

*Original Research***Detection and Quantification of *Bluetongue* Virus Serotype 23 by Evagreen Based Real Time PCR**

Shabir Ahmad Bhat, Jaynudin Khorajiya*, Bilal Ahmad Malla, Pervaiz Ahmad Dar, Karam Pal Singh, Sukdeb Nandi and Mudasir Ahmad Shah

Centre for Animal Disease Research and Diagnosis (CADRAD), ICAR-Indian Veterinary Research Institute, Izatnagar 243 122, Bareilly, Uttar Pradesh, INDIA

*Corresponding author: jainudeen_1990@rediffmail.com

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Abstract

Bluetongue, an economically important arthropod-borne viral disease of livestock especially sheep. Presently, there are 27 serotypes of bluetongue virus prevalent worldwide. Traditional bluetongue virus typing methods such as virus isolation and Serum Neutralization Tests (SNT) are cumbersome, time-consuming, and many times inconclusive. The 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. Serotype specific RT-PCR and qRT-PCR based amplification of BTV Seg-2 are most reliable, less time consuming and high specificity for serotype typing. In the present study, standardization of EVA-green based qRT-PCR was done for amplification and quantification of BTV-23 serotype from infected BHK-21 cell culture; the results showed high sensitivity as well specificity of realtime PCR for diagnosis of BTV-23.

Key words: *Bluetongue* virus, Cell Culture, EVA-Green, Serotype

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Introduction

Bluetongue (BT) is an arthropod borne non-contagious viral disease of domestic and wild ruminants. It is caused by the *bluetongue* virus (BTV), a member of the genus *Orbivirus* in the family *Reoviridae* (Pringle, 1999), and is transmitted by *Culicoides* spp. Clinical signs of BTV infection are more severe and confined to mostly sheep and white-tailed deer (Howerth *et al.*, 1988; Johnson *et al.*, 2006). It is characterized by fever, facial oedema, ulceration of oral mucosa, congestion of tongue and coronary band, debility, decreased production and variable mortality (Maclachlan *et al.*, 2009). Cattle, goat and buffalo act as

reservoir for the disease. The disease is enzootic in areas where reservoirs (cattle and wild ruminants) and vectors exist for the BT virus (BTV). The preliminary diagnosis of BT is based on by clinical signs, post-mortem findings, and epidemiology and it is further confirmed by laboratory examination (Afshar, 1994). For the diagnosis of BT, isolation of BTV is the most reliable technique. Samples of choice for laboratory examination include blood in anticoagulant, serum, post-mortem samples like spleen, lymph node, bone marrow, lung, liver, heart and brain from aborted fetuses (Afshar, 1994; Tweedle and Mellor, 2002). Many mammalian (BHK-21, Vero and CPAE cell line) and insect cell line such as KC - derived from *Culicoides sonorensis* and C6/36 from *Aedes albopictus* can also be used for the isolation of virus (Girard *et al.*, 1967; McPhee *et al.*, 1982; Wechsler *et al.*, 1989; Mecham, 2006). Traditional typing methods such as virus isolation and serum neutralization tests (SNT) were initially used for identification of BTV serotypes. But these methods are slow (taking weeks, depend on availability of reference virus-strains or antisera) and can be inconclusive. Currently, nucleotide sequence analyses and phylogenetic comparisons of genome segment 2 (Seg-2) encoding BTV outer-capsid protein VP2 (the primary determinant of virus serotype) are used for BTV serotype typing. The resulting Seg-2 database has been used to develop rapid (within 24 h) and reliable RT-PCR-based typing assays for each BTV type. This provides a rapid, sensitive and reliable method for the identification and differentiation of the 27 BTV serotypes, and can be updated periodically to maintain their relevance to current BTV distribution and epidemiology.

Materials and Methods

Virus Propagation

The *bluetongue virus* serotype-23 was received from the Virology Laboratory, Center for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute (IVRI), Izatnagar. The 75cm² flasks of BHK-21 cell showing 80% confluent monolayer were infected with 0.3ml master seed of BTV-23. This was incubated at 37 °C and the flask containing BHK-21 cells with the virus was tilted at 15 min interval. After one hour of incubation the inoculum was decanted, washed two times with 2 ml maintenance medium (MM) and 15 ml of MM was added. Uninfected control cells were also kept and flasks were incubated at 37 °C until CPE developed. BHK-21 cell showing 90% CPE were harvested by freeze-thaw method. The suspension was centrifuged at 3000 rpm for 10 min. Supernatant was collected by leaving about 3 ml of medium with the infected cell pellet. The cell pellet was suspended in 2 mM Tris-HCl buffer (pH 8.8), freeze-thawed three times, and sonicated for 60 sec at 30 micron amplitude in ultrasonic processor (Sonics, USA). It was centrifuged again at 3000 rpm for 10 min and the supernatant was collected and kept by suspending in Tris HCl buffer at 4°C. The identity of serotype was checked by RT-PCR amplification of the serotype-specific genome segment-2 by serotype specific primers

developed by Maan *et al.* (2012).The details of primers used for serotyping in this study have been given in Table 1.

Table 1: Details of Primers

BTV Primer designation	Sequence (5' to 3')	Predicted Product Size (bp)
BTV-23/2/384-402F	GCGCACACGAGAACGCGAG	1548
BTV-23/2/1932-1914R	GTTTAACRTGCATACTCAG	
BTV-23/S2/60-81 F	GCGGARYTGTTAGATGGCTATG	88
BTV-23/S2/148-126R	GGAATTTGWGYRACRTCATGACG	

Viral RNA Extraction

Total RNA was extracted from 250 µl infected BHK-21 cells suspension with 750 µl Trizol-LS reagent (Life Technology, USA), following the method recommended by the manufacturer. RNA was precipitated with isopropanol and washed with 70% ethanol. Double-stranded RNA (dsRNA) was purified with 2M LiCl differential precipitation of ssRNA described previously (Wilson *et al.*, 2000). The dsRNA was dried in dry bath and resuspended in 30µl DEPC treated water. The quality of the dsRNA was assessed by 1% agarose gel electrophoresis (AGE) in TAE buffer containing 0.5 mg/ml of ethidium bromide. The concentration of dsRNA was determined by measuring of optical density at 260 nm by using a Nanodrop spectrophotometer (Thermo Scientific, USA). The extracted RNA was stored at -80°C until cDNA synthesis. The purity and integrity of viral RNA was analysed in spectrophotometry and by running in 1.5% agarose gel for detecting segmented nature of BTV RNA, respectively.

cDNA Synthesis by RT-PCR

1µg of purified BTV RNA was converted to cDNA and used for standard dilution curve preparation. A standard log10 dilution curve was generated using tenfold serial dilution of known copies of cDNA. The master mix contained 1µg of total RNA, 1µl of 0.5µg/µl random hexamer and nuclease free water (NFW) to make a volume of 12.5µl. The mixture was incubated at 75°C for 5 min followed by snap chilling on ice. To the above mixture, 4 µl of 5× reverse transcriptase buffer, 2 µl dNTP mix (10mM), 0.5µl RNase inhibitor (40units/µl), and 1µl RevertAid (reverse transcriptase) (200 units/µl) (Fermentas, Lithuania) were added according to manufacturer's instruction. Reverse transcription was carried out at 42 °C for 1 h followed by enzyme inactivation at 70 °C for 10 min.

Amplification by RT-PCR and qRT-PCR

The reverse transcription was checked by conventional PCR using BTV-23 serotype specific primers and 1µl of cDNA as the template. The PCR master mix consisted of 2.5 µl of 10X magnesium free PCR buffer (10mM Tris-HCl, 50mM KCl, 0.1% Triton X), 1.5 µl of 25 mM MgCl₂, 1.0 µl of 10 mM each of

four dNTP, 1.0 µl each of (20 pmol) forward and reverse primers, 0.5 µl of 1.0 U/µl Taq DNA polymerase (promega, USA), 1 µl of cDNA and rest NFW. The PCR was carried out by GeneAmp® PCR system 9700 thermal cycler machine (Applied biosystems®, USA). The cycling conditions were initial denaturation at 95°C for 5 min, 35 cycles of 94°C/30sec denaturation, 56°C/30sec primer annealing and 72°C/1 min extension followed by final extension of 72°C for 7 min. The RT-PCR product was resolved on 1% agarose gel in 1X TAE buffer. 100 bp plus DNA ladder (Fermentas, USA) was used as marker to determine the size of RT-PCR products. Gel was stained with ethidium bromide (life technologies, USA) and visualized under UV Transilluminator using Gel Documentation System (Gel Doc™ XR⁺, imaging system, BIORAD, USA).

Quantitative real-time PCR was performed with Evagreen qPCR MasterMix-Low ROX (G-Biosciences) using Mx3005P Real Time Thermal Cycler System (Stratagene, Agilent Technologies, USA). Each qPCR reaction was put in duplicate in a total volume of 10 µl, which contained 5 µl of 2x Evagreen qPCR MasterMix, 0.125 µl each of 10 pmol forward and reverse primer, 1 µl of cDNA template and rest NFW. Nuclease free filter tips were used for taking the individual reaction components and preparation of reaction mixture. Forty cycles of denaturation for 15 sec at 95 °C, annealing and extension for 1min at 60 °C were performed after an initial denaturation at 95°C for 15 min and last cycle at 95 °C for 30 sec, 65 °C for 30 sec, and gradual increment from 65 °C to 95 °C @ 0.580 C/sec and continuous fluorescence measurement, and a final cooling down to 4°C. After the run, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the “EVA green (with dissociation curve)” method. No template control (NTC), in which no cDNA added, was kept to rule out reagents contamination. For each sample, a dissociation curve was generated after completion of amplification and analyzed in comparison to negative controls to determine the specificity of PCR reaction.

Results and Discussion

Bluetongue virus was propagated in BHK-21 cells and cytopathic effect was observed at 48-72 hours post infection (hpi). After 24 hpi, there were shrinking and rounding up, increased granularity and production of optically dense nuclei of the cells. After 48 h, vacuoles appeared on the cell sheet as presented in Fig. 1 and 2. Finally by 72-96 h post infection, the cells were separated and floated in the medium. BHK-21 cell showing 90% CPE were harvested by freeze-thaw method. The identity of BTV-23 serotype was checked by using serotype specific primers in RT-PCR and qRT-PCR. The products of PCR were run on 1% and 2.5% agarose gel respectively, and visualized in gel documentation system and was found to be of expected size of 1548 bp and 88 bp respectively as presented in Fig. 3 and 4. The amplification and dissociation curves of BTV-23 genome in realtime PCR are presented in Fig. 5. A standard log₁₀ dilution

curve was generated using tenfold serial dilution of known copies of cDNA and corresponding C_t values. Regression analysis of C_t values from each dilution produced R^2 of 0.99 as presented in Fig. 6.

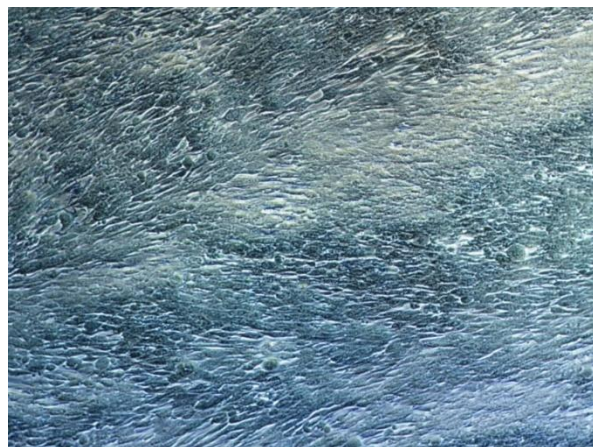


Fig. 1: BHK-21 cells showing confluent growth after 48 h at 37°C under 5 % CO₂ tension in a humidified CO₂ incubator.

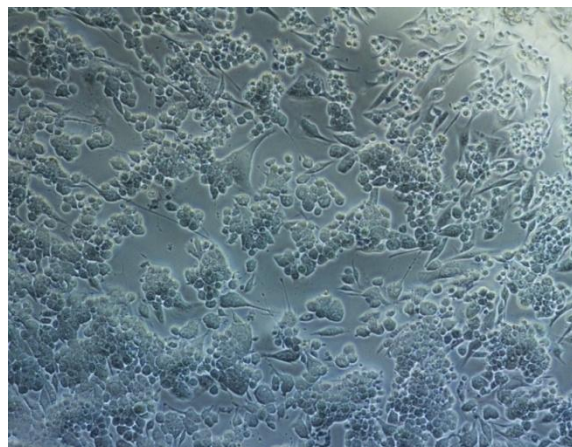


Fig. 2: CPE observed in BHK-21 cells after inoculation of BTV serotypes and incubated for 48 h at 37°C under 5 % CO₂ tension in a humidified CO₂ incubator.

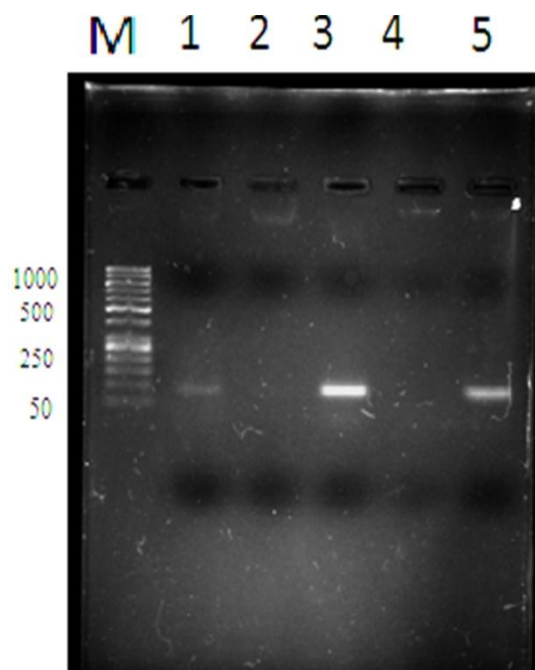


Fig. 3: Agarose gels showing the amplicon obtained from RT-PCR with BTV-23 serotype used in 500 the study. **Lane M:** 50 bp DNA ladder; **Lane 1, 3, 5:** 88 bp RT-PCR product of BTV-23 serotype; **Lane 2, 4:** NTC

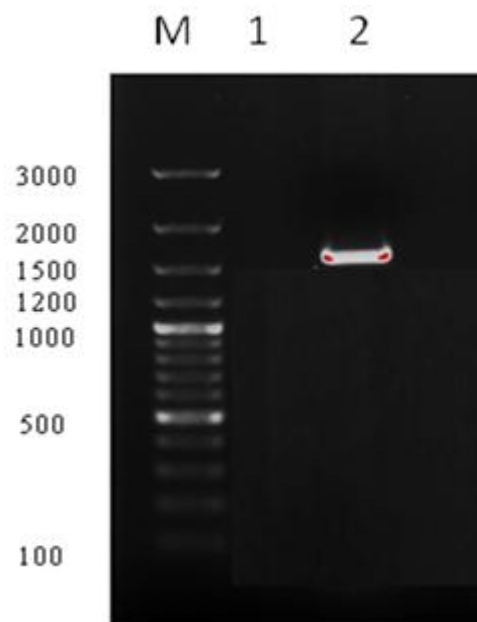


Fig. 4: Agarose gels showing the amplicon obtained from RT-PCR with BTV-23 serotype used in the study. **Lane M:** 100 bp plus DNA ladder; **Lane 1 :** NTC; **Lane 2 :** 1548 bp RT-PCR product of BTV-23 serotype.

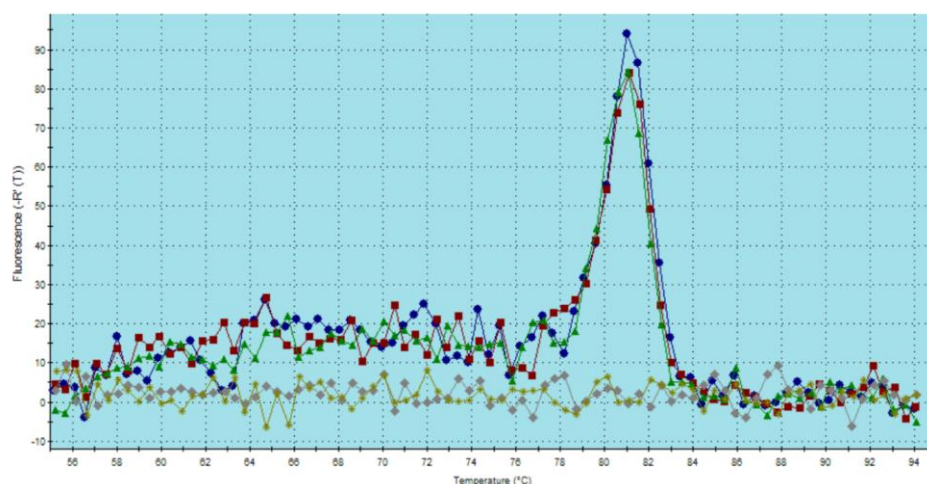


Fig.5A: Amplification plots

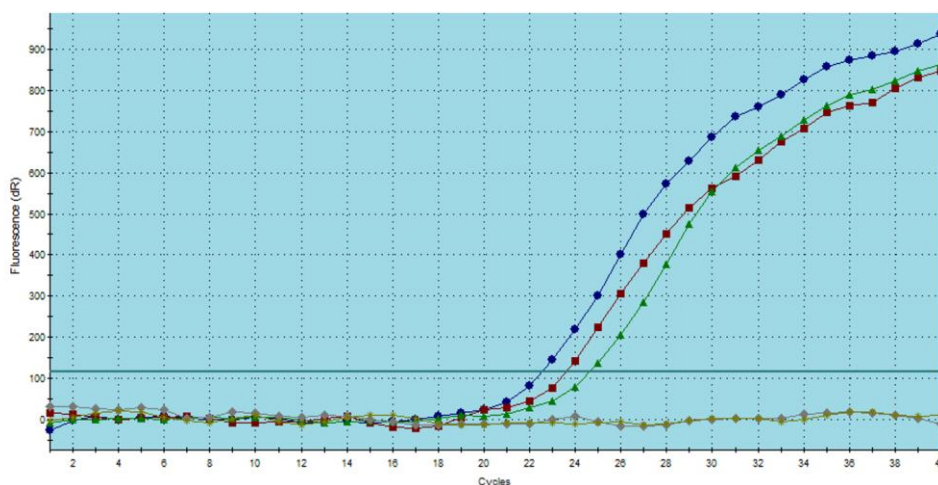


Fig. 5B: Dissociation curves of BTV-23 genome in realtime-PCR.

BT is a vector-borne viral disease of ruminants especially sheep, with outbreaks during monsoon season have been reported every year from the Indian subcontinent. This is an economically important disease causing huge losses to the marginal and landless farmers who rear small ruminants for their livelihoods in terms of trade restrictions and disease treatment. Currently there are 27 serotypes of *bluetongue virus* worldwide and furthermore, there is lack of cross-reactivity among serotypes. Thus there is a need for serotype specific, reliable and quick diagnostic methods for successfully controlling the disease with development of serotype specific vaccines based on serotype prevalence in the region.

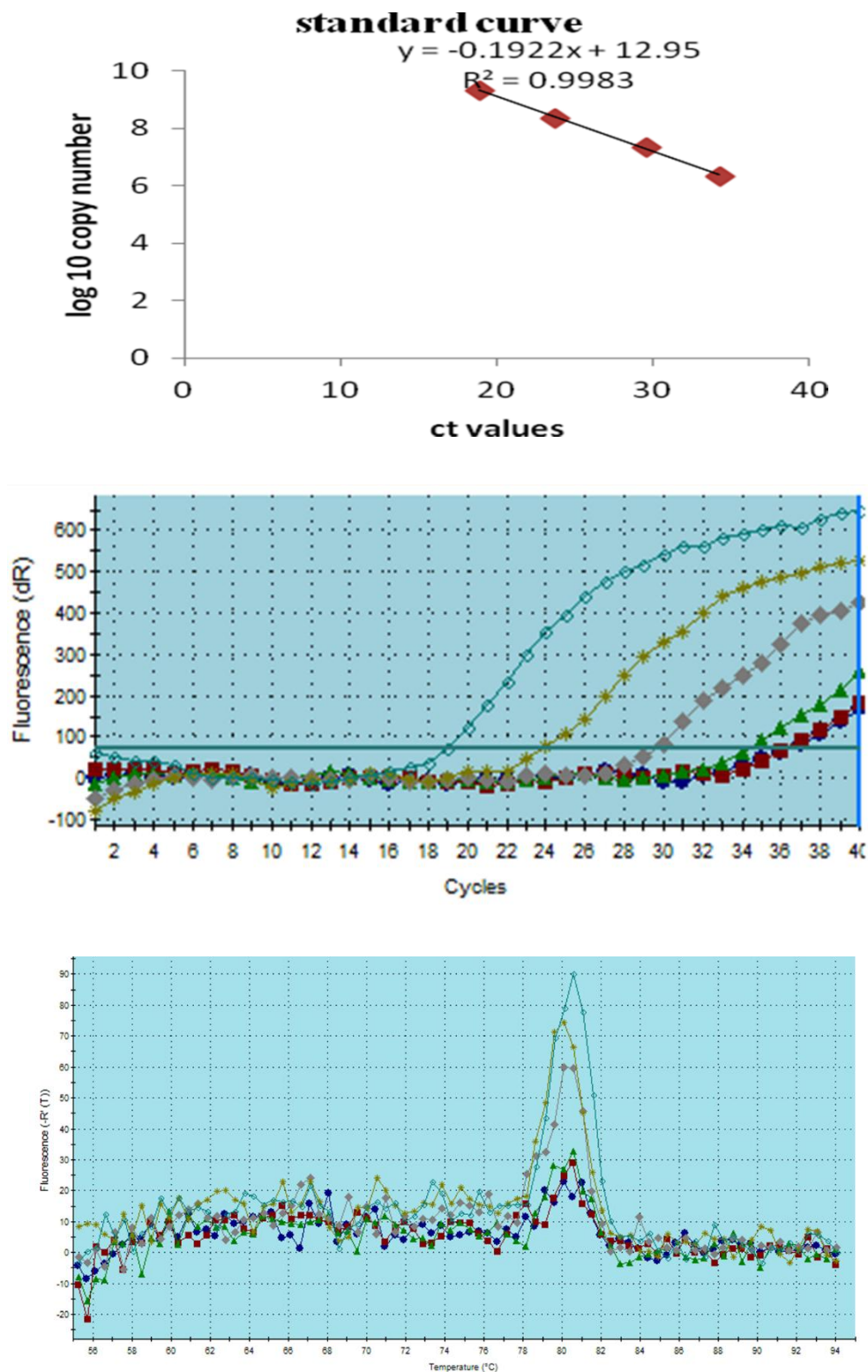


Fig. 6 (A, B, C): Determination of sensitivity of EVA green Real Time PCR in detecting and quantifying genome of BTV-23

The preliminary diagnosis of BT is based on by clinical signs, post-mortem findings, and epidemiology and it is further confirmed by laboratory examination (Afshar, 1994). For the diagnosis of BT, isolation of BTV is the most reliable technique. Samples of choice for laboratory examination include blood in anticoagulant, serum, post-mortem samples like spleen, lymph node, bone marrow, lung, liver, heart and brain from aborted fetuses (Afshar, 1994; Tweedle and Mellor, 2002). Many mammalian (BHK-21, Vero and CPAE cell line) and insect cell line such as KC - derived from *Culicoides sonorensis* and C6/36 from *Aedes albopictus* can also be used for the isolation of virus (Girard *et al.*, 1967; McPhee *et al.*, 1982; Wechsler *et al.*, 1989; Mecham, 2006). Traditional typing methods such as virus isolation and serum neutralization tests (SNT) were initially used for identification of BTV serotypes. But these methods are slow (taking weeks, depend on availability of reference virus-strains or antisera) and can be inconclusive. 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. Currently, nucleotide sequence analyses and phylogenetic comparisons of genome segment 2 (Seg-2) encoding BTV outer capsid protein VP2 (the primary determinant of virus serotype) are used for BTV serotype typing. The resulting Seg-2 database has been used to develop rapid (within 24 h) and reliable RT-PCR-based typing assays for each BTV type. To overcome these limitations, RT-PCR was developed and evaluated as a suitable diagnostic method for direct detection of BTV in clinical samples (Maan *et al.*, 2004; Mertens *et al.*, 2007; Ayanur *et al.*, 2016). RT-PCR, which is more sensitive than serological assays, is used to detect and/or differentiate BTV serotypes. Only a few reports have evaluated real-time PCR for detection of BTV burden in clinical samples (Lanciotti and Kerst, 2001; Shaw *et al.*, 2007; Hoffmann *et al.*, 2009; Steinrigl *et al.*, 2010).

Conclusion

In this study, we evaluated RT-PCR and real-time RT-PCR assay using primers specifically for a highly conserved region in segment 2 of BTV. The primers for both RT-PCR and qRT-PCR were standardized and annealing temperature of 56°C giving distinct and expected PCR product size of 1548bp and 88bp respectively were selected. The standardized primers were used for amplifying and quantifying the genome of BTV-23 in real-time PCR. The early and serotype specific diagnosis of bluetongue can be helpful in controlling the disease by preventing its further spread with use of insecticides and also by developing serotype specific monovalent vaccine.

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