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The development of an accelerated reverse-transcription loop mediated isothermal amplification for the serotype specific detection of bluetongue virus 8 in clinical samples

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A B S T R A C T

In 2006 bluetongue virus serotype 8 (BTV 8) was identified for the first time in the Netherlands causing a major epidemic in sheep and cattle that quickly spread to neighbouring Belgium, Germany and beyond to France and the UK. This resulted in severe animal health and welfare problems as well as substantial economic losses to the agrifood industries of these countries. Given that the early diagnosis of BTV infection 'in-the-field' is extremely useful to its subsequent management and control, this study was established to design a novel, sensitive and rapid nucleic acid diagnostic test for the serotype-specific detection of BTV 8, which could be used without the use of advanced laboratory support and equipment. Primers for the detection of BTV 8 were based on genome segment 2 of the virus, the VP2 gene. The assay was assessed using a full panel of BTV reference strains and clinical samples. Positive amplification was observed using a fluorescent detection reagent. The sensitivity of the RT-LAMP assay was 102 copies of RNA. The assay did not amplify the closely related orbivirus EHDV. This novel RT-LAMP offers a sensitive, specific and rapid method of detecting BTV 8. The approach is inexpensive and easy to use and could potentially be used in a 'pen-side' setting 'in the field' or by smaller less well-equipped laboratories in developing countries.

1. Introduction

Bluetongue is a non-contagious viral disease affecting domestic and wild ruminants including sheep, cattle, goats, deer, antelope and camelids (Leblanc et al., 2010). It is caused by bluetongue virus (BTV), an economically important orbivirus within the *Reoviridae* family. BTV is an arbovirus transmitted by the adult females of certain *Culicoides* species (biting midge) (Chaignat et al., 2009). Twenty-six distinct virus serotypes are recognised (Maan et al., 2012). In 2006 bluetongue virus serotype 8 (BTV 8) occurred for the first time in the Netherlands causing a major epidemic (Elbers et al., 2009). From here the virus quickly spread to neighbouring Belgium, Germany and beyond to France and UK (Hund et al., 2012). The virus that caused the outbreak had subsequently been identified as being closely related phylogenetically to a 1982 West Africa Strain (Meiswinkel et al., 2007). Given that the control of BTV epidemics is based on early detection of infected animals, this recent epidemic highlighted the need for a novel, rapid and sensitive assay for detection of BTV 8 'in-the-field'. Specific detection of BTV currently relies on reverse transcription-polymerase chain reaction (RT-PCR) and although this method is highly sensitive, it has the disadvantages of requiring high-cost precision instruments (thermal cyclers) and highly trained technicians. In addition, detection by RT-PCR can take up to 2–4 h plus the transport time required for the sample to reach the laboratory before a diagnostic result can be achieved. Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method offering rapid, accurate and cost effective diagnosis of infectious diseases (Kono et al., 2004). The main advantages of LAMP are the ability of the technique

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to amplify specific DNA/RNA sequences under isothermal conditions, usually between 63 °C and 65 °C, and the fact that results can be achieved rapidly, within 60 min (Chen et al., 2009). LAMP uses two inner and two outer primers that recognise six different target sequence regions (Jaroenram et al., 2009). Accelerated LAMP uses an extra set of primers (termed loop primers), six primers in total, and recognising eight regions of target DNA. Previous published studies have demonstrated the effective use of LAMP for diagnostic purposes (Jaroenram et al., 2009; Le Roux et al., 2009; Nimitphak et al., 2008; Puthawibool et al., 2009).

In this study, a one-step accelerated RT-LAMP assay was designed to target a region within the gene encoding the serotype specific VP2. The assay was tested for specificity on all BTV strains and on the closely related epizootic haemorrhagic disease virus (EHDV) which is also an insect-transmitted orbivirus that produces clinical signs indistinguishable from BTV (Aradaib et al., 2003). In addition, the diagnostic sensitivity of the assay was determined using clinical samples from a sheep infected experimentally with BTV 8. To our knowledge, this is the first report of use of the LAMP method for diagnosis of BTV 8 infection.

2. Materials and methods

2.1. Virus strains and samples used

BTV 8 RNA transcripts were used for the optimisation of the time and temperature of this assay. Full genomic quantified BTV 8 RNA, which was serially diluted from 5×10^5 copies to 5×10^{-2} copies, was used to test the analytical sensitivity. BTV reference strains 1–24 and BTV 26 were supplied at the Friedrich-Loeffler-Institute (FLI), Greifswald, Germany. The BTV 8 sample used was a strain from France during the northern European outbreak. Sample BTV 8 PIR was supplied by The Pirbright Institute, Surrey, UK, to the FLI and originated from South Africa. A positive control was supplied with RT-LAMP kit (Eiken, Tochigi, Japan). Negative controls throughout contained the BTV 8 LAMP primer mix and nuclease free water as template.

2.2. RNA extraction

The viral RNA was extracted using the MagAttract® Virus Mini M48 kit (Qiagen, Hilden, Germany) according to manufacturer's instructions on a KingFisher Flex platform (ThermoFisher, Schwerte, Germany).

2.3. LAMP primer design and selection

Full-length BTV 8 genome sequences from segment 2 (VP2) were aligned using all currently published BTV 8 strains on the GenBank database (NCBI, MD, USA) (accession nos.: FJ183375, AM498052, AJ585184, AJ585183, and AJ585129). The BTV 8 sequences were aligned using Gendoc (Version 2.6.001, MA, USA). A total of six primers comprising of two outer primers, known as forward outer (F3) and backward outer (B3), two inner primers, known as forward inner primer (FIP) and backward inner primer (BIP) and two loop primers, forward loop LpF and backward loop LpB, that recognise eight distinct regions on the target sequence were designed using the LAMP primer designing support software program (Primer Explorer V4, Fujitsu, Tokyo, Japan) (http://primerexplorer.jp/elamp4.0.0/index.html). FIP and BIP are high-performance liquid chromatography-purified primers containing two recognition sequences. The two loop primers were designed to accelerate the amplification reaction. All primers were synthesised by Eurofins MWG (Ebersberg, Germany). The nucleotide sequences and locations of primers are shown in Table 1.

2.4. RT-LAMP assay

The reaction mixture had a total volume of 25 μl containing 40 pmol each of the inner primers (FIP and BIP), 20 pmol each of the loop primers (LpF and LpB), and 5 pmol each of the outer primers (F3 and B3). 12.5 μl of the supplied 2× reaction mix from the RNA amplification kit, Loopamp (Eiken, Tochigi, Japan) was added to the reaction. In addition, 1 μl of the supplied enzyme mix and 1 μl of fluorescent detection reagent (Eiken, Tochigi, Japan) was added to the reaction. Distilled water was added to a total volume of 20 μl. 5 μl heat-denaturised RNA was added as template for the reaction. Sterile water was used as the negative control template. A standard time and temperature of 63 °C for 1 h with termination at 95 °C for 2 min was recommended by the manufacturer. The positive control in each reaction was supplied with the RNA amplification kit, Loopamp (Eiken, Tochigi, Japan).

2.5. Analysis of RT-LAMP products

The RT-LAMP products were analysed by 3 different methods. The addition of a fluorescent dye (Eiken, Tochigi, Japan) to the master mix allows for the detection of amplified products under UV light. The products in this study were photographed in tubes and in a 96 well plate using a conventional digital camera under handheld UV light and software-driven mounted camera under UV light (Bio-Rad, Hertfordshire, UK). These photographs were taken differently to demonstrate the difference observed in sample analysis between each UV method. Amplified fragments were also observed by electrophoretic separation in 2% agarose gel with ethidium bromide staining. This revealed a ladder-like banding pattern typical of a positive LAMP reaction.

2.6. Optimisation of the RT-LAMP

To determine the optimal temperature for amplification, the LAMP reactions were performed at 61 °C, 63 °C or 65 °C for 1 h, followed by 95 °C for 2 min to terminate the reaction. The optimal time for amplification was determined by performing the LAMP assay for 15, 30, 45 or 60 min and then terminating the reaction by increasing the temperature to 95 °C for 2 min.

2.7. Analytical sensitivity of the BTV 8 RT-LAMP assay

The sensitivity of the RT-LAMP was analysed using ten-fold serial dilutions of quantified BTV 8 RNA ranging from 5×10^5 copies to 5×10^{-2} copies. This was compared with a pan real-time RT-PCR assay capable of detecting all serotypes of BTV and targeting the segment 5 (NS1) gene (Toussaint et al., 2007). The samples were tested in duplicate.

2.8. Specificity of the RT-LAMP assay

The specificity of the RT-LAMP assay was examined using four different strains of EHDV. The strains included EHDV Type 1 (USA 1955/01), a strain originating from New Jersey, USA (accession no.: AM744978); EHDV Type 2 (CAN 1962/01) a strain originating from Alberta USA/Canada (accession no.: AM744998); EHDV Type 6 (318) a strain originating from cattle in Bahrain (accession no.: AM745068); EHDV Type 7 (ISR 2006/01) a strain originating from Jordon Valley, Israel. In addition, 5 μl of undiluted reference RNA from all available serotypes of BTV (BTV serotypes 1–24 and BTV 26) were also analysed to confirm the specificity of the LAMP assay.