



One-step single-tube accelerated quantitative nucleoprotein gene-specific reverse transcription loop-mediated isothermal gene amplification (RT-LAMP) assay for rapid, real-time & reliable clinical detection of Ebola virus

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Background & objectives: Due to the absence of specific drugs or vaccines for Ebola virus disease, rapid, sensitive and reliable diagnostic methods are required to control the transmission chain of the disease and for better patient management. Isothermal amplification of nucleic acids has emerged as a promising alternative in which rapid and efficient amplification is achieved at a constant temperature without the thermal cycling required in PCR.

Methods: A one-step single-tube accelerated quantitative reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed by targeting the NP gene of 2014 Zaire Ebola virus (ZEBOV). The RT-LAMP assay was found to be specific for ZEBOV, without having any cross-reactivity with related haemorrhagic fever viral agents.

Results: The comparative evaluation of Ebola virus NP gene-specific RT-LAMP assay with reverse transcription (RT) - PCR and TaqMan real-time RT-PCR demonstrated that RT-LAMP was 10-1000 folds more sensitive than TaqMan real-time RT-PCR and conventional RT-PCR, respectively, with a detection limit of 1 copy number. In the absence of real-world clinical samples, the feasibility of Ebola virus RT-LAMP assay for clinical diagnosis was evaluated with different body fluids including serum, urine, saliva, semen and stool samples from healthy human volunteers spiked with gamma-irradiated ZEBOV 2014 obtained from Robert Koch Institute, Berlin, Germany, through the European Network for Diagnostics of Imported Viral Diseases. The Ebola virus RT-LAMP assay could correctly be picked up the spiked samples up to 1 copy of viral RNA without having any matrix interference. The monitoring of gene amplification can also be visualized with the naked eye by using SYBR Green I fluorescent dye.

Interpretation & conclusions: Thus, due to easy operation without a requirement of sophisticated equipment and skilled personnel, the RT-LAMP assay reported here is a valuable tool as a point-of-care diagnosis for the rapid and real-time detection of Ebola virus in resource-limited healthcare settings of developing countries.

Key words Molecular diagnosis - point-of-care diagnosis - real-time RT-PCR - RT-LAMP - spiked samples - Zaire Ebola virus

The Ebola virus (EBOV) is a member of a unique negative-stranded RNA virus family, the *Filoviridae*, and causes a severe fatal haemorrhagic fever with a high case fatality rate of up to 90 per cent¹. EBOV consists of four species, Zaire, Sudan, Ivory Coast and Reston EBOV, which were first isolated in the Democratic Republic of Congo, Sudan, Ivory Coast and the Philippines respectively². The genome of EBOV virus is 19 Kb, single-stranded, negative-sense RNA molecules encoding a total of seven genes (*NP*, *VP35*, *VP40*, *GP*, *VP30*, *VP24* and *L*) separated by intergenic regions of varying lengths and flanked by untranslated regions (UTRs) at the 3' and 5' ends³. The 2014 Ebola epidemic is the largest in history, affecting multiple countries in West Africa spreading between countries starting in Guinea then spreading across land borders to Sierra Leone and Liberia⁴. The World Health Organization (WHO) declared the epidemic a public health emergency². The Zaire species of Ebola virus, discovered in an outbreak in Zaire (the present Democratic Republic of the Congo) in 1976, is the causative agent of the 2014 epidemic in West Africa with 70 per cent case fatality^{5,6}. The spread of the virus to other countries through travellers led to serious public health emergency of international concern. Once the individual becomes ill or dies, the virus then spreads to others who come into direct contact with their blood, skin or other body fluids. The ICMR-National Institute of Virology (NIV) reported follow up of a man who recovered from Ebola virus disease (EVD) and was monitored for 165 days after he was declared Ebola free⁷. On rare occasions, EVD has resulted from accidental laboratory infections, and there is concern that the virus might be used as an agent of bioterrorism⁸. Moreover, EBOV remains a concern due to their high lethality rates, the lack of effective countermeasures and the threat they pose to national security if weaponized. The US Centers for Disease Control and Prevention (CDC) has classified these as 'Category A Bioterrorism Agents' as these could be easily transmitted, resulting in high mortality, cause major public health impact and panic and require special action for public health preparedness⁹.

Ebola virus disease (EVD), formerly known as Ebola haemorrhagic fever (EHF), is a severe, often fatal illness in humans. The virus is transmitted to people from wild animals and spreads in the human population through human-to-human transmission¹⁰. Humans are usually infected with EBOV through close contact with the contaminated blood, tissues and/or

excretions of viraemic animals, including patients. After an incubation period of 4-10 days, infected individuals abruptly develop flu-like symptoms characterized by fever, chills, malaise and myalgia. Subsequently, patients usually develop the signs and symptoms such as vomiting, diarrhoea, rash, symptoms of impaired kidney and liver function and, in some cases, both internal and external bleeding (*e.g.*, oozing from the gums and blood in the stools)¹¹. Laboratory findings include low white blood cell and platelet counts and elevated liver enzymes.

There is no proven treatment available for EVD. However, a range of potential treatments including blood products, immune therapies and drug therapies are currently being evaluated¹². No licensed vaccines are available yet, but potential vaccines are undergoing human safety testing¹³. Although there are no approved specific therapies for EVD, it is essential to make the diagnosis as early as possible, in order to initiate supportive measures before the development of irreversible shock and to institute infection control procedures.

Traditionally, the laboratory diagnosis of EHF has been accomplished through virus isolation or serologic assays. However, because of the biosafety hazards associated with the handling and testing of EBO virus, these assays can be performed in only a few specialized laboratories worldwide. Several diagnostic tests are available for detection of EVD. These include virus isolation, reverse transcription-polymerase chain reaction (RT-PCR), including real-time quantitative RT-PCR, antigen-capture enzyme-linked immunosorbent assay (ELISA), antigen detection by immunostaining and IgG- and IgM-ELISA using authentic virus antigens¹⁴⁻¹⁷. Diagnosis by the detection of virus antigens is suitable for patients in the early stage of illness, while serological diagnosis by the detection of specific IgM and IgG antibodies is suitable for patients in a relatively late stage of illness. New diagnostic systems for EHF have been developed recently, using EBOV recombinant proteins including IgM-ELISA, immunofluorescence techniques and antigen-capture ELISAs with monoclonal antibodies induced by recombinant proteins¹⁸. These assays, first developed at the U.S. Army Medical Research Institute of Infectious Diseases, Maryland, USA in 1990⁹ and adopted by the CDC, utilize viral antigens prepared from inoculated cell cultures to bind antibodies present in patient serum, but these tests cannot be used for

early diagnosis. Molecular diagnostics for EHF and Marburg haemorrhagic fever (MHF) have been proven to be sensitive, specific and efficacious for early confirmatory diagnosis¹⁹. SYBR Green and TaqMan probe-based real-time quantitative RT-PCR methods for EHF and MHF were also developed for detection as well as quantification of virus load in clinical samples with high degree of selectivity and sensitivity^{20,21}. The WHO has accepted the first Ebola *in vitro* diagnostic product is RealStar® Filovirus Screen RT-PCR Kit manufactured by Altona Diagnostics. However, this Kit has several limitations including high cost owing to the use of large number of primer and probe sets. In addition to 95 and 100 per cent sensitivity and specificity, respectively, and a confidence level of 95 per cent, the requirement of expensive real-time PCR equipment also restricts its application only to few referral laboratories with good financial resources. Overall, real-time RT-PCR test system is expensive and time consuming as it requires 2-3 h. LAMP is an innovative, new generation gene amplification methodology that possesses the ability to amplify target nucleic acid sequences with a high degree of selectivity, sensitivity and specificity under isothermal conditions with a set of six specially designed primers that recognize eight distinct sequences of the target²². The whole procedure is simple and rapid wherein amplification can be obtained in less than one hour by incubating all of the reagents in a single tube with reverse transcriptase and Bst DNA polymerase at 63°C. The monitoring of gene amplification can be accomplished through naked eye either as turbidity or in the form of colour change. The LAMP assay has emerged as a powerful gene amplification tool for rapid identification of microbial infections and is being increasingly used by various investigators for rapid detection and typing of emerging viruses, namely Swine flu (H1N1), West Nile (WN), severe acute respiratory syndrome, dengue (DEN), Japanese encephalitis and chikungunya²³⁻²⁵.

In the present study, RT-LAMP assay was employed to detect Zaire Ebola virus (ZEBOV) in clinical specimens with diagnostic accuracies corresponding to that of the standard RT-PCR test. In addition, the assay could detect positive samples within 15 min. These findings have raised the prospects for developing a rapid molecular diagnostic test for EVD. With further improvements in methods for pre-treatment of clinical specimens, the RT-LAMP assay may provide a highly accurate rapid diagnostic test for EVD at point-of-care

in endemic rural areas. In the present study, we report the development of a one-step single-tube real-time RT-LAMP assay for rapid and real-time detection of Ebola virus RNA in clinical specimens by targeting the *NP* gene.

Material & Methods

Primer design: A set of six primers comprising two outer, two inner and two loop primers was designed that recognize eight distinct regions spanning more than 181 base pair (bp) conserved regions of the structural *NP* gene of the 2014 ZEBOV (Gene Bank Accession No. KP120606.1). The two outer primers were described as the forward outer primer (F3) and the backward outer primer (B3). The inner primers were described as the forward inner primer (FIP) and the backward inner primer (BIP). Further, two loop primers, forward loop primer (FLP) and backward loop primer (BLP), were designed to accelerate the amplification reaction. The usefulness of the selected primer sets for correctly identifying all strains and isolates of EBV was established through sequence alignment of all available *NP* gene sequences in the GenBank, using DNASIS software (MiraiBio, Yokohama, Japan). Cross-reaction with other haemorrhagic fever viruses such as DEN, West Nile and yellow fever virus was also evaluated. The details of the primer sequences with regard to genome position are depicted in Table I.

Virus: The virus tested in this study was received from Robert Koch Institute, Berlin, Germany, is a ZEBOV 2014 inactivated by heat and gamma irradiation. It was certified to be safe and non-biohazard material by the European Network for Diagnostics of Imported Viral Diseases (ENIVD). The assay was tested and evaluated in different matrices spiked with gamma-irradiated ZEBOV and results were compared with CDC-reported qRT-PCR. Detailed schematic representation is shown in Supplementary Figure.

RNA extraction: The genomic viral RNA was extracted from 140 µl of spiked body fluids (stool, saliva, urine, semen and serum) using QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols. RNA was also extracted from Kyasanur forest disease (KFD), Crimean-Congo haemorrhagic fever (CCHF), DEN, yellow fever, *etc.* to check the cross-reactivity of the assay. We used cDNA of KFD virus obtained from ICMR-NIV, Pune, CCHF synthetic plasmid, yellow fever and DEN virus RNA samples to check the specificity of EBOLA RT-LAMP. All the samples were handled in BSL-3 laboratory.

Table I. Comparative sensitivity of reverse transcription loop-mediated isothermal gene amplification (RT-LAMP) as well as Centers for Disease Control and Prevention (CDC)-recommended real-time reverse transcription–polymerase chain reaction (RT-PCR) assay for detection of Ebola virus

Serial number	Matrices	10 ⁶ copies	10 ⁵ copies	10 ⁴ copies	10 ³ copies	10 ² copies	10 copies	1 copy
1	Serum	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2	Saliva	+ve	+ve	+ve	+ve	+ve	+ve	+ve
3	Semen	+ve	+ve	+ve	+ve	+ve	+ve	+ve
4	Stool	+ve	+ve	+ve	+ve	+ve	+ve	-ve
5	Urine	+ve	+ve	+ve	+ve	+ve	+ve	-ve

The DEN and yellow fever viruses used in this study were obtained from the Institute of Tropical Medicine, Nagasaki, Japan and were propagated in Vero cells and then used in this study.

Reverse transcription-polymerase chain reaction (RT-PCR): In order to compare the sensitivity and specificity of the RT-LAMP one-step RT-PCR was performed with EBOLA GP-specific primers targeting 580 bp amplicon (forward primer 5'-ATGGGCTGAAAATTGCTACAATC-3' and reverse primer 5'-TAGTTTCCCAGAAGGCCCACT-3'). The amplification was carried out in a total reaction volume of 25 µl by using the Sigma RT-PCR kit (Sigma Aldrich, Germany) with 10 pmole of forward and reverse primers each and 2.5 µl of RNA as template. The thermal profile of RT-PCR was 48°C for 45 min and followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for two minutes and final extension time of 15 min²¹.

Real-time RT-PCR: Prior to RT-LAMP assay, all the samples were analyzed by the CDC-recommended real-time RT-PCR based on TaqMan assays (Applied Biosystems, USA) that involve a panel of oligonucleotide primers and a dual labelled hydrolysis probe using the SuperScript III Platinum One-Step quantitative kit (Invitrogen, USA). The amplification was carried out in a 25 µl reaction volume according to the manufacturer's instructions and the recommended thermal profile¹⁹.

One-step RT-LAMP: The amplification of the NP gene of Ebola virus was carried out in a total 25 µl reaction volume using 12.5 µl of in-house buffer [20 mmol/L Tris-HCl pH 8.8, 10 mmol/L KCl, 10 mmol/L (NH₄)₂SO₄, 8 mmol/L MgSO₄, 1.4 mmol/L dNTPs, 0.8M betaine and 0.1 per cent Tween-20];

3.75 µl of cDNA mix (5x buffer, 10 mmol/L dNTP mix and 0.25 µl of RNasin inhibitor); 1 µl of primer mix (50 pmole each of the primers FIP and BIP, 5 pmole each of the outer primers F3 and B3 and 25 pmole each of loop primers FLP and BLP); 1 µl (8 units) of the Bst DNA polymerase (New England Biolabs, Ipswich, MA); 0.25 µl of MMLV-RT; 1.5 µl of nuclease-free water and 5 µl of RNA at 37°C for 30 min. The gene amplification was accomplished by incubating the reaction mixture at 63°C for 60 min in a both real-time turbidimeter as well as routine laboratory water bath. To rule out contamination issues, proper negative controls, including no template, no primer and no enzyme, were always kept alongside each run.

Real-time monitoring of RT-LAMP: The real-time monitoring of RT-LAMP was observed through spectrophotometric analysis by recording optical density at 400 nm every six seconds with the help of a Loopamp real-time turbidimeter (LA-200, Teramecs, Kyoto, Japan). The cut-off value for positivity by the real-time RT-LAMP assay was determined by taking into account the time of positivity (Tp), in minutes, at which the turbidity increases above the threshold value, fixed at 0.1, which is two times more than the average turbidity value of the negative controls of several replicates.

Naked-eye visualization: Following amplification, the tubes were inspected by naked eye for white turbidity after pulse spins to deposit the precipitate at the bottom of the tube. The inspection for amplification was also performed by observation of colour change following the addition of 1 µl of SYBR Green 1 dye (Cambrex, East Rutherford, NJ, USA) to the tube. In cases of positive amplification, the original orange colour of the dye changes to green and can be seen in natural light and under UV light (302 nm). If there

is no amplification, the original orange colour of the dye is retained. However, to rule out bias in borderline cases, the result was recorded by three observers in the laboratory, and the sample was considered positive if the score was judged to be positive by more than two observers.

Determination of copy number and analytical sensitivity: In order to check the detection limit of RT-LAMP, the targeted region of the NP gene was amplified by RT-PCR using forward outer (F3) and backward outer (B3) primers as described above. The 181bp amplicon was then purified using a QIAGEN gel extraction kit and cloned into the pCR4-TOPO vector system (Invitrogen, USA) according to the manufacturer's specifications. The plasmid DNA was subjected to *in vitro* transcription (IVT) using T7 Transcription Kit (Fermentas, USA) at 37°C for two hours. The IVT products were then treated with 1U of DNase I and incubated at 37°C for 15 min to remove the remaining DNA followed by inactivation of DNase I at 70°C for 15 min. The excess nucleotides and pyrophosphate remaining inside the IVT products were then removed by sodium acetate/ethanol precipitation. The RNA pellet was re-suspended in diethyl pyrocarbonate (DEPC)-treated water. The amount of the IVT-generated Ebola virus RNA fragment was determined by spectrophotometric reading and converted to molecular copies by using the following formula²⁶:

$$Y \text{ (molecules/}\mu\text{l)} = X \text{ (g/}\mu\text{l)} \text{ transcript length (bp)} \times 340 \times 6.02 \times 10^{18}.$$

Further, ten-fold serial dilutions of the RNA transcript were used for detection of sensitivity and for construction of standard curve using the T_p values obtained against the known concentration of serially diluted standard RNA.

Evaluation of RT-LAMP: As cases of EVD are not present in India, the Ebola virus RNA was spiked into various body fluids of healthy volunteers. A total of 10 samples of stool, saliva, urine, semen and serum each were spiked with serial ten-fold dilutions of virus starting with 2×10^6 plaque-forming units (pfu)/ml. These spiked samples were then treated like clinical samples and were tested by RT-PCR, real-time RT-PCR and RT-LAMP. Five different human samples were collected (including saliva, serum, urine, stool and semen) from Gajra Raja Medical College, Gwalior. They have taken the patient consent and

ethical approval of the samples. These specimens were spiked with known copy numbers of ZEBOV strain received from Robert Koch Institute, Berlin, Germany, in triplicates and processed by following the entire standard SOP for RNA extraction and amplification reactions in a BSL-3+ laboratory. All these different healthy human samples having virus copy numbers of 10^5 to 10^0 , making six dilutions in each matrix, were tested. Negative controls including healthy human samples and samples spiked with DEN haemorrhagic fever virus were included. A panel of 10 negative serum samples was selected for further evaluation by RT-LAMP assay. Following spiking, all of the samples were processed for RNA extraction with a QIAamp viral RNA mini kit (QIAGEN) and screened by RT-LAMP and RT-PCR simultaneously for detection of viral RNA as described above.

Results

A one-step, real-time, accelerated quantitative RT-LAMP assay was standardized for rapid detection of Ebola virus. A set of six primers, comprising two outer primers (F3 and B3), two internal primers (FIP and BIP) and two loop primers (FLP and BLP), was designed against NP gene of circulating 2014 ZEBOV, epidemic strain (Gene Bank Accession No. KP120616.1). The monitoring of gene amplification was accomplished by real-time monitoring of turbidity, which was recorded at 400 nm every six seconds with the help of a Loopamp real-time turbidity meter (LA-500, Teramecs, Los Angeles, CA). The result indicated that the minimum time required for the initiation of amplification was 20 min. It was also observed that there was continuous amplification of the target sequence, as revealed by the increased turbidity. However, in the negative control, the turbidity remained fixed at about 0.01, well below the threshold value (Fig. 1). None of the spiked samples, which were tested multiple times, showed increased turbidity after 45 min. Therefore, a sample having T_p values of 45 min or less and turbidity above the threshold value of 0.1 or more was considered to be positive.

Sensitivity and specificity of RT-LAMP: The comparative limit of detection (LOD) of Ebola virus NP RT-LAMP as well as WHO-approved RT-PCR and CDC-recommended real-time RT-PCR was determined by using serial ten-fold dilutions of extracted RNA from the spiked samples in triplicate. A standard curve was generated by plotting a graph between different concentrations of gene copy

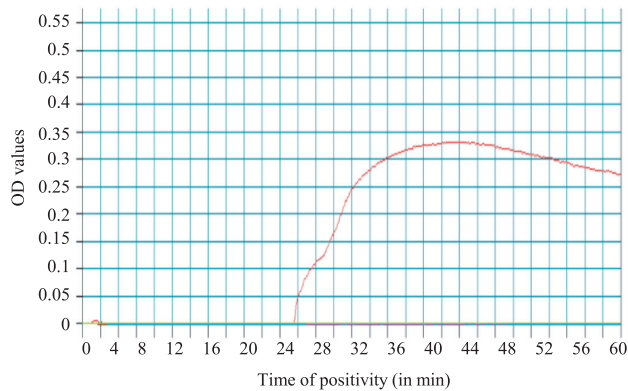


Fig. 1. Real-time amplification plot (turbidity curve) of Zaire Ebola virus NP gene-specific reverse transcription loop-mediated isothermal gene amplification (RT-LAMP) assay. The X-axis depicts the time of positivity, and the Y-axis shows the turbidity value in terms of the optical density at 400 nm recorded every six seconds with the help of real-time turbidimeter (LA-500). The negative template control did not show amplification.

numbers (10^5 to 10^0) and T_p in minutes through real-time monitoring (Fig. 2). The quantification of the virus load in the positive samples was extrapolated on the basis of their T_p by using the standard curve. The LOD for the Ebola virus NP RT-LAMP as well as CDC-recommended real-time RT-PCR assay was found to be copy number 1 in spiked samples from different matrices except stool sample where real-time RT-PCR could detect only 10 copies (Table I). Comparative sensitivity of RT-LAMP *vs.* conventional RT-PCR *vs.* RT-qPCR is represented in Figure 3A-C. is able to detect only 10^3 copies in all the spiked samples. Further specificity of the RT-LAMP primers for the NP gene of ZEBOV was established by ruling out the cross-reactivity with that of related haemorrhagic fever viral agents, namely KFD, CCHF, DEN and yellow fever. The Ebola virus NP gene-specific RT-LAMP primers showed a high degree of specificity for the Ebola virus by amplifying the NP gene of Ebola virus only while yielding negative results for other viruses tested as well as for healthy negative samples (Table II). Further confirmation of the amplified products was also carried out by nucleotide sequencing; the sequences we obtained perfectly matched the expected nucleotide sequences (data not shown).

The field applicability of RT-LAMP assay was also validated by using the SYBR Green I-mediated naked-eye visualization test. Following incubation at 63°C for 30 min in the water bath, the monitoring of RT-LAMP amplification was accomplished by

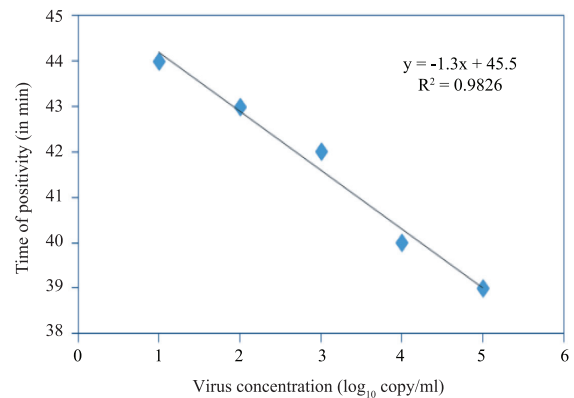


Fig. 2. Standard curve for a Zaire Ebola virus-specific RT-LAMP assay generated from the amplification plots between serial ten-fold dilutions of different virus RNA transcript concentrations (copies) ranging from 10^5 to 10^0 and time of positivity.

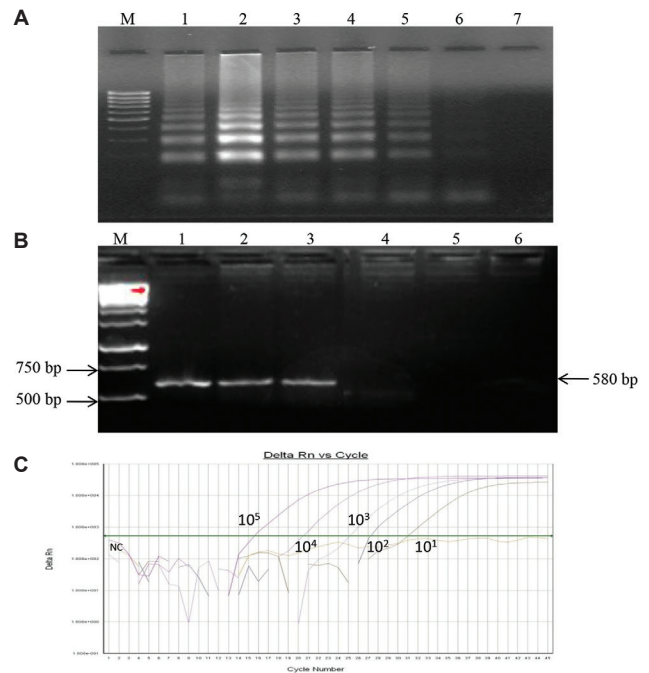


Fig. 3. Comparative sensitivity of RT-LAMP, RT-PCR and TaqMan RT-PCR. (A) Sensitivity of RT-LAMP assay: Lane M, 100-bp DNA ladder; lanes 1 to 6, different concentrations of virus, ranging from 10^5 to 1 copy; lane 7, negative control having no template; (B) Sensitivity of the RT-PCR assay revealing a 580 bp amplicon with a detection limit of 1000 copies. Lane M, 1000-bp DNA ladder; lanes 1 to 5, different concentrations of virus, ranging from 10^5 to 10^0 copies; lane 6, negative control having no template; (C) Sensitivity of real-time TaqMan RT-PCR.

naked-eye observation enabled by the addition of $1\ \mu\text{l}$ of SYBR Green I dye (1:1000) to the amplified products either in natural or UV light (Fig. 4). End point detection of amplification with agarose gel electrophoresis exhibited a characteristic ladder-like

Table II. Comparative evaluation of Ebola *NP* gene-specific isothermal gene amplification assay with WHO-approved, CDC-recommended TaqMan real-time RT-PCR assay and conventional RT-PCR assay for the detection of *NP* gene of Zaire Ebola virus in spiked human samples

Matrix (n=18)	Conventional RT-PCR	Real-time RT-PCR	RT-LAMP
Saliva	12	15	15
Urine	12	15	15
Serum	12	15	15
Stool	12	15	18
Semen	12	15	15

KFD, Kyasanur forest disease; CCHF, Crimean–Congo haemorrhagic fever

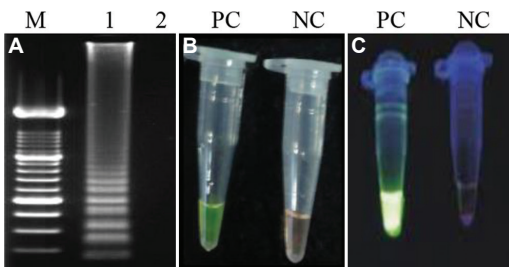


Fig. 4. End point detection of amplification in loop-mediated isothermal gene amplification reaction. (A) Agarose gel electrophoresis of LAMP reaction products, Lane M: 1 Kb DNA ladder, Lane 1: Amplification with 100 copies of Ebola virus, Lane 2: No Template Control. (B) SYBR Green I-based detection under normal light: SYBR Green I retains orange colour in negative samples, while its colour changes to greenish yellow in positive reaction; (C) SYBR Green I-based detection under UV light: Positive reaction results in green fluorescence while no fluorescence in negative reaction.

pattern (Fig. 4A). Amplification in the reaction tubes incubated in heat block was detected under normal and UV light by adding 1 µl of 1:10 diluted SYBR Green I (1:10,000, Invitrogen, country) after completion of the reaction. The colour of SYBR Green I changed from orange to greenish yellow in amplification-positive reaction, while in amplification-negative reactions, the dye retained its original orange colour when viewed under normal light (Fig. 4B). LAMP-positive reactions exhibited green fluorescence, whereas negative reaction did not fluoresce under UV light (Fig. 4C).

Discussion

The Ebola epidemic is one of the largest in history, affecting multiple countries in West Africa. The spread of the virus to other countries through travellers has

led to serious public health emergency of international concern. The emergence of EBOV in the form of an unprecedented outbreak in West Africa further confirmed the necessity of rapid diagnosis³.

In the current epidemic scenario, confirmation of EHF is generally made by using WHO-approved CDC-recommended real-time RT-PCR based on TaqMan chemistry. However, one of its limitations is that the test system is expensive that can be afforded only by referral laboratories with good financial resources. Another limitation of real-time RT-PCR test system is that it is time consuming as it requires 2-3 h and also limited availability of reagents and technical expertise required for interpretation for routine laboratory diagnosis. It is, therefore, critical to develop simple and economical molecular tests that can be used in field conditions, especially in peripheral healthcare settings as a routine test without requirement of any sophisticated high-end equipment.

The present study describes the development of a one-step, single-tube isothermal RT-LAMP gene-amplification assay for rapid detection of Ebola viruses. This is an alternative real-time test system for rapid detection and for identification of Ebola viruses. The assay is based on a simple isothermal gene amplification tool using a specially designed primer set that specifically amplifies the *NP* gene of Ebola virus only. Unlike PCRs, in which the target gene is flanked by two primers, in the case of LAMP, a set of six primers flank a small target of 181 bp through recognition of eight different sequences of the target gene. LAMP is, therefore, selective and highly target specific. Unless all of the eight different sequences are identified, the amplification will not proceed. Therefore, a sequence homology of 95 per cent or higher is required for LAMP, making the assay extremely specific.

As already shown in results, the RT-LAMP assay has demonstrated similar sensitivity compared to TaqMan RT-PCR in all samples. The detection limit of RT-LAMP assay was found to be 1 copy number in different matrices. The higher sensitivity of the RT-LAMP reaction can be attributed to continuous amplification under isothermal conditions. This leads to substantial reduction in time required for the confirmation of results by RT-LAMP assay (in 30 min compared to two hours in the case of RT-PCR). The higher amplification efficiency of the RT-LAMP reaction yields a large amount of a by-product, pyrophosphate ion, leading to white precipitate of

magnesium pyrophosphate in the reaction mixture. Since the increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the RT-LAMP reaction can be achieved by real-time measurement of turbidity²⁴.

Southern *et al.*²⁷ evaluated three diagnostic assays including FilmArray and two qRT-PCR assays for detection of ZEBOV from whole blood specimens spiked with inactivated virus at known titers. The result revealed 100 per cent agreement at virus dilutions ranging from 4×10^7 to 4×10^2 TCID₅₀/ml. It has already been reported that inactivation process affects nucleic acid integrity and shifts the detection limit, usually about 10-100 folds higher, compared to that obtained with viable virus. Studies performed by BioFire Defense, LLC on the BT-E panel indicated a LOD of 6×10^5 PFU/ml of whole blood using inactivated ZEBOV²⁸. BioFire Defense, LLC also reported successful BT-E panel detection of synthetic ZEBOV *L*-gene RNA template up to a concentration of 100 genome equivalents. Studies at the CDC documented a LOD for both the NP and VP40 qRT-PCR assays of 30 TCID₅₀/reaction (~ 5400 TCID₅₀/ml) using inactivated ZEBOV in both whole blood and urine^{16,17}. These marked differences are primarily linked to variations in the harshness of the protocols used to inactivate the virus in the analyte preparations. The remarkably low detection limit of RT-LAMP assay even with inactivated sample confirms the sensitivity of the detection system and its utility for molecular detection in epidemic setting at remote locations. One-step single-tube real-time RT-LAMP assay for rapid and real-time detection of Ebola virus RNA in clinical specimens by targeting the *NP* gene was developed. The *NP* gene-specific RT-LAMP assay reported in this study was found to be a simple, rapid, reliable and inexpensive method for detection as well as identification of Ebola virus without requiring any sophisticated detection equipment and fluorescent probes. Thus, the RT-LAMP assay reported in this study will be useful for clinical diagnosis and surveillance of EBOV in developing nations across WHO networks.

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Conflicts of Interest: None.

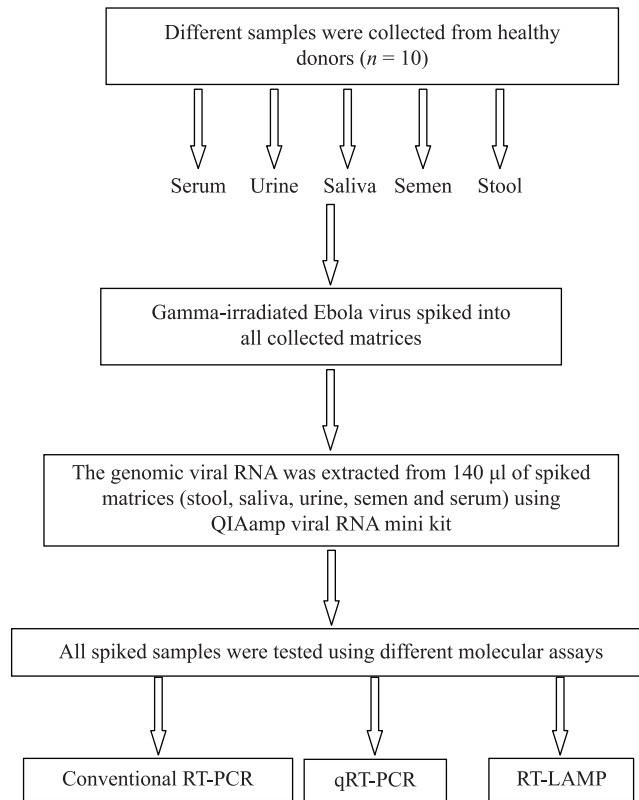
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Supplementary Material



Supplementary Figure. Schematic representation.