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A new multiplex RT-PCR assay for serotyping of bluetongue virus

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Among all viral diseases affecting small ruminants, bluetongue is the one that affects adversely to an alarming extent. The current available diagnosis/serotyping of bluetongue virus (BTV) is time consuming, costly, and requires to screen individually for each of the 29 distinct serotypes. The present study was conducted with the objective of developing a multiplex reverse transcription PCR (mRT-PCR) assay for serotyping of BTV, especially for serotypes BTV-1, 2, 9, 12, 16, 21 and 23 predominantly circulating in India. The type specific primers for the selected BTV serotypes were designed targeting the serotype specific segment-2 region of BTV based on the reference serotype sequences of Indian isolates available in GenBank. The mRT-PCR was conducted in two groups - group A for BTV-1, 9, 12, 21 and group B for BTV-2, 16 and 23. A panel of 25 BT suspected clinical samples were typed by mRT-PCR. The results were further validated by the gold standard serum neutralization test (SNT). A seroprevalence of 60% for BTV- 2, 10% for BTV- 9, 15% for BTV- 1, 10% for BTV- 16 and 5% for BTV- 23 were observed. Further, we noticed that there was a mixed serotype infection in 10% of BTV positive samples. In conclusion, we report the development of a novel mRT-PCR assay for a rapid and cost-effective nucleic acid based serotyping of BTV having the specificity same as SNT.

Keywords: Bluetongue virus; multiplex reverse transcription PCR; molecular serotyping

Introduction

Bluetongue (BT) is an enzootic, infectious, non-contagious, arthropod-borne viral disease of ruminants and camelid species caused by bluetongue virus (BTV), a prototype member of the Orbivirus genus in the Reoviridae family[1]. It is transmitted almost exclusively by adult female hematophagous midges that belong to the Culicoides genus[2]. The disease is mainly characterized by fever, facial edema, hemorrhages, ulceration on the oral mucosa and coronitis[3]. The genome encodes seven structural (VP1 - VP7) and five non-structural proteins (NS1, NS2, NS3, NS3/A, NS4)[4]. There are 29 distinct serotypes of BTV recognized based on the ability of antibodies generated during infection of the mammalian host to neutralize only the homologous virus type[5-6]. The genome segments of BTV were grouped into 'eastern' or 'western' topotypes, where the eastern group includes isolates from China, South Asia, South-East Asia and Australia and the western group includes viruses from America, Africa, Europe and the Middle East countries[7]. The serotype specificity is determined by the variation in the outer capsid proteins more specifically the VP2 protein[8]. Out of the 29 serotypes recognized worldwide, 24 serotypes are known to be existing in India, either by serology or virus isolation[9-12]. At present nine serotypes of BTV (BTV-1, 2, 3, 9, 10, 12, 16, 21 and 23) were in circulation mostly from southern states of India[13].

Bluetongue is traditionally diagnosed by virus isolation, serotyping by neutralization assays, and antibody detection in convalescent animals[14]. Virus neutralization assays were used for determination of prior infection with different serotypes of BTV[15]. Nucleic acid based detection of different conserved segments (all segments except segment 2 and 6) can be used for diagnosis of BTV[16-20], whereas segment 2 based PCR will determine the serotype of the virus[21]. Having 29 serotypes of BTV, it is often challenging to detect the serotype by neutralization assays as it will be time consuming. For effective control of BT disease, an early diagnosis and effective prophylactic measures are much needed. This hints the need for the development of cost effective and early diagnostic techniques for serotyping of BTV.

Multiplex PCR has emerged as a highly efficient and sensitive molecular tool for nucleic acid based diagnosis of multiple serotypes in a single reaction[22].

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The present study was aimed at standardization of multiplex reverse transcription PCR (mRT-PCR) for serotyping majorly circulating BTV (BTV- 1, 2, 9, 12, 16, 21 and 23) in India.

Materials and Methods

Cell lines, Virus Isolates and Study Samples

The viral isolates, BTV serotypes 1 and 23 collected from Vaccine Research Centre, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), serotype 2 (isolated at Chittoor district in 1995[23]), serotype 9 MBN and K8 (isolated at Mahabubnagar and Khammam districts, respectively in 2002), serotype 12 (isolated at Adilabad district in 2011), serotype 16 (collected from VBRI, Hyderabad), serotype 21 KMN 07/05 (isolated at Karimnagar district in 2005) were used in the study. Apart from these, 25 unknown samples suspected for BTV were used for the study. These blood samples were collected from different field outbreaks occurred in Andhra Pradesh during 2012-13. All of these virus isolates were propagated in Vero cells.

Genotyping of Bluetongue Virus

Serotype specific primers (Table 1) were designed for BTV- 1, 2, 9, 12, 16, 21 and 23 by using serotype specific VP2 segment sequence targeting a region of 2000 bp to 2500 bp of VP2. These primers were designed in a way to achieve optimum annealing with a varying amplicon product length for each serotype. The serotypes were grouped into two: group A (BTV-1, 9, 12 and 21), group B (BTV-2, 16 and 23). Primers targeting NS1 (segment 5) of BTV that can amplify and detect all the known serotypes of BTV served as internal control[24].

Multiplex RT-PCR and Serum Neutralization Test

The viral nucleic acid was extracted using Trizol LS reagent (Invitrogen) as per the protocol described by the manufacturer. The cDNA was synthesized from genomic RNA by using random hexamers and M-MLV reverse transcriptase kit (Invitrogen) following the manufacturer's protocol. PCR was standardized for serotypes of BTV- 1, 2, 9, 12, 16, 21 and 23 using each pair of primers. The reaction mix consisted of 2.5 µL of 10X PCR buffer, 1.25 U *Taq* DNA polymerase, 10 mM of dNTPs, 10 pmols each of forward and reverse primers and 5 µL appropriate template cDNA (corresponding to 30 ng of RNA) or nuclease free water as a negative control were added to the reaction.

The multiplex RT-PCR for both groups (group A, group B) was carried out by targeting specific genetic portion of cDNA for each serotype with variable product sizes. The reaction was set for a final volume of 50 µl for each group individually. The reaction mix for group A consisted of 5 µl cDNA each of BTV- 1, 9, 12 and 21 and for group B consisted 5 µl cDNA each of BTV- 2, 16 and 23 as a template, 2.5 U of *Taq* DNA polymerase, 5 µl of 10X DNA polymerase buffer, 1 µl of 10 mM dNTP mix, and 5 pmols each of multiplex primers.

The conditions were set as initial denaturation at 94°C for 3 min followed by 35 cycles each at 94ºC for 30 sec, 50°C for 30 sec, 72°C for 3 min, and the final extension at 72°C for 10 min. The gold standard

Table 1 — The list of primers used in the study

SI. No	Serotype	Primer name	Primer sequence
1	BTV - 1	BTV - 1F	5' CCGCGCAATACACCTAGA 3'
2	(KF563934.1)	BTV - 1R	5' TCTAACCGTTCGTCTCCGTT 3'
3	BTV - 2	BTV - 2F	5' AGGCGGATCCATTGGAAGAG 3'
4	(KP339165.1)	BTV - 2R	5' CGATCACTACCTACTGCGCT 3'
5	BTV - 9	BTV - 9F	5' CGGTGATGGGATCAACCCTA 3'
6	(KP339185.1)	BTV - 9R	5' CATCGYTTGACGAGCGAATGA 3'
7	BTV - 12	BTV - 12F	5' CGCACTGAAGGTATATGCGG 3'
8	(MK516489.1)	BTV - 12R	5' GCGAACCTTGTGTAAGTGArC 3'
9	BTV - 16	BTV - 16F	5' GCRGTTAAACAGACGCCATC 3'
10	(MG710531.1)	BTV - 16R	5' GCTCAGYCTGTCCCGACTTT 3'
11	BTV - 21	BTV - 21F	5' CGTGGGAAATGTyCTGACGA 3'
12	(KP339235.1)	BTV - 21R	5' GCGTTCTGGCGTCTCTCATA 3'
13	BTV - 23	BTV - 23F	5' CAGATGACCCTGCCGTAGC 3'
14	(KP696691.1)	BTV - 23R	5' AACGACTCGTCCAAACCGTG 3'

serum neutralization tests (SNT) was performed to validate the results in accordance with the protocol described in the World Organisation for Animal Health (OIE manual)[25]. The wells are scored for the degree of cytopathic effects (CPE) observed. A CPE of 75-100% was considered as negative for serum neutralization.

Results and Discussion

Bluetongue, an enzootic, infectious viral disease caused by BTV is mainly characterized by fever, facial edema, hemorrhages, and ulceration on the oral mucosa and coronitis. The presence of 29 distinct serotypes complicates the diagnosis of BTV. Though there were several diagnostic aids such as virus isolation, serotyping by neutralization assays, antibody detection in convalescent animals and nucleic acid based detection of different conserved segments[26], they are often time consuming, costly and involves several reactions. The present study has been taken up with an objective to develop a diagnostic assay based on mRT-PCR.

Multiplex PCR involves the use of multiple pairs of specific primers in the same reaction, which can simultaneously amplify multiple targeted regions of multiple DNA templates at one time. The VP2 gene of BTV was targeted in the study for designing the multiplex PCR primers. VP2 is the most variable outer capsid layer protein encoded by segment-2 of the genome and is the serotype determinant of BTV[27-28]. It is also reported to be the potential target for development of serotype specific RT-PCR[29]. VP2 is a viral hemagglutinin that is responsible for cell attachment during the initiation of infection[30]. Based on the phylogenetic relationship[31] the serotypes chosen in the current study can be grouped into two: group A (BTV-1, 9, 12 and 21) and group B (BTV- 2, 16 and 23) for convenience and specificity in multiplex PCR. NS1 is the most abundantly expressed cytoplasmic protein during the BTV infection. Further, the NS1 is highly conserved between the BTV serotypes and OIE recommended it as a target for group specific RT-PCR[32]. Hence, we used NS1 as an internal control in the present study.

Standardization of RT-PCR

Initially, RT-PCR was setup for each primer pairs to optimize the PCR conditions and to avoid the non-specific amplification and to validate the working concentrations of primers. The RT-PCR of BTV serotypes 1, 2, 9, 12, 16, 21 and 23 generated amplicons of sizes 206 bp, 196 bp, 396 bp, 390 bp, 500 bp, 546 bp and 590 bp, respectively (Fig. 1). There was no non-specific amplification seen, and the amplification is seen at respective length for each serotype. The previous study performed by Mertens and colleagues (2007) for evaluation of different primer pairs for serotype specific RT-PCR also reported that VP-2 is ideal for nucleic acid based

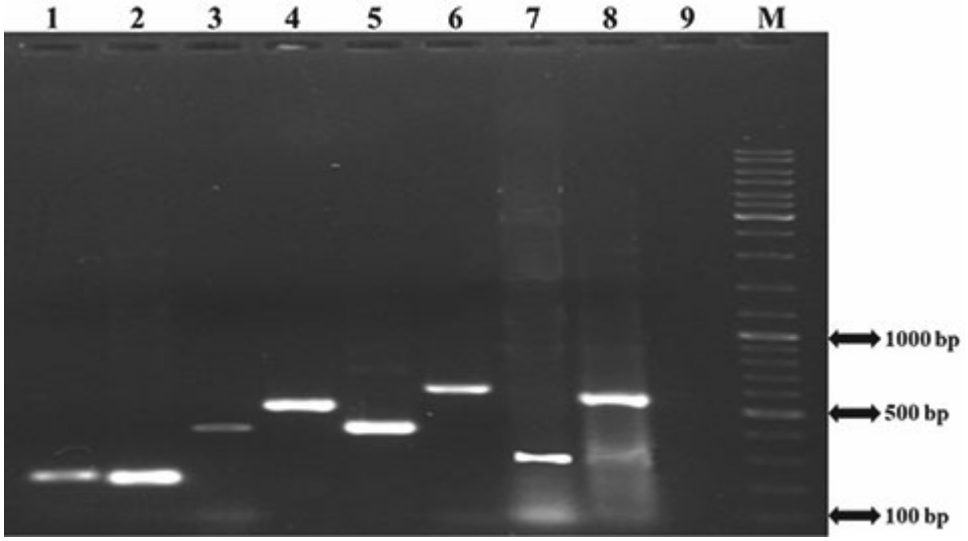

Fig. 1 — Uniplex RT-PCR of known reference BTV serotypes. Lane 1 show amplification of 206 bp for BTV-1, Lane 2 show amplification of 196 bp for BTV-2, Lane 3 show amplification of 396 bp for BTV-9, Lane 4 show amplification of 500 bp for BTV-12, Lane 5 show amplification of 390 bp for BTV-16, Lane 6 show amplification of 591 bp for BTV-21, Lane 7 show amplification of 274 bp for NS1, Lane 8 show amplification of 548 bp for BTV-23, Lane 9 show no amplification for negative control and Lane M is a 1 kb DNA marker.

serotyping. However, their study is limited to evaluation of different primer pairs for serotyping by uniplex RT-PCR.

Standardization of Multiplex RT-PCR

Multiplex PCR for simultaneous detection of BTV and epizootic hemorrhagic disease virus were previously reported[33]. The multiplex PCR can be an efficient way for detecting the serotypes of BTV in a cost effective and single reaction. Hence, after optimizing the conditions in RT-PCR, the multiplex RT-PCR was standardized for BTV serotyping in two reaction groups. The group-A mRT-PCR showed amplification of 196 bp, 396 bp, 500 bp, 591 bp and 274 bp (Fig. 2) indicating presence of BTV- 1, 9, 12, 21 and NS1, respectively. The group B mRT-PCR show amplification of 206 bp, 390 bp, 546 bp and 274 bp (Fig. 2) indicating BTV- 2, 16, 23 and NS1, respectively. The primers show only a specific amplification with the respective BTV serotypes. A similar kind of study was previously performed by Johnson and colleagues[34] for identification of BTV- 2, 10, 11, 13 and 17 serotypes and was found to be a reliable technique.

Typing of Field BTV Isolates by Multiplex RT-PCR (mRT-PCR)

It is more important to test the clinical samples to validate the test. Hence, a panel of 25 BTV suspected clinical samples collected from sheep during the BTV outbreaks in the year 2012 - 2013 were typed using the mRT-PCR as described above. Out of 25 samples screened, 20 showed an amplification of 274 bp for NS1 (Fig. 3). This indicates that 80% of the cases were infected with BTV. Different seroprevalence rates were reported previously with a seroprevalence ranging from 21.4% to 63%[35-41]. Climatic factors which influence the size of vector populations and periods of their seasonal activity play an important role in the occurrence of BTV infection in animals[42].

In addition to it, there was an amplification of 196 bp specific for BTV- 2 in 10 samples, 396 bp specific for BTV- 9 in one sample, 206 bp specific for BTV- 1 in one sample, 390 bp specific for BTV- 16 in one sample, and 548 bp specific for BTV- 23 in one sample (Fig. 3). It was also observed that there was a mixed serotype infection in 3 samples; with an amplification of 206 bp and 390 bp specific for BTV-1 and 16 in one sample (WGL - 1026), 196 bp and 396 bp specific for BTV-2 and 9 in the second sample (WGL - 1041) and 206 bp and 196 bp specific for BTV- 1 and 2 in the third sample (NLG - 1955) (Fig. 3). In countries endemic for BT like India, mixed serotype infections are common. The previous reports[43] also suggest the superinfection or circulation of multiple serotypes in the sampled flocks. Further, we noticed that three samples positive for BTV NS1 turned out to be negative for tested BTV serotypes screened in the present study (Fig. 3). This may be due to infection with serotype/s other than the ones tested in this study.

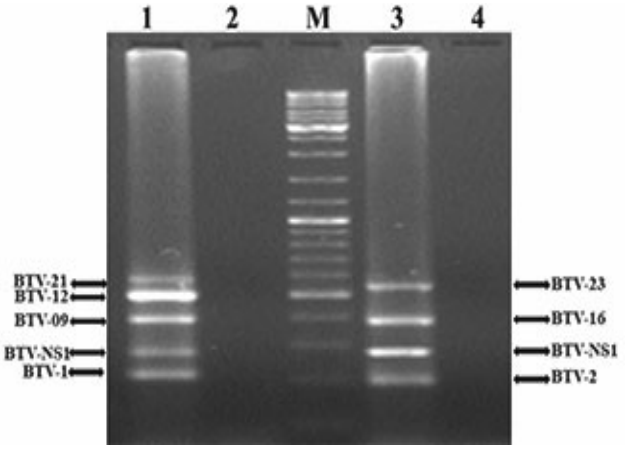

Fig. 2 — Multiplex RT-PCR of known reference BTV serotypes. Lane 1 show the group A PCR products having amplicon lengths 206 bp, 274 bp, 396 bp, 500 bp, 591 bp for BTV-1, NS1, BTV-9, BTV-12, BTV-21 respectively. Lane 2 show no amplification for negative control with group A primers. Lane 3 show the group B PCR products having amplicon lengths 196 bp, 274 bp, 390 bp, 548 bp for BTV-2, NS1, BTV-16, BTV-23 respectively. Lane 4 show no amplification for negative control with group B primers. Lane M is a 1 kb DNA marker.

Taken together, we noticed a seroprevalence of 50% for BTV- 2, 5% for each of BTV- 1, BTV- 16, BTV- 23, BTV- 9, BTV- 1 & 2, BTV- 2 & 9, BTV- 1 & 16, and 15% for other serotypes (Fig. 3 & 4). A recent report from India showed seroprevalence of 65.27% for BTV- 1, 26.38% for BTV- 16, 20.83% for BTV- 10, 13.88% for BTV- 9 and 23, and 6.94% for BTV- 2 (De *et al.* 2019). This suggests that the serotypes prevalent in India were varying from year to year and region to region. In addition, the results of mRT-PCR typing of BTV isolates were validated by SNT with hyperimmune sera for each of the specific BTV serotype. The results of SNT correlated with that of the mRT-PCR with 100% specificity. This indicates that the multiplex RT-PCR can be an effective replacement for other serotyping tests having great specificity, economic, and less time consuming.

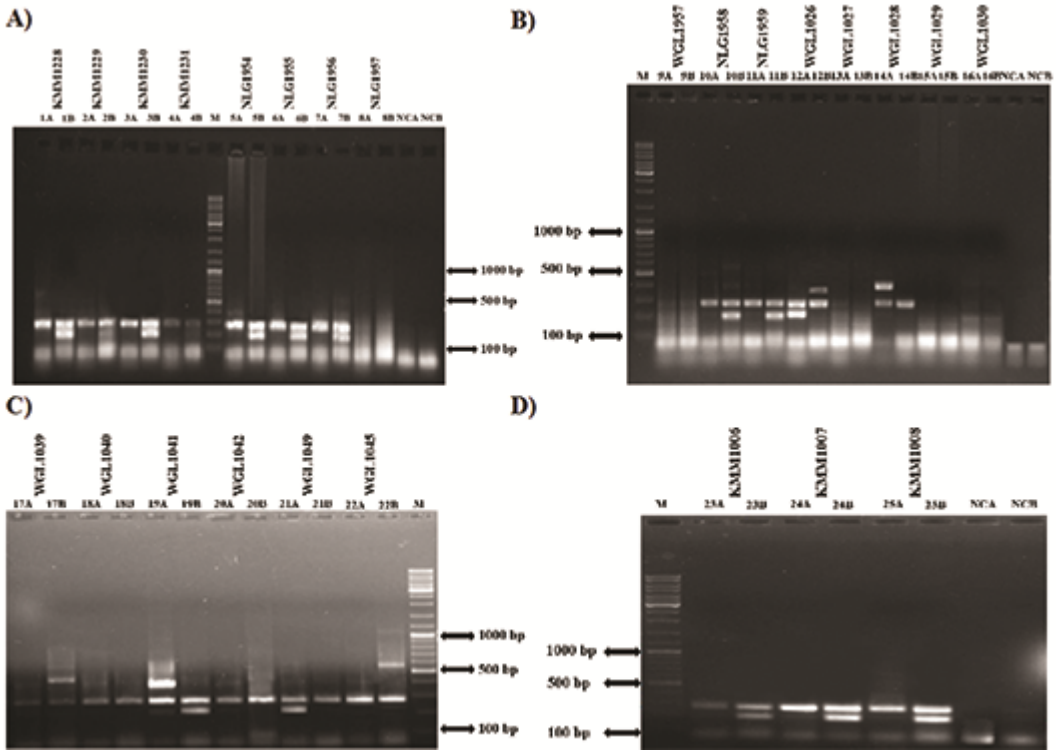

Fig. 3 — Screening of BTV suspected samples by multiplex RT-PCR. The letter beside no. indicate the set of primers used, NCA and NCB indicates negative control for group A and B respectively that show no amplification, M indicate 100 bp DNA marker. A) Sample No. 1-8; KMM-1228, KMM-1229, KMM-1230, NLG-1954, NLG-1956 show amplification of 196 bp and 274 bp; KMM-1231 show amplification of 274 bp; NLG-1955 show amplification of 196 bp, 206 bp and 274 bp; N1957 show no amplification. B) Sample No. 9-16; NLG-1958, NLG-1959 show amplification of 196 bp and 274 bp; WLG-1026 show amplification of 206 bp, 274 bp and 390 bp; WGL-1028 show amplification of 274 bp and 396 bp; WGL-1957, WGL-1027, WGL-1029, WGL-1030 show no amplification. C) Sample No. 17-22; WGL-1039 show amplification of 274 bp and 390 bp; WGL-1040, WGL-1042 show amplification of 274 bp; WGL-1049 show amplification of 206 bp and 274 bp; WGL-1045 show amplification of 274 bp and 548 bp; WGL-1041 show amplification of 196 bp, 274 bp and 396 bp. D) Sample No. 23-25; KMM-1006, KMM1007, KMM-1008 show amplification of 196 bp and 274 bp.

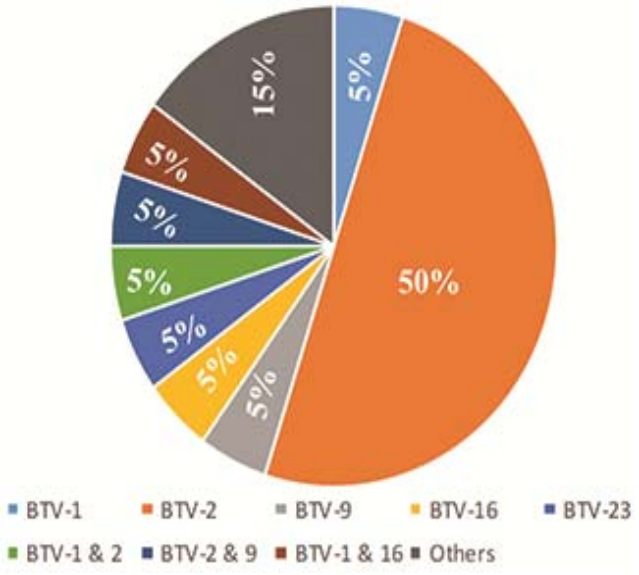

Fig. 4 — Seroprevalence of BTV serotypes in the year 2012-13 in Andhra Pradesh. The bar indicates the percentage of prevalence for each serotype.

In conclusion, we standardized a VP2 based multiplex RT-PCR based diagnostic tool for BTV which gives a direct correlation for variation in serotypes. This novel multiplex RT-PCR is more reliable, cost effective, and rapid test for typing BTV serotypes without compromising the specificity of serum neutralization test. This assay may also be useful in epidemiological surveillance and can be made as part of a regular diagnostic regime for testing cattle, sheep, and goats. Further, we acknowledge the need for screening a large number of clinical samples for the test to be validated at a large scale.

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References

1 ICTV. Reoviridae - dsRNA Viruses - dsRNA Viruses (2011) - International Committee on Taxonomy of Viruses (ICTV).

2019 [cited 2019 Nov 8]. Available from: https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsrna-viruses-2011/w/dsrna_ viruses/ 188/reoviridae.

2 Du Toit R, The transmission of bluetongue and horse-sickness by *Culicoides*, *Onderstepoort J Vet Sci Anim Ind*, 19 (1944) 7-16.

3 Elbers A R W, Backx A, Meroc E, Gerbier G, Staubach C, *et al*, Field observations during the bluetongue serotype 8 epidemic in 2006 I. Detection of first outbreaks and clinical signs in sheep and cattle in Belgium, France and the Netherlands, *Prev Vet Med*, 87 (2008) 21-30.

4 Rao P P, Hegde N R, Reddy Y N, Krishnajyothi Y, Reddy Y V *et al*, Epidemiology of bluetongue in India, *Transbound Emerg Dis*, 63 (2016) e151-e164.

5 Maan S, Maan N S, Nomikou K, Batten C, Antony F *et al,* Novel bluetongue virus serotype from Kuwait, *Emerg Infect Dis*, 17 (2011) 886-889.

6 Zientara S, Sailleau C, Viarouge C, Hoper D, Beer M *et al,* Novel bluetongue virus in goats, Corsica, France, 2014, *Emerg Infect Dis,* 20 (2014) 2123-2125.

7 Maan S, Maan N S, van Rijn P A, van Gennip R G P & Sanders A, Full genome characterisation of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains, *PLoS One,* 5 (2010) e10323.

8 Huismans H & Erasmus B J, Identification of the serotype-specific and group-specific antigens of bluetongue virus, *Onderstepoort J Vet Res*, 48 (1981) 51-58.

9 Bommineni Y R, Reddy Y N, Rao P P, Sudheer D, Susmitha B *et al,* Isolation of bluetongue virus serotypes 2, 9 and 15 from sheep in India, *Vet Rec,* 163 (2008) 545-546.

10 Susmitha B, Sudheer D, Rao PP, Uma M, Prasad G *et al,* Evidence of bluetongue virus serotype 21 (BTV-21) divergence, *Virus Genes,* 44 (2012) 466-469.

11 Krishnajyothi Y, Maan S, Kandimalla K, Maan N S, Tutika R B *et al,* Isolation of BT virus 24 from India– An exotic serotype to Australasia, *Transbound Emerg Dis*, 63 (2016) 360-364.

12 Hemadri D, Maan S, Chanda M M, Rao P P, Putty K *et al,* Dual infection with bluetongue virus serotypes and first time isolation of serotype 5 in India, *Transbound Emerg Dis*, 64 (2017) 1912-1917.

13 De A, Das T K, Chand K, Debnath B C, Dey S *et al,* Seroprevalence of bluetongue and presence of viral antigen and type-specific neutralizing antibodies in goats in Tripura, a state at Indo-Bangladesh border of North Eastern India, *Trop Anim Health Pro*, 51 (2019) 261-265.

14 Worwa G, Hilbe M, Chaignat V, Hofmann MA, Griot C *et al,* Virological and pathological findings in bluetongue virus serotype 8 infected sheep, *Vet Microbiol*, 144 (2010) 264-273.

15 Afshar A, Thomas F C, Wright P F, Shapiro J L & Anderson J, Comparison of competitive ELISA, indirect ELISA and standard AGID tests for detecting bluetongue virus antibodies in cattle and sheep, *Vet Rec*, 124 (1989) 136-141.

16 Dangler C A, De Mattos C A, De Mattos C C, & Osburn B I, Identifying bluetongue virus ribonucleic acid sequences by the polymerase chain reaction, *J Virol Methods*, 28 (1990) 281-292.

17 Akita G Y, Chinsangaram J, Osburn B I, Ianconescu M & Kaufman R, Detection of bluetongue virus serogroup by polymerase chain reaction, *J Vet Diagn Invest*, 4 (1992) 400-405.

18 Jiménez-Clavero M A, Agüero M, Miguel E S, Mayoral T, López M C *et al,* High throughput detection of bluetongue virus by a new real-time fluorogenic reverse transcription-polymerase chain reaction: Application on clinical samples from current Mediterranean outbreaks, *J Vet Diagn Invest*, 18 (2006) 7-17.

19 Vishwaradhya T M, Minakshi P, Ranjan K, Supriya A, Kumar P *et al,* Sensitive detection of novel Indian isolate of BTV 21 using *ns1* gene based real-time PCR assay, *Vet World*, 6 (2013) 554-557.

20 Lakshmi I K, Putty K, Raut S S, Patil S R, Rao P P *et al,* Standardization and application of real-time polymerase chain reaction for rapid detection of bluetongue virus, *Vet World*, 11 (2018) 452-458.

21 Shad G, Wilson W C, Mecham J O & Evermann J F, Bluetongue virus detection: A safer reverse-transcriptase polymerase chain reaction for prediction of viremia in sheep, *J Vet Diagn Invest*, 9 (1997) 118-24.

22 Elnifro E M, Ashshi A M, Cooper R J & Klapper P E, Multiplex PCR: Optimization and application in diagnostic virology, *Clin Microbiol Rev*, 13 (2000) 559-570.

23 Sreenivasulu D & Subba Rao MV, Clinical, virological and serological responses of sheep and goat inoculated with bluetongue virus type 2, *Indian J Anim Sci*, 70 (2000) 151-153

24 Yin H Q, Zhang G P, Zhang H & Zhang J G, A pair of novel primers for universal detection of the NS1 gene from various bluetongue virus serotypes, *Virol Sin*, 23 (2008) 68-72.

25 OIE, Chapter 3.1.3, Bluetongue in *Manual of diagnostic tests and vaccines for terrestrial animals*, 2019.

26 Maan S, Maan N S, Belaganahalli M N, Potgieter A C, Kumar V *et al,* Development and evaluation of real time RT-PCR assays for detection and typing of bluetongue virus, *PloS One*, 11 (2016) e0163014.

27 Roy P, Bishop D H, LeBois H & Erasmus B J, Long lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: Evidence for homologous and partial heterologous protection, *Vaccine*, 12 (1994) 805-811.

28 Mertens P P C, Orbiviruses and coltiviruses-general features in *Encyclopaedia of Virology*, 2nd edn, Edited by R G Webster & A Granoff (Academic Press, Lodon) 1999, 1043-1061.

29 Mertens P P C, Maan N S, Prasad G, Samuel A R, Shaw A E *et al,* Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: Differentiation of field and vaccine strains, *J Gen Virol*, 88 (2007) 2811-2823.

30 Kahlon J, Sugiyama K & Roy P, Molecular basis of bluetongue virus neutralization, *J Viral*, 48 (1983) 627-632.

31 Maan S, Maan N S, Samuel A R, Rao S, Attoui H *et al,* Analysis and phylogenetic comparisons of full-length VP2 genes of the twenty-four bluetongue virus serotypes, *J Gen Virol*, 88 (2007) 621-630.

32 Ayanur A, Singh K P, Cherian S, Singh V, Nandi S *et al,* Sero-epidemiology and molecular detection of bluetongue virus in Indian ruminants, *Vet Ital*, 52 (2016) 305-311.

33 Aradaib I E, Smith W L, Osburn B I & Cullor J S, A multiplex PCR for simultaneous detection and differentiation of North American serotypes of bluetongue and epizootic hemorrhagic disease viruses, *Comp Immunol Microb*, 26 (2003) 77-87.

34 Johnson D J, Wilson W C & Paul P S, Validation of a reverse transcriptase multiplex PCR test for the serotype determination of US isolates of bluetongue virus, *Vet Microbiol*, 76 (2000) 105-115.

35 Joardar S N, Barkataki B, Halder A, Lodh C & Sarma D, Seroprevalence of bluetongue in north eastern Indian state-Assam, *Vet World*, 6 (2013) 196.

36 Sreenivasulu D, Subba Rao M V, Reddy Y N & Gard G P, Overview of bluetongue disease, viruses, vectors, surveillance and unique features: the Indian sub-continent and adjacent regions, *Vet Ital*, 40 (2004) 73-77.

37 Khezri M & Azimi S M, Seroprevalence of bluetongue disease in sheep in west and northwest provinces of Iran in Veterinary Research Forum, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, Vol. 4, No. 3, 2013, 195.

38 Lundervold M, Milner-Gulland E J, O'callaghan C J & Hamblin C, First evidence of bluetongue virus in Kazakhstan, *Vet Microbiol*, 92 (2003) 281-287.

39 Gur S, A serologic investigation of blue tongue virus (BTV) in cattle, sheep and gazella subgutturosa in southeastern Turkey, *Trop Anim Health Pro*, 40 (2008) 217-221.

40 Yousef M R, Al-Eesa A A & Al- Blowi M H, High seroprevalence of bluetongue virus antibodies in sheep, goats, cattle and camel in different districts of Saudi Arabia, Vet World, 5 (2012).

41 Akhtar S, Djallem N, Shad G & Thieme O, Bluetongue virus seropositivity in sheep flocks in North West Frontier Province, Pakistan, *Prev Vet Med*, 29 (1997) 293-298.

42 Ward M P & Thurmond M C, Climatic factors associated with risk of seroconversion of cattle to bluetongue viruses in Queensland, *Prev Vet Med*, 24 (1995) 129-136.

43 Sairaju V, Susmitha B, Rao P P, Hegde N R, Meena K *et al*, Type-specific seroprevalence of bluetongue in Andhra Pradesh, India, during 2005-2009, *Indian J Virol*, 24 (2013) 394-397.