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

S. Maan[a,*], N.S. Maan[a,f], K. Batra[a], A. Kumar[a], A. Gupta[a], Panduranga P. Rao[b], Divakar Hemadri[c], Yella Narasimha Reddy[d], M. Guimera[e], M.N. Belaganahalli[e], P.P.C. Mertens[e]

[a] Department of Animal Biotechnology, College of Veterinary Sciences, LLR University of Veterinary and Animal Sciences, Hisar, Haryana, India
[b] Ella Foundation, Genome Valley Hyderabad, 500 078 T.S, India
[c] National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Hebbal, Bengaluru 560024 K.A, India
[d] College of Veterinary Science, Acharya N.G. Ranga Agricultural University, Rajendra Nagar, Hyderabad 500 030, T.S, India
[e] The Vector-Borne Viral Diseases Programme, The Pirbright Institute, Pirbright, Woking GU24 0NF Surrey, United Kingdom
[f] Resource Faculty, Department of Animal Biotechnology, College of Veterinary Sciences, LLR University of Veterinary and Animal Sciences, Hisar, Haryana, 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

* Corresponding author.
E-mail addresses: sushilamaan105@gmail.com, sushila.maan@pirbright.ac.uk (S. Maan).

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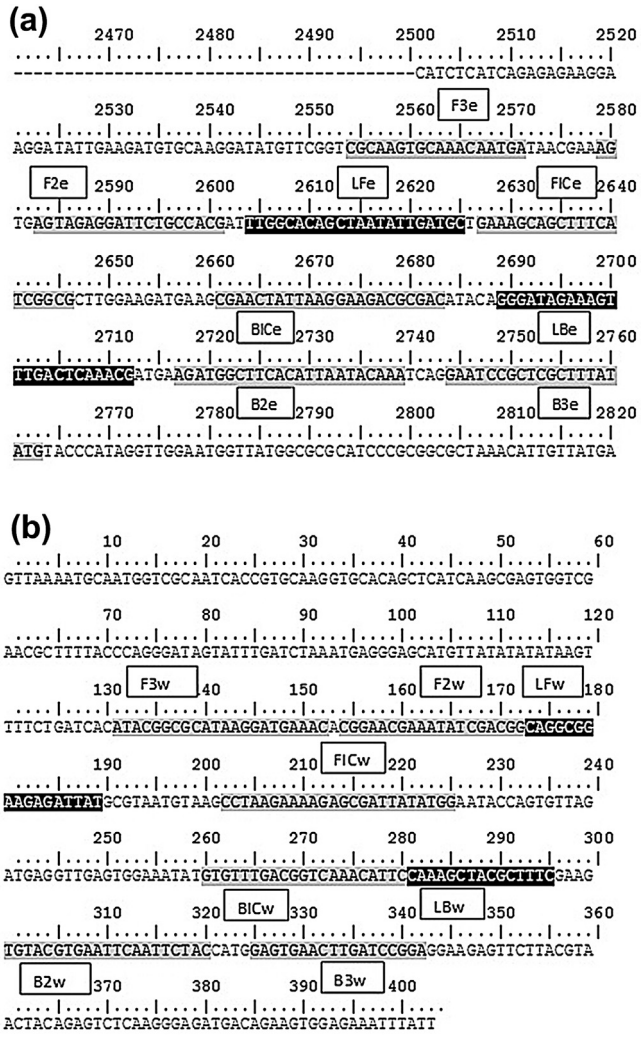

Fig. 1. (a) Location of BTV eRT-LAMP assay primers on genome segment 1. (b)Location of BTV wRT-LAMP assay primers on genome segment 1.

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