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Reverse transcription loop-mediated isothermal amplification assays for rapid identification of eastern and western strains of bluetongue virus in India

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A B S T R A C T

Bluetongue virus (BTV) infects all ruminants, including cattle, goats and camelids, causing bluetongue disease (BT) that is often severe in naïve deer and sheep. Reverse-transcription-loop-mediated-isothermal-amplification (RT-LAMP) assays were developed to detect eastern or western topotype of BTV strains circulating in India. Each assay uses four primers recognizing six distinct sequences of BTV genome-segment 1 (Seg-1).

The eastern (e)RT-LAMP and western (w)RT-LAMP assay detected BTV RNA in all positive isolates that were tested (n = 52, including Indian BTV−1, −2, −3, −5, −9, −10, −16, −21 −23, and −24 strains) with high specificity and efficiency. The analytical sensitivity of the RT-LAMP assays is comparable to real-time RT-PCR, but higher than conventional RT-PCR. The accelerated eRT-LAMP and wRT-LAMP assays generated detectable levels of amplified DNA, down to 0.216 fg of BTV RNA template or 108 fg of BTV RNA template within 60–90 min respectively. The assays gave negative results with RNA from foot-and-mouth-disease virus (FMDV), peste des petits ruminants virus (PPRV), or DNA from Capripox viruses and Orf virus (n = 10), all of which can cause clinical signs similar to BT. Both RT-LAMP assays did not show any cross-reaction among themselves. The assays are rapid, easy to perform, could be adapted as a 'penside' test making them suitable for 'front-line' diagnosis, helping to identify and contain field outbreaks of BTV.

1. Introduction

Bluetongue (BT) is an infectious but non-contagious disease affecting many species of domestic and wild ruminants that is often severe in naïve deer and sheep. It is caused by the bluetongue virus (BTV), which is classified within *Bluetongue virus,* the prototype species of the genus *Orbivirus*, family *Reoviridae* (Maclachlan et al., 2009; Mertens et al., 2009b). Bluetongue virus is transmitted to ruminants primarily by biting midges of the genus *Culicoides* (Mellor et al., 2000), but the virus can also be transmitted vertically or horizontally (via an oral route or by direct contact) (Backx et al., 2009; Batten et al., 2014; De Clercq et al., 2008; Menzies et al., 2008). Bluetongue virus occurs throughout temperate and tropical areas of the world, between latitudes 40°S and 53°N, coincident with the distribution and activity of vector-competent *Culicoides* spp. midges (Gibbs and Greiner, 1994; Tabachnick, 2004).

Bluetongue virus is a small (~90 nm diameter) icosahedral virus with a genome of approximately 19,200 base pairs, composed of ten linear segments of double-stranded RNA (dsRNA), packaged within a three layered icosahedral protein-nucleocapsid composed of seven structural proteins (VP1 to VP7). The virus also codes for at

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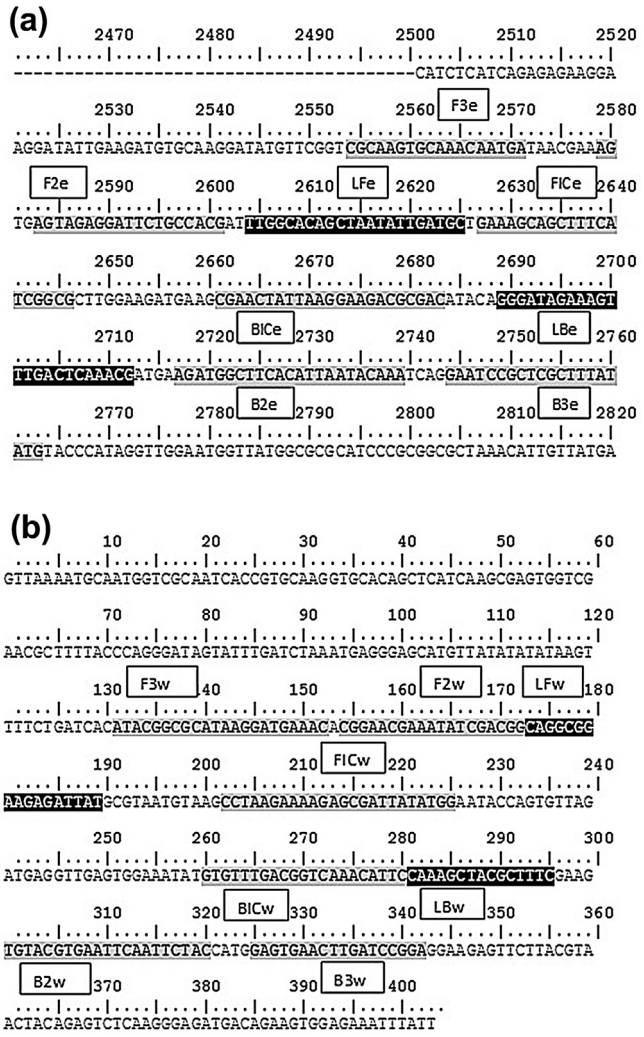

Fig. 1. (a) Location of BTV eRT-LAMP assay primers on genome segment 1. (b)Location of BTV wRT-LAMP assay primers on genome segment 1.

least four non-structural proteins (NS1, NS2, NS3/NS3a, and NS4) (Attoui et al., 2009; Belhouchet et al., 2011; Firth, 2014; Ratinier et al., 2011; Roy and Noad, 2006).

Twenty-seven immunologically distinct BTV serotypes have been confirmed worldwide (Zientara et al., 2014). However, additional BTV strains have been identified that may represent further new serotypes (Wright, 2013; Peter Mertens – personal communication).

Full genome sequence data for BTV have identified large genetic and phenotypic variations, even within an individual serotype, that are related to the geographic origin of the isolate (BTV topotypes) (Gould and Pritchard, 1990). These include the major 'eastern' and 'western' lineages (topotypes) identified by Maan and associates (Maan et al., 2011a,b, 2010). The 'eastern' BTV topotype includes isolates from South East Asia, India, China, Australia and Europe, while the 'western' topotype includes viruses from Africa, Europe, Mediterranean region and North or South America (Maan et al., 2009, 2011b). Some recent studies have reported that BTV serotypes –1e, –2e, –2w –3e, –5w, –9e, –10w, –12w, –16e, –21e –23e and –24w are currently circulating in India. These include strains belonging to both eastern and western topotypes in individual genome segments (Maan S. et al., 2015; Rao et al., 2014). The ability of BTV to reassort genome segments (even between topotypes) adds to the overall genetic variability of the virus (Maan et al., 2012b; Maan et al., 2010; Shaw et al., 2013) and may even result in BTV strains with enhanced virulence or transmission characteristics (Waldvogel et al., 1987), or increased abilities to adapt to new ecological zones (Maan et al., 2010).

Historically, laboratory diagnosis and identification of BTV serotype, were carried out by serological methods, including antigen capture, agar gel immunodiffusion (AGID), ELISA, or virus neutralisation (VN) assays (Patton et al., 1994). BTV in clinical samples can often be grown in embryonated chicken eggs (ECE), and/or 'isolated' in insect or mammalian cell cultures (e.g. KC cells or BHK-21 and Vero cells) (OIE, 2011) and can be detected by ELISA, immunofluorescence, dot immunobinding assays (DIA), western blotting, or immuno-electronmicroscopy.

However, 'molecular assays' to detect the viral RNA are increasingly being used as primary diagnostic tools for serogrouping, serotyping and epidemiological investigations of BTV. These methods, which can detect and identify BTV RNA include conventional gel-based reverse transcription polymerase chain reactions (RT-PCR) (Anthony et al., 2007; Maan et al., 2012a; McColl and Gould, 1991; Stallknecht et al., 2015; Zientara et al., 2004) and real-time RT-PCR (qRT-PCR) assays (Shaw et al., 2007; Toussaint et al., 2007). Although these molecular methods are more sensitive, specific and reliable than serological diagnostic techniques but require a well-equipped laboratory with sophisticated PCR equipments.

Loop-mediated isothermal amplification (LAMP), is a simple autocycling, strand displacement, DNA synthesis method that does not require special equipment (Notomi et al., 2000). Because LAMP recognizes the target using six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity. The assay can detect the viral RNA rapidly (even <60 min), and the result could be visualized either by resolving a 'ladder pattern' of LAMP amplicons after agarose gel electrophoresis, or can be observed directly with the naked eye after addition of an intercalating dye (SYBR green I, picogreen etc.) to the reaction products (Parida et al., 2008).

Recently, a single step RT-LAMP assay has been developed targeting Seg-5 which has been validated with only seven serotypes of BTV circulating in India (Mohandas et al., 2015). However, the assay cannot identify topotypes and it was not tested against all circulating serotypes in the country. Hence these studies describe development and evaluation of two separate 'accelerated' RT-LAMP assays (eRT-LAMP and wRT-LAMP) for the rapid and accurate detection and differentiation of RNA from either 'eastern' or 'western' BTV strains circulating in India.

This assay is expected to be particularly suitable for use in 'front-line' diagnostic facilities and mobile diagnostic units. It has potential for adaptation as a 'penside' test to help in early diagnosis and containment of field outbreaks caused by eastern or western topotypes of BTV on the sub-continent.

2. Material and methods

2.1. Primer design

The nucleotide sequences of genome segment 1 (Seg-1) from a total of 110 eastern, and 105 western BTV strains, were aligned using CLUSTAL X ver 2.0 (Larkin et al., 2007) (Table 1S). RT-LAMP primers from the consensus sequences were designed using the Primer Explorer software V4 (http://primerexplorer.jp/e/) (Table 1). The primer sequences were also tested *in silico* (for specificity) with Seg-1 sequences of other related *orbiviruses* particularly Epizootic haemorrhagic disease virus (EHDV).

Two sets of four primers each including F3, B3, FIP, and BIP were designed by targeting highly conserved sequences in Seg-1 of the 'eastern' and 'western' BTV strains separately (eRT-LAMP and wRT-LAMP). The primers for both assays were designed using

Table 1
List of primers used for BTV LAMP assays.

Primer name	5′ position	Length	Sequence (5′-3′)
BTV eastern strain specific primers			
F3e	57	18	CGCAAGTGCAAACAATGA
B3e	247	20	CATATAAAGCGAGCGGATTC
FIPe	F1C, 130 F2, 86	39	CGCCGATGAAAGCTGCTTTC-AGTAGAGGATTCTGCCACG
BIPe	B1C, 164 B2, 220	45	CGAACTATTAAGGAAGACGCGAC-TTGTATTAATGTGAAGCCATCT
F2e	86	19	AGTAGAGGATTCTGCCACG
B2e	220	22	TTGTATTAATGTGAAGCCATCT
LFe	107	22	GCATCAATATTAGCTGTGCCAA
LBe	192	24	GGGATAGAAAGTTTGACTCAAACG
BTV western strain specific primers			
F3w	132	22	ATACGACGTATAAGGATGAAAC
B3w	326	18	TCCGGATCAAGTTCACTC
FIPw	F1C, 203 F2, 155	43	CCATACAATCGCTCTTTCCTCAAA-TGGAACGAAATATCGACGG
BIPw	B1C, 261 B2, 301	42	GTGTTTGATGGCCAAACGTTC-GCAAAATTGAGTTCACGTACA
F2w	155	19	TGGAACGAAATATCGACGG
B2w	301	21	GCAAAATTGAGTTCACGTACA
LFw	174	17	ATAATCTCTTCCGCCTG
LBw	282	16	CAAAGTTACGCTTTTG

default parameters. The eastern and western strain specific two outer primers 'F3′ and 'B3′ help to displace the primary strand during the reaction.

Two additional eastern and western-strains specific 'loop forward' (LF) and 'loop backwards' (LB) primers were also designed, so that their inclusion in the reaction mix would accelerate amplification reaction and are complementary to sequences between the F1 and F2 regions and between the B1 and B2 regions, respectively. Additionally, for the loop primers of wRT-LAMP, some of the design parameters were modified in the software to achieve specificity.

The length, genome position, and sequence of primers used for RT-LAMP amplification of segment 1 of the BTV genome are presented in Table 1 and Fig. 1a,b. Oligonucleotide primers were synthesised from Sigma Aldrich (Bengaluru, India).

2.2. Virus isolates

A panel of 52 Seg-9 specific qRT-PCR positive Indian isolates of BTV, collected from 1982 onwards, representing various BTV serotypes, as well as over 50 clinical specimens (blood, nasal and lachrymal swabs) were tested during an initial validation of the LAMP assays (Maan N. et al., 2015). Individual strains are identified by a unique reference collection number, composed of 'country code', year, and the number of the isolate in that year from that country. Full details of these isolates are available on the *Orbivirus* reference collection (ORC) website (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-Nos.htm) and are listed in Table 2. Some of the isolates are stored in the Indian 'satellite' reference collections located at Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar; Ella Foundation Hyderabad and National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru (linked to ORC at Pirbright).

Representative isolates of other viruses, including foot and mouth disease virus (FMDV) serotype O, peste des petits ruminants virus (PPRV), goat pox virus (GTPV), sheep pox virus (SPPV) and Orf virus (ORFV) were used to assess the specificity of the group specific assays.

2.3. RNA extraction

RNA was extracted from the cell culture grown viruses and field samples using a QIAamp Viral RNA Mini Kit (Qiagen) and by Trizol Reagent (Invitrogen) (Attoui et al., 2000), according to the manufacturer's instructions. Similarly RNA from uninfected tissue culture supernatants and uninfected sheep and cattle blood was also extracted in triplicates for use as negative controls.

2.4. Standardization of LAMP assays

'Eastern' and 'Western' BTV strains specific LAMP assays (eRT-LAMP and wRT-LAMP) were evaluated using RNA from different Indian BTV isolates as listed in Table 2 An initial standardization of each assay was carried out using a set of four topotype-specific primers targeting Seg-1, including two outer and two inner primers (F3, FIP, B3 and BIP) in each case, using checkerboard titrations and concentrations of 0.2–0.8 μM of each primer. Following the initial standardization of the e & w RT-LAMP assays, they were further developed in order to enhance the amplification reaction and reduce detection times, as 'accelerated' assays (aRT-LAMP), by adding pairs of topotype-specific loop-primer (LF and LB), at a concentration of 0.1–0.8 μM. The optimal ratio of primers (inner-outer-loop) concentrations for the RT-LAMP reaction was found to be 4:1:4, and 2:1:1 at 10 μM concentration per reaction for eRT-LAMP and wRT-LAMP, respectively.

For each e & w aRT-LAMP reaction other parameters were also optimised that included temperature range (60 to 70 °C), time interval (30 min–120 min) reaction volume (12.5 μl and 25 μl), concentrations of various LAMP components like $MgSO_4$ (4–16 μM), betaine (1–3 μM) and concentration of *Bst* 2 DNA polymerase (4–16 units). Changes to the reaction volume (12.5 μl–50 μl) had no detectable effect on the efficiency of the assay, and no significant improvements were observed in the reaction kinetics when primer, enzyme or template concentrations were increased. Optimum results were obtained using 1 M betaine and 12 mM $MgSO_4$ in each assay.

2.5. Analytical sensitivity and specificity

In order to estimate the analytical sensitivity of the RT-LAMP technique, serial 10-fold dilutions (ranging from 10^{-1} to 10^{-9} dilution) of BTV RNA [IND2003/10–BTV-1 strain having eastern Seg-1 and IND2004/01–BTV-10 strain having western Seg-1, each having starting concentrations of 0.216 μg/μl and 1.08 μg/μl respectively] were prepared and tested. The analytical specificity

Table 2
BTV isolates used for testing the BTV accelerated e & w RT-LAMP assays.

Sample No.	Virus Species-serotype	Topotype of Seg-1 sequence [accession number]	ORC isolate number
1.	BTV-1	E [KP696507]	IND1985/01
2.	BTV-1	E [KP696517]	IND1988/01
3.	BTV-1	E [JQ282770]	IND1992/01
4.	BTV-1	E [KP696526]	IND1992/02
5.	BTV-1	E [KP696535]	IND1999/01
6.	BTV-1	E [KP696544]	IND2001/01
7.	BTV-1	E [KP696552]	IND2003/04
8.	BTV-1	E [KP696562]	IND2003/05
9.	BTV-1	E [KP696572]	IND2003/10
10.	BTV-2	E [JQ713557]	IND1982/01
11.	BTV-2	E [KP696582]	IND1993/01
12.	BTV-2	E [KP268774]	IND1994/01
13.	BTV-2	W [KP696592]	IND2003/01
14.	BTV-2	W [JQ681257]	IND2003/02
15.	BTV-2	W [KP696602]	IND2003/03
16.	BTV-3	E [–]	IND2003/07
17.	BTV-3	E [JQ771813]	IND2003/08
18.	BTV-3	E [–]	IND2004/05
19.	BTV-3	E [–]	IND2004/06
20.	BTV–5 + 2	– –	IND2010/02
21.	BTV-9	E [–]	IND2003/11
22.	BTV-9	E [KP696612]	IND2004/02
23.	BTV-9	E [–]	IND2004/03
24.	BTV-9	E [KP696622]	IND2004/04
25.	BTV-9	E [–]	IND2005/07
26.	BTV-9	E [KP696632]	IND2005/01
27.	BTV-9	E [KP696641]	IND2005/02
28.	BTV-9	E [KP696651]	IND2005/03
29.	BTV–9 + 2	E [–]	IND2005/06
30.	BTV-10	W [–]	IND2003/06
31.	BTV-10	W [JQ740771]	IND2004/01
32.	BTV-10	W [–]	IND2005/04
33.	BTV-10	W [–]	IND2005/05
34.	BTV-12	E [KC662612]	IND2012/01
35.	BTV-16	E [–]	IND2007/01
36.	BTV-16	E [–]	IND2007/02
37.	BTV-16	E [–]	IND2007/03
38.	BTV-16	E [–]	IND2007/04
39.	BTV-16	E [–]	IND2007/06
40.	BTV-16	E [–]	IND2007/07
41.	BTV-16	E [–]	IND2007/08
42.	BTV-16	E [–]	IND2007/09
43.	BTV-16	E [–]	IND2014/01
44.	BTV-21	E [–]	IND2007/09
45.	BTV-23	E [JQ771823]	IND1988/02
46.	BTV-23	E [–]	IND1988/03
47.	BTV-23	E [KP696661]	IND1997/01
48.	BTV-23	E [JQ771823]	IND1998/02
49.	BTV-23	E [KP696670]	IND2004/07
50.	BTV-23	E [KP696680]	IND2004/08
51.	BTV-23	E [KP696690]	IND2004/09
52.	BTV-24	– [–]	IND2010/01
53.	PPRV	NA	PPRV/IND2013/01
54.	PPRV	NA	PPRV/IND2013/02
55.	FMDV	NA	FMDV type O
56.	SPPV	NA	CaPV/IND2013/01
57.	SPPV	NA	CaPV/IND2013/02
58.	SPPV	NA	CaPV/IND2013/03
59.	GPPV	NA	CaPV/IND2013/04
60.	ORFV	NA	ORFV/IND2014/01
61.	Uninfected sheep blood	NA	–
62.	Uninfected cattle blood	NA	–

of these assays were also evaluated by testing RNA from FMDV and PPRV and DNA from ORFV, GTPV and SPPV, which show clinical signs in animals that are similar to bluetongue disease.

2.6. Conventional RT-PCR

For comparison of the sensitivity of the RT-LAMP method, 'conventional' RT-PCR were also performed using the two outer primers, F3 and B3 (which were used for the LAMP amplification), as forward and reverse primers, in accordance with a standard protocol, as described by Maan et al. (2012a). Briefly, the protocol used SuperScript™ III one-step RT-PCR system (Invitrogen) with high fidelity platinum®Taq, which is designed for sensitive, high fidelity end-point detection and analysis of RNA templates extracted from blood or cell culture supernatant. After the reaction, 5 μl of each amplified cDNA product was analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide (0.5 μg/mL) then visualised under UV light.

2.7. Real time RT-PCR (qRT-PCR)

Real-time RT-PCR assays (qRT-PCR), based on Seg-1 and Seg-9 were carried out as described previously (Maan et al., 2014; Maan N. et al., 2015; Shaw et al., 2007).

3. Results

3.1. Eastern (e) RT-LAMP

The eRT-LAMP reaction was carried out in 25 μl, containing 2.5 μl isothermal buffer (New England Biolabs), 0.2 μM each of F3 and B3, 0.8 μM each of FIP and BIP primers, 3U of Thermoscript reverse transcriptase (Invitrogen), 8U of hot start *Bst* 2 polymerase (New England Biolabs), 1 M betaine (Sigma Aldrich), 8 mM $MgSO_4$, 1.2 mM dNTP and 1 μl BTV RNA. The reaction was accelerated (aRT-LAMP) using 0.8 μM each of LF and LB primers in the reaction mixture. The BTV RNA was denaturated by incubation of samples for 5 min at 99 °C then cooling to 4 °C before adding to the LAMP mix. The plate was then transferred to a thermal cycler (Veriti™ Thermal Cycler, Applied Biosystems) and the reaction was carried out at 50 °C for 30 min followed by 65 °C–68 °C for 30 min before inactivation at 85 °C for 5 min. RT-LAMP products were analyzed by 1.5% agarose gel electrophoresis.

3.2. Western (w) RT-LAMP

The wRT-LAMP reaction uses the same reagents and protocol as described for eRT-LAMP with the exception that it uses 0.4 μM each of FIP and BIP primers, 0.4 mM dNTP and 0.2 μM each of LF and LB primers for aRT-LAMP at 50 °C for 30 min followed by 65 °C–68 °C for 60 min before inactivation at 85 °C for 5 min.

Lamp amplification: A positive result leading to cDNA amplification was observed as a ladder-like pattern after agarose gel electrophoresis (Fig. 2a). Positive gene amplification was also detected by addition of intercalating Picogreen dye and visualisation of a colour change from orange to green in positive samples, either by the naked eye or under UV light (Fig. 2b and c).

An unaccelerated eastern & western RT-LAMP assays amplified the 190 and 194 bp target sequences of Seg-1 from BTV e & w strains to detectable levels in 90 min (Fig. 3b and c) and 120 min (data not shown) respectively at 65 °C to 68 °C giving a ladder like pattern (as detected by agarose gel electrophoresis). However, the accelerated eRT-LAMP and wRT-LAMP assays amplified the target sequences in 60 min (Fig. 3d) and 90 min (data not shown) respectively. This time includes 30 min of reverse transcription at 50 °C followed by amplification at 65 °C.

All the BTV samples (isolates as well as field specimens) that had tested positive by Seg-9 based real time RT-PCR (Maan N. et al., 2015), also gave positive results either by eRT-LAMP or by wRT-LAMP and there was no cross reaction detected between eRT-LAMP or by wRT-LAMP assays. However, the RNA extracted from FMDV and PPRV samples, as well as the RNAs from blood of uninfected sheep or cattle (negative controls), gave negative results (Table 2). Similarly, the DNA extracted from SPPV, GTPV and ORFV were tested negative in eRT-LAMP and wRT-LAMP assays (Table 2).

3.3. Analytical sensitivity and specificity of LAMP assays and comparison to conventional PCR

Serial 10-fold dilutions (ranging from 10^{-1} to 10^{-9} dilution) of purified BTV RNA ([IND2003/10–BTV-1 strain containing an 'eastern' Seg-1] and [IND2004/01–BTV-10 strain containing a 'western' Seg-1]) that had previously been quantified using a Qubit® 2.0 Fluorometer (Invitrogen) (starting concentrations of 0.216 μg/μl and 1.08 μg/μl respectively) were tested in respective accelerated RT-LAMP assays.

The accelerated eRT-LAMP assay generated detectable levels of amplified DNA, down to 0.216 fg of BTV RNA template (10 copies) within 30 min (data not shown). In contrast using the same dilution series, the conventional RT-PCR assay using F3 and B3 primers had a detection limit of 216 pg of viral RNA (indicated by the detection of a 190-bp amplicon), showing that the accelerated eRT-LAMP assay was 1000 fold more sensitive than the conventional eRT-PCR (data not shown). Similar sensitivity data was obtained for eRT-LAMP assay with other eastern strains of BTV (IND2014/01) (Fig. 4).

The accelerated wRT-LAMP assay generated detectable levels of amplified DNA, down to 108 fg of BTV RNA template (5000 copies) within 60 min (Fig. 5a). In contrast using the same dilution series, the conventional RT-PCR assay using F3 and B3 primers had a detection limit of 1.08 pg of viral RNA (indicated by the detection of a 194 bp amplicon), showing that the accelerated wRT-LAMP assay was only 10-fold more sensitive than the conventional wRT-PCR using F3 and B3 primers (Fig. 5b).

The eRT-LAMP assays showed perfect agreement with the qRT-PCR targeting Seg-9 (0.126 fg, (Maan N. et al., 2015) indicating a comparable high efficiency for BTV RNA detection, even though only a limited range of representative samples were used in the initial testing described here – Table 2.

Negative results were obtained using either set of the RT-LAMP primers in assays containing nucleic acids from other unrelated viruses (including FMDV, PPRV, SPPV, GTPV and Orf virus, which cause clinical symptoms similar to bluetongue disease). However positive results were consistently obtained either in accelerated eRT-LAMP or wRT-LAMP assays containing RNA from additional eastern-topotype or western-topotype strains of BTV from India, confirming their specificity (Table 2). Mixed isolates have also been frequently detected and they have shown positive reactions in both assays. The lack of cross reactivity with other viruses or between eastern and western BTV strains showed that both RT-LAMP assays are highly specific (Fig. 6a,b).

4. Discussion

Rapid and accurate diagnosis plays an important role in the implementation of effective measures to control the spread of any disease. The substantial economic impact of BTV infection on livestock industry is well documented. Recent outbreaks of BTV in the southern part of the Indian sub-continent and in other regions of the world demonstrate the importance of efficient protocols for surveillance and early detection. Bluetongue diagnosis usually involves detection and identification of BTV-specific antigens, antibodies or RNA in diagnostic samples using virus isolation, serological or molecular assays to identify the virus serogroup and serotype (Mertens et al., 2009a). BTV exists as a number of major and minor topotypes that are represented by many different virus strains. The segmented nature of the genome also allows high frequency of reassortment between these different viruses, generating further genetic diversity between individual virus isolates, even within a single region or outbreak.

The simple amplification method of RT-LAMP assays described here detected viral RNA in all the BTV positive samples tested, which included both 'eastern' strains of BTV serotypes −1, −2, −3, −9, −16, −21 and −23 and 'western' strains of BTV-2, −5, −10, −12 and −24 that are currently circulating in India (Maan et al. – unpublished data). No cross-reaction was observed between the eastern and western strains that were originally topotyped by sequencing and phylogenetic analyses of Seg-1, indicating that the assays are both BTV and topotype specific.

Amplification was detected as a ladder-like pattern after agarose gel electrophoresis, due to the formation of stem-loop cDNAs with different stem lengths and 'cauliflower-like' structures with multiple loops (Fig. 2a). Addition of an intercalating Picogreen dye allowed gene-amplification in the reaction to be visualised by the naked eye, generating a colour change (from orange to green) in positive samples either with or without UV light (Figs. 2 b,c and 4 c). Other DNA intercalating dyes such as SYBR green I or propid-

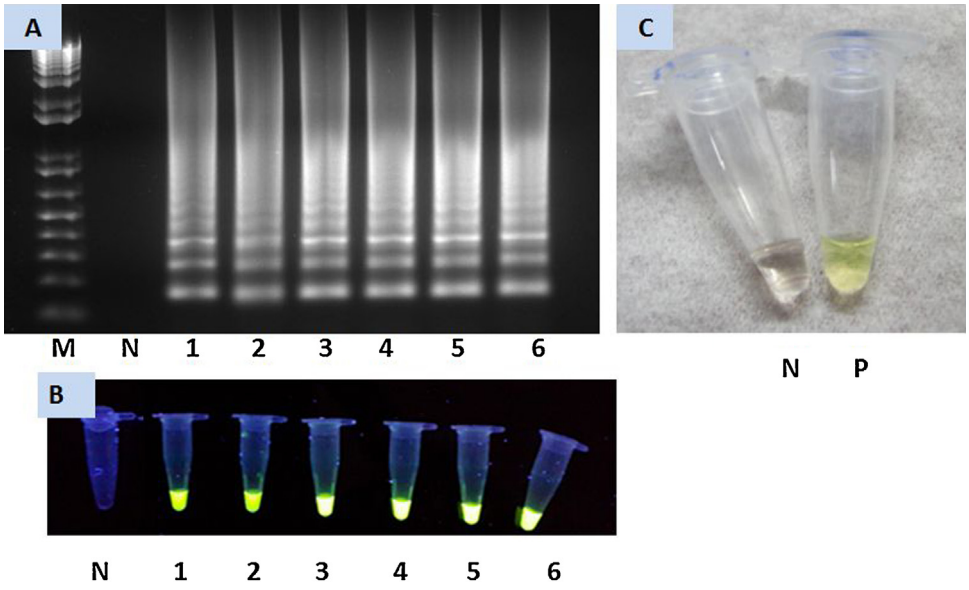

Fig. 2. Analysis of amplified BTV cDNA products from eRT-LAMP.
(Panel A): Agarose gel electrophoresis: Lane M: molecular weight marker; Lane 2: Negative control; Lanes 1–6: Indian BTV strains IND2003/10, IND1982/01, IND2004/06, IND2005/01, IND1998/01. (Panel B): products visualised under the UV light after addition of Picogreen. Tube N: Negative control; Tubes 1–6: Indian BTV strains IND2003/10, IND1982/01, IND2004/06, IND2005/01, IND1998/01. (Panel C) products of BTV visualised by naked eye after addition of Picogreen. Tube N: Negative control; Tube P: Positive control.

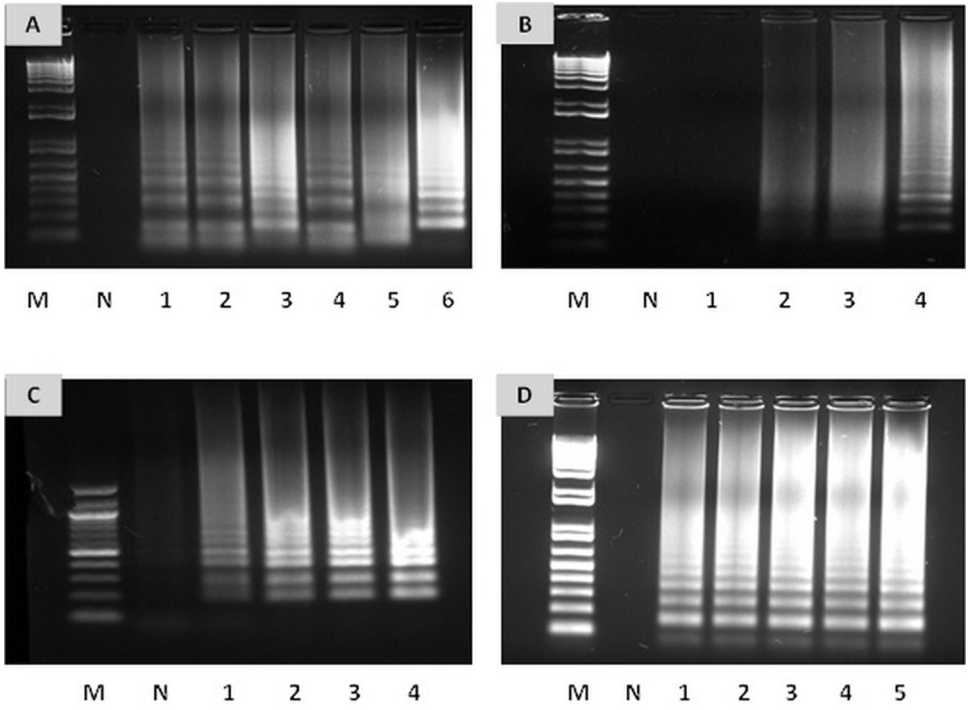

Fig. 3. Agarose gel electrophoresis of eRT-LAMP products amplified from BTV RNA (strain IND2003/10).
Lane M: molecular weight markers; Lane 2: Negative control; (panel A): products generated at different temperatures. Lanes 1–6: temperature 60–65° centigrade in 60 min. (panel B and C): products generated at different time intervals, without loop primers. B lanes 1–4: 15, 30, 45 and 60 min; C lanes 1–4: 60, 80, 100 and 120 min. (Panel D): products generated at different time intervals, with loop primers. D lanes 1–5: Time 30, 45, 60, 90, and 120 min. Times given are for LAMP amplification at 65 °C only. Total time includes reverse transcription time of 30 min at 50 °C.

ium iodide could also be added to the solution after the reaction is completed. However, analysis of LAMP products using agarose gel electrophoresis or colorimetric assays using intercalating dyes both require the reaction tubes to be opened, increasing the risk of subsequent contamination of other LAMP reaction solutions. Care needs to be taken to avoid such contamination, including the use of separate rooms for LAMP setup and analysis.

The addition of hydroxy naphthol blue (HNB—an indicator for calcium and a colorimetric reagent for alkaline earth metal ions) to the pre-reaction solution, for colorimetric detection of the LAMP reaction, generates a colour changes from violet (negative reaction)

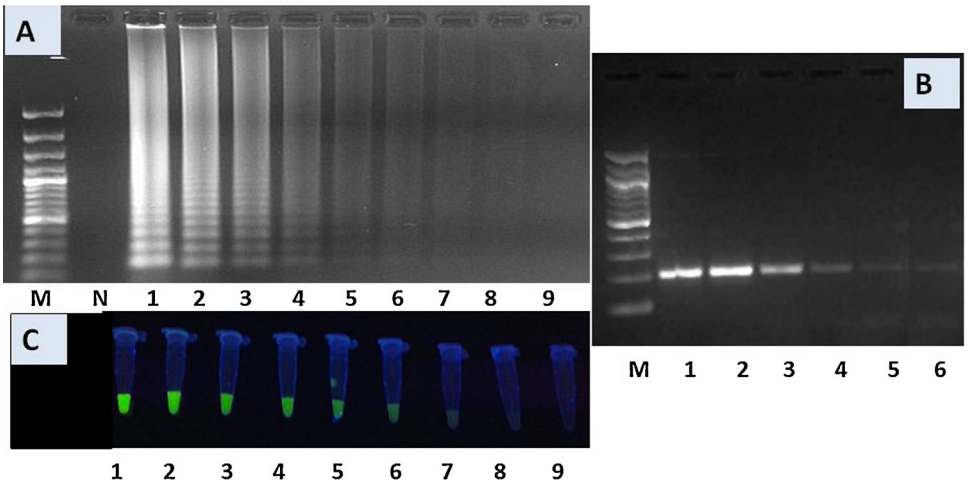

Fig. 4. Detection limit of 'accelerated' eRT-LAMP for BTV RNA (BTV-16 strain IND2014/01).
(Panel A) Accelerated eRT-LAMP performed on a serial 10 fold dilution of BTV RNA, detected down to dilution 1×10^{-9} (0.216 fg) of BTV RNA. (Panel B) conventional RT-PCR using eRT-LAMP specific F3 and B3 primers, using a serial 10 fold dilution series of BTV RNA, detected down to dilution 1×10^{-5} (216 pg) of BTV RNA. M: 1Kb marker; Lane 1: Undiluted RNA as template (0.216 μg); Lane 2: dilution 1×10^{-1}; Lane 3: dilution 1×10^{-2}; Lane 4: dilution 1×10^{-3}; Lane 5: dilution 1×10^{-4}; Lane 6: dilution 1×10^{-5}.
(Panel C) eRT-LAMP products (serial 10 fold dilution of BTV RNA (strain IND2014/01)) of BTV visualised under UV light after addition of Picogreen.

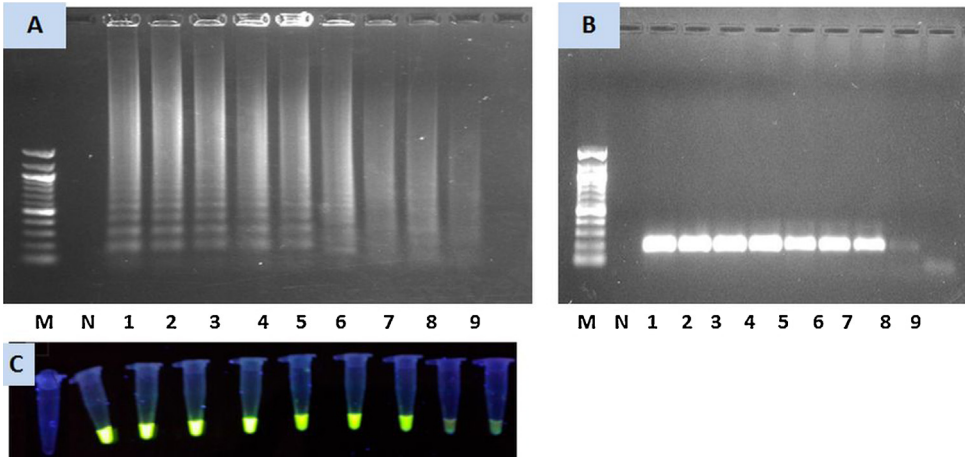

Fig. 5. Detection limit of 'accelerated' wRT-LAMP for BTV RNA (BTV-10 strain IND2004/01).
(Panel A) Accelerated wRT-LAMP performed on a serial 10 fold dilution of BTV RNA, detected down to dilution 1×10^{-8} (108 fg) of BTV RNA. (Panel B) conventional RT-PCR using wRT-LAMP specific F3 and B3 primers, using a serial 10 fold dilution series of BTV RNA, detected down to dilution 1×10^{-7} (1080 fg) of BTV RNA. M: 1Kb marker; N: Negative control; Lane 1: Undiluted RNA as template (1.08 μg); Lane 2: dilution 1×10^{-1}; Lane 3: dilution 1×10^{-2}; Lane 4: dilution 1×10^{-3}; Lane 5: dilution 1×10^{-4}; Lane 6: dilution 1×10^{-5}; Lane 7: dilution 1×10^{-6}; Lane 8: dilution 1×10^{-7}; Lane 9: dilution 1×10^{-8}.
(Panel C) wRT-LAMP products (serial 10 fold dilution of BTV RNA (strain IND2004/01)) of BTV visualised under UV light after addition of Picogreen.

to sky blue (positive reaction). This reduces the contamination risks, and facilitates high-throughput DNA or RNA detection (Goto et al., 2009).

Amplification of RNA by RT-LAMP required both reverse transcriptase and *Bst* DNA polymerase, since no amplification was observed when either of these two enzymes was omitted from the reaction mixture. The optimum temperature required for efficient amplification by the RT-LAMP assay were evaluated by incubating the reaction mixture at temperatures ranging from 60 to 70 °C. The results indicated an optimum temperature for both e & w RT-LAMP reactions is 65 °C–68 °C.

An initial analysis of the real-time kinetics of the RT-LAMP reaction at 65 °C, showed the detection of amplified cDNAs, in positive reactions, with or without the loop primers, after 30–60 min respectively for eRT-LAMP (Fig. 3b–d). The time taken by wRT-LAMP was slightly longer at 60 and 90 min with or without the loop primers respectively. This confirms that the loop primers do 'accelerate' the amplification mechanism.

The RT-LAMP reaction has higher amplification efficiency than conventional RT-PCR, rapidly generating a relatively large amount of a pyrophosphate ion reaction by-product during incubation. These ions react with Mg^{2+} ions to form the insoluble product magnesium pyrophosphate, which accumulate as a white precipitate in positive reactions, progressively increasing their turbidity (compared to negative control assays containing no BTV RNA template) (Fig. 2b and c). The increased turbidity of the reaction mixture correlates with the amount of DNA synthesized, which can be measured using a real-time turbidimeter (Mori et al., 2004, 2001). However, the use of expensive equipment for reaction confirmation, would decrease the versatility of LAMP and would potentially limit the wider use of this procedure, especially in developing countries. Since Mg^{2+} ion concentration decreases as the LAMP reaction progresses, the LAMP reaction can also be quantified by measuring the Mg^{2+} ion concentration in the reaction solution by adding calcein, a fluorescent metal indicator, to the pre-reaction solution reducing contamination risks (Tomita et al., 2008).

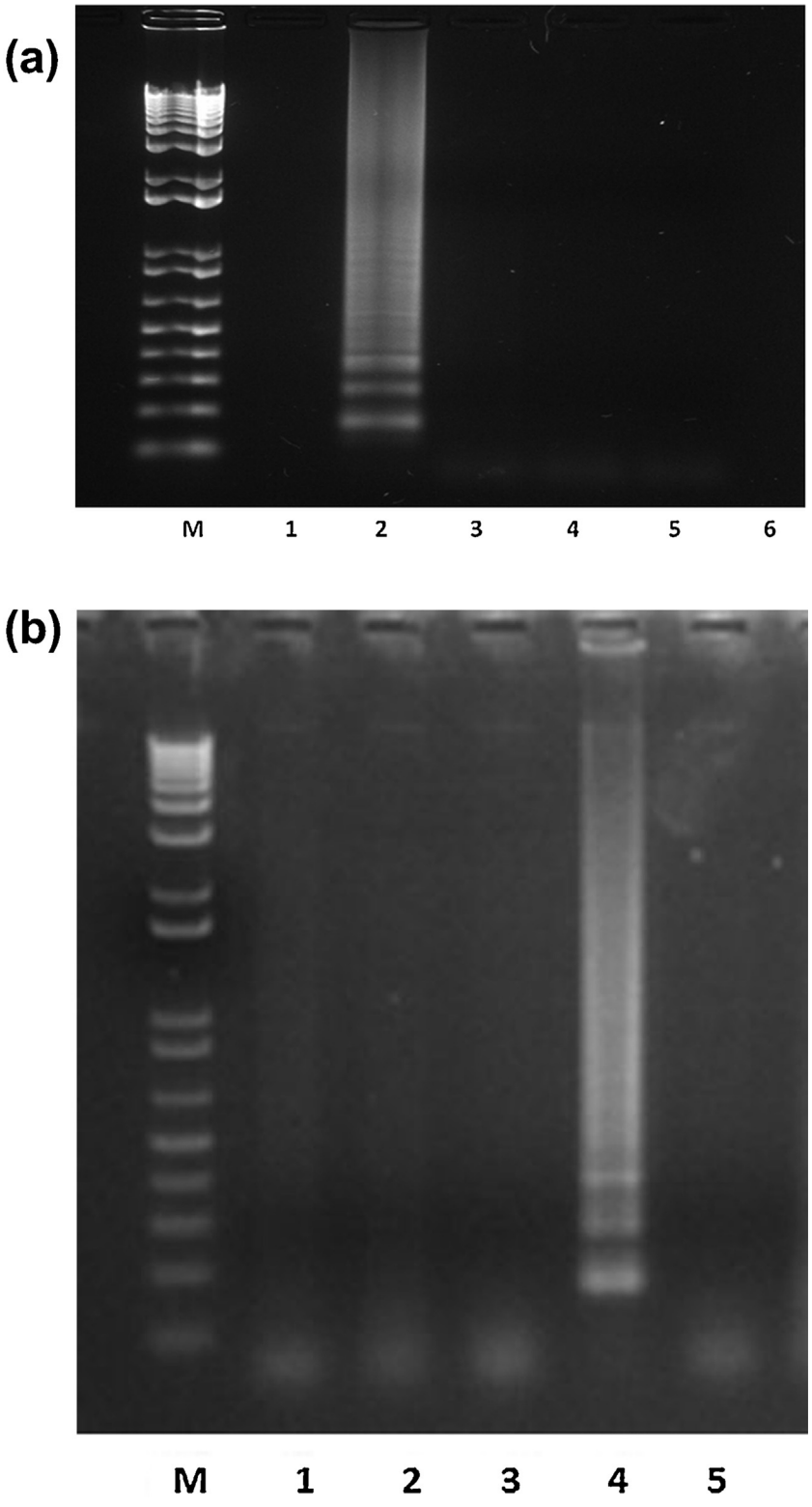

Fig. 6. (a) Specificity of 'accelerated' eRT-LAMP. (b) Specificity of 'accelerated' wRT-LAMP.
M: 1 Kb marker; Lane 1: RNA from uninfected sheep blood; Lane 2: RNA from BTV strain IND2003/10; Lane 3: PPRV/IND2013/01; Lane 4: FMDV type O; Lane 5: CaPV/IND2013/01; Lane 6: RNA from BTV strain IND2004/01.
M: 1 Kb marker; Lane 1: RNA from uninfected sheep blood; Lane 2: PPRV/IND2013/01; Lane 3: FMDV type O; Lane 4: RNA from BTV strain IND2004/01; Lane 5: RNA from BTV strain IND2003/10.

Each diagnostic method has its strengths and limitations, which need to be understood to ensure that it is used properly and with the appropriate samples to give meaningful results. Molecular techniques such as qRT-PCRs, which are available for detection and typing of BTV RNA, are rapid and more sensitive and can give positive results at an earlier stage of post-infection (p.i.) (e.g.

after 1–3 days) than methods which rely on detection of BTV-specific antibodies in the infected host (e.g. at 7–10 days) (Maan N. et al., 2015; Mertens et al., 2007, 2009a; Toussaint et al., 2007; Vandenbussche et al., 2010). These methods can amplify target nucleic acids within few hours, down to a detection limit of less than 10 copies. Despite their advantages, the requirement for sophisticated precision-equipment and appropriate laboratory facilities has prevented these powerful methods from being more widely used as a routine diagnostic tool.

Performing RT-LAMP reactions and detection of amplification is simple as compared to real-time qRT-PCR assays. It requires less expensive detection methods/equipment and is therefore can be more readily adaptable to use in the field. Since turbidity and colour change in the RT-LAMP assay can be confirmed visually, the only device required for the reaction is a water bath or heating block to provide a constant temperature of 65 °C. Substantially less time is required for generation of results using the RT-LAMP assay (i.e., usually 1–2 h, down to as little as 30 min), compared to 3–4 h for qRT-PCR.

The suitability of RT-LAMP assays for detection of eastern as well as western BTV strains was evaluated with a number of representative isolates from India, however warrants further evaluation on other serotypes and clinical samples. The LAMP assays described in this study are rapid, cost-effective, highly sensitive, and specific, suggesting that they have considerable potential for further development as 'field based'/pen-side tests. In combination with other established methods these LAMP assays could also be used for comprehensive BTV detection within a diagnostic laboratory setting to form part of an 'early warning system' facilitating the speedy implementation of disease control measures.

Conflict of interest

The authors declared "no conflict of interest".

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2016.04.002.

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