTV-12 and BTV-16 were confirmed by conventional RT-PCR in which desirable size four products respective to BTV serotypes (Fig. 1) were observed that is for BTV-12 Product A (1,133 bp), Product B (681 bp), Product C (847 bp) and Product D (800 bp) and for BTV-16 Product A (1,214 bp), Product B (1,044 bp), Product C (918 bp) and Product D (851 bp).

The PCR amplified and purified products of Seg-2 of IND2016/98 were sequenced and phylogenetically analyzed. The sequences of Seg-2 of this isolate (Accession No. ON692402 and ON675589) were found to be clustered within nucleotype G along with reference BTV-12 (RSArrrr/AJ585133) and BTV-22 (RSArrrr/AJ585143) of South Africa confirming the serotype BTV-12 infection and in nucleotype B along with BTV-16 (RSArrr/AJ685137), BTV-13 (RSArrrr/AJ134), and BTV-3 (RSArrrr/AJ124) reference strains of South Africa confirming presence of BTV-16 serotype, respectively (Fig. 2).

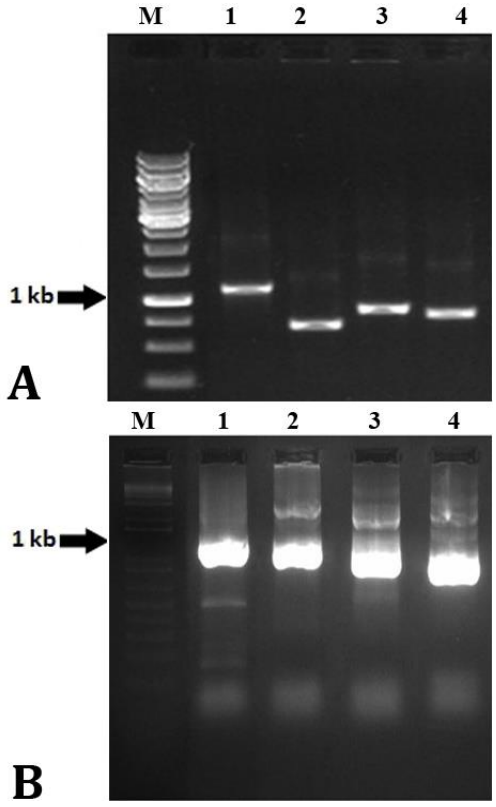

Fig. 1. A) Conventional polymerase chain reaction amplified and purified products of Seg-2 of IND2016/98 isolate (BTV-12). Lane M: 1 kbp DNA ladder, Lane 1: Product A (1,133bp), Lane 2: Product B (681bp), Lane 3: Product C (847bp) and Lane 4: Product D (800bp) and **B)** Conventional polymerase chain reaction amplified and purified products of Seg-6 of IND2016/98 isolate (BTV-16). Lane M: 100 bp DNA ladder, Lane 1: Product A (1214 bp), Lane 2: Product B (1044 bp), Lane 3: Product C (918 bp) and Lane 4: Product D (851bp).

The BTV Seg-2 encodes a major immuno-dominant, outer capsid protein 1 i.e. VP2 which determines the virus serotype. Phylogenetic analysis via tree topology of Seg-2 of IND2016/98 (Accession No. ON692402) indicated its distribution under the nucleotype G, distinctly rooted from rest of 11 nucleotypes confirming the serotype of isolates as BTV-12.[15] It showed 99.40% nucleotide (nt) identity with BTV-12 isolate NMO1/11(KC662613) from India; 96.40 - 96.60% nt identity with BTV-12 isolate ON90-2 from Japan; 97.00% nt identity with BTV-12 isolate (AJ585185.1) from Kenya; 97.10% nt identity BTV-12 isolate RSArrrr/12 (AJ585133.1) from South Africa. However, with a western topotype BTV-12 isolate 75,005 (JX272500.1), it showed 97.20 - 97.50% nt identity. This feature might have some significance regarding the origin of BTV Seg-2 from western topotypes. Similar pattern of inter-relationship was noted in Seg-2 sequence analysis of NMO1/11.[30]

The Seg-2 sequence (Accession No. ON675589) based phylogenetic anlaysis grouped IND2016/98 isolate within nucleotype B along with other BTV-3, -13 and other previously published BTV-16 isolates from southern India. Nucleotide identity pattern of Seg-2 of IND2016/98 BTV-16 isolate was 99.40% with BTV16IND2010-AP06 (KP339205.1) and 99.30% with BTV16IND2011-NR82 (KP339215.1) published BTV-16 sequences from southern India, highlighting the origin of Seg-2 of the isolate from eastern topotype.[29,30]

The BTV genome segment-6 encoding a structural protein VP5, a minor outer capsid protein being involved in antibody neutralization reaction, is another serotype determining segment along with segment-2. However, it is less variable compared to segment-2 and there are similarities in some serotypes. So, it is less frequently used to determine the serotypes of BTV. On phylogenetic and sequence analysis of Seg-6 the BTV isolate IND2016/98, in tree topology was found to be grouped with nucleotype E for BTV-12 along with BTV-22 and with nucleotype B for BTV-16 along with BTV-21, -3 and others BTV-16 published sequences (Fig. 3).

Briefly, the isolate Seg-6 sequence (Accession No. ON692403) gave 99.10 - 99.40% nt identity with BTV-12 isolate NMO1/11 (KC662617.1) from India; 96.20 - 96.70% nt identity with BTV-12 isolate ON90-2 (AB686230.1) from Japan; 98.00% nt identity with BTV-12 isolate (AJ586712.1) from Kenya; 97.50 - 97.90% nt identity BTV-12 isolate RSArrrr (AJ586711.1) from South Africa. However, with a western topotype BTV-12 isolate 75,005 (JX272504.1), the isolate demonstrated 97.50 - 97.80% nt identity. Similar to Seg-2, this feature was quite strange and might have some significance.

The Seg-6 sequence (Accession No. ON675588) analysis of IND2016/98 isolate of present study also showed 99.80% nt identity with BTV-16 isolates IND2010-VC07 (KP339229.1), IND2010-AP06 (KP339209.1) and IND2011-NR82 (KP339219.1) from southern India.

Discussion

In Indian subcontinent, BT was first reported in Pakistan in 1959, whereas, in India, with the introduction of exotic breeds of sheep from abroad and their subsequent cross-breeding with the native breeds, the disease was first reported in the year 1964 in the state of Maharashtra. The BTV is endemic in India, because of favorable climatic conditions and density of natural host population, which are essential for survival of Culicoides vector and BTV. Dramatic changes in climate, deforestation, anthropogenic factors and global warming resulted in emergence of novel BTV serotypes outbreaks with variable pathology, which warns the livestock rearing community and arboviral researchers. At present, 23 out of 28 BTV serotypes have been found to be prevalent in the geo-ecological regions of India which were detected either on the basis of virus isolation or serology.[11,13,17,31-33]

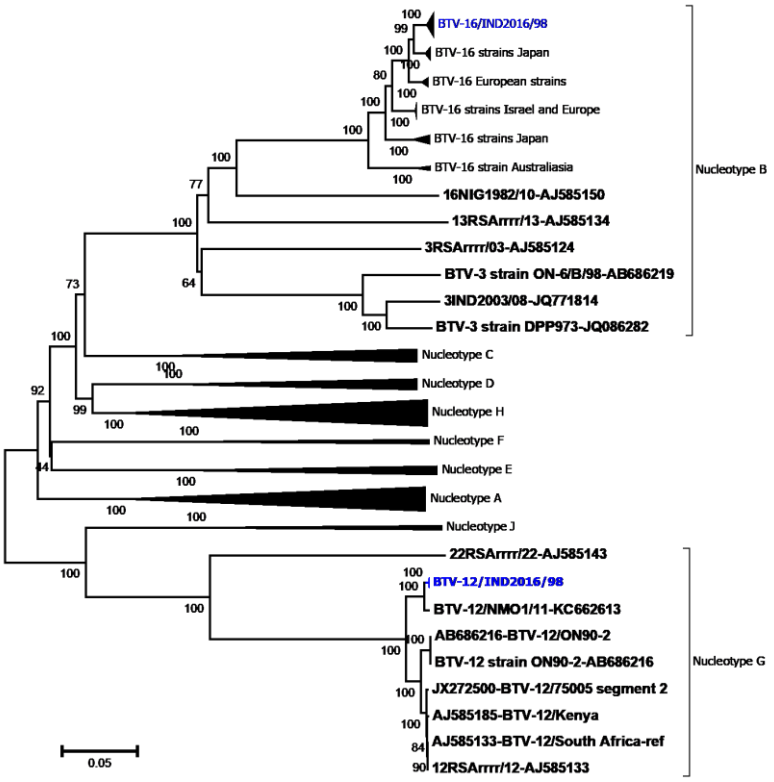

Fig. 2. Unrooted neighbour joining tree for Seg-2 of bluetongue virus (BTV) isolates showing relationships of BTV isolate IND2016/98 from Haryana with those of global isolates. The tree was generated with MEGA Software (version 6.0; Biodesign Institute, Tempe, USA) using default parameters.

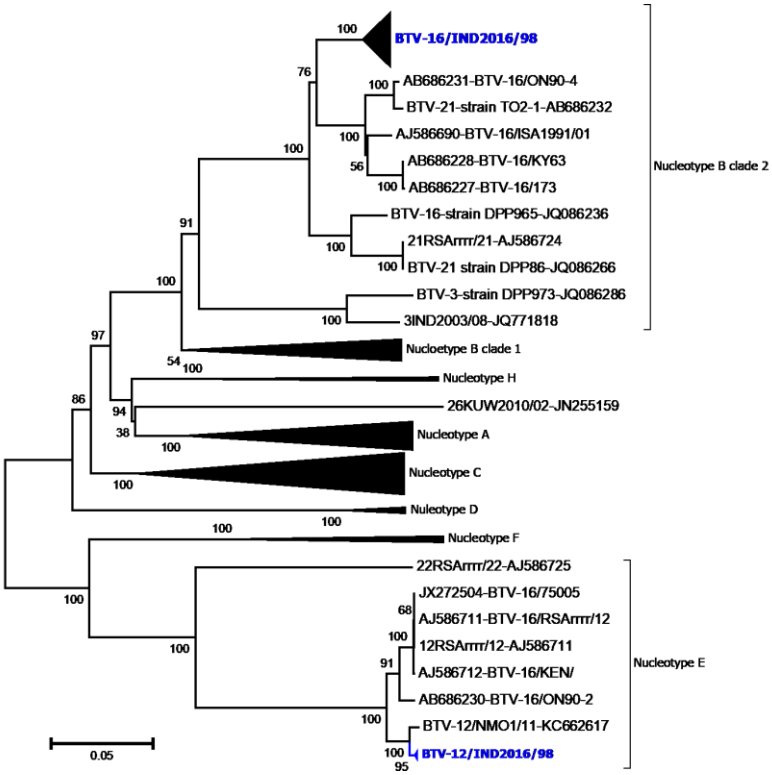

Fig. 3. Unrooted neighbour joining tree for Seg-6 of bluetongue virus (BTV) isolates showing relationships of BTV isolate IND2016/98 from Haryana with those of global isolates. The tree was generated with MEGA Software using default parameters.

Previously, BT had been more frequently reported in the southern states of Andhra Pradesh, Karnataka and Tamil Nadu, very severe during 2004 - 2006. The BTV-10 was reported for the first time in the east of India, in Kolkata during 2004, although there were no reports of BT in the north-eastern states.[31,32] Recently, BT has also been reported from northern state Haryana as a co-infection with PPRV.[11] In past, only two BTV serotypes were isolated namely BTV-1 and BTV-4.[19,23] However, serological evidence of BTV-2, -8, -12 and -16 from Haryana has been reported earlier.[31]

Previously, dual infection of BTV-2 and -5 in Karnataka has been reported in sheep.[34] During sequence analysis by phylogenetic tree construction, Seg-2 of BTV-2 serotype was found to be of eastern origin and that of BTV-5 of western origin. Similarly, the findings of the present study supported the origin of Seg-2 of BTV-12 to be western and that of BTV-16 to be eastern topotype. The first isolation and second complete genome sequencing of BTV-12, NMO1/11 (KC662612-KC662621) was reported from Andhra Pradesh[30] after a Taiwan BTV-12 whole genome sequence.[9] Serologically, anti-BTV-12 antibodies have been reported in sheep and cattle from Andhra Pradesh, Gujarat and Haryana.[31] However, there have been no reports about existence of eastern topotype for BTV-12 serotype. The Asian and African isolates are closely related and it has been speculated that these isolates of BTV-12 have a recent common ancestry. The isolate IND2016/98 in the present study gave 99.40% nt identity with BTV-12, NMO1/11 (KC662612-KC662621) from Andhra Pradesh, signifying the movement of virus from southern to northern India (Haryana) in small ruminants.

The BTV-16 serotype was reported for the first time from Maharashtra in 1967, 1970 and 1973 and Himachal Pradesh in 1973.[19,24] During the period of 2006 to 2011, BTV-16 was found to be involved in most of the outbreaks reported from different parts of southern peninsular India i.e., Andhra Pradesh, Tamil Nadu and Karnataka. Several isolates were obtained and a few were characterized genetically.[31,35] Although western topotype of BTV-16 has been reported recently,[36] all BTV-16 isolates from Asia and Europe belong to eastern topotype.[11] Kumar *et al.* sequenced a reassortant BTV-16 from Tamil Nadu, south India where except seg-5, all the genome segments of BTV were of eastern topotype in origin.[29] The present study also governed the same pattern of prevalence of BTV-16 from Haryana as that of southern India isolates. All segments of the isolate of present study showed 99.30 - 99.80% identity with already published sequences of BTV-16 isolates IND2010-VC07, IND2010-AP06, and IND2011-NR82 from southern states of India. Therefore, it could be said that reassortant strains of BTV-16 with nine segments from eastern lineage and one (Seg-5) from western lineage are circulating in India. A very high sequence identity in all genome segments of BTV-16 strains from southern states of India and the strains studied here from northern state of India (Haryana) showed that same virus is circulating probably via movements of nomadic animal which may be subclinically infected with BTV.

The above data signified the potential of different BTV serotypes to co-infect the same host. The presence of mixed serotypes in individual sample and cell culture recovered BTV isolates has also been reported previously.[37,38] This may be due to the co-circulation of multiple serotypes in BTV endemic areas. Not only dual infection but involvement of three serotypes (BTV-4, -9 and -16) in an outbreak were reported in Greece during 1999.[39] The spread of BTV serotypes to new geographical areas (southern to northern India) has occurred due to the movement of carrier or reservoir or subclinically infected host animals or insect vectors, in addition to illegal importation of live animals, embryos or semen or use of imported live vaccines from South Africa and /or USA.

Continuous sero-surveillance programs are needed to monitor the endemicity, emerging and re-emerging status of BTV serotypes in India. This will help to identify the most commonly circulating serotypes in endemic areas, which will be utilized for development of multivalent vaccines leading to control and eradication of BTV.

Acknowledgments

The authors would like to acknowledge funding support from BBSRC-DBT (BB/L004690/1; BT/IN/Indo-UK/FADH/46/SM/2013), DBT Bio-CARe grant (BT/Bio-CARe/04/261/2011-12) and Rashtriya Krishi Vikas Yojana from Government of India. The authors would also like to thank all collaborators of BBSRC-DBT project and various staff of Animal Husbandry Department of Haryana for their direct or indirect help.

Conflict of interest

The authors declare no conflict of interest.

References

1. Maan S, Kumar A, Gupta AK, et al. Concurrent infection of Bluetongue and Peste-des-petits-ruminants virus in small ruminants in Haryana State of India. Transbound Emerg Dis 2018; 65(1): 235-239.
2. Savini G, Puggioni G, Melon G, et al. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. Infect Genet Evol 2017; 51: 108-117.
3. Maan S, Maan NS, Nomikou K, et al. Complete genome characterisation of a novel 26th bluetongue virus serotype from Kuwait. PLoS One. 2011; 6(10): e26147. doi: 10.1371/journal.pone.0026147.
4. Schulz C, Bréard E, Sailleau C, et al. Bluetongue virus serotype 27: detection and characterization of two

novel variants in Corsica, France. J Gen Virol 2016; 97(9): 2073-2083.
5. Bumbarov V, Golender N, Jenckel M, et al. Characterization of bluetongue virus serotype 28. Transbound Emerg Dis 2020; 67(1):171-182.
6. Sun EC, Huang LP, Xu QY, et al. Emergence of a novel Bluetongue virus serotype, China 2014. Transbound Emerg Dis 2016; 63(6): 585-589.
7. Maan S, Maan NS, Ross-smith N, et al. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. Virology 2008; 377(2): 308-318.
8. Maan S, Maan NS, van Rijn PA, et al. Full genome characterisation of Bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. PLoS One 2010; 5(4): e10323. doi: 10.1371/journal.pone.0010323.
9. Breard E, Belbis G, Viarouge C, et al. Evaluation of adaptive immune responses and heterologous protection induced by inactivated bluetongue virus vaccines. Vaccine 2015; 33(4): 512-518.
10. Saminathan M, Singh KP, Khorajiya JH, et al. An updated review on bluetongue virus: epidemiology, pathobiology, and advances in diagnosis and control with special reference to India. Vet Q 2020; 40(1): 258-321.
11. Rao PP, Hegde NR, Reddy YN, et al. Epidemiology of Bluetongue in India. Transbound Emerg Dis 2016; 63(2): e151-e164.
12. Reddy YK, Brindha K, Ganesan PI, et al. Occurrence of Bluetongue in ruminants in Tamil Nadu, South India. Vet Ital 2016; 52(3-4): 293-297.
13. Sapre SN. An outbreak of 'bluetongue' in goats and sheep in Maharashtra State. Indian Vet Rev (M&B) 1964; 15:69-71.
14. Gollapalli SR, Mallavarapu S, Uma M, et al. Sequences of genes encoding type-specific and group-specific antigens of an Indian isolate of bluetongue virus serotype 10 (BTV-10) and implications for their origin. Transbound Emerg Dis 2012; 59(2): 165-172.
15. Rao PP, Reddy YN, Ganesh K, et al. Deep sequencing as a method of typing bluetongue virus isolates. J Virol Methods 2013; 193(2): 314-319.
16. Rao PP, Reddy YN, Hegde NR. Complete genome sequence of bluetongue virus serotype 9: implications for serotyping. J Virol 2012; 86(15): 8333. doi: 10.1128/JVI.01101-12.
17. Krishnajyothi Y, Maan S, Kandimalla K, et al. Isolation of bluetongue virus 24 from India - an exotic serotype to Australasia. Transbound Emerg Dis 2016; 63(4): 360-364.
18. Maan S, Maan NS, Belaganahalli MN, et al. Full-genome sequencing as a basis for molecular epidemiology studies of bluetongue virus in India. PloS One 2015; 10(6): e0131257. doi: 10.1371/journal.pone.0131257.
19. Uppal P, Vasudevan B. Occurrence of bluetongue in India. Indian J Comp Microbiol Immunol Infect Dis 1980; 1: 18-20.
20. Oya Alpar H, Bramwell VW, Veronesi E, et al. Bluetongue virus vaccines past and present. In: Mellor PS, Baylis M, Mertens PPC (Eds). Bluetongue. London, UK: Elsevier Academic Press 2009; 397-428.
21. Kakker N K, Prasad G, Bhatnagar P, et al. Sero-prevalence of bluetongue virus infection in cattle in Haryana, Himachal Pradesh, Punjab and Rajasthan. Indian J Comp Microbiol Immunol Infect Dis 2003; 23: 147-151.
22. Shaw AE, Monaghan P, Alpar HO, et al. Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. J Virol Methods 2007; 145(2):115-126.
23. Jain NC, Sharma R, Prasad G. Isolation of bluetongue virus from sheep in India. Vet Rec 1986; 119(1): 17-18.
24. Prasad G, Garg AK, Minakshi P, et al. Isolation of bluetongue virus from sheep in Rajasthan state, India. Rev Sci Tech 1994; 13(3): 935-938.
25. Maan S, Maan NS, Belaganahalli MN, et al. Development and evaluation of real time RT-PCR assays for detection and typing of bluetongue virus. PLoS One 2016;11(9): e0163014. doi: 10.1371/journal.pone.0163014.
26. OIE Terrestrial Animal Health Code and OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Available at: https://www.woah.org/app/uploads/2021/03/3-01-03-bluetongue.pdf
27. Attoui H, Billoir F, Cantaloube JF, et al. Strategies for the sequence determination of viral dsRNA genomes. J Virol Methods 2000; 89(1-2): 147-158.
28. Maan NS, Maan S, Belaganahalli MN, et al. Identification and differentiation of the twenty six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. PLoS One 2012; 7(2): e32601. doi: 10.1371/journal.pone.0032601.
29. Kumar L, Batra K, Chaudhary D, et al. Full-genome sequence analysis of a Reassortant strain of bluetongue virus serotype 16 from Southern India. Genome Announc 2016; 4(4): e00783-16. doi: 10.1128/genomeA.00783-1.
30. Rao PP, Reddy YV, Hegde NR. Isolation and complete genome sequencing of bluetongue virus serotype 12 from India.Transbound Emerg Dis 2015; 62(5):e52-e59.
31. Minakshi P, Singh R, Ranjan K, et al. Complete genome sequence of bluetongue virus serotype 16 of goat origin from India. J Virol 2012; 86(15): 8337-8338.
32. Sreenivasulu D, Subba Rao MV, Reddy YN, et al. Overview of bluetongue disease, viruses, vectors, surveillance and unique features: the Indian sub-continent and adjacent regions. Vet Ital 2004; 40(3): 73-77.
33. Ranjan K, Minakshi P, Prasad G. Bluetongue: Indian perspective. Acta Virol 2015; 59(4): 317-337.

34. Hemadri D, Maan S, Chanda MM, et al. Dual infection with bluetongue virus serotypes and first-time isolation of serotype 5 in India. Transbound Emerg Dis 2017; 64(6): 1912-1917.
35. Shafiq M, Minakshi P, Bhateja A, et al. Evidence of genetic reassortment between Indian isolate of bluetongue virus serotype 21 (BTV-21) and bluetongue virus serotype 16 (BTV-16). Virus Res 2013; 172(2): 336-343.
36. Mertens PP, Maan NS, Belaganahalli MN, et al. Full genome sequence of a Western reference strain of bluetongue virus serotype 16 from Nigeria. Genome Announc 2013; 1(5): e00684-13. doi: 10.1128/ genomeA.00684-13.
37. Maan S, Maan NS, Batra K, et al. Reverse transcription loop-mediated isothermal amplification assays for rapid identification of eastern and western strains of bluetongue virus in India. J Virol Methods 2016; 234: 65-74.
38. Brenner J, Oura C, Asis I, et al. Multiple serotypes of bluetongue virus in sheep and cattle, Israel. Emerg Infect Dis 2010; 16(12): 2003-2004.
39. Orrù G, Ferrando ML, Meloni M, et al. Rapid detection and quantitation of bluetongue virus (BTV) using a molecular Beacon fluorescent probe assay. J Virol Methods 2006; 137(1): 34-42.