Published in final edited form as:

Microbiol Immunol. 2011 January ; 55(1): 44–50. doi:10.1111/j.1348-0421.2010.00286.x.

Rapid detection of Lassa virus by reverse transcription-loop-mediated isothermal amplification

Aiko Fukuma^{1,2,3}, Yohei Kurosaki^{1,2}, Yuko Morikawa³, Allen Grolla⁴, Heinz Feldmann^{4,5,6}, and Jiro Yasuda^{1,2,*}

¹First Department of Forensic Science, National Research Institute of Police Science, Kashiwa 277-0882, Japan

²CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan

³Kitasato Institute for Life Sciences and Graduate School for Infection Control, Kitasato University, Tokyo 108-8641, Japan

⁴National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba R3E 3R2, Canada

⁵Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada

⁶Laboratory of Virology, Division of Intramural Research, National Institutes of Allergy and Infectious Diseases Rocky Mountain Laboratories, Hamilton, Montana, USA

Abstract

In this study, a simple one-step reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for rapid detection of Lassa virus (LASV) was established. The two primer sets were designed to detect LASV circulating in Sierra Leone and northeastern Nigeria. The RT-LAMP assay using these primer sets was able to detect 100 copies of the *in vitro* transcribed artificial LASV RNA within 25 min. The assay was also evaluated using intact viral RNA extracted from cell culture-propagated viruses and confirmed to be highly specific for LASV. The RT-LAMP assay developed in this study is rapid, simple, and highly specific for the detection of LASV, although its sensitivity is slightly lower than that of real-time RT-PCR. In addition, because the RT-LAMP assay does not require the use of sophisticated equipment, it would be advantageous for clinical diagnosis of LASV infection in developing countries. It might also be employed in cases of deliberate release during bioterrorism attacks or in epidemiological surveillance for disease outbreaks.

Keywords

detection; Lassa virus; RT-LAMP

Lassa virus belongs to the family *Arenaviridae*, genus *Arenavirus*. The single-stranded arenavirus genome consists of two segments, a small RNA fragment of 3.4 kb (S) and a large RNA fragment of 7 kb (L). The S segment encodes the NP and the viral GPC, which is posttranslationally cleaved into the envelope glycoproteins GP1 and GP2. The L segment

encodes the viral polymerase (L protein) and a small, Z protein. The genes are located on the RNA in opposite orientations separated by an intergenic region folding into a stable secondary structure. The terminal 19 nucleotides at the 3' and 5' ends of the RNA segments, which are believed to function as binding sites of the viral polymerase, are complementary to each other and highly conserved among all arenaviruses (1).

LASV is the causative agent of a hemorrhagic fever endemic to the West African countries Sierra Leone, Liberia, Guinea, and Nigeria, and the Central African Republic. The natural host of LASV is the rodent *Mastomys natalensis*, which is indigenous to most of sub-Saharan Africa (2). Humans presumably become infected through close contact with infected rodent excreta, and human-to-human transmission can also occur (3). Lassa fever causes severe morbidity and mortality, with an estimated 100,000 to 300,000 infections and approximately 5000 deaths occurring annually in West Africa (4, 5). Recently, cases of Lassa fever imported into countries outside the endemic region have been reported in Europe (5, 6). Although the clinical symptoms of Lassa fever in the initial phase are characterized by fever, muscle aches, sore throat, nausea, vomiting, and chest and abdominal pain, these symptoms are often similar to those of other viral, bacterial, or parasitic infections (1, 5). Ribavirin therapy is most effective if administered soon after infection occurs (8). Therefore, the development of a rapid and accurate diagnostic system that is appropriate for use in rural endemic area hospitals is important.

The laboratory diagnosis is generally made by virus isolation in cell culture and immunoassays such as ELISA, IFA, and RT-PCR (6). Virus culture is influenced very little by virus variability and allows detailed characterization. However, it requires BSL-4 facilities and the associated infrastructure in addition to a viral cultivation period of 7–10 days. Although antigen detection by ELISA capture is robust and reliable, it is less sensitive than other methods (9). RT-PCR and real-time PCR techniques are sensitive methods for rapid detection of LASV (10–15). However, as these methods require expensive equipment and expertise, they have not been adopted by many small and frontline laboratories.

Loop-mediated isothermal amplification is a novel nucleic acid amplification method originally reported by Notomi *et al.* (16) that can amplify DNA rapidly, efficiently, and with high specificity under isothermal conditions. This simple and rapid technique is based on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment with high strand displacement activity. The reaction is conducted under isothermal conditions ranging from 60°C to 65°C, and therefore expensive thermocycling equipment is not required. As there is no time loss in thermal changes, the amplification efficiency of the LAMP method is extremely high. The reaction is highly specific for the target sequences, because a set of specific primers recognize at least six distinct sites on them. The LAMP reaction can be further enhanced by addition of loop primers (17). This modification reduces the reaction time and has the potential to increase sensitivity. Moreover, the LAMP method generates an increase in turbidity in positive samples, allowing detection by real-time monitoring based on the turbidity of the reaction mixture as well as agarose gel electrophoresis (18). Therefore, the LAMP assay has advantages in specificity and rapidity over other nucleic acid amplification methods. Recently, this method has been used successfully to detect human pathogenic RNA viruses (19–22).

In this study, we developed a LASV-specific RT-LAMP assay as a rapid, simple, sensitive, and specific molecular test for the detection of LASV.

MATERIALS AND METHODS

Viruses

The LASV strains used in this study were the Josiah strain belonging to the Sierra Leone lineage, and the Pinneo strain belonging to the northeastern Nigeria lineage. The viruses used for cross-reactivity studies were *Zaire ebolavirus* strains Mayinga and Zaire 95, *Sudan ebolavirus*, *Reston ebolavirus*, *Cote d'Ivoire ebolavirus*, and *Lake Victoria marburgvirus* strains Musoke, Ozolin, Ravn, and Angola. All virus strains were propagated in Vero E6 cells. Viral RNAs were extracted manually from virus suspensions as described previously (19). All infectious materials were handled in the BSL-4 facility of the National Microbiology Laboratory of the Public Health Agency of Canada.

Preparation of *in vitro* transcripts of the LASV targeting sequence

To determine the detection limit of RT-LAMP, plasmids containing the target sequence were constructed. The complete S segments of Josiah and Pinneo strains were amplified by PCR using the primers (for Josiah strain, forward: 5'-GCTAGCGCACAGTGGATCCTAGG-3', reverse: 5'-GCGGCCGCGCACAGTGGATCCTAGG-3'; for Pinneo strain, forward: 5'-GCTAGCGCACAGTGGATCCTA GCGATTGGATTGCGCTTTG-3', reverse: 5'-GCTAGCGCACAGTGGATCCTAGGCATTTAGGATTG-3') and a Prime STAR RT-PCR Kit (Takara, Shiga, Japan). The amplified products were cloned into the pGEM3XZf (+) vector (Promega, Madison, WI, USA). Artificial LASV RNA possessing the complete S segment sequence of Josiah or Pinneo strain was synthesized using the T7 RiboMAX expression large-scale RNA production system (Promega) according to the manufacturer's protocol. The reaction-mixtures were incubated at 37°C for 30 min with 1 µg of the linearized plasmid. One unit of RQ1 RNase-free DNase, (Promega) was added followed by incubation for a further 15 min. The transcripts were extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), and resuspended in 100 µL of RNase-free water. The concentration of RNA transcript was determined by measuring the OD.

Primer design

Two sets of RT-LAMP primers for northeastern Nigeria and Sierra Leone LASV were designed from the NP gene. The nucleotide sequence of the NP gene of the LASV strains Josiah and Pinneo were retrieved from Gen-Bank (accession numbers J04324 and AY628207, respectively) and aligned with the available NP gene sequences of other strains (98 sequence data) to identify the conserved regions using Genetyx software (Genetyx, Tokyo, Japan). The potential target regions were selected from the aligned sequences, and RT-LAMP primers were designed. Six primers comprising two outer, two inner, and two loop primers that recognize eight distinct regions on the target sequence were designed employing the LAMP primer designing support software program (Primer Explorer ver. 3; Net Laboratory, Tokyo, Japan; <http://primerexplorer.jp/e/>). The FIP and BIP primers were purified by high-performance liquid chromatography, and the others were purified using oligonucleotide purification cartridges. The details of the primers are shown in Table 1.

Reverse transcription-loop-mediated isothermal amplification

The RT-LAMP reactions were performed using a Loopamp RNA amplification kit (Eiken Chemical, Tokyo, Japan). The 25 µL reaction mixture contained 40 pmol of each of the inner primers FIP and BIP, 5 pmol of each of the outer primers F3 and B3, 20 pmol of each of the loop primers F and B, 12.5 µL of 2 × reaction mix, 1 µL of enzyme mix, and 5 µL of RNA sample. The reaction mixture was incubated at 63°C for 60 min in a Loopamp Realtime Turbidimeter (LA-200; Teramecs, Kyoto, Japan). Analysis of each sample was

performed in duplicate and the lowest concentration of genome copies was taken as the limit when all of the duplicate samples were positive. A no-template negative control was included in each assay. Real-time monitoring of amplification of the virus genome was performed by spectrophotometric analysis by recording the OD at 400 nm every 6 s using the Loopamp Realtime Turbidimeter. The cutoff value for positivity by real-time RT-LAMP assay was determined by taking into account the time of positivity (in min) at which the turbidity increase above the threshold value was fixed at 0.1. Then, 1 µL of the amplified product from the RT-LAMP assay was electrophoresed on a 3% NuSieve 3:1 agarose gel (Bio Whittaker Molecular Applications, Rockland, ME, USA) in Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized under UV light. To confirm the specificity of RT-LAMP-amplified products, the RT-LAMP products of northeastern Nigeria strain or Sierra Leone strain were digested with the restriction enzyme *StuI* or *Tsp509I* (New England Biolabs, Ipswich, MA), respectively. After 2 h of digestion, RT-LAMP products were analysis by electrophoresis on 3% agarose gels, stained with ethidium bromide, and visualized under UV light.

Real-time reverse transcription polymerase chain reaction

To compare the sensitivities of the RT-LAMP with those of the real-time RT-PCR assays, real-time RT-PCR was performed with LASV-specific primers described previously, 80F2 (5'-ATATAATGATGACTGTTGTTCTTTGTGCA-3') and 36E2 (5'-ACCGGGGATCCTAGGCATT-3') (10). Amplification was carried out in a total reaction volume of 25 µL using a One Step SYBR RT-PCR Kit (Takara) with 5 pmol of forward and reverse primers and 5 µL of RNA sample. The thermal profile was 50°C for 20 min and 95°C for 5 min, followed by 40 cycles of 95°C for 5 s, 56°C for 10 s, and 72°C for 25 s. Thermal cycling and quantification were performed using a Smart Cycler II System (Cepheid, Sunnyvale, CA, USA).

RESULTS

Detection limits of the reverse transcription-loop-mediated isothermal amplification assay for Lassa virus

For specific amplification of the genomic RNA of LASV circulating in Sierra Leone and northeastern Nigeria, we designed primer sets for either the Sierra Leone or northeastern Nigeria group based on alignment analysis of LASV genomic sequences (Table 1). We analyzed 100 sequence data of Lassa virus NP gene. Highly conserved sequences in the NP coding region were selected as the target sequences. Alignment of the target sequences of representative strains of each lineage is shown in Figure 1.

To determine the detection limit of the RT-LAMP assay with the primer set, assays were carried out using serial 10-fold dilutions of *in vitro* transcribed RNA, which has the complete sequence of the S segment from the Pinneo strain of the northeastern Nigeria lineage or Josiah strain of the Sierra Leone lineage (Fig. 2). The RT-LAMP reactions were monitored by real-time turbidity detection. The detection limits of both assays using the northeastern Nigeria primer set or the Sierra Leone primer set were 100 copies per reaction. Each reaction could detect 100 copies per reaction within 25 min for northeastern Nigeria or 17 min for Sierra Leone. The detection limits of the assays were also confirmed by observing the fluorescence of the solutions under a UV light source. Clear fluorescence signals were seen at concentrations ranging from 100 to 10⁶ copies per reaction, at each reaction, as well as turbidity detection (data not shown). With the same RNA template and conventional primers, real-time RT-PCR could detect 10 copies for Josiah and 100 copies for Pinneo (data not shown), indicating that the sensitivity of RT-LAMP using these primer sets was equivalent to, or 10-fold less, than that of RT-PCR.

Cross-reactivity of the reverse transcription-loop-mediated isothermal amplification assay for Lassa virus

To determine whether the RT-LAMP with these primer sets was specific for LASV amplification, the RT-LAMP assay was evaluated with LASV and other hemorrhagic fever viruses, including *Zaire ebolavirus*, *Sudan ebolavirus*, *Reston ebolavirus*, *Cote d'Ivoire ebolavirus*, and *Lake Victoria marburgvirus* (Musoke, Ozolin, Ravn, and Angola strains). Like LASV, these viruses are causative agents of hemorrhagic fever and can also grow in Vero E6 cells, which is used for the propagation of LASV. Viral RNA preparations extracted from these viruses propagated in Vero E6 cells were used for the assay as negative controls, since some cellular agents, such as cellular RNA and DNA, might be capable of inducing false-positive reaction. The assays were performed using viral RNA from viruses propagated in cell culture by real-time turbidity detection. The results are summarized in Table 2. RT-LAMP using the northeastern Nigeria LASV-specific primer set could detect the viral RNA of Pinneo strain only at more than 1 ng per reaction within 23.9 min, while the assay using the Sierra Leone-specific primer set could detect the viral RNA of Josiah strain only at more than 10 pg per reaction within 18.2 min. Both RT-LAMP assays amplified only the corresponding LASV strain, but not other hemorrhagic fever viruses, indicating that the RT-LAMP assay is highly specific to each group of LASV. Detection limits of real-time RT-PCR for the same viral RNA templates were 100 pg for Pinneo and 1 pg for Josiah (Table 2), indicating that the sensitivities of both RT-LAMP assays were 10-fold lower than those of real-time RT-PCR. Quantitative real-time RT-PCR analyses indicated that 100 pg of Pinneo viral RNA sample and 1 pg of Josiah viral RNA sample contain 20 and 38 copies of intact viral RNA, respectively (data not shown), indicating that each RNA sample contains different contents of intact viral RNA. These results are very similar to those of the experiments using the *in vitro* transcribed RNA.

The RT-LAMP products amplified from LASV RNA showed a visibly distinct ladder-like pattern on 3% agarose gels (Fig. 3). To confirm specific amplification, amplicons were digested with the restriction enzyme *StuI* for northeastern Nigeria or *Tsp509I* for Sierra Leone, and separated by electrophoresis on 3% agarose gels. Two *StuI*-digested DNA fragments of 73 and 138 bp, and two *Tsp509I*-digested DNA fragments of 112 and 119 bp, corresponding to the sizes predicted from the expected DNA structures, were observed (Fig. 3). Thus, it was confirmed that both the RT-LAMP assays for Sierra Leone isolates and for northeastern Nigeria isolates amplified only the corresponding LASV strain and showed no cross-reactivity with other hemorrhagic fever viruses and cellular RNA.

DISCUSSION

In this study, we developed the RT-LAMP assay as a genetic detection method for LASV.

Most diagnostic LASV PCR assays that have been developed to date target the S RNA segment encoding GPC and NP (10–14). Both ends of the S segment and the intergenic region are highly conserved among arenaviruses. However, these regions are too short to design LAMP primers, whereas the NP gene sequences are long enough for LAMP primer design and have been determined in many strains, including rodent and human isolates. The sequence data of the L and Z genes encoded in the L RNA segment are much fewer than that of the NP gene. In addition, the L and Z genes are generally less conserved than the NP gene (23). Therefore, we selected the NP gene as the LAMP target. NP genes are relatively highly conserved among LASV strains. Nevertheless, it is very difficult to design a primer set for all LASV strains isolated to date, because the NP gene sequences are diverse among different strains. Phylogenetic analyses of partial NP gene sequences showed that LASV consists of four lineages—three in Nigeria isolates (lineages I–III) and one in the Guinea, Liberia, and Sierra Leone isolates (lineage IV) (24). LASV genetic distances correlate with

geographic distance rather than time. In this study, we designed primer sets for isolates from Sierra Leone (lineage IV) and from northeastern Nigeria (lineage I). The primer regions for detection of Sierra Leone or northeastern Nigeria isolates are highly conserved among LASV strains isolated from human or rodents in Sierra Leone from 1975 to 1996, or in northeastern Nigeria, respectively.

The RT-LAMP assay using each primer set could specifically detect LASV strains belonging to each group and showed no cross-reactivity with other severe hemorrhagic fever viruses (Table 2). In addition, the RT-LAMP products showed a characteristic ladder-like pattern upon gel electrophoresis and were further confirmed to be specific amplification products by restriction enzyme digestion (Fig. 3). These results indicate that the assay possesses reliable specificity. Although we examined only one virus strain from each Sierra Leone group and northeastern Nigeria group in this study, the target sequences are highly conserved among the strains of each group, and very diverse between Lassa virus groups (Fig. 1). Therefore, the assay could detect only Lassa virus strains of the particular lineages without the potential for cross-reactivity for strains belonging to other lineages.

We also determined the detection limits of RT-LAMP by 10-fold serial dilutions of *in vitro* transcripts and RNA from viruses propagated in cell culture and compared with real-time RT-PCR. Both RT-LAMP assays for northeastern Nigeria and Sierra Leone lineages could detect 100 copies of *in vitro* transcribed RNA per reaction. For viral RNAs from viruses propagated in cell culture, detection limits of the assays were 1 ng (20 copies of intact viral RNA) for the Pinneo strain of the northeastern Nigeria lineage and 10 pg (38 copies of intact viral RNA) for the Josiah strain of the Sierra Leone lineage. The sensitivities of both RT-LAMP assays for northeastern Nigeria and Sierra Leone lineages were almost 10-fold lower than those of real-time RT-PCR. Nevertheless, the sensitivity of the RT-LAMP would be sufficient for clinical diagnosis and epidemiological surveillance, because clinical specimens appear to contain high concentrations of LASV. Drosten *et al.* reported that an initial concentration of 10^6 viral RNA molecules/ml serum was found on day 6 after onset (11).

The LAMP method has many advantages by virtue of its high specificity and simple methodology. In addition, the assay results can be monitored by various methods, such as viewing increased turbidity levels with the naked eye, spectrophotometrically monitoring for increased UV fluorescence or turbidity, or by agarose gel electrophoresis, or determining turbidity values. Furthermore, the LAMP method is more rapid and cost-effective than PCR and real-time PCR, as the only equipment required is a water bath or a heat block. Real-time RT-PCR requires at least 1 h, while RT-LAMP yields results within 25 min. Therefore, RT-LAMP is a more rapid method for the detection of LASV compared to conventional methods, such as RT-PCR, real-time RT-PCR, and ELISA.

Thus, the RT-LAMP assay developed in this study might be suitable for clinical diagnosis of LASV infection. This might be especially advantageous for use in developing countries where expensive and technically demanding equipment and techniques are not readily available, and could also be employed in response to the deliberate release of virus in the event of a bioterrorism attack, as well as for routine epidemiological surveillance to detect or confirm disease outbreaks and limit their spread.

Acknowledgments

This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan, the Japan Society for the Promotion of Science, and the Japan Science and Technology Agency.

List of Abbreviations

BIP	backward inner primer
FIP	forward inner primer
GPC	glycoprotein precursor protein
HPLC	high-performance liquid chromatography
IFA	immunofluorescence assay
L	large RNA fragment of arenavirus
LAMP	loop-mediated isothermal amplification
LASV	Lassa virus
NP	nucleoprotein
OD	optical density
RT-LAMP	reverse transcription-loop-mediated isothermal amplification
S	small RNA fragment of arenavirus
Z	zinc-binding

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Fig. 1. Sequence alignment of the target region of RT-LAMP for LASV NP gene
The target sequences of representative strains of each lineage are aligned. Nucleotides identical to those of Josiah strain are represented by dots. Asterisks show the nucleotides conserved among all strains. Primer regions are boxed. Accession numbers of the aligned strains (top to bottom) are J04324, AY179173, AF182246, AF182252, AF182261, AF182263, X52400, AF181854, AF181853, AY628208, and AY628207.

Fig. 2. Kinetics of RT-LAMP reaction for *in vitro* transcribed LASV RNA

Serial 10-fold dilutions of *in vitro* transcripts RNA from (a) Pinneo or (b) Josiah strains were subjected to RT-LAMP using (a) northeastern Nigeria or (b) Sierra Leone primer sets, respectively, and monitored by measurement of turbidity. A measurement of >0.1 was determined as a cutoff for a positive result. NC, negative control.

Fig. 3. Analysis of RT-LAMP products by 3% agarose gel electrophoresis

Lane M, 100-bp DNA ladder; Lane 1, RT-LAMP products with viral RNA of Pinneo strain using northeastern Nigeria primers; Lane 2, RT-LAMP products from Lane 1 after digestion with *Stu*I. The product sizes were predicted to be 73 and 138 bp. Lane 3, RT-LAMP products with viral RNA of Josiah strain using Sierra Leone primers; Lane 4, RT-LAMP products from Lane 3 after digestion with *Tsp*509I. The product sizes were predicted to be 112 and 119 bp.

Table 1

Primer sets for detection of Lassa virus

Lineage	Primer name	Genome position ^a	Sequence (5'–3')
Northeastern Nigeria	F3	1211–1231	CCTATTCTCAACTGATGACAC
	B3	1426–1405	GATCTGTAACATCTATCCCATG
	FIP (F1c+TTTT+F2)	1299–1280, 1239–1258	TTCAGGCCTGCCCTCAATATTTTGTATGTTACAGTTGGACC
	BIP (B1+TTTT+B2c)	1303–1324, 1396–1377	CCAGTGGAGATTGCTTTGTTCTTTTAGCATCCTGTTTGAATTGC
	Loop F	1279–1259	CTATCCATGTTTGTACTTG
	Loop B	1355–1374	TTAGAGAACCTACTGACCTT
Sierra Leone	F3	1200–1219	ATTCTCAGCTGATGACCCTC
	B3	1411–1390	GTCTGTGACATCAATCCCATGT
	FIP (F1c+TTTT+F2)	1296–1273, 1220–1238	TCCACTGGATCTTCAGGTCTTCCTTTTAAGGATGCAATGCTGCAAC
	BIP (B1+TTTT+B2c)	1301–1324, 1380–1362	GCCCTCTATCAACCAAGTTCAGGCTTTGCATCCTGCTTGAAGTCT
	Loop F	1270–1250	AATGTCCATCCAGGTCTTAGC
	Loop B	1325–1346	TGCTACATACACTTCTTCCGTG

^aPosition numbers are based on the sequences of Pinneo strain (GenBank accession No. AY628207) for Northeastern Nigeria lineage and Josiah strain (GenBank accession No. J04324) for Sierra Leone lineage.

Cross-reactivity and detection limit of RT-LAMP using virus genome RNA extracted from viruses propagated in cell culture

Table 2

Genus	Species	Strain	Quantity	Real-time RT-PCR	RT-LAMP Tt (min) ^a	
					Northeastern Nigeria	Sierra Leone
Lassa virus		Pinneo	10 ng	+	18.2	-
			1 ng	+	23.9	-
			100 pg	+	-	ND
		Josiah	10 pg	-	-	ND
			10 ng	+	-	11.6
			1 ng	+	-	13.0
Ebola virus	<i>Zaire ebolavirus</i>	Mayinga	100 pg	+	ND	14.7
			10 pg	+	ND	18.2
			1 pg	+	ND	-
		Zaire95	100 fg	-	-	-
			600 pg	-	-	-
			600 pg	-	-	-
Marburg virus	<i>Reston ebolavirus</i> <i>Sudan ebolavirus</i> <i>Cote d'Ivoire ebolavirus</i>	Pennsylvania	600 pg	-	-	-
			600 pg	-	-	-
			600 pg	-	-	-
		Musoke	600 pg	-	-	-
			600 pg	-	-	-
			600 pg	-	-	-
		Ravn	600 pg	-	-	-
			600 pg	-	-	-
		Angola	600 pg	-	-	-
			600 pg	-	-	-

^aTt, average threshold time; ND, not done.