



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 Recherche 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 (GCGCACC GGGGATCCTAGGC) 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 | CACAGATAGTGGTTGTGCACTC | | | |
| | LVLforw2 | AGCTCATCTAGTGCCAGATGC | TaqMan | 130 bp | |
| | LVL rev2 | GCTGAGACAGTTGAGACACA | | | |
| | LVLprb1a | R6 G -AgTTCTgCAAgAgTTgCTggTTTgAgAAC - BHQ1 | | | |
| | LVLprb1b | R6 G -CCgCAATTCTgCAAgAgCTgTTggTTTgCg-BHQ1 | | | |
| IC-STI87-rec | LVLprb1c | R6 G -AAGAgCTgCTggTTCgAAAACAAgggCtc- BHQ1 | TaqMan | 135 bp | Artificial target |
| | STI-87f | N/A | TaqMan | 117 bp | |
| | STI-87r | N/A | | | |
| | 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- β -D-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 derivative 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-FI (AmpliSens, Russia) exogenous IC was added to the reagent mixture. The IC-FI 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-FI 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

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 | <i>H. sapiens</i> | 2012 | Nigeria | n/A | 10 ³ |
| LVL2 | LASV046-NIG-2009 | KM822009 | <i>H. sapiens</i> | 2009 | Nigeria | n/A | 10 ³ |
| LVL3 | LASV274-NIG-2010 | KM822056 | <i>H. sapiens</i> | 2010 | Nigeria | n/A | 10 ³ |
| LVL4 | LASV975-NIG-2009 | KM822075 | <i>H. sapiens</i> | 2009 | Nigeria | n/A | 10 ⁴ |
| LVL5 | G3151-SLE-2013 | KM821893 | <i>H. sapiens</i> | 2013 | Sierra-Leone | IV | 5 ² 10 ⁴ |
| LVL6 | Josiah | JN650518 | <i>H. sapiens</i> | 1976 | Sierra-Leone | IV | 10 ³ |
| LVL7 | G3148-SLE-2013 | KM821891 | <i>H. sapiens</i> | 2013 | Sierra-Leone | IV | 10 ⁵ |
| LVL8 | LM765-SLE-2012 | KM822116 | <i>H. sapiens</i> | 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 10³ copies/ml and 10⁵ copies/ml (depending on the ARP).

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 μ L, extracted using the RIBO-prep extraction kit (AmpliSens, Russia, in accordance with the manufacturer's instructions), and then tested using the LASV-FI 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 10⁶ 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 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 | <i>Filoviridae</i> | Ebolavirus | RNA |
| 2 | Sudan ebolavirus | SUDV | <i>Filoviridae</i> | Ebolavirus | RNA |
| 3 | Marburg virus | MARV | <i>Filoviridae</i> | Marburgvirus | RNA |
| 4 | Tahyna virus | TAHV | <i>Peribunyaviridae</i> | Orthobunyavirus | RNA |
| 5 | Batai virus | BATV | <i>Peribunyaviridae</i> | Orthobunyavirus | RNA |
| 6 | Inkoo virus | INKV | <i>Peribunyaviridae</i> | Orthobunyavirus | RNA |
| 7 | Crimean-Congo hemorrhagic fever virus | CCHFV | <i>Nairoviridae</i> | Orthohairovirus | RNA |
| 8 | Dhori virus | DHOV | <i>Orthomyxoviridae</i> | Thogotovirus | RNA |
| 9 | Flu A/H1N3 | FLUAV(H1N3) | <i>Orthomyxoviridae</i> | Influenzavirus A | RNA |
| 10 | Flu A/H3N2 | FLUAV(H3N2) | <i>Orthomyxoviridae</i> | Influenzavirus A | RNA |
| 11 | Flu B | FLUBV | <i>Orthomyxoviridae</i> | Influenzavirus B | RNA |
| 12 | Yellow fever virus | YFV | <i>Flaviviridae</i> | Flavivirus | RNA |
| 13 | West Nile virus | WNV | <i>Flaviviridae</i> | Flavivirus | RNA |
| 14 | Zika virus | ZIKV | <i>Flaviviridae</i> | Flavivirus | RNA |
| 15 | Tick borne encephalitis virus | TBEV | <i>Flaviviridae</i> | Flavivirus | RNA |
| 16 | Sindbis virus | SNDBV | <i>Togaviridae</i> | Alphavirus | RNA |
| 17 | Chikungunya virus | CHIKV | <i>Togaviridae</i> | Alphavirus | RNA |
| 18 | Rubella virus | RUBV | <i>Togaviridae</i> | Rubivirus | RNA |
| 19 | Kemerovo virus, strain 21/10 | KEMV-21/10 | <i>Reoviridae</i> | Orbivirus | RNA |
| 20 | Tribec virus, strain Tr19 | TRBV-Tr19 | <i>Reoviridae</i> | Orbivirus | RNA |
| 21 | Human Rotavirus A | RVA | <i>Reoviridae</i> | Rotavirus | RNA |
| 22 | Enteric Cytopathic Human Orphan virus 11 | ECHO11 | <i>Picornaviridae</i> | Enterovirus | RNA |
| 23 | Human immunodeficiency virus 1 | HIV-1 | <i>Retroviridae</i> | Lentivirus | RNA |
| 24 | Rabies virus | RABV | <i>Rhabdoviridae</i> | Lyssavirus | RNA |
| 25 | Human Cytomegalovirus 5 | HCMV-5 | <i>Herpesviridae</i> | Cytomegalovirus | DNA |
| 26 | Human parvovirus B19 | B19 | <i>Parvoviridae</i> | Erythroparvovirus | DNA |
| 27 | Middle East respiratory syndrome coronavirus | MERS | <i>Coronaviridae</i> | 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 $5 \times 10^2–10^2$ 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

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.

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-FI assay and positive by VLHV) was observed.

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

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

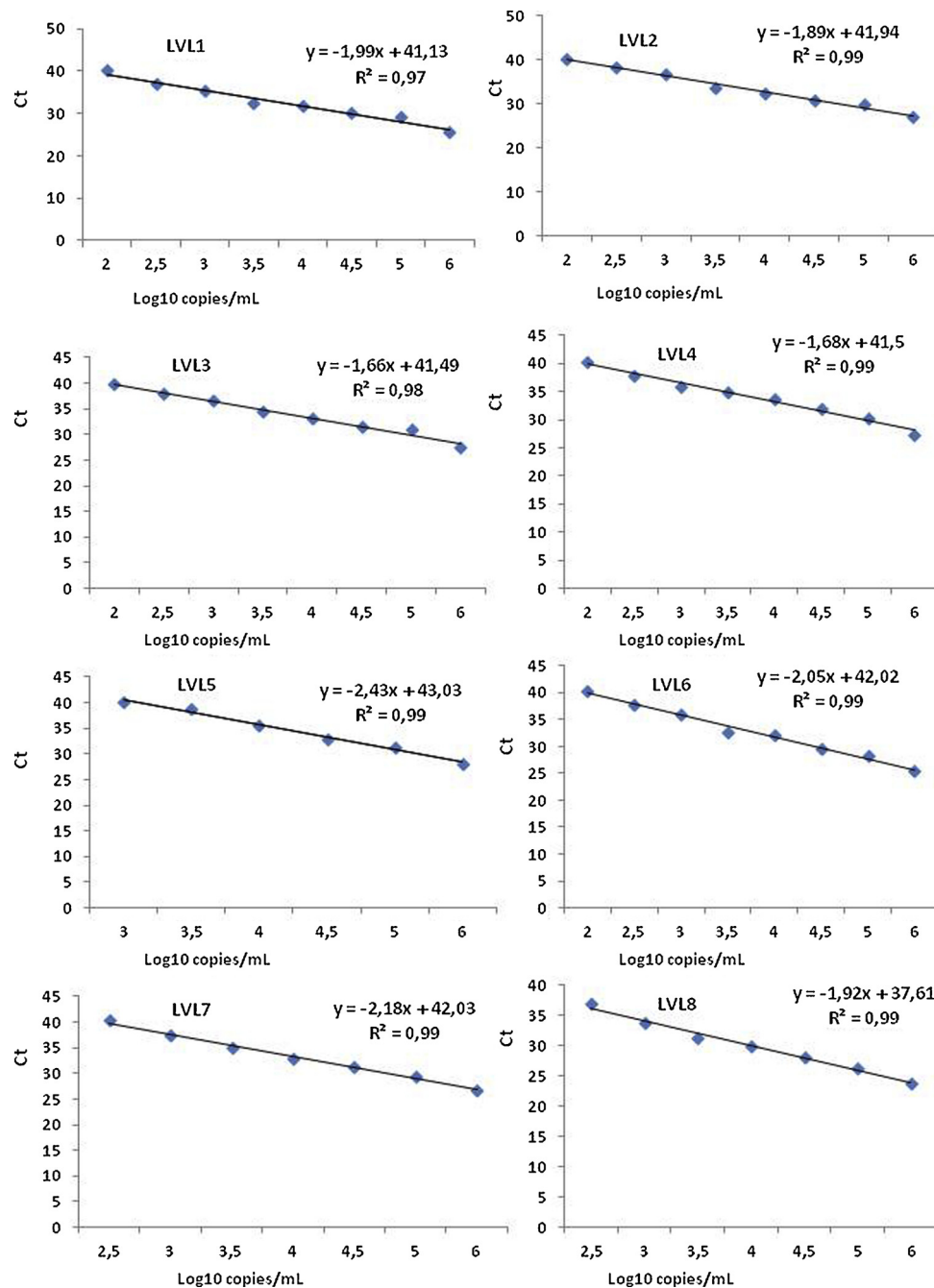


Fig. 1. The assay LOD and assay standard curves assessed using ARPs.

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-FI 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 Recherche 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 human serum were provided by the Centre de Recherche 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

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jviromet.2019>.

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