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Comparative sensitivity of sandwich ELISA, RT-PCR, and real-time RT-PCR for detection of bluetongue virus

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Bluetongue virus (BTV) is an insect-transmitted, double stranded RNA virus that infects domestic and wild ruminants. BTV is the prototype virus of the genus *Orbivirus* in the family Reoviridae (Mertens *et al*. 2004). The BTV genome consists of 10 linear double-stranded RNA (dsRNA) segments that code for seven structural (VP1–VP7) and four non-structural (NS1, NS2, NS3/NS3a and NS4) proteins (Ratinier *et al*. 2016). There are 27 serotypes of BTV worldwide and 22 serotypes of the virus have been reported in India (Chand *et al.* 2015). Routine diagnosis of BTV infection is done by immunodiffusion, ELISA, virus isolation, PCR and real-time PCR (Afshar 1998, Danglar *et al.* 1990, Maan *et al*. 2016, Chand *et al.* 2017). In the present study, we have measured and compared the sensitivity of a polyclonal antibody-based s-ELISA, PCR and real-time RT-PCR for detection of virus in the cell culture.

Virus: BTV–23 serotype available in the BTV repository, ICAR-Indian Veterinary Research Institute (IVRI), Mukteswar was used for the study.

Primers and probe: For RT-PCR, forward primer (5'-GTT CTC TAG TTG GCA ACC ACC–3') and reverse primer (5'-AAG CCA GAC TTG TTC CCG AT-3') were used (OIE 2004).

For one step qRT-PCR, forward primer (5'-ATG CTA TCC GGG CTG ATC C-3'), reverse primer (5'- ACA TCA TCA CGA AAC GCT TC-3') and the probe (5'6-FAM - GCT GCA TTC GCA TCG TAC GC-3'TAMRA) were used (LeBlanc *et al.* 2010).

Virus propagation and titration: BTV- 23 was grown in baby hamster kidney-21 (BHK-21) cells (in 75 cm^2 flasks) and the virus was harvested at complete cytopathic effect (CPE). The supernatant was used for RNA extraction and as an antigen in the s-ELISA. The titer of the virus was measured as per Reed and Muench (1938).

Sandwich-ELISA for antigen detection: The s-ELISA used in this study is a polyclonal antibody-based assay developed at IVRI, Mukteswar (Chand *et al.* 2009). The sensitivity (LOD of cell culture-grown BTV-23) of the test was measured by assaying aliquots of the serially diluted virus. The virus was diluted 10- fold up to 10^{-7} with culture medium and 50 µl of each dilution was tested in s-ELISA. A test sample was classified as positive if the OD was greater than or equal to twice that of negative antigen control (i.e. positive to negative(P/N) ratio ≥2).

RT-PCR: A NS1 gene (Segment-5) based group-specific RT-PCR was done to detect BTV in the infected cell culture as described in OIE manual (2004) with some modifications. Infected culture supernatant was serially diluted 10-fold and total RNA was extracted from 50 µl aliquot. The entire RNA was used for cDNA synthesis. The total 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 at 72°C for 5 min and the amplicons were checked on 1.5% agarose gel.

One step real-time RT-PCR: A Taqman probe based one step qRT-PCR assay was optimized to detect BTV from infected cell culture in this study. The oligonucleotide primers and probe were designed for NS3/NS3A conserved region. Infected culture supernatant was serially diluted 10-fold and total RNA was extracted from 50 µl aliquot. The Taqman probe based one step real-time RT-PCR assay was carried out in a total volume of 20 µl in 0.1 ml optical tube using real time PCR machine (Applied Biosystems version 2.2.3). The entire RNA was added to a primer mix containing 0.2 µM of forward primer, 0.4 µM of reverse primer and denatured at 90°C for 5 min. One step real-time RT-PCR was carried out using TaqMan® Fast Virus 1-Step Master Mix (Life Technologies) containing 0.4 µM probe. The real-time thermo cyclic conditions were as follows: reverse transcription at 50°C for 5 min, RT inactivation and initial denaturation 95°C for 20 sec followed by 45 cycles of amplification at 95°C for 3 sec and 60°C for 30 sec. Cycle threshold (Ct) value was measured during the exponential phase at which fluorescence exceeded the baseline fluorescence. The LOD was taken as the highest dilution showing the Ct value.

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The titer of the BTV-23 was found to be $10^{6.7}$ $TCID_{50}$/ml. In s-ELISA, virus was detectable by the assay in 50 µl volume up to 10^{-3} dilution and the LOD was equivalent to $10^{2.4}$ $TCID_{50}$ (Table 1). The measured sensitivity was comparable to the findings of Thevasagayam *et al.* (1996) where the LOD was $10^{2.6}$ $TCID_{50}$ for BTV-1. The sensitivity of the s-ELISA depends on the antigen preparations against which the capture and detection antibodies have been developed. With an unpurified virus, like infected cell culture antigen and its cognate antibody, the limit of detection was approximately 10^4 $TCID_{50}$/ml (Mecham 1993). The sensitivity can be improved with a limit of detection up to 10^2 $TCID_{50}$, if a polyclonal antibody is used for capturing antigen and a monoclonal antibody for detection (Portanti *et al.* 2005).

On PCR amplification, expected size (273 bp) amplicon was observed up to 10^{-5} dilution in agarose gel which was equivalent to $10^{0.4}$ $TCID_{50}$/ml (Table 1). The PCR detected less than 10 infectious units of BTV-23 in the infected cell culture. This sensitivity is comparable to the detection limit of 10 infectious particles as reported by Prasad *et al.* (1999), but higher than the findings of Subhadra *et al.* (2014), where LOD was$10^{0.4}$ $TCID_{50}$/ml.

One step qRT- PCR was standardized and the Ct value decrease with increase in the dilution of virus. The LOD of Taqman probe based one step qRT- PCR for BTV-23 was 0.004 $TCID_{50}$/ml (Table 1). The sensitivity was comparable to findings of Polci *et al.* (2007), where LOD for BTV-16 was 0.005 $TCID_{50}$/ml. However, sensitivity was slightly lower than the findings of Subhadra *et al.* (2014) where LOD was 3.16×10^{-4} $TCID_{50}$/ml.

Table 1. Comparative sensitivity of RT-PCR, real-time RT-PCR and s-ELISA for detection of BTV-23

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)	+ (1.38)
10^{-2}	3.4	+	+ (14.01)	+ (0.44)
10^{-3}	2.4	+	+ (16.14)	+ (0.20)
10^{-4}	1.4	+	+ (19.88)	+ (0.08)
10^{-5}	0.4	+	+ (23.8)	- (0.08)
10^{-6}	0.04	-	+ (25.71)	- (0.08)
10^{-7}	0.004	-	+ (26.86)	-
10^{-8}	0.0004	-	- (undetermined)	-

[a]Fifty microlitre infected culture supernatant was tested by RT-PCR, real-time RT-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 0.16. + denotes positive and - denotes negative.

SUMMARY

In the present study, sensitivity of sandwich enzyme-linked immunosorbent assay (s-ELISA), reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (qRT-PCR) was measured and compared for detection of bluetongue virus (BTV) in infected cell culture. The limit of detection (LOD) of the s-ELISA was $10^{2.4}$$TCID_{50}$/ml. The RT-PCR detected less than 10 infectious units ($10^{0.4}$ $TCID_{50}$/ml) of BTV. The LOD of the real- time RT-PCR was 0.004 $TCID_{50}$/ml. Thus, real time RT-PCR is 100 times more sensitive than RT-PCR and at least 10000 times more sensitive than s-ELISA for the detection of BTV in cell culture. Due to the higher sensitivity, qRT-PCR can be used to detect virus, especially from the blood of infected ruminants with low viremia, where s-ELISA and RT-PCR may fail to detect the virus.

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