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Rapid one-step real-time RT-PCR assay for the detection and quantitation of bluetongue virus

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ABSTRACT

Bluetongue virus (BTV) infects domestic and wild ruminants, but it is primarily a disease of sheep. In the present study, a rapid one-step real-time RT-PCR (RT-qPCR) assay based on SYBR green chemistry was optimized by targeting the conserved region of genome segment-10 (encoding NS3). The assay was able to detect BTV-1, 2, 9, 10, 16, 21 and 23 serotypes. The sensitivity of the assay using the RNA transcribed *in vitro* was 10^2 copies with 94.25%, efficiency. The sensitivity of the assay was compared to sandwich-ELISA (s-ELISA) and RT-PCR. The sensitivity of s-ELISA, RT-PCR and one step RT-qPCR for detection of BTV-1 was equivalent to $10^{2.4}$ $TCID_{50}$/ml, $10^{0.4}$ $TCID_{50}$/ml and $10^{0.04}$ $TCID_{50}$/ml, respectively and the assay was specific to BTV. Further, the assay was validated with whole blood samples from sheep and goats used to evaluate the assay performance. The assay provides an important tool for early and rapid detection of all serotype of BTV.

Key words: Bluetongue virus, One-step RT–qPCR, SYBR green, Segment 10

Bluetongue (BT) is an arthropod-borne viral disease of domestic and wild ruminants. The causative agent is the bluetongue virus (BTV) which is transmitted by certain *Culicoides* species (Mellor 1990). At present, worldwide 27 distinct serotypes of BTV have been recognized (Zientara *et al*. 2014). The BTV genome consists of ten segmented double-stranded RNA (dsRNA) which codes for 7 structural (VP1-VP7) and 4 non-structural (NS1, NS2, NS3/NS3A and NS4) proteins (Belhouchet *et al*. 2011). BTV is endemic in India and 23 serotypes of the virus have been reported based on serology and virus isolation; out of which 13 serotypes (BTV 1–4, 6, 9, 10, 12 16–18, 21 and 23) have been isolated (Rao *et al.* 2016).

Laboratory confirmation of BTV infection is carried out by virus isolation using cell culture or embryonated chicken egg (ECE) followed by immuno-peroxidase or immunofluorescent staining. Virus isolation is tedious and may take up to 5 weeks for completion. Consequently, alternative methods of virus detection have been sought which include polymerase chain reaction (PCR) and real-time PCR to detect BTV nucleic acid (Dangler *et al*. 1990, Shaw *et al.* 2007), and antigen capture or sandwich ELISA (s-ELISA) to detect BTV antigen (Chand *et al*. 2009, Mecham 1993). These techniques, singly or in various combinations, have been applied for the detection of BTV in cell cultures, eggs, insect vectors and ruminants infected naturally or experimentally. ELISA and PCR have some drawbacks, such as low sensitivity and as a result, it can detect antigen or nucleic acid only in animals with high viraemia (Stanislawek *et al.* 1996, Subhadra *et al.* 2014). To overcome these problems, RT-qPCR assays (two-step) were developed and evaluated for the detection of BTV serotypes based on nucleotide sequences of different genome segments (seg-1 and seg-2) (Shaw *et al*. 2007, Maan *et al*. 2016). The RT-qPCR is a highly sensitive assay that can detect all BTV serotypes present in the country. In this paper, we describe development of a rapid and sensitive SYBR green-based one-step RT-qPCR for the detection of BTV targeting genome segment-10.

MATERIALS AND METHODS

Viruses and samples: BTV serotypes 1, 2, 9, 10, 16, 21 and 23 serotypes available in the BT repository of the institute were used for the optimization of the assay. The blood sample from sheep and goat origin were also used for evaluation of the assay.

RNA extraction: Total RNA was extracted from 300 µl of the supernatant of BTV infected BHK-21 cells using TRIzol LS reagent (Sigma, USA) or from whole blood using TRIzol BD reagent (Sigma, USA) according to manufacturer's instructions.

Primer design: The oligonucleotide primer pair was designed by targeting segment 10 (encodes NS3) with conserved region of the BTV-1 genome (GenBank Accession no. KU234266). Group specific alignment of 3 sequences of each 24 serotype was carried out using Lasergene software (DNASTAR, Inc.). The position of primer was selected to ensure efficient amplification and

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detection of all serotypes of BTV. The nucleotide sequence of forward primer (BTV_S10_F_1-19) was 5'-ATGCT-ATCCGGGCTGATCC-3' and the reverse primer (BTV_S_10_R_285-266) was 5'-ACATCATCACGAAAC-GCTTC-3'.

One step real-time RT-PCR: The SYBR green-based one-step RT-qPCR was carried out in 20 µl in 0.1 ml optical tubes (Applied Biosystems, USA). Sample RNA (5 µl) was added to primer mix containing 0.5 µM of each forward and reverse primers and denatured at 95ºC for 5 min and snap chilled on ice. Thereafter 10 µl of VeriQuest SYBR Green One-Step RT-qPCR master mix (2×), 0.2 µl of VeriQuest 100× RT Enzyme mix (Affymetrix, USA) and 3.8 µl of nuclease-free water was added. The reaction mixtures were combined and RT-qPCR was carried out in one-step Plus Real-Time PCR System (Applied Biosystems, USA). The RT-qPCR cyclic conditions were as follows: reverse transcription 50ºC for 30 min, 95ºC for 10 min followed by 40 cycles of amplification: 95ºC for 15 sec, 60ºC for 1 min (acquired real-time fluorescence data). This was followed immediately by melt-curve analysis with ramping of temperature from 60ºC to 95ºC with auto increments of 1ºC to confirm specific amplification of the target. Cycle threshold (Ct) was determined at which the fluorescence exceeded the baseline fluorescence.

Assay sensitivity and specificity: The sensitivity of one-step RT-qPCR was evaluated by detecting standard template R/pGEM–T diluted serially and RNA from BTV-1 at different concentrations. A 285 nt cDNA fragment from segment-10 of BTV-1 was cloned in pGEMT-easy (Promega) according to manufacturer's guideline and RNA was transcribed using TranscriptAid T7 High Yield Transcription kit (Thermo, USA). Transcribed RNA was purified, quantified and 10 fold serial dilution was made to test the sensitivity of the assay.

The sensitivity of the RT-qPCR was also compared with sandwich-ELISA (s-ELISA) and RT-PCR. To evaluate the sensitivity, baby hamster kidney-21 (BHK-21) cells were infected with BTV-1 and supernatant was used for RNA extraction and as the antigen in the s-ELISA. The titer of the virus was measured by the method of Reed and Muench (1938) and to compare the sensitivity 10-fold serial dilution were prepared. The aliquot of each dilution was tested by s-ELISA (Chand *et al*. 2009), RT- PCR (OIE 2004) and RT-qPCR.

In the s-ELISA, the ELISA plates (Maxisorp Nunc A/S, Rosklide, Denmark) were coated with the capture antibody in carbonate bicarbonate buffer, pH 9.6 at 37°C for 1 h. The plates were blocked by adding blocking buffer (4% skim milk powder and 2% gelatin in PBS). The plates were washed three times with washing buffer (PBS containing 0.05% Tween-20). After adding the test sample, plates were incubated, washed and then detection antibody was added. After incubation for 1 h and washing, anti-guineapig HRP conjugated antibody (Dako Glostrup, Denmark) was added. After incubation and final washing step, freshly prepared substrate/chromogen mixture (hydrogen peroxide/ orthophenylene diamine) was added to all wells. Colour was developed for 10 min and the reaction was stopped using 1 M H_2SO_4. The OD value were measured at 492 nm wavelength on an ELISA plate reader. A value twice (or more) the mean OD value of the negative antigen control was considered as the positive/negative cut-off value (i.e. positive to negative (P/N) ratio ≥2).

An NS1 gene (Segment-5) based group-specific RT-PCR was carried out to detect BTV in the infected cell culture as per OIE protocol. Briefly, infected culture supernatant was diluted 10-fold serially and total RNA was extracted from 50 µl aliquot. The total RNA was used for cDNA synthesis. The cDNA was used for PCR under the following conditions: initial denaturation at 95°C for 5 min, denaturation at 95°C for 20 sec, annealing at 53°C for 20 sec and extension at 72°C for 20 sec for 35 cycles. The final extension was carried out at 72°C for 5 min and the amplicons were checked in 1.5% agarose gel. To evaluate and compare the performance of RT-qPCR, 38 blood samples of sheep (2) and goat (36) which were already available in the laboratory were randomly selected and tested by s-ELISA, RT-PCR and one step RT-qPCR. The specificity of the assay was determined by inclusion of peste-des-petits ruminants virus (PPRV). Further BTV serotypes available in the BT repository 1, 2, 9, 10, 16, 21, 23 were also assessed by the one-step RT-qPCR.

RESULTS AND DISCUSSION

RNA extracted from the BHK-21 cells infected with BTV-1 serotype was used for optimization of one step RT-qPCR. Each primer was tested at 0.10, 0.25, 0.5 and 1.0 µM. The best combination of forward and reverse primer was found at 0.5 µM with significant fluorescence in the quantitation and melting curve (Tm ~ 80°C). Primers also worked at 0.25 µM concentration, but with significantly lower fluorescence in the quantitation and melting curve. The primer concentration below that fails to emit fluorescence in the quantitation curve but emits in a melting curve (Tm ~70°C) indicates fluorescence emit by primer-dimer. The sensitivity of the assay for BTV-1 RNA transcript was up to 100 copies. A strong linear negative correlation (R^2=0.997) was seen between the quantity of RNA and the Ct value (threshold: 0.652022) and the slope of a standard curve obtained was –3.468 with an efficiency of 94.25%.

The sensitivity of qRT- PCR for the detection of BTV was compared with s-ELISA and RT-PCR. The titer of the BTV-1 was $10^{6.7}$ $TCID_{50}$/ml. In s-ELISA, the virus was detectable by the assay up to 10^{-3} dilution and the LOD is equivalent to $10^{2.4}$ $TCID_{50.}$ On PCR, expected size (273 bp) amplicon could be observed up to 10^{-5} dilution in agarose gel which was equivalent to $10^{0.4}$ $TCID_{50}$/ml. The LOD of one step qRT- PCR for BTV-1 was $10^{0.04}$ $TCID_{50}$/ml (Table 1). The one-step real-time RT-PCR was effective in detecting BTV serotypes 1, 2, 9, 10, 16, 21 and 23 serotypes. The Ct-value was different among all the serotype tested and the melting curve showed Tm ~80°C. The assay did not amplify the RNA of PPRV. Among the 38 blood

sample tested, 6 goat samples were found positive by RT-qPCR with Ct value ranging from 25 to 31.

Bluetongue is an epidemic disease in India and there is a need for an automated sensitive diagnostic assay that can test large numbers of samples and also identify all the serotypes circulating in the country. The assay should be sensitive and specific or superior to currently used laboratory assays. In India, different assays like s-ELISA, RT-PCR and RT-qPCR are used for detection of virus antigen and nucleic acid. The sensitivity of s-ELISA, RT-PCR and RT-qPCR for virus-spiked culture medium was $10^{2.4}$ $TCID_{50}$/ml, 10^3 $TCID_{50}$/ml and 10°C $TCID_{50}$/ml respectively (Chand *et al.* 2009, Lakshmi *et al*. 2018). The limitations of s-ELISA and RT-PCR are inability to detect virus in animals with low viremia. Real-time PCR, particularly one step RT-qPCR will be a potential assay as compared to conventional PCR for rapid and sensitive detection of BTV nucleic acid in cell culture and clinical samples as there is no need of detection of amplified product in an agarose gel and no separate step for cDNA synthesis that prevents chance of cross contamination. One step RT-qPCR is considerably shorter than conventional PCR and amplified product is detected during the PCR cycles. In the present study, one step qRT- PCR was optimized that targets the conserved region of genome segment-10 that can be used to detect all serotype of BTV. Also to reduce the test time, RT and PCR were executed in a single step. Thus, real-time RT-PCR developed was able to quickly identify (~3 h) all the serotypes of BTV examined. The samples including serotypes prevalent in the country (1, 2, 9,10, 15, 16, 21 and 23) were effectively detected using the optimized assay. The amplification of BTV specific target was confirmed by melting curve analysis directly after the completion of real-time RT-PCR. The data produced after the amplification of different serotypes indicates that there is variation in the capacity of the assay to detect the different serotype. However, this could be due to variation in the virus concentration of the samples tested. The sensitivity of the assay is 100 target copies of RNA, i.e. comparable to the sensitivity measured by other researchers for RNA genome segment 1 and 10 (Leblanc *et al.* 2010, Wilson *et al*. 2009). However, that low number could not be detected in blood samples possibly due to the presence of PCR inhibitors in the samples. In comparison, the sensitivity of qRT PCR was 10 times higher than the RT-PCR and 1000 times higher than s-ELISA, which is comparable but slightly lower than the TaqMan probe-based real-time assay (Polci *et al*. 2007).

Table 1. Comparative sensitivity of RT-PCR, one- step qRT-PCR and s-ELISA

Dilution	Equivalent infectious units ($Log_{10}TCID_{50}$[a])	RT-PCR	Real-Time RT-PCR (Ct Value)	s-ELISA (OD 492 nm)
10^{-1}	4.4	+	+ (9.97)	+ (1.12)
10^{-2}	3.4	+	+ (14.01)	+ (0.48)
10^{-3}	2.4	+	+ (16.14)	+ (0.28)
10^{-4}	1.4	+	+ (19.88)	+ (0.11)
10^{-5}	0.4	+	+ (23.8)	- (0.10)
10^{-6}	0.04	-	+ (25.71)	- (0.11)
10^{-7}	0.004	-	- (39.10)	-
10^{-8}	0.0004	-	- (undetermined)	-

Fifty microliter infected culture supernatant was tested by RT-PCR, qRT-PCR and s-ELISA from each dilution. At 10^{-1} dilution, 50 µl contained an infectious units of $10^{4.4}$ $TCID_{50}$ while the initial titer was $10^{6.7}$ $TCID_{50}$/ml. The P/N cut-off OD was 0.20; + denotes positive; - denotes negative.

In conclusion, the present study produced a competent one-step RT-qPCR assay for the detection of different BTV serotypes. The use of assay is significant in surveillance of BTV for laboratories in the country where the disease is endemic. The specificity of the assay should be studied with epizootic hemorrhagic disease virus (EHDV) since the virus is closely related to BTV; however, till now EHDV is not reported in the country.

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