Original Article

Comparison of bluetongue virus detection and quantitation methods in south India

Subhra Subhadra[1], Subrat Kumar[2], Veluvarthy VS Suryanarayana[3], Daggupati Sreenivasulu[1]

[1] *Department of Veterinary Microbiology, College of Veterinary Science, SVV University, Tirupati, Andhra Pradesh, India*
[2] *School of Biotechnology, Campus-XI, KIIT University, Patia, Bhubaneswar, Orissa, India*
[3] *Indian Veterinary Research Institute, Hebbal, Bangalore, Karnataka, India*

Abstract
Introduction: Bluetongue (BT), a vector-borne viral disease, primarily affects sheep. Of the 26 serotypes of BTV identified so far, 22 are reported to be circulating in India. Due to an increase in vector population and delays in disease diagnosis, the BT control program heavily relies on rapid and confirmatory diagnosis. Polymerase chain reaction (PCR)-based real-time detection assays may be an ideal method to detect the BTV genome in animal blood at an early stage of infection.
Methodology: In this study, a SYBR green-based real-time RT-PCR assay was evaluated, validated, and compared with conventional RT-PCR. The specificity and sensitivity of an assay using BTV-2 RNA extracted from tenfold serially diluted (starting from 1.0 $TCID_{50}$/mL) cell culture virus was also evaluated.
Results: While conventional RT-PCR could detect 3.16×10^2 $TCID_{50}$ of virus/mL, the real-time PCR test had a detection limit of 3.16×10^{-4} $TCID_{50}$/mL. Melting curve analysis indicated the absence of non-specific amplification ($R^2 = 0.987$). Out of the 32 infected blood samples examined, 24 tested positive for BTV RNA. Seven that were found negative through conventional PCR tested positive through real-time PCR.
Conclusions: These results showed that the SYBR green-based real-time PCR assay is rapid, sensitive, and equally specific in the diagnosis of BT in BTV-affected animals.

Key words: bluetongue virus; sheep; NS3 gene; RT-PCR; real-time PCR.

Introduction

Bluetongue (BT) disease is one of the economically important diseases of livestock, causing an annual economic loss of about USD 3 billion worldwide [1,2]. It is an arthropod-borne, non-contagious viral disease affecting domestic and wild ruminants [3]. BT is caused by a virus (BTV), a type of species in the genus *Orbivirus* under the family *Reoviridae* [4]. Twenty-six distinct serotypes of the virus have been identified to date [5-7]. Outbreaks of BT are suspected from clinical symptoms such as fever, depression, excessive drooling, nasal discharges, oral lesions, facial edema, hyperemia of coronary bands, muscle weakness, and reproductive disorders [8]. On an average, 2%–30% of the animals infected by BT die, but the mortality rate can reach as high as 100% in highly susceptible sheep. BT has been listed as a notifiable disease by Office International des Epizooties (OIE), also known as the World Organization for Animal Health [9]. Apart from causing losses through reduced productivity of farm animals, BT infection can also negatively affect the trade of such animals, due to the strict restrictions on the movement of animals and animal products from BT-endemic countries to BT-free countries.

BT is a vector-borne disease transmitted by the *Culicoides* vector. *Culicoides* shows a seasonal variation [10], with the incidence of BT being the highest after the rainy season when conditions are favorable for the multiplication of such vectors. Due to the abundance of the *Culicoides* vector, favorable conditions, and susceptible sheep populations, outbreaks of BT have become a major concern in South India. To control the rampant spread of the disease, a sensitive, reliable, and rapid diagnostic method for detecting BTV in clinical samples is needed. Due to the high amount of antigenic diversity and 22 serotypes of BTV circulating in India,

developing an effective control method for BT has become a problem. Therefore, the present study aimed to develop and validate a rapid and sensitive diagnostic test for early detection of the disease. Real-time PCR was developed and evaluated for detection and quantitation of BTV from naturally infected sheep so that appropriate and timely control measures can be implemented before the disease spreads to other susceptible populations.

Methodology

BTV serotypes and virus titration

BTV serotypes (2, 9, and 10), maintained in BHK_{21} cell line at Department of Veterinary Microbiology, College of Veterinary Science, Tirupati, were used in this study. BTV-23 maintained at College of Veterinary Science, Hyderabad, was procured and propagated in the laboratory for use in the examination. Virus titer was determined by micro-titration assay using BHK_{21} cells cultured in Eagle medium (EMEM) supplemented with L-glutamine, antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), and 2% fetal calf serum. $TCID_{50}$ and 100 $TCID_{50}$ were calculated using the method of Reed and Muench [11].

Clinical samples from field outbreak

A total of 32 blood samples were collected from sheep suspected to be infected by BT during 2010-2011 in Andhra Pradesh, India. A blood sample (~5.0 mL) was collected from each sheep in vials with EDTA (5-10 mg). Blood samples (~5.0 mL) collected from healthy sheep (maintained in the Department of Animal Nutrition, College of Veterinary Sciences, Tirupati) served as negative controls.

Extraction of viral RNA and cDNA synthesis

RNA from all the four BTV-infected cell culture supernatants with titers ranging from 4.5 to 5.5 $TCID_{50}$/mL was extracted using TRI Reagent (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions. Total RNA from whole blood was extracted using the procedure described by Billinis *et al*. [12]. Necessary precautions were taken to avoid RNAse contamination during extraction as described by Sambrook *et al*. [13]. Extracted RNA was suspended in 20 µL of nuclease-free water and stored at −80°C until further use. cDNA synthesis was done from total RNA using random hexamers as primer for the standard protocol. Briefly, total RNA in 3 µL was mixed with 1.0 µL (2 µg) of primer (Fermentas Life Sciences Pittsburgh, USA) and

incubated at 65°C for 5 minutes in Eppendorf Master Cycler before snap cooling on ice. To the primer-template mixture, 4 µL of 5× RT buffer (250mM Tris-HCL, 250 mM KCl, 20 mM MgCl2, 50mM DTT), 40 units of RNAse inhibitor, 10 mM dNTP, and 20 units of M-MuLV RT enzyme were added in a reaction volume of 20 µL and incubated at 42°C for an hour. The reaction was stopped by heating at 75°C for 15 minutes and the cDNA thus obtained was stored at −20°C for further use.

Polymerase chain reaction (PCR)

The sequences, corresponding to BTV-S10 segment, encoding for NS3 protein of 24 isolates (available in the National Center for Biotechnology Information database), were aligned using the Clustal W program. The highly conserved region was identified and oligos custom-synthesized. The primers used for amplification were BTV NS3 F – 5' GCGGGATCCATGCTATCCGGCTGAT and BTV NS3 R – 5' GCGTACGATGCGAATGCAGC as forward and reverse primers, respectively. PCR was performed in 25 µL reaction mixture containing 2 µL of reverse transcriptase reaction, 2.5 µL of 10×PCR buffer (100mM Tris-HCL; 500mM KCl; 15mM MgCl2), 20 pmol of each primer (BTV NS3F and NS3R), 10 mM of each dNTP, 1.5 mM $MgCl_2$, and 1U of Taq DNA polymerase (Fermentas Life Sciences). PCR cycling conditions were as follows: initial denaturation of 94°C for 3 minutes, followed by 35 cycles of 94°C, 60°C, and 72°C for 30 seconds each, followed by 1 cycle of 10 minutes at 72°C. All the PCR products were subjected to 2% agarose gel electrophoresis to confirm the positive samples.

Real-time RT-PCR

BT viral load was quantitated by real-time RT-PCR assay with SYBR green. The reactions were carried out in 25 µL reaction mixture containing 2 µL of cDNA, 5 pmol of each primer, 12.5 µL of SYBR Green Jump Start Taq Ready Mix (Sigma-Aldrich, Missouri, USA), and 0.3 µL of reference dye. PCR cycling conditions were as follows: initial denaturation of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, followed by 1 cycle of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. Real-time PCR assay was carried out in Applied Biosystems 7300 (Applied Biosystems, New York, USA) and the data was analyzed by 7300 SDS software version 1.3.1. Appropriate controls (RT enzyme control, negative and positive controls) were included in the assay. Minimum detection limits of

real-time and conventional PCR assays were determined using tenfold dilutions of BTV-2 RNA extracted from cell culture-grown virus.

Results

Conventional PCR

The PCR assay was optimized using the NS3 gene-specific primers. A single amplified product of 250 bp was observed in the ethidium bromide stained agarose gels for all the four serotypes of BTV (Figure 1, lanes 2-5). The negative control did not show any

amplification (Figure 1, lane 6). A similar amplified product of 250 bp was also observed with the cDNA prepared from the RNA extracted from tenfold dilutions of BTV-2 infected cell culture supernatant (Figure 2, lanes 3-5). The minimum detectable limit for conventional PCR was found to be 3.16×10^2 $TCID_{50}$/mL (Figure 2, lane 5).
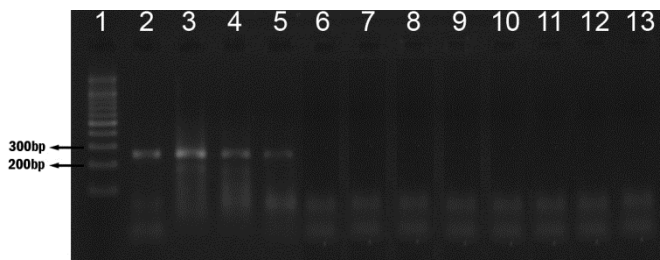
Real-time PCR assay

Real-time PCR was standardized using different dilutions of $TCID_{50}$/mL of BTV-2. The C_T values

Figure 1. 2% gel electrophoresis of BTV RNA amplified by RT-PCR in tissue culture fluid. Gel image showing 250 bp product of the NS3 gene of BTV RNA. Lane 1: 100 bp molecular ladder; lanes 2-5: positive control (BTV serotypes 2, 9, 10, and 23); lane 6: negative control.
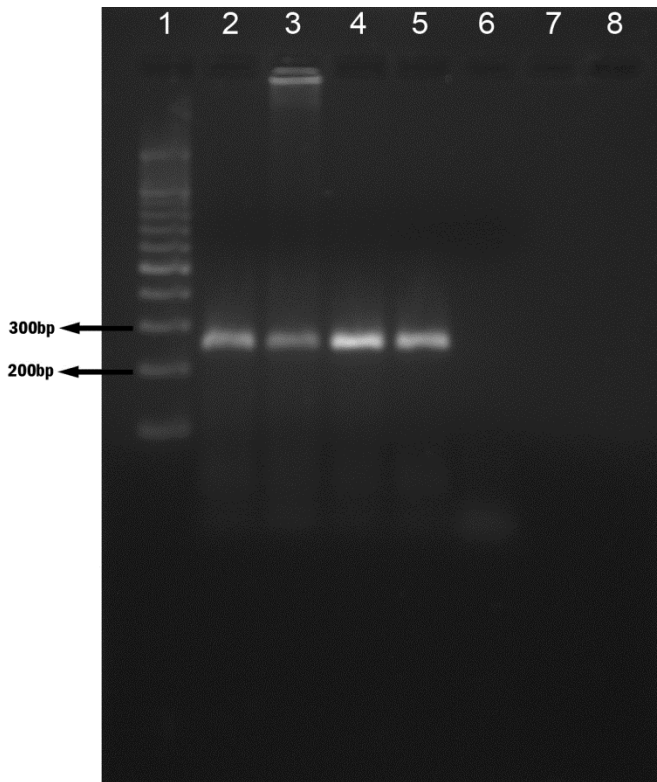
Figure 2. 2% gel electrophoresis of BTV RNA amplified by RT-PCR in the tissue culture fluid. Lane 1: 100bp molecular ladder; lane 2: positive control; lanes 3-12: different dilutions of $TCID_{50}$; lane 13: negative control.

Figure 3. Real-time PCR assay for detection of BTV. (A) amplification plot; (B) melting curve; (C) standard curve.
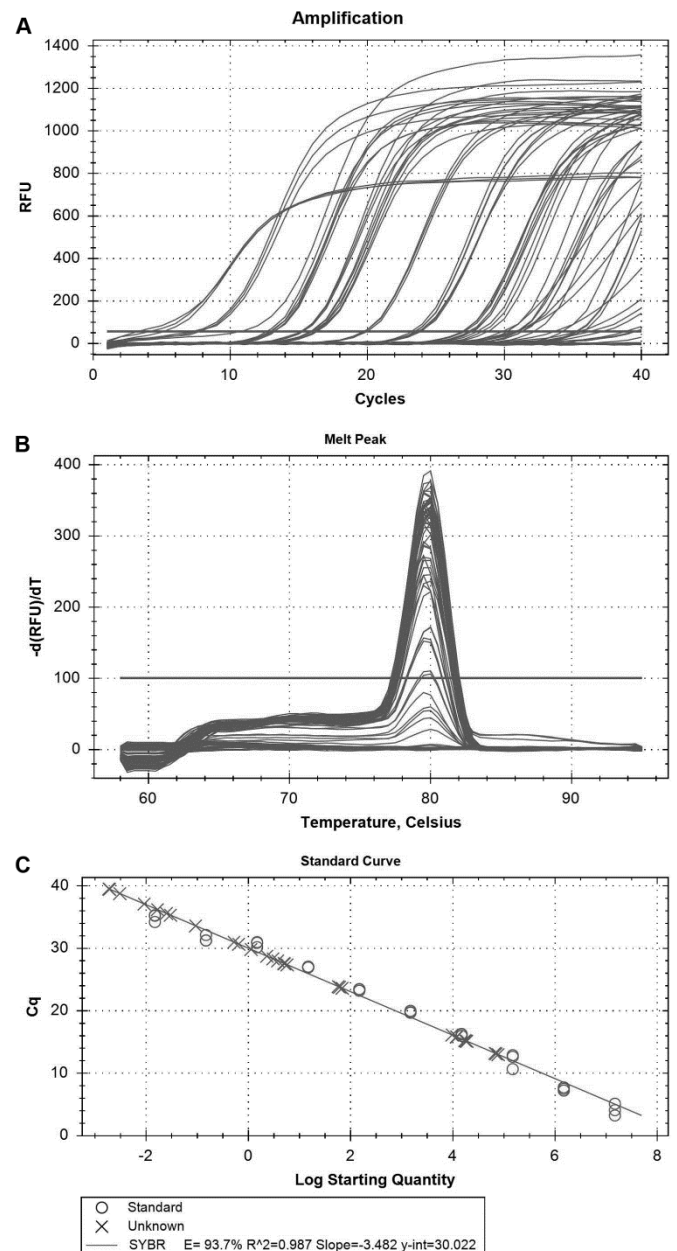
decreased proportionally with the increase in the titer of the virus. The amplification plot for the standards is also shown in Figure 3A. A standard curve was generated by interpolating the crossing point of each standard and plotting it against the logarithmic value of the titer of the virus. No C_T value was observed in no template control (NTC). The specificity of the amplification was carried out by melt curve analysis of the amplified products in real-time RT-PCR (Figure 3B). The standard curve was linear, ranging from 3.16×10^4 to 3.16×10^{-4} $TCID_{50}$/mL with a regression value (R^2) of -0.987 (Figure 3C). The melting temperature (Tm) of the PCR products generated from the standards ranged from 79.5°C to 80°C. This showed that the developed real-time RT-PCR assay was specific. The minimum detectable limit for real-time PCR assay was 3.16×10^{-4} $TCID_{50}$/mL.

Clinical samples

Of the 32 blood samples screened, 17 were found to be positive for BTV-RNA through conventional PCR, while 24 tested positive through real-time PCR assay. The Tm values of the 24 positive samples ranged from 79.5°C to 80°C. Seven samples which tested negative through conventional PCR were found to be positive through real-time PCR.

Discussion

BT is an arthropod-borne viral disease that affects ruminants. Its outbreaks are reported every year from the Indian subcontinent, particularly in the southern part of the country, causing huge economic losses to the marginal and landless farmers who rear small ruminants for their livelihoods. The presence of multiple serotypes of the virus across the world and their rapid spread in the subcontinent as a result of an increase in the vector population underline the need for reliable and quick diagnostic methods. Traditional diagnostic methods for the BT disease involve isolation of the virus using embryonated chicken eggs and/or cell culture adaptation; confirmation is done by serological tests using serotype-specific sera. These involve cumbersome and time-consuming processes. Hence, other diagnostic methods, such as antigen-capture enzyme-linked immunosorbent assay (ELISA), dot immunobinding assay, and immuno-electron microscopy, have been developed. All these techniques have limited sensitivities and thus are unable to detect the virus at a sub-clinical level. Serological methods fail to detect BTV in the blood of clinically BTV-positive ruminants. However, virus antigen can be detected only in those animals showing

a heightened viremia [14]. To overcome these limitations, RT-PCR was developed and evaluated as a suitable diagnostic method for direct detection of BTV in clinical samples [15-17]. RT-PCR, which is more sensitive than serological assays, is used to detect and/or differentiate BTV serotypes. However, conventional RT-PCR is only a qualitative test and cannot estimate the viral load in the clinical sample. Post-amplification procedures (agarose gel electrophoresis) associated with RT-PCR make it unsuitable for large-scale screening of samples. To overcome these problems, real-time PCR methods have been standardized and applied for the diagnosis of various human and animal diseases [18]. Real-time RT-PCR shows a high degree of sensitivity and specificity in comparison with conventional PCR and is therefore used for simultaneous detection and quantification of the etiological agent in the sample. Only a few reports have evaluated real-time PCR for detection of BTV burden in clinical samples [19-21]. A few researchers have targeted the NS3 gene for the development of a real-time assay [22,12,23]. In this study, we developed and evaluated a real-time RT-PCR assay using primers specifically for a highly conversed region in segment 10 (NS3 gene) of BTV. As suggested by Innis and Gelfand [24], we optimized the annealing temperature and $MgCl_2$ for the PCR assay, and we found that $MgCl_2$ at 1.5 mM and annealing temperatures of 60°C for 30 seconds yielded a specific band without any non-specific amplification for all the BTV serotypes tested in the study. When we compared the minimum detectable limit of the conventional RT-PCR assay and the real-time RT-PCR assay using tenfold dilutions of the $TCID_{50}$ of BTV-2/mL of infected cell culture fluid, the detection limit was found to be 3.16×10^2 $TCID_{50}$/mL and 3.16×10^{-4} $TCID_{50}$/mL, respectively. This is comparable to the detection limit of 10^1-10^2 $TCID_{50}$/mL reported by Zientara *et al.* [25]. We also applied the current assay for detection and quantification of BTV in the blood samples of infected animals during natural outbreaks. When the 32 blood samples collected from infected sheep running a temperature of 104°F were analyzed, 17 tested positive through conventional RT-PCR assay, while 22 tested positive through real-time RT-PCR. The titer of the virus in the blood samples ranged from 1.1×10^{-2} to 7.7×10^4 $TCID_{50}$/mL. On melt curve analysis, no amplification was observed in NTC or RT control. The C_T values decreased proportionally with an increase in the titer of the virus, from 3.16×10^4 $TCID_{50}$/mL to 3.16×10^{-4} $TCID_{50}$/mL of BTV-2. The results were in

agreement with previous studies [26,16]. The real-time PCR assay developed by us could detect not only all the four serotypes of BTV – BTV-2, 9, 10, and 23 – used in this study, but also the presence of the virus in seven PCR-negative samples. Hence, the real-time PCR assay developed in our study is more sensitive in the detection of BTV in blood samples compared to the conventional PCR assay reported by Jimenez-Clavero *et al.* [26] and Toussaint *et al.* [27].

Conclusions

The real-time PCR using NS3-specific primers reported in this study was found to be specific, sensitive, and rapid in the detection of BTV in blood samples. However, more blood samples from animals during natural outbreaks must be subjected to the real-time assay developed by us to correlate viral load with disease severity. Furthermore, this assay should be evaluated to understand both the viremic status of BTV-infected sheep and asymptomatic carriers such as cattle and buffalo.

Acknowledgements

References
1. Mertens PP, Diprose J, Maan S, Singh KP, Attoui H, Samuel AR (2004) Bluetongue virus replication, molecular and structural biology. Vet Ital 40: 426-437.
2. Mellor PS, Carpenter S, Harrup L, Baylis M, Mertens PP (2008) Bluetongue in Europe and the Mediterranean Basin: history of occurrence prior to 2006. Prev Vet Med 87: 4-20.
3. Verwoerd DW, Erasmus BJ (2004) Bluetongue. In Coetzer JAW, Tustin RC, editors. Infectious Diseases of Livestock, 2nd edition. Cape Town: Oxford Press. 1201-1220.
4. Pringle CR (1999) Virus Taxonomy. The universal system of virus taxonomy updated to include new proposals ratified by International Committee on Taxonomy of Viruses during 1998. Archives of Virol 144: 422-429.
5. Chaignat VG, Scherrer W, Hilbe N, Ehrensperger M, Batten F, Corteyn C, Hofmann M, Thuer MB (2009) Toggenburg orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. Vet Microbiol 138: 11-19.
6. Maan S, Maan NS, Nomikou K, Batten C, Antony F, Belaganahalli MN, Samy AM, Reda AA, Al-Rashid SA, El BM, Oura CA, Mertens PP (2011) Novel bluetongue virus serotype from Kuwait. Emerg Infect Dis 17: 886-889.
7. Batten CA, Henstock MR, Bin-Tarif A, Steedman HM, Waddington S, Edwards L, Oura CAL (2012) Bluetongue virus serotype 26: Infection kinetics and pathogenesis in Dorset Poll sheep. Vet Microbiol 157: 119-124.
8. Maclachlan NJ, Crafford JE, Vernau W, Gardner IA, Goddard A, Guthrie AJ, Venter EH (2008) Experimental reproduction of severe bluetongue in sheep. Vet Pathol 45 310-315.
9. World Organization for Animal Health (2011) OIE Listed Diseases 2011. Paris: OIE.
10. Maclachlan NJ, Guthrie AJ (2010) Re-emergence of bluetongue, African horse sickness, and other Orbivirus diseases. Vet Res 41: 35.
11. Reed LJ, Muench H (1938) A simple method of estimating fifty percent end points. Am J Hyg 27: 493-497.
12. Billinis C, Koumbati M, Spurou V, Nomiku K, Manganao Panagiotidis CA, Papadopoulos O (2001) Bluetongue virus diagnosis of clinical cases by a duplex reverse-transcription – PCR: a comparison with conventional methods. J Virol Methods 98: 77-89.
13. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory. 1626 p.
14. Singer RS, MacLachlan NJ, Carpenter TE (2001) Maximal predicted duration of viremia in bluetongue virus-infected cattle. J Vet Diagn Invest 13: 43-49.
15. Maan S, Maan NS, Singh KP, Samuel AR, Meretens PPC (2004) Development of reverse transcriptase–polymerase chain reaction based assays and sequencing for typing European strains of bluetongue virus and differential diagnosis of field and vaccine strains. Veterinaria Italiana 40: 552-561.
16. Polci A, Camma C, Serini S, Di Gialleonardo L, Monaco F, Savini G (2007) Real-time polymerase chain reaction to detect bluetongue virus in blood samples. Veterinaria Italiana 43: 77-87.
17. Mertens PP, Maan NS, Prasad G, Samuel AR, Shaw AE, Potgieter AC, Anthony SJ, Maan S (2007) 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: 2811-2823.
18. Lanciotti RS, Kerst AJ (2001) Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. J Clin Microbiol 39: 4506-4513.
19. Shaw AE, Monaghan P, Alpar HO, Anthony S, Darpel KE, Batten CA, Guercio A, Alimena G, Vitale M, Bankowska K, Carpenter S, Jones H, Oura CAL, King DP, Elliott H, Mellor PS, Mertens PPC (2007) Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. J Virol Methods 145: 115-126.
20. Hoffmann B, Eschbaumer M, Beer M (2009) Real time qRT-PCR assays specifically detecting bluetongue virus serotypes 1, 6 and 8. J Clin Microbiol 47: 2992-2994.
21. Steinrigl A, Revilla-Fernandez S, Eichinger M, Koefer J, Winter P (2010) Bluetongue virus RNA detection by RT-qPCR in blood samples of sheep vaccinated with a commercially available inactivated BTV-8 vaccine. Vaccine 28: 5573-5581.
22. Bonneau KR, Zhang N, Zhu J, Zhang F, Li Z, Zhang K, Xiao L, Xiang W, MacLachlan NJ (1999) Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. Virus Research 61: 153-160.
23. Devi BV, Sreenivasulu D, Chetty MS (2006) Detection of bluetongue virus in clinical samples by NS3 specific RT-

PCR. Indian J of Comp Micro, Immunology and Infectious Diseases 27: 70-73.

24. Innis MA, Gelfand DH, (1990) Optimization of PCR in PCR protocols – A guide to methods and applications. Edited by Michael A Innis, David H Gelfand, John J Sninsky and Thomas J White 1990. 482 p.

25. Zientara S, Breard E, Sailleau C (2004) Bluetongue diagnosis by reverse transcription polymerase chain reaction. Veterinaria Italiana 40: 531-537.

26. Jimenez-Clavero MA, Aguero M, San Miguel E, Mayoral T, Lopez MC, Ruana MJ, Romero E, Monaco F, Polci A, Savini G, Gomez-Tejedor C (2006) 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 Diagnostic Invest 18: 7-17.

27. Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K (2007) Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J Virol Methods 140: 115-123.

Corresponding author
Subhra Subhadra, M.V.Sc.
Department of Veterinary Microbiology
College of Veterinary Science, SVV University
Tirupati, Andhra Pradesh, India
Phone: +91-8458045043
Fax: 0877-2249563
Email: ssubhadra@yahoo.com

Conflict of interests: No conflict of interests is declared.