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Detection of bluetongue virus by reverse transcription-loop-mediated isothermal amplification

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Summary

A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was applied for the detection of the RNA of bluetongue virus (BTV). A primer set that targets conserved segment 1 of the BTV genome was used. The assay detected the viral RNA in all archival BTV-positive samples. Results of the study show that the sensitivities of the RT-LAMP and real-time RT-PCR assays were equal, and the detection limit for both methods was the 1/160 dilution of BTV-infected blood samples. RNA isolated from blood samples taken from healthy uninfected cattle (negative control) was not detected in this assay. No cross-reactivity of the primers with the genes of symptomatic look-alike diseases, such as foot-and-mouth disease (FMDV) and peste des petits ruminants (PPR), was found. Including the time required for the extraction of RNA, its presence in archival EDTA-treated blood samples could be detected within 2 hours. RT-LAMP is a very fast, sensitive, and specific technique for the detection of BTV in biological samples. Therefore it can be a valuable tool complementing the routine diagnostic procedure for BTV diagnosis.

Keywords: bluetongue virus, RT-LAMP, detection

Bluetongue virus (BTV), the prototype of the genus *Orbivirus*, within the family *Reoviridae*, is an infectious but non-contagious causative agent of bluetongue disease (BT) in many species of domestic and wild ruminants (18). Twenty-six immunologically distinct serotypes of BTV have been identified worldwide to date (4, 17, 30). BTV is a small (about 70 nm in diameter) icosahedral virus with a genome of approximately 19 200 base pairs, composed of ten linear segments of double-stranded RNA (dsRNA), which is packaged within an icosahedral nucleocapsid composed of seven structural proteins (29). BTV is transmitted to ruminants by biting midges of the genus *Culicoides* (20), but it can sometimes also be transmitted either via the oral route or vertically (2, 7, 21). The occurrence of BTV closely matches the distribution of *Culicoides* midges and climate conditions that support a large population of these insects. BTV is therefore endemic in many tropical, sub-tropical, and temperate regions of the world, between latitudes 40°S and 53°N, during times of the year that are optimal for vector activity (20).

The rapid spread of BTV-8 outbreaks in north-western Europe in 2006-2008 highlighted the need for all diagnostic laboratories dealing with BTV to be capable of rapid and reliable detection of this pathogen. The laboratory diagnosis and identification of the BTV serotype involved, is nowadays carried out by various antibody detection methods, including antigen capture, agar gel immunodiffusion (AGID), ELISA, and virus neutralisation (VN) assays (27). BTV in clinical samples is detected by virus isolation in cell cultures (BHK-21, Vero) or in embryonated chicken eggs (ECE), ELISA, immunofluorescence, dot immunobinding assays (DIA), and immunoelectron microscopy (25). The viral RNA is usually detected by various conventional gel-based reverse transcription–polymerase chain reactions (RT-PCR) (1, 8, 36) or real-time RT-PCR (rRT-PCR) assays (31, 33, 35). Recently, the rRT-PCR technique for the detection and typing of BTV serotypes was introduced in our laboratory (23).

Loop-mediated isothermal amplification (LAMP), a novel gene amplification method, is an autocycling and strand displacement DNA synthesis method (24). Two or three primer pairs are used to amplify the template, which gives a long-stem loop product under isothermal conditions. The result can be visualised by gel-electrophoresis or by real-time fluorogenic analysis with a thermal cycler, or it can be observed directly with the naked eye upon the addition of an intercala-

ting dye (SYBR green I, calcein etc.) (26). LAMP is simple and no special equipment is required. There is an interest in this technique as a method of detecting a variety of pathogens in bacterial, fungus and viral infections (5, 12, 34).

The aim of this study was to determine the diagnostic value of reverse transcription – LAMP (RT-LAMP) for the simple and rapid detection of BTV in blood samples collected from BTV infected animals.

Material and methods

Sample origin. Four panels of rRT-PCR positive blood samples (each of 8 samples) collected in 2008-2011 for the purposes of a ring trial for the BTV genome and antibody detection were tested. A sample of EDTA-treated blood collected from uninfected sheep and provided by the European Union Reference Laboratory for BT (EURL BT), Pirbright, UK, was used as a negative (K–) control. Moreover, samples of RNA extracted from foot and mouth disease virus (FMDV) serotype O: O_1 Manisa/TUR/69, serotype A: A_{22}/IRQ/24/64, serotype Asia 1: Asia 1/Shamir/97, and peste des petits ruminants virus (PPRV) strains: PPR CI 89 (lineage I) and PPR 75-1 wild type (lineage II) were used.

RNA extraction and denaturation. RNA was extracted from the EDTA blood samples by means of a QIAamp Viral RNA Mini Kit (Qiagen) according to the method recommended by the manufacturer. Extracted RNA was denaturated by the incubation of the samples for 5 min at 100°C, and then cooled to 4°C (22).

Oligonucleotide primers. A set of four primers F, B, FIP and BIP were designed by targeting conserved segment 1 of the BTV genome. Segment 1 nucleotide sequences of the representative isolates of BTV were retrieved from GenBank and aligned by the software program Primer Explorer V4 (http://primerexplorer.jp/e/). The two outer primers (F3 and B3) help to displace the primary strand. The inner primers (FIB and BIP) each have two distinct sequences corresponding to the sense and antisense sequence of the target. The length, genome position, and sequence of primers used for RT-LAMP amplification of segment 1 of the BTV genome are presented in Tab. 1. Oligonucleotide primers were prepared in the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw.

RT-LAMP reaction. The RT-LAMP reaction was carried out in a 25 µl mixture containing 15 µl Isothermal Master Mix (OptiGene Ltd.), 0.2 µM each of F3 and B3, 1.6 µM each of FIP and BIP primers, 4.5 U AMV reverse transcriptase (Invitrogen) and 5 µl BTV RNA. The plate was transferred to a thermal cycler (7300 Real Time PCR System, Applied Biosystems) and the reaction was carried out at 63°C for 60 min (45 cycles of 60 s at 63°C) (6-carboxyfluoroscein – FAM) and inactivation at 80°C for 2 min. Fluorescence was measured at the end of the 63°C annealing/extension step. Additionally, RT-LAMP products after heating at 80°C for 10 min were analyzed by 2% agarose gel electrophoresis. The real-time RT-PCR was carried out as described previously (22).

Analytical sensitivity and specificity. In order to estimate the analytical sensitivity of the RT-LAMP technique, 8 serial two-fold dilutions (1/5 – 1/320) of blood samples collected from BTV-8 infected sheep were prepared. The analytical specificity of the assay was evaluated by testing RNA from FMDV and PPRV agents causing symptomatic look-alike diseases.

Tab. 1. Details of oligonucleotide primers used for the RT-LAMP amplification of segment 1 of the BTV genome

Primer name	Type	Lenght	Genome position	Sequence (5'-3')
F3	forward outer	18-mer	289-306	ACGCTTTTGAGGTGTACG
B3	reverse outer	18-mer	470-487	GCACTCAGTTCGTTGATG
FIP	forward inner	43-mer	F1c, 367-390; F2, 307-325	CTTCGGTCATCTCCCTTGAAACTC TGAACTCAATTTTGCCGTG
BIP	reverse inner	44-mer	B1c, 402-422; B2, 444-466	ATCGAGTTCCGCGCTAAAAAC CAACACCATACTTTAATGGGTAT

Results and discussion

Rapid and accurate diagnosis plays an important role in the implementation of effective measures to control the spread of disease. Conventional BTV laboratory diagnostic methods, such as virus isolation, ELISA, and RT-PCR, can be time-consuming and laborious. Virus isolation techniques require a long time before the results are available and are especially laborious (6). ELISA is very rapid and easy to perform, but usuitable for the detection of BTV in blood samples (11, 20). The conventional RT-PCR method requires agarose gel electrophoresis, which is time-consuming, less sensitive, non-quantitative, and subjective, which limits the number of samples that can be tested during a day (36). The real-time PCR assay is going to replace conventional PCR methods, as it is faster and has proved to be more sensitive (35).

In this study, the utility of the RT-LAMP technique for rapid and accurate detection of BTV RNA in EDTA-treated blood samples was investigated. Using this assay, we detected the viral RNA in archival BTV-positive blood samples supplied by the EURL BT for the purposes of the ring trial for the BTV genome and antibody detection. The set of four specific LAMP primers targeting conserved segment 1 of the BTV genome is well designed to detect all serotypes of BTV.

The real-time monitoring of RT-LAMP amplification can be accomplished through fluorescence analysis with the automatic thermocycler routinely used for rRT-PCR reactions (Fig. 1). The amplification products of RT-LAMP reactions can also be visualized by agarose gel electrophoresis (Fig. 2). Positive RT-LAMP products were observed as a "smear" pattern due to the formation of a mixture of stem-loop DNAs of various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternatively

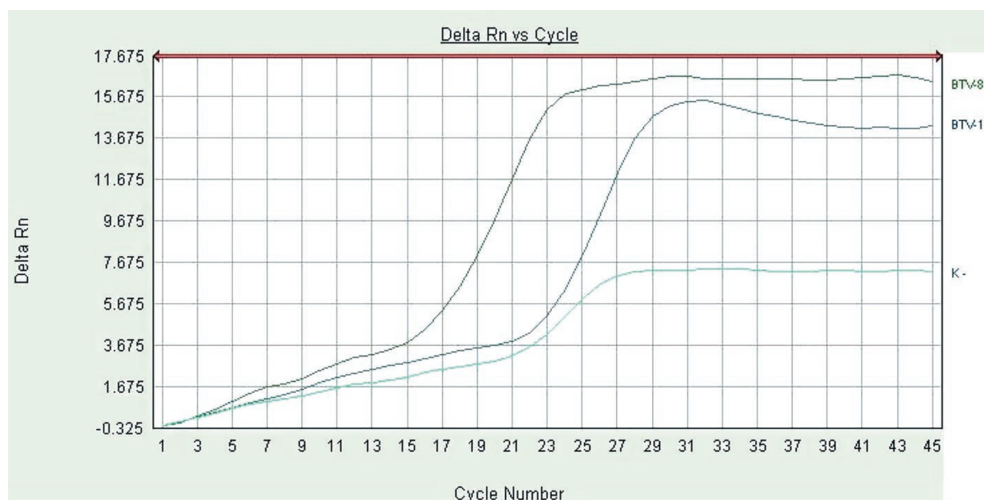

Fig. 1. Real-time monitoring of RT-LAMP amplification resulting from the determination of BTV RNA in archival EDTA blood samples

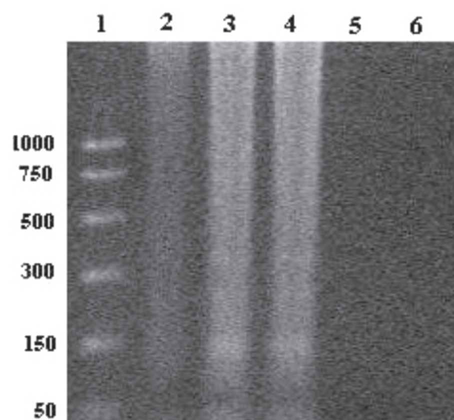

Fig. 2. Agarose gel electrophoresis of specific RT-LAMP products of BTV. Lane 1: molecular size marker; lane 2: blood from healthy, uninfected sheep (negative control); lane 3: BTV-8; lane 4: BTV-1; lane 5: FMDV O_1 Manisa/TUR/69; lane 6: PPR CI 89 (lineage I)

inverted repeats of a target sequence in the same strand. All archival rRT-PCR positive blood samples were recognised as positive by RT-LAMP. FMDV and PPRV RNA, as well as RNA isolated from the epithelium taken from uninfected sheep (negative control), were not detected in this assay (Fig. 2, Tab. 2).

The results of this study show that the sensitivities of rRT-PCR and RT-LAMP assays are equal and there is no cross-reactivity of the primers with the genes of symptomatic look-alike diseases such as FMD and PPR. The detection limit by both methods was a blood dilution of 1/160; six subsequent dilutions up to 160 were scored positive by both techniques. Only a 1/320 dilution was scored negative by both methods (Tab. 2). The results indicate that the RT-LAMP assay was specific for BTV; neither FMDV nor PPRV genes were detected by this method (Tab. 2). The equal sensitivity of rRT-PCR and RT-LAMP was also observed previously for other viral pathogens, such as PPRV (16), African swine fever virus (ASFV) (13), goose parvovirus (GPV) (14), FMDV (9), and Rift Valley fever virus (RVFV) (28). The detection sensitivity of RT-LAMP was even higher than that of rRT-PCR in cases of enterovirus 71 (32) and pseudorabies virus (PRV) (10). RT-LAMP for the detection of swine vesicular disease virus (SVDV) in faecal samples was shown to be more sensitive than rRT-PCR, possibly because the LAMP assay is less sensitive to inhibitory substances (3). However, compared to rRT-PCR, RT-LAMP is simpler and easier to apply for diagnosis. By using isothermal mastermix containing a novel GspSSD polymerase, it was possible to detect BTV RNA within less than 20 min (Fig. 1). Including the time required for the extraction of viral RNA, its detection in archival EDTA-treated blood samples could be achieved within 2 hours. The amplification efficiency of the RT-LAMP method is high because its isothermal reaction eliminates the time loss for thermal change (15). However, primer design for the RT-LAMP technique is more complex than that for the RT-PCR assay, and specialized training and software are required for their design.

Apart from a high level of diagnostic accuracy and speed of detection, another important practical advan-

Tab. 2. Result comparison of RT-LAMP and rRT-PCR assays

Pathogen	Serotype (isolate)	Results (positive number/number tested)	
		RT-LAMP	rRT-PCR
BTV	BTV-8	+ (10/10)	+ (10/10)
	BTV-1	+ (9/9)	+ (9/9)
	BTV-2	+ (3/3)	+ (3/3)
	BTV-4	+ (4/4)	+ (4/4)
	BTV-6	+ (3/3)	+ (3/3)
	BTV-16	+ (3/3)	+ (3/3)
	BTV-8 (undiluted)	+	+ (21.82)*
	BTV-8 (dil. 1/5)	+	+ (24.48)*
	BTV-8 (dil. 1/10)	+	+ (26.35)*
	BTV-8 (dil. 1/20)	+	+ (28.74)*
	BTV-8 (dil. 1/40)	+	+ (31.86)*
	BTV-8 (dil. 1/80)	+	+ (33.28)*
	BTV-8 (dil. 1/160)	+	+ (37.21)*
	BTV-8 (dil. 1/320)	–	– (> 40)*
FMDV	O, A, Asia 1	– (3/3)	– (3/3)
PPRV	PPR CI 89, PPR 75-1	– (2/2)	– (2/2)

Explanation: + positive; – negative; * C_T value

tage of the RT-LAMP technique is that it can be carried out with relatively simple and inexpensive equipment, such as a heating block or a water bath. However, for the real-time monitoring of RT-LAMP amplification, an automated thermal cycler is required. In conclusion, it can be stated that RT-LAMP is a very fast, specific, and sensitive technique for the detection of BTV in biological samples. The RT-LAMP method can therefore be considered as a valuable tool complementing the routine diagnostic procedure for BTV diagnosis and shows promise as a practical laboratory approach for the detection of BTV.

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