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Development and Evaluation of a One-Step Quantitative RT-PCR Assay for Detection of Lassa Virus

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ABSTRACT

Lassa fever is a severe viral hemorrhagic illness caused by Lassa virus. Based on estimates, the number of LASV infections ranges from 300,000 to 500,000 cases in endemic areas with a fatality rate of 1%. Development of fast and sensitive tools for the control and prevention of Lassa virus infection as well as for clinical diagnostics of Lassa fever are crucial.

Here we reported development and evaluation of a one-step quantitative RT-qPCR assay for the Lassa virus detection – LASV-Fl. This assay is suitable for the detection of lineages I-IV of Lassa virus. The limit of detection of the assay ranged from 10^3 copies/ml to 10^5 copies/ml and has 96.4% diagnostic sensitivity, whereas analytical and diagnostic specificities both were 100%. Serum, whole blood and tissue are suitable for use with the assay. The assay contains all the necessary components to perform the analysis, including an armored positive control (ARC+) and an armored internal control (IC). The study was done during the mission of specialized anti-epidemic team of the Russian Federation (SAET) in the Republic of Guinea in 2015-2018.

Based on sequencing data, LASV-specific assay was developed using synthetic MS2-phage-based armored RNA particles, RNA from Lassa virus strain Josiah, and further, evaluated in field conditions using samples from patients and Mastomys natalensis rodents.

1. Introduction

Lassa virus (LASV) is a single-stranded ambisense RNA virus belonging to the Arenavirus genus in the *Arenaviridae* family (Radoshitzky et al., 2015). LASV causes an acute hemorrhagic illness in humans—Lassa fever disease (LFD), which is characterized by fever, muscle aches, sore throat, nausea, vomiting, chest, and abdominal pain (Ogbu et al., 2007). However, the mild manifestation of LFD and asymptomatic infection with LASV is common (Frame et al., 1970, Ajayi et al., 2013). The natural host of LASV is the multimammate mouse (*Mastomys* natalensis, person-to-person transmission was reported, particularly in nosocomial settings). The role of *M. erythroleucus* as a natural host of LASV is under discussion. These rodents are ubiquitous and highly commensal in Africa (Keenlyside et al., 1983, Monath et al., 1974). Despite the wide distribution of *M. natalensis,* LASV is endemic only in West African countries, including Nigeria, Sierra Leone, Liberia, Guinea, Benin, Mali, and Côte d'Ivoire (Frame et al., 1970, Safronetz et al., 2013). Based on estimates, the number of LASV infections ranges from 300,000 to 500,000 cases in endemic areas (Siddle et al., 2018) with a fatality rate of 1%. (Ogbu et al., 2007). Humans primarily become infected with LASV through inhalation or ingestion of infected rodent excreta. In addition, the infection can occur due to handling and preparation of infected *M. natalensis* for grilling, as they are considered delicious in the West African region (Ter Meulen et al., 1996). Person-to-person transmission was also reported, especially in nosocomial settings (Monath et al., 1973).

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Given the high incidence and fatality, LFD is a severe burden for regional health care. Moreover, LFD is one of the most prominent exotic viral hemorrhagic fever disease in Africa (Macher and Wolfe, 2006). Therefore, fast and sensitive tools to control and prevent LASV infection, as well as for clinical diagnosis of LFD, are crucial.

Traditionally, the diagnosis of LFD is done by real-time polymerase chain reaction (RT-PCR), lateral flow immunoassays, and enzyme-linked immunosorbent assay (ELISA). However, RT-PCR is most suitable for the detection of LASV, particularly in the early stage of illness, because of its high sensitivity and ease of implementation in the study (Asogun et al., 2012). Therefore, the development of RT-PCR assays, particularly based on quantitative RT-PCR (RT-qPCR) technique, is highly crucial for improving LFD diagnosis and for LASV surveillance and epidemiological control.

This study aimed to develop and evaluate a one-step RT-qPCR assay for the detection of LASV—LASV-Fl, targeting the L gene. This assay may be suitable for the detection of various lineages of LASV.

2. Materials and Methods

2.1. Samples Used in the Study

The LASV strain Josiah provided by the Virology and Biotechnology Centre "Vector," was propagated using Vero E6 cell culture, which is commonly available in Russian cell culture collections; the concentration of viral particles was evaluated and inactivation was performed at the Virology and Biotechnology Centre "Vector," Novosibirsk, Russia under BSL4 conditions. Armored RNA particles (ARPs) were synthesized by the biotechnology branch at the Central Research Institute for Epidemiology, Moscow, Russia.

Both the LASV strain Josiah and ARPs were used to assess the limit of detection (LOD) of the assay.

Whole blood samples from *M. natalensis* (n = 20 from individuals and n = 8 pools of three animals) were provided by the Virology Laboratory of Hemorrhagic Fevers Research Project of Gamal Abdel Nasser University of Conakry, Guinea. Viral RNA was extracted from 140 µL of the whole blood of *M. natalensis* using the QIAmp viral RNA kit (Qiagen, Germany, in accordance with the manufacturer's instructions. Another part of biological samples (the lung and spleen tissue) from *M. natalensis* (n = 27) and serum from humans (n = 37) were collected by the staff of the Centre de Recherché en Epidemiologie, Microbiologie et Soins Medicaux (CREMS) in Kindia, Guinea. The lung and spleen tissue samples were homogenized in Hanks' buffered salt solution using a Tissue Lyser LT (Qiagen, Germany). Suspensions were centrifuged at 2000 rpm for 10 min to precipitate debris, and 100 µL of the supernatant was then used for viral RNA extraction.

The extraction of nucleic acids from the samples collected by CREMS (including samples from *M. natalensis* and serum from humans) was performed using the RIBO-prep extraction kit (AmpliSens, Russia) as per the manufacturer's instructions.

Viral RNAs were examined for Lassa immediately after extraction by the staff of the Virology Laboratory of Hemorrhagic Fevers Research Project of Gamal Abdel Nasser University of Conakry, Guinea and were then used to assess diagnostic sensitivity and specificity.

One-step RT-PCR assay targeting GP, with primers OWS-1-fwd (GCGCACCGGGGATCCTAGGC) and OWS-1000-rev (AGCATGTCACAA-AAYTCYTCATCATG) was used for LASV detection (Ehichioya et al., 2011).

2.2. Identification of Conserved Sites

All sequences of LASV L gene available in GenBank (NCBI) were aligned to identify conserved sites. The alignment was performed using the BioEdit 7.2.5 software package (Ibis Biosciences, USA). A 119-nt fragment of the L gene (nt positions 106–225 in the reference sequence of LASV, strain Josiah, GenBank accession number JN650518) was selected as a target for amplification using PLOTCON (http://emboss.bioinformatics.nl/cgi-bin/emboss/plotcon).

2.3. Design of Primers and Probe for RT-qPCR

The primers and probes were designed in accordance with the current guidelines, regarding TaqMan primers and probes for RT-PCR techniques (Tyagi and Kramer, 1996, Van Pelt-Verkuil et al., 2008). The melting temperature for the primers was calculated using the oligonucleotide properties calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html) (Kibbe, 2007). In addition, the oligonucleotide properties calculator and MFOLD were used to assess the thermodynamic characteristics of the probes and the probability of the appearance of secondary structures (http://unafold.rna.albany.edu/?q=mfold/download-mfold). Specifically, the LASV-specific probes were covalently attached to the fluorescent reporter dye rhodamine 6 G and black hole quencher 1 at the 5′ and 3′ ends, respectively. The primers and probes were synthesized by the branch associated with bioorganic synthesis at the Central Research Institute for Epidemiology, Moscow, Russia.

2.4. Reaction Mixture and Amplification Mode

The total volume of the RT-qPCR reaction mix was 25 µL, including the following: 10 µL of the RNA sample, 0.2 µM of each primer and probe (LVL-forw1, LVL-forw2, LVL-rev1, LVL-rev2, LVL-prb1a, LVL-prb1b, and LVL-prb1c) and 0.12 µM of each IC-detection primer and the probe (IC-forwA, IC-revA, and IC-prbA), 2.5 µL of dNTPs (1.76 mM; AmpliSens, Russia), 5 µL of RT-PCR-mix 2 FEP/FRT (AmpliSens, Russia), 0.25 µL of MMLV reverse transcriptase (AmpliSens, Russia), 0.25 µL of RTG-mix 2 (AmpliSens, Russia), and 0.5 µL of TaqF polymerase (AmpliSens, Russia). The thermal cycling parameters were as follows: 50 °C for 15 min, 95 °C for 15 min, and then 42 cycles of 95 °C for 10 s and 55 °C for 20 s. Fluorescence was observed at 55 °C in a Rotor-Gene 6000 (Qiagen, Germany) in the yellow and green channels for specific signals and IC signals, respectively. The threshold value of fluorescence was chosen as the middle of the linear increase in the positive-control fluorescence expressed in the logarithmic units. Amplification results were considered positive if the level of fluorescence crossed the threshold.

To control all stages of the RT-qPCR reaction, an external positive control for PCR (C+) and an armored recombinant positive control for reverse transcription (ARC+) were developed. The commercially available IC (IC-Fl, AmpliSens, Russia) was used to monitor RNA extraction; for this purpose, IC-Fl-specific primers and probe were added to the reaction mixture. In addition, a negative control for extraction (EC−) and PCR (C−) were used to exclude false-positive results due to possible or inadvertent cross-contamination.

2.5. Generation of Positive-Control Samples

The cDNA region (126 bp) equivalent to L gene of LASV (strain Josiah) that included the primer and probe target sequences was generated using step-out amplification, as previously described (Dedkov et al., 2016). The final PCR product was purified using a MinElute Gel Extraction kit (Qiagen, Germany), ligated into the pGEM-T plasmid vector (Promega, USA), and transformed into Escherichia *coli* (XL1-Blue strain) (Maniatis et al., 1989). Recombinant plasmids from individual clones were purified using a Plasmid Miniprep kit (Axygen, USA), and the orientation and absence of mutations in the cloned PCR fragment were evaluated by Sanger sequencing using an ABI-Prism 3500 XL (Applied Biosystems, USA). The diluted plasmid solutions of known concentrations were used as C+.

Furthermore, the same cDNA region was used to prepare ARC + based on a previously described procedure for MS2-phage-based ARPs (Cheng et al., 2006, Pasloske et al., 1998) with certain minor

Table 1
Features of the primers and the probes used in the Lassa assay.

Species	Primer/probe	Sequence (5'-3')	Probe type	Product size	Gene target
LASV	LVLforw1	AGAGCCAGCCTGATCCCAGA	TaqMan	113 bp	L
	LVL rev1	CACAGATAGTGGTTGTTGCACTC			
	LVLforw2	AGCTCATCTAGTGCCAGATGC			
	LVL rev2	GCTGAGACAGTTGAGACACA	TaqMan	130 bp	
	LVLprb1a	R6 G –AgTTCTgCAAgAgTTgCTggTTTgAgAAC – BHQ1			
	LVLprb1b	R6 G –CCgCAATTCTgCAAgAgCTgTTggTTTgCg–BHQ1			
	LVLprb1c	R6 G –AAgAgCTgCTggTTCgAAAACAAgggCtc– BHQ1	TaqMan	135 bp	
IC-STI87-rec	STI-87f	N/A	TaqMan	117 bp	Artificial
	STI-87r	N/A			target
	STI-87-prb	Cy5-$(X)_{25}$-BHQ2			

The primers and probes were designed in accordance with the current guidelines, regarding TaqMan primers and probes for RT-PCR techniques The LASV-specific probes were covalently attached to the fluorescent reporter dye rhodamine 6 G and black hole quencher 1 at the 5′ and 3′ ends, respectively.

modifications. In brief, the PCR fragment containing the target region and additional flanking nucleotides (see above) was ligated into a linearized in-house plasmid vector containing the MS2 coat protein gene. After verifying by DNA sequencing, the generated recombinant plasmid was transformed into *E. coli* (strain B21) and protein expression was induced with isopropyl-L-thio-D-galactopyranoside. After induction, the cells were collected, lysed using a method combining lysozyme and freeze-thawing, and treated with DNase I (Fermentas, USA) and RNase A (Fermentas, USA). The derivate was then purified using CsCl gradient centrifugation, quantified, and diluted in RNAlater Stabilization solution (Life Technologies, USA). The absence of residual DNA in the treated sample was verified using the developed qPCR assay without the reverse transcription step. The C + and ARC + concentrations were measured with a QX100 system (Bio-Rad) using a PCR Supermix for Probes kit (Bio-Rad), a One-step ddPCR Supermix for Probes kit (Bio-Rad), specific primers, and suitable probes as per the manufacturer's instructions (Table 1).

2.6. Internal Control Samples

To assess the efficiency of RNA extraction, an IC-Fl (AmpliSens, Russia) exogenous IC was added to the reagent mixture. The IC-Fl is specifically an artificial RNA sequence (150–170 nt, GC content 50%) mixture, surrounded by an MS2-derived protective protein coat.

2.7. Limit of detection

The LOD of the LASV-Fl assay was determined using LASV strain Josiah, which was provided by the Center of Virology and Biotechnology "Vector" as a part of their collection.

In addition, LOD was assessed using a series of 10-fold dilutions of ARPs. For this purpose, eight LASV sequences of a maximal number of mismatches in the targeting region of L gene were selected (including a sequence of the strain Josiah, which was also used for the generation of the positive controls) and generated for the production of ARPs as described above (Table 2). Moreover, the concentrations of ARPs were measured with a QX100 system (Bio-Rad) using a One-step ddPCR Supermix for Probes kit (Bio-Rad), specific primers, and suitable probes as per the manufacturer's instructions.

LOD was assessed using a series of 10-fold dilutions of ARPs and LASV strain Josiah.

In brief, ARPs and LASV particles of known concentrations were diluted 10-fold using RNase-free elution buffer (AmpliSens, Russia), added to intact human serum to make the final volume of 100 µL, extracted using the RIBO-prep extraction kit (AmpliSens, Russia, in accordance with the manufacturer's instructions), and then tested using the LASV-Fl assay to establish the standard curves and limit of detection (LOD). The LOD was set as the minimal dilution detected in three replicates (Cherpillod et al., 2016).

2.8. Assay Cross-Reactivity

Potential cross-reactivity was assessed using the high-titer solutions (more than 10^6 copies/ml) of viral RNA and DNA from 27 viral species belonging to 13 viral families, which are part of the collection of Central Research Institute for Epidemiology. The summary of RNA or DNA used in the study is shown in Table 3 .

2.9. Diagnostic Sensitivity and Specificity

The assay sensitivity and specificity were determined using serum samples from patients with the clinical symptoms of fever who were found Lassa positive (n = 18) and Lassa negative (n = 19) and using whole blood and tissue samples from *M. natalensis* (n = 37 positive and n = 18 negative).

The 95% confidence interval for a proportion was calculated according to R. Newcombe derived from a procedure outlined by E. Wilson (Newcombe, 1998, Wilson 1927).

Table 2
ARPs used for evaluation of the assay LOD.

ID of ARP	Strain	Access no	Origin	Isolation date	Country	Lineage	LOD, copies/ml
LVL1	ISTH-2358-NIG-2012	KM821995	*H. sapiens*	2012	Nigeria	n/A	10^3
LVL2	LASV046-NIG-2009	KM822009	*H. sapiens*	2009	Nigeria	n/A	10^3
LVL3	LASV274-NIG-2010	KM822056	*H. sapiens*	2010	Nigeria	n/A	10^3
LVL4	LASV975-NIG-2009	KM822075	*H. sapiens*	2009	Nigeria	n/A	10^4
LVL5	G3151-SLE-2013	KM821893	*H. sapiens*	2013	Sierra-Leone	IV	$5*10^4$
LVL6	Josiah	JN650518	*H. sapiens*	1976	Sierra-Leone	IV	10^3
LVL7	G3148-SLE-2013	KM821891	*H. sapiens*	2013	Sierra-Leone	IV	10^5
LVL8	LM765-SLE-2012	KM822116	*H. sapiens*	2012	Sierra-Leone	IV	10^4

Eight LASV sequences of a maximal number of mismatches in the targeting region of L gene were selected and generated for the production of ARPs. The LOD assessed using ARP dilutions was between 10^3 copies/ml and 10^5 copies/ml (depending on the ARP).

Table 3
List of viral species used evaluation of the assay analytical specificity.

N	Species	Acronym	Family	Genus	Type of nucleic acid
1	Zaire ebolavirus	EBOV	*Filoviridae*	Ebolavirus	RNA
2	Sudan ebolavirus	SUDV	*Filoviridae*	Ebolavirus	RNA
3	Marburg virus	MARV	*Filoviridae*	Marburgvirus	RNA
4	Tahyna virus	TAHV	*Peribunyaviridae*	Orthobunyavirus	RNA
5	Batai virus	BATV	*Peribunyaviridae*	Orthobunyavirus	RNA
6	Inkoo virus	INKV	*Peribunyaviridae*	Orthobunyavirus	RNA
7	Crimean-Congo hemorrhagic fever virus	CCHFV	*Nairoviridae*	Orthonairovirus	RNA
8	Dhori virus	DHOV	*Orthomyxoviridae*	Thogotovirus	RNA
9	Flu A/H1N3	FLUAV(H1N3)	*Orthomyxoviridae*	Influenzavirus A	RNA
10	Flu A/H3N2	FLUAV(H3N2)	*Orthomyxoviridae*	Influenzavirus A	RNA
11	Flu B	FLUBV	*Orthomyxoviridae*	Influenzavirus B	RNA
12	Yellow fever virus	YFV	*Flaviviridae*	Flavivirus	RNA
13	West Nile virus	WNV	*Flaviviridae*	Flavivirus	RNA
14	Zika virus	ZIKV	*Flaviviridae*	Flavivirus	RNA
15	Tick borne encephalitis virus	TBEV	*Flaviviridae*	Flavivirus	RNA
16	Sindbis virus	SNDBV	*Togaviridae*	Alphavirus	RNA
17	Chikungunya virus	CHIKV	*Togaviridae*	Alphavirus	RNA
18	Rubella virus	RUBV	*Togaviridae*	Rubivirus	RNA
19	Kemerovo virus, strain 21/10	KEMV-21/10	*Reoviridae*	Orbivirus	RNA
20	Tribec virus, strain Tr19	TRBV-Tr19	*Reoviridae*	Orbivirus	RNA
21	Human Rotavirus A	RVA	*Reoviridae*	Rotavirus	RNA
22	Enteric Cytopathic Human Orphan virus 11	ECHO11	*Picornaviridae*	Enterovirus	RNA
23	Human immunodeficiency virus 1	HIV-1	*Retroviridae*	Lentivirus	RNA
24	Rabies virus	RABV	*Rhabdoviridae*	Lyssavirus	RNA
25	Human Cytomegalovirus 5	HCMV-5	*Herpesviridae*	Cytomegalovirus	DNA
26	Human parvovirus B19	B19	*Parvoviridae*	Erythroparvovirus	DNA
27	Middle East respiratory syndrome coronavirus	MERS	*Coronaviridae*	Betacoronavirus	RNA

Potential cross-reactivity was assessed using the high-titer of viral RNA and DNA from 27 viral species belonging to 13 viral families, which are part of the collection of Central Research Institute for Epidemiology.

3. Results

The multiple alignments (Suppl. Fig. 1) of the sequences of LASV available in the GenBank at the beginning of the study allowed the identification of highly conserved regions required for the designing of the LASV-specific primers and respective probes (Table 1). On the basis of the sequencing data, oligonucleotide primers and fluorescent probes were designed and synthesized, and the LASV-specific assay was developed. The developed assay included all components required for RT-qPCR. The advantage of this assay is that it allows the verification of all steps of the analysis, including extraction, reverse transcription and PCR. In addition, using EC − and C−, the risk of false-positive results because of cross-contamination was minimized. The LOD assessed using ARP dilutions was between 10^3 copies/ml and 10^5 copies/ml (depending on the ARP), and the LOD measured using LASV strain Josiah was 10 PFU/ml (Table 4). Standard detection was linear ranging from 10^6 copies/ml (C_t = 25.5–28.1) to 5×10^2–10^2 copies/ml (C_t = 37.9–38.4) of the LASV ARPs (R^2 = 0.97–0.99; Fig. 1). The potential for cross-reactivity was assessed using high-titer RNA or DNA from 27 viral species. None of the 27 different viral species showed a positive reaction with the LASV RT-qPCR. Consequently, the evaluated analytical specificity was 100%.

In addition, a total of 92 biological samples (positive n = 55 and negative n = 37) examined for Lassa previously by the Virology Laboratory of Hemorrhagic Fevers Research Project of Gamal Abdel Nasser University of Conakry, Guinea were tested using the LASV RT-PCR assay, with 53 samples testing positive and 39 negative (Tables 5 and 6). Discordance in the two samples (negative by the LASV-Fl assay and positive by VLHV) was observed.

The Ct − values of the positive samples ranged from 21.9–39.2 cycles. Thus, the diagnostic sensitivity and specificity of LASV-Fl RT-qPCR assay were 96.4% (53/55) (95% CI, 86.4–99.0%) and 100% (37/37), respectively.

Table 4
LOD assessment performed using LASV strain Josiah of known concentrations.

Concentration, PFU/ml	Replicates		
	Ct	Ct	Ct
10^4	28,2	29.0	28.6
10^3	32.0	31.9	31.7
10^2	35.8	36.1	35.9
10^1	39.5	39.8	39.1
5	40.4	N/D	N/D

LOD was assessed using a series of 10-fold dilutions of LASV strain Josiah, which was provided by the Center of Virology and Biotechnology “Vector” as a part of their collection.

4. Discussion

LASV antigen can be detected by ELISA using LASV-specific antibodies (Bausch et al., 2000, Niklasson et al., 1984, Jahrling et al., 1985). However, this method has a relatively low sensitivity (ranging from 10^2–10^5 PFU/ml) (Jahrling et al., 1985). This peculiarity limits its usage, particularly at an early stage of LFD because of the low viral load in the biological fluids of patients. In addition, serological methods based on specific antibody detection in the case of LFD cannot be used during the initial days of the onset of the disease because a considerable concentration of LASV-specific IgM appears during 10–20 days (Salvato et al., 2018). However, a lack of an antibody response has been reported in some fatal cases of Lassa fever (Wulff and Johnson, 1979). Moreover, differing antibody responses depending on the virus strain used (Emmerich et al., 2008). Therefore, RT-qPCR technique is most suitable for the diagnosis of LASV (Salvato et al., 2018), particularly during the initial days after LFD onset and is the preferred method for the rapid and early diagnosis of Lassa fever because of its high sensitivity and easy implementation (Gunther and Lenz, 2004). In this paradigm, a number of RT-PCR assays for LASV detection were developed and evaluated. Most of them targeted the S-segment encoding the glycoprotein precursor and nucleoprotein as limited information is available regarding LASV sequences (Drosten et al., 2002, Trappier

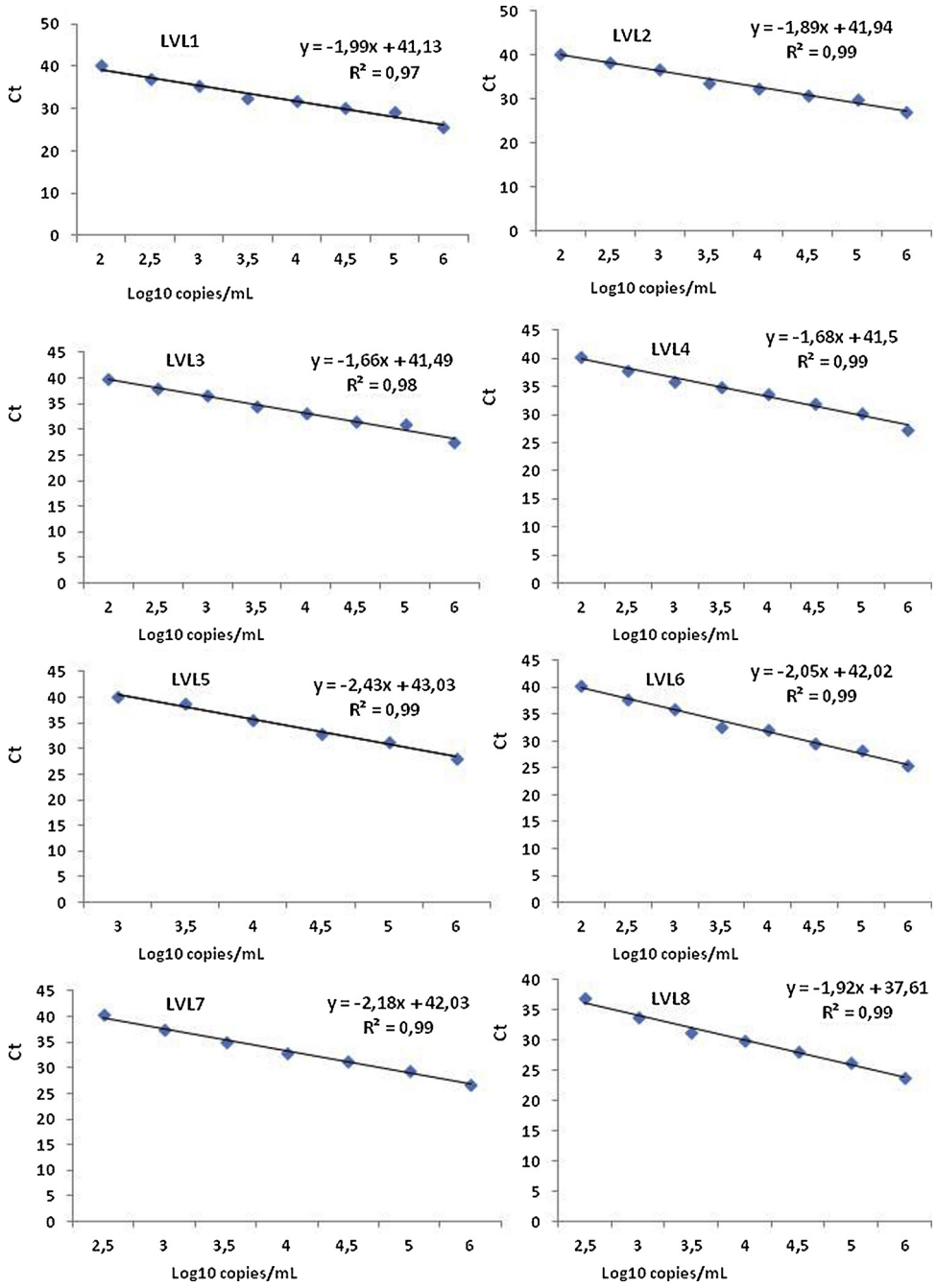

Fig. 1. The assay LOD and assay standard curves assessed using ARPs.
Standard detection was linear ranging from 10^6 copies/ml to 5×10^2–10^2 copies/ml of the LASV ARPs (depending on the ARP). The LOD was set as the minimal dilution detected in three replicates.

et al., 1993, Demby et al., 1994, Trombley et al., 2010). However, some LASV strains were not detected using the developed RT-PCRs (Trappier et al., 1993). From the new information regarding the sequences of the S-segment, LASV is a virus of high genetic variability and designing reliable LASV-specific primers and probes is problematic (Bowen et al., 2000). The appearance of a sufficient number of the L-gene sequences facilitated in designing an assay that could be more reliable for LASV detection because RNA polymerases that are encoded by the L-gene share conserved amino acid motifs even between different virus families (Poch et al., 1990, Poch et al., 1989). During the past 10 years, several LASV genomes have been completely sequenced, and the L-gene sequences of LASV also demonstrate high genetic variability even within one genetic lineage. Therefore, the genetic diversity of LASV is a natural peculiarity, which most likely will be a limitation in the usage of RT-qPCR in the diagnosis of LASV.

In this study, we developed an assay that is suitable, in equal measure, to detect the broad range of Lassa strains. However, the LOD of the assay considerably differs and depends on the LASV strain (Table 2). Two discordant samples of *M. natalensis* (negative by the LASV-Fl assay and positive by VLHV) found in the assessment of diagnostic sensitivity supporting this assumption.

However, we successfully used it in our routine practice to detect LASV in Guinea using various types of samples, including whole blood and tissue of *M. natalensis,* as well as the serum samples from humans collected at 3–7 days after the onset of LFD. Thus, our assay can be used for LFD diagnosis and surveillance in the case of LASV endemic in

Table 5
List of samples from *M. natalensis* used for assessing diagnostic sensitivity of the assay.

N	ID	Region	Sample type	Assessed by	Ct	Confirm
1	21	Faranah	Whole blood	VLHV	39.1	positive
2	38	Faranah	Whole blood	VLHV	35.6	positive
3	39	Faranah	Whole blood	VLHV	33.4	positive
4	40	Faranah	Whole blood	VLHV	38.9	positive
5	44	Faranah	Whole blood	VLHV	36.4	positive
6	50	Faranah	Whole blood	VLHV	29.7	positive
7	81	Faranah	Whole blood	VLHV	33.3	positive
8	110	Faranah	Whole blood	VLHV	32.9	positive
9	Pool2	Faranah	Whole blood	VLHV	35.9	positive
10	103	Faranah	Whole blood	VLHV	negative	positive
11	104	Faranah	Whole blood	VLHV	negative	negative
12	105	Faranah	Whole blood	VLHV	negative	negative
13	161	Faranah	Whole blood	VLHV	32.4	positive
14	163	Faranah	Whole blood	VLHV	negative	negative
15	173	Faranah	Whole blood	VLHV	negative	negative
16	174	Faranah	Whole blood	VLHV	38.0	positive
17	175	Faranah	Whole blood	VLHV	negative	negative
18	104dl	Faranah	Whole blood	VLHV	negative	negative
19	195	Faranah	Whole blood	VLHV	negative	negative
20	106	Faranah	Whole blood	VLHV	34.8	positive
21	108	Faranah	Whole blood	VLHV	negative	negative
22	109	Faranah	Whole blood	VLHV	negative	positive
23	111	Faranah	Whole blood	VLHV	negative	negative
24	107	Faranah	Whole blood	VLHV	37.2	positive
25	99	Faranah	Whole blood	VLHV	25.4	positive
26	63	Faranah	Whole blood	VLHV	28.0	positive
27	65	Faranah	Whole blood	VLHV	37.2	positive
28	94	Faranah	Whole blood	VLHV	33.0	positive
29	11276	Mamou	Tissue	CREMS	22.6	positive
30	11102	Mamou	Tissue	CREMS	25.2	positive
31	11309	Mamou	Tissue	CREMS	24.6	positive
32	11092	Mamou	Tissue	CREMS	21.8	positive
33	11095	Mamou	Tissue	CREMS	24.7	positive
34	11087	Faranah	Tissue	CREMS	24.2	positive
35	11269	Faranah	Tissue	CREMS	27.4	positive
36	11098	Faranah	Tissue	CREMS	22.7	positive
37	12356	Macenta	Tissue	CREMS	24.9	positive
38	13251	Macenta	Tissue	CREMS	negative	negative
39	13252	Macenta	Tissue	CREMS	25.0	positive
40	13253	Macenta	Tissue	CREMS	26.3	positive
41	13254	Macenta	Tissue	CREMS	negative	negative
42	13255	Macenta	Tissue	CREMS	23.5	positive
43	13256	Macenta	Tissue	CREMS	21.9	positive
44	13257	Macenta	Tissue	CREMS	negative	negative
45	13258	Macenta	Tissue	CREMS	25.1	positive
46	13259	Macenta	Tissue	CREMS	negative	negative
47	13260	Macenta	Tissue	CREMS	25.7	positive
48	13261	Nzerekore	Tissue	CREMS	25.2	positive
49	13262	Nzerekore	Tissue	CREMS	negative	negative
50	13263	Nzerekore	Tissue	CREMS	negative	negative
51	13283	Nzerekore	Tissue	CREMS	negative	negative
52	13284	Nzerekore	Tissue	CREMS	negative	negative
53	13285	Nzerekore	Tissue	CREMS	negative	negative
54	13280	Nzerekore	Tissue	CREMS	26.5	positive
55	13286	Nzerekore	Tissue	CREMS	24.7	positive

The samples from *M. natalensis* were provided by the Virology Laboratory of Hemorrhagic Fevers Research Project of Gamal Abdel Nasser University of Conakry Guinea and by the Centre de Recherché en Epidemiologie, Microbiologie et Soins Medicaux (CREMS) in Kindia, Guinea. All samples were previously tested for LASV using one-step RT-PCR assay targeting GP (Ehichioya et al., 2011).

Guinea. The developed assay may be used for LASV strains circulated in other endemic territories. However, this suggestion needs to be confirmed in further studies.

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Table 6
List of samples from humans used for assessing diagnostic sensitivity of the assay.

N	ID	Sex	Age	Region	Sample type	Day after onset	Ct	Confirm
1	2136	F	40	Kankan	Serum	4	28.3	positive
2	2137	M	60	Kankan	Serum	3	35.4	positive
3	2140	M	60	Kankan	Serum	5	31.8	positive
4	2143	F	42	Kankan	Serum	5	25.6	positive
5	2144	F	61	Kankan	Serum	4	33.0	positive
6	3334	M	56	Nzerekore	Serum	6	neg	neg
7	3335	M	36	Nzerekore	Serum	7	neg	neg
8	3336	M	36	Nzerekore	Serum	10	39.2	positive
9	3337	F	61	Nzerekore	Serum	4	34.2	positive
10	3338	M	40	Nzerekore	Serum	6	neg	neg
11	3339	M	55	Nzerekore	Serum	2	36.9	positive
12	3340	M	28	Nzerekore	Serum	5	neg	neg
13	3341	M	28	Nzerekore	Serum	6	neg	neg
14	3342	F	7	Mamou	Serum	4	28.3	positive
15	3343	M	60	Mamou	Serum	3	30.8	positive
16	3344	M	7	Mamou	Serum	5	27.6	positive
17	3345	F	7	Mamou	Serum	6	24.3	positive
18	3346	M	11	Mamou	Serum	7	neg	neg
19	3347	M	39	Kindia	Serum	8	neg	neg
20	3348	M	32	Kindia	Serum	4	neg	neg
21	3349	M	39	Kindia	Serum	3	neg	neg
22	3350	F	36	Mamou	Serum	7	neg	neg
23	3351	F	25	Kankan	Serum	10	38.9	positive
24	3352	F	n/A	Kankan	Serum	3	neg	neg
25	3358	M	18	Faranah	Serum	5	25.9	positive
26	3367	M	23	Faranah	Serum	4	36.4	positive
27	3369	M	43	Faranah	Serum	3	28.6	positive
28	3372	M	24	Faranah	Serum	5	neg	neg
29	3373	M	15	Faranah	Serum	3	neg	neg
30	3375	M	23	Faranah	Serum	5	27.9	positive
31	3378	F	n/A	Faranah	Serum	6	26.2	positive
32	3391	M	n/A	Faranah	Serum	8	neg	neg
33	3392	M	n/A	Mamou	Serum	6	neg	neg
34	3394	F	26	Mamou	Serum	9	neg	neg
35	3396	F	35	Mamou	Serum	11	neg	neg
36	3398	M	37	Mamou	Serum	4	neg	neg
37	3400	M	41	Mamou	Serum	5	neg	neg

The samples of humam serum were provided by the Centre de Recherché en Epidemiologie, Microbiologie et Soins Medicaux (CREMS) in Kindia, Guinea. All samples were previously tested for LASV using one-step RT-PCR assay targeting GP [Ehichioya et al., 2011].

Ethical considerations

The study has been evaluated and approved by local Ethics Committees of the Pasteur Institute, Saint-Petersburg, Russia and Comité National D'Ethique pour la Recherche en Santé, Guinea

7. Competing interests

The authors declare that they have no competing interests

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Appendix A. Supplementary data

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