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Determination of Chikungunya Virus RNA by Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Assay

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Abstract

As a matter of fact, infectious diseases hamper everyone's life and produce a lifelong threat to everyone neutral of age, sex, lifestyle and socio-economic status. Nowadays, come into the sight of Chikungunya viral (CHIKV) infection injured many Asian and African countries, also deliberated threat in rising countries and also low socio-economic countries. CHIKV is a positive-sense, enveloped single-stranded, RNA virus belonging to the genus *Alphavirus*, family *Togaviridae*. As the Dengue & Chikungunya viruses are spread simultaneously at the same time, so it is tough to identify them. In our resource-limited countries, swift detection of CHIKV by RT-LAMP is the simplest molecular technique in low-equipment settings without the use of any expensive decoration. Heat-treated centrifuged and uncentrifuged samples were used in this study and they showed the same result (100%). Different instruments like heat block, water bath, conventional thermal cycler & real-time thermal cycler were used to amplify the CHIKV RNA and they indicated that 100% samples were identified by all four instruments. The amplified products were visualized by turbidity test, color change by HNB, step-ladder band pattern in agarose gel electrophoresis and amplification curve in real-time thermal cycler naked eye, here the results also showed 100% samples were determined by all visualized methods. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a recent technique for amplifying RNA under limited temperature, with high tangibility, quickness and competency. To identify the CHIKV RNA Reverse transcription loop-mediated isothermal amplification was fabricated and validated, and the results were also compared with reverse

transcription polymerase chain reaction (RT-PCR). The sensitivity was 95.71% and specificity was 100%, these results indicate that RT-LAMP is a feasible method for quick detection of CHIKV RNA.

Keywords

CHIKV, Chikungynea, RT-LAMP, Infectious Diseases, Viral Infection, CHIKV-RNA

1. Introduction

CHIKV is an acute arthropod-borne *Alphavirus*, 1st isolated in Tanzania in 1953. It is infected by a transmitted virus by a bit of an infected female adult *Aedes aegypti* and *Aedes albopictus* mosquito. Human acts as an efficient reservoir for this virus, so it is most comprehensive in civic areas and epidemics are maintained by the man-mosquito-man cycle.

CHIKV is a positive-sense, enveloped single-stranded, RNA virus belonging to the genus *Alphavirus*, family *Togaviridae*. Serologically CHIKV is arranged in categories as a member of the Semliki Forest antigenic complex, closely related to *O'nyong-nyong*, *Sindbis* virus, *Mayaro* virus and *Ross River virus* due to sequence similarities [1]. The RNA genome of CHIKV (approximately 11.8 kb), capped at the 5' and with a poly (A) tail at the 3' end, comprises two open reading frames (ORFs) interrupted by an untranslated region (UTR) and the junction region (J). The ORF at the 5' end enciphers four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) and the other ORF encodes five structural proteins, including the capsid (C), envelope proteins E3, trans-membrane glycoproteins (E2 and E1) are occurring in pairs and stationed as trimeric spikes on its surface and small 6K protein [2] [3].

CHIKV expresses a sickness in humans that is identified by a sudden onset of fever, headache, fatigue, nausea, vomiting, rash, myalgia and arthralgia. Symptoms are generally self-centered and last for 1 - 10 days.

Diagnosing the CHIKV infection in the laboratory is censorious, particularly in regions where dengue and CHIKV co-existed. There are very few probable laboratory parameters for discrepant diagnoses of CHIKV and dengue viral fever [1] [4]. Recently reliable diagnostic tests used for CHIKV diagnosis in most CHIKV endemic countries include IgM and IgG-based Enzyme immunoassay (EIA) and Immunochromatographic test (ICT). Antibody against CHIKV specific IgM displays within 5 - 7 days of sickness which is inappropriate for early diagnosis and it cross-reacts with other related *Alphavirus* e.g. *Ross River virus*, *Sindbis virus* and clinically similar diseases producing *Flavi virus* (dengue 1 - 4, *Japanese Encephalitis virus, Yellow Fever virus*, *West Nile virus*) [5]. Other old diagnostic methods that were used for the detection of CHIKV infections are virus isolation, and genome detection by Polymerase Chain Reaction (PCR) based methods.

Another molecular technique, the Loop-mediated isothermal amplification

(LAMP)) represents a unique gene amplification procedure where the reaction can be prepared at a suitable constant temperature by strand displacement activity of *Bacillus stearothermophilus* (Bst) DNA polymerase enzyme [6] [7]. It is an isothermal, quick, easy, sensitive and unique gene amplification method. Extraction of nucleic acid is the first step in many molecular biology research; LAMP is a method that does not need this step, in order to reduce sample processing periods and expenses, definite virus detection avoids nucleic acid extraction by practicing a simple heat-treatment of serum is possible by LAMP assay [8] [9]. LAMP has the capacity to enhance nucleic acids with potential efficacy under constant conditions (63˚C) using simple incubators such as a water bath or heating block for 60 minutes creating this method suitable for territory region & ground work. Direct amplification from heat-treated samples by visual observation was done during the entire amplification process within 60 minutes [8]. This assay can amplify with a sensing limit of 20 copies of nucleic acid/reaction [1]. The method uses 6 - 8 primers that recognize 6 - 8 individual regions of the aimed DNA which facilitate to eliminating of nonspecific binding and improve the specificity of this assay. The amplification efficacy in LAMP is extremely high as no period is needed for thermal change and the reaction is performed at a fixed temperature. The LAMP product can be tracked out by gel electrophoresis, turbidity, color change by Hydroxynapthol blue (HNB) dye and amplification curve in real-time LAMP. Colorimetric detection by using HNB dye is one of the most enchanting features of the LAMP assay. HNB dye allows the test to be interpreted with the naked eye [10] [11]. LAMP is easy to use, convenient, affordable, and time-saving method that requires minimal equipment and easy detection methods. As opposed to LAMP, molecular detection by PCR is sophisticated technology-based method requiring expensive reagents and instruments to amplify.

To diagnose communicable diseases, Nucleic Acid Amplification Tests (NAATs) are just solely performed in principle & a few laboratories exercise sophisticated instruments and efficient technologists. In the case of Neglected Tropical Diseases (NTD), there is very limited opportunity to reach out to a faithful & reliable diagnosis. In the last few years in Bangladesh, dengue and Chikungunya both are spreading simultaneously and they show similar characteristics; thus, the diagnosis of CHIKV is very challenging. So, CHIKV RT-LAMP (Reverse transcription loop-mediated isothermal amplification) for identification of CHIKV RNA may be norm weapon to diagnoses regularly CHIKV infection in clinical samples in controlled laboratory set-up. Therefore, the present study aimed to optimize and validate CHIKV RT-LAMP for rapid detection of CHIKV.

The aim of this study was to mold a prompt, impressible and specific genuine time method to detect CHIKV in symptomatic patients' serum samples. Loop-mediated isothermal amplification (LAMP) is a new nucleic acid amplification method and has the probability to limit the use of expensive PCR technique as this newly discovered method is simple, quick, specific and cheap. It is a potent gene amplification tool for speedy identification of different infectious organisms. In this

present study, a single-step, solo tube & swift RT-LAMP assay was ascertained by selecting the immunodominant envelope (E1) gene for fast detection of CHIKV and compared with the results of qRT-PCR. The result of the CHIKV RT-LAMP assay is measured using different instruments like; heat block, water bath, conventional thermal cycler and real-time thermal cycler & to compare CHIKV RT-LAMP assay using heat treated centrifuged and heat treated uncentrifuged serum samples.

2. Methodology

2.1. Study Design

This was an analytical research leading from July 2018 to June 2019 at the Department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU).

2.2. Study Population

In this study, two groups of samples were used. In the first group, one known CHIKV positive control, 20 Dengue IgM positive samples and 10 healthy controls were selected for optimizing CHIKV RT-LAMP. Different amplification and detection techniques were used to optimize the CHIKV RT-LAMP.

In the second group, 70 archived CHIKV specific IgM positive serum samples, 20 archived CHIKV specific IgM negative serum samples, 20 archived CHIKV IgG negative samples and 10 healthy controls were used for detection of CHIKV RNA using optimized amplification methods and detection techniques.

2.3. Laboratory Methods

Primer Selection:

The oligonucleotide primers were used for CHIKV RT-LAMP amplification objecting the E1, here the E1 gene of the S-27 African prototype strain from GenBank (accession no-AF36902) nucleotide sequence was used. A set of six primers comprising two inner, two outer and two loop primers that recognize eight distinct regions on the target sequences was designed. The two outer primers (forward and backward) were used for CHIKV qRT-PCR. The dynamic target region of 205 bp resembling to the genome positions 10,294 to 10,498 was selected.

The details of primers are s listed in Table 1.

Control Selection:

One known CHIKV CDC positive control was to optimize this assay.

Sample Processing:

All the samples were processed following the standard procedure [6] [7] (Figure 1 and Figure 2, Table 2).

Reagents:

RT-LAMP Assay:

Optimization of CHIKV RT-LAMP Assay:

To optimize the CHIKV RT-LAMP, several experiments were leading with

Table 1. The nucleotide sequences for the CHIKV RLAMP [1].

Primer	Genome position	Length of Oligonucleotide (bp)	Sequences (5'-3')
Forward outer (F3)	10,294 - 10,312	19	ACGCAATTGAGCGAAGCAC
Backward outer (B3)	10,498 - 104,80	19	CTGAAGACATTGGCCCCAC
Forward inner (F1c)	10,378 - 10,357	22	CGGATGCGTATGAGCCCTGTA
Forward inner (F2)	10,316 - 10,335	20	TGGAGAAGTCCGAATCATGC
Backward inner (B1c)	10,391 - 10,413	23	TCCGCGTCCTTTACCAAGGAAAAT
Backward inner (B2)	10,472 - 10,453	20	TTGGCGTCCTTAACTGTGAC
Forward loop (FLP)	10,355 - 10,339	17	GCTGATGCAAATTCTGT
Backward loop (BLP)	10,430 - 10,446	17	CCTATGCAACGGCGAC

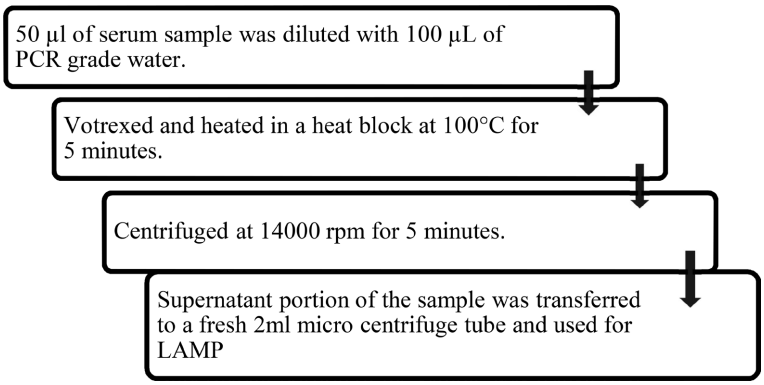

Figure 1. Preparation of heat-treated centrifuge serum samples.

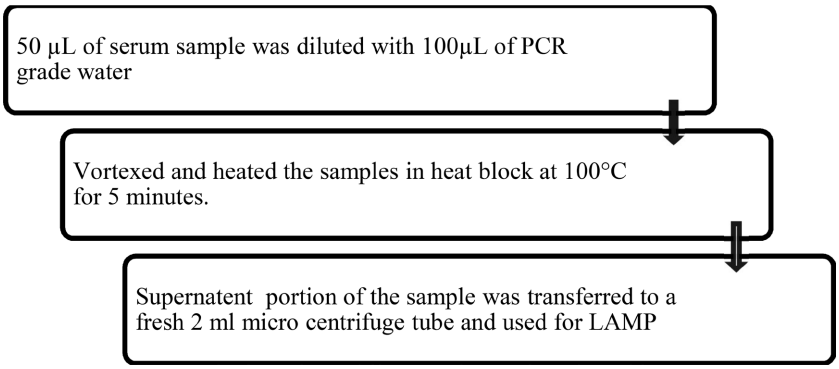

Figure 2. Preparation of heat-treated uncentrifuged serum samples.

different concentration of required reagents and time duration *i.e.* Bst enzyme (6U and 8U), betaine (0.2 mM, 0.4 mM, 0.6 mM) and $MgSO_4$ (4 mM, 6 mM, 8 mM) in different amplification instruments *i.e.* heat block, water bath, conventional thermal cycler and real-time thermal cycler. To set optimized temperature, CHIKV RT-LAMP was performed with different temperatures (61˚C, 62˚C and 63˚C). To observe the color change, CHIKV RT-LAMP was carried out with different concentration of HNB dye (10 mM, 20 mM and 40 mM) in different

Table 2. CHIKV RT-LAMP reagents [1].

Reagents	Quantity	Final concentration
1. Thermopol buffer (10×)	2.5 µL	1×
2. $MgSO_4$ (100 mM)	2 µL	8 mM
3.dNTP mix (20 mM)	1.75 µL	1.4 mM
4. F3 primer	0.5 µL	0.2 mM
5. B3 primer	0.5 µL	0.2 mM
6. FIP primer (F1c and F2)	1.25 µL	1.6 µM
7. BIP primer (B1c and B2)	1.25 µL	1.6 µM
8. FLP primer	0.625 µL	0.8 µM
9. BLP primer	0.625 µL	0.8 µM
10. Betaine (5 M)	2 µL	0.4 mM
11. Bst polymerase (8000 U/ml)	1 µL	320 U/mL
12. Reverse transcriptase (200 U/ml)	0.1 µL	8 U/mL
13. EVA Green (20×)	1 µL	1×
14. HNB dye	1.5 µL	120 µM
15. Sample	5 - 10 µL	
16. PCR grade water	Up to 25 µL	

amplification instruments mentioned above (heat block, water bath, conventional thermal cycler and real-time thermal cycler).

Preparation of In-House CHIKV RT-LAMP Master Mix:

Prior to master mix preparation, all reagents preserved at −20˚C, were thawed and kept on ice pack. After optimization of the different components of CHIKV RT-LAMP, the master mix was prepared in total 25 µL of volume using the optimized concentration. All the reaction components and primers were added in a micro centrifuged tube and briefly centrifuged. Ten microliter of preserved sample (heat-treated centrifuged/heat-treated uncentrifuged) (Appendix 10) added in each CHIKV RT-LAMP reaction and in every test positive control and negative control were included. Ten microliter of distilled water was added in replace of serum sample in negative control.

Preparation of Agarose Gel:

To observe the amplified products, prepare 100 ml of 2% agarose gel, 1.8 gram of agarose was melted in 90 ml of distilled water and 9 ml of 1× TBE buffer in a 250 ml conical flask. The flask was heated at 100˚C for 2 minutes in a microwave oven. Then 7 µL of ethidium bromide was added to the cooled gel. After the gel was completely set (approximately 45 minutes at normal temperature), the comb was carefully shifted and the agarose gel tray carrying the gel was mounted into the electrophoresis tank containing 1× TBE buffer.

Amplification Techniques and Reaction Cycle of CHIKV RT-LAMP:

The reactions of amplification were performed either in a heat block or wa-

ter bath or conventional thermal cycler (Infinigen Biotech, USA) or real-time CHIKV RT-LAMP (Applied Bio system, USA).

1) Water Bath:

Temperature of the water bath was increased and fixed at 62˚C. After taking the LAMP reaction mixture in a micro centrifuge tube, the cap of micro centrifuge tube was closed. The closed micro centrifuge tube was placed in water bath using a floating rack for 45 - 60 minutes. Reaction was conducted in 2 ways:

a) Using HNB dye with reaction mixture for detection of color change.

b) Without HNB dye for detection of turbidity.

2) Heat Block:

Temperature was fixed at 62˚C & the tube was placed in a heat block for 1 hour. Reaction for each sample was conducted in 2 micro centrifuge tube with HNB (Hydroxynapthol Blue) dye and without dye.

3) Conventional Thermal Cycler:

Using the same reaction mixture CHIKV RT-LAMP was performed in two tubes with each sample with or without use of HNB.

4) Real-time CHIKV RT-LAMP:

To detect real-time CHIKV RT-LAMP, 1 µL of 20× Eva green was added into every reaction tube. The reaction was set at the following temperature 62˚C for 1 min, 60 cycles of 30 seconds at 62˚C, 30 seconds at 63˚C and 82˚C for 2 minutes.

Detection and Visualization of CHIKV RT-LAMP Products:

1) Visual Detection of CHIKV RT-LAMP Products by Turbidity:

By placing the each CHIKV RT-LAMP reaction tubes on a black paper, turbidity was observed in each reaction.

2) Visual Detection of CHIKV RT-LAMP Products by Color Change:

Each CHIKV LAMP was observed for color change by placing the reaction tube on a white paper. A positive reaction was showed by color change and it was from violet to sky blue.

3) Detection of CHIKV RT-LAMP Products by Amplification Curve:

Amplification curve was plotted against Y-axis (ΔRn) versus X-axis (cycle). ΔRn was formed from fluorescence action of EVA green dye and threshold value was set at 0.1 line was set X-axis showed real-time amplification of CHIKV LAMP. Sixty cycle reaction was performed in real-time CHIKV RT-LAMP. To complete every run, 1 minute was required and the time of positivity was same as the cycle number in the amplification plot.

4) Detection of CHIKV RT-LAMP Products in Agarose Gel

Amplified products from heat block, water bath and conventional thermal cycler was visualized in ethidium bromide stained agarose gel by gel documentation system using UV Tran's illuminator. Using a micropipette, 10 µL aliquot of CHIKV LAMP amplified products was loaded in 2% agarose gel slowly and carefully onto the wells of the submerged gel. A voltage of 100 volts for 40 minutes was supplied.

CHIKV qRT-PCR Assay:

Selection of Primers:

The sensitivity and specificity of this assay was assimilated & for this purpose, qRT-PCR was performed by using the two outer primer pairs of RT-LAMP (Table 3) [1].

Extraction of CHIKV RNA:

Extraction of nucleic acid from serum was performed manually (Geneproof, Taiwan) according to the manufacturer's protocol.

cDNA (Complementary DNA) Synthesis:

For cDNA synthesis, the extracted RNA was analyzed by Nano drop 2000 UV-Vis spectrophotometer (Thermo Scientific, USA) and from each sample 250 ng of RNA was taken for cDNA synthesis.

CHIKV qRT-PCR:

The E1 gene was targeted for CHIKV qRT-PCR and expression of the gene was analyzed by Step One Real-time PCR machine (Applied Biosystems, USA) using the HOT FIREPOL Eva Green qPCR Mix plus.

Test Procedure:

Reaction mixture for qRT-PCR was prepared in PCR tube, in every run of qRT-PCR contained negative control and positive control. After reaction mix was prepared, the PCR cycle was performed following PCR cycling parameter (Table 4).

qRT-PCR Data Analysis:

After each successful run, the data was collected setting the threshold value at 0.1 for all sets of PCR run.

qRT-PCR Assay:

In this study, to parallelism the sensitivity and specificity of the RT-LAMP assay, RT-PCR was carrying out by using two outer primers of CHIKV RT-LAMP. The reaction was performed in 20 µL volume containing 0.5 µL of each forward and backward outer primers and 150 ng of cDNA.

2.4. Statistical Analysis

The data was imported into IBM SPSS Statistics 22 software (USA) for analysis

Table 3. The nucleotide sequence of RT-PCR [1].

Primer	Sequences (5'-3')	Position
Forward outer	ACGCAATTGAGCGAAGCAC	10,294 - 10,312
Backward outer	ACGCAATTGAGCGAAGCACGCCCCAC	10,498 - 10,480

Table 4. PCR cycling parameters.

Cycle step	Temperature	Time	Cycle
Initial denaturation	95˚C	12 minutes	1 cycle
Denaturation	95˚C	15 seconds	40 cycles
Annealing	60˚C	20 seconds	
Elongation	72˚C	20 seconds	

(Table 5).

3. Result

In this present study, out of 70 CHIKV IgM +ve samples, CHIKV RNA were detected in all IgM +ve samples by four amplification techniques *i.e.* heat block, water bath, conventional thermal cycler and real-time thermal cycler, heat treated centrifuged and uncentrifuged samples showed same results. CHIKV RNA was not detected in CHIKV IgM –ve and IgG +ve samples, CHIKV IgM and IgG both negative samples and healthy controls by any of the four amplification techniques mentioned above (Table 6). Heat-treated centrifuged and heat-treated uncentrifuged serum samples were observed to detect CHIKV RNA by same amplification instruments and detection methods and all positive samples showed same efficacy in these two forms of sample preparation in different instruments and detection methods (Table 7). Corresponding Figure 3 and Figure 4.

Table 5. CHIKV qRT-PCR reagents [1].

Component	Volume
1. 5× Hot Fire pol Eva Green qPCR mix plus	4 µL
2. Forward primer (10 µM)	0.5 µL
3. Backward primer (10 µM)	0.5 µL
4. cDNA template	5 µL
5. PCR grade water	10 µL
6. Total	20 µL

Table 6. Detection of CHIKV RNA by CHIKV RT-LAMP using different instruments used for amplification.

Serum samples	Number	Number(%) positive for CHIKV RNA using			
		Heat block	Water bath	Conventional thermal cycler	Real-time thermal cycler
CHIKV IgM +ve	70	70 (100%)	70 (100%)	70 (100%)	70 (100%)
CHIKV IgM –ve and IgG +ve	20	00 (00%)	00 (00%)	00 (00%)	00 (00%)
CHIKV IgM –ve and IgG –ve	20	00 (00%)	00 (00%)	00 (00%)	00 (00%)
Healthy control	10	00 (00%)	00 (00%)	00 (00%)	00 (00%)

Table 7. Detection of CHIKV RNA by CHIKV RT-LAMP using heat-treated centrifuged and heat-treated uncentrifuged samples.

Serum samples	Number	Number (%) positive for CHIKV RNA by	
		Heat-treated centrifuged	Heat-treated uncentrifuged
CHIKV IgM +ve	70	70 (100%)	70 (100%)
CHIKV IgM-ve and IgG +ve	20	00 (00%)	00 (00%)
CHIKV IgM –ve and IgG –ve	20	00 (00%)	00 (00%)
Healthy control	10	00 (00%)	00 (00%)

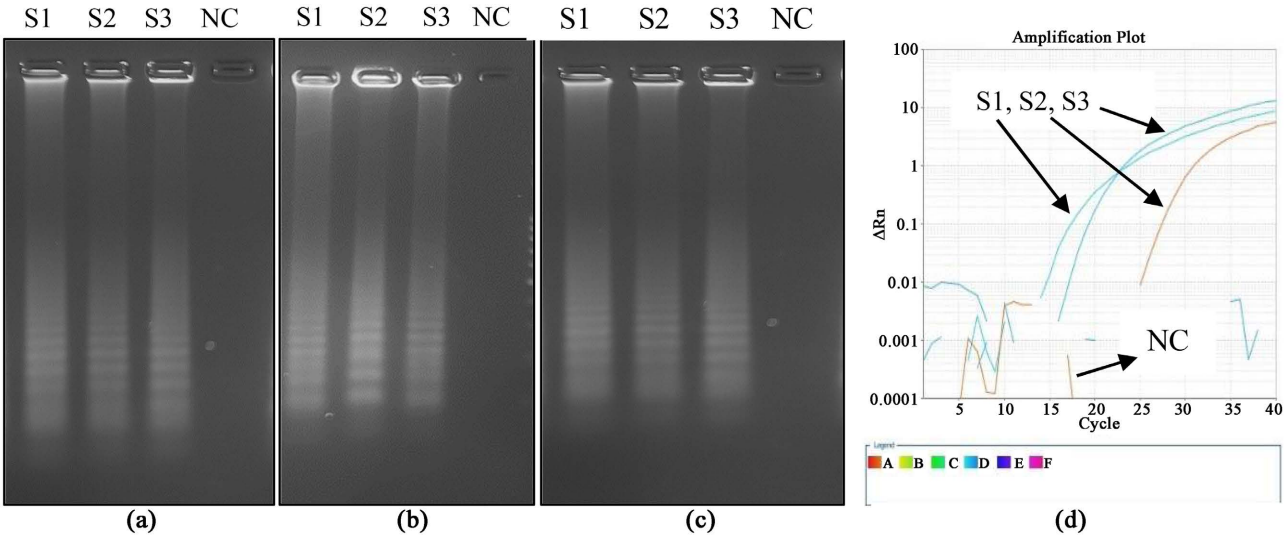

Figure 3. Demonestrates the comparison of heat-treated centrifuged samples in different instruments. (a) Water bath; (b) Heat block; (c) Conventional thermal cycler; (d) Real-time thermal cycler.

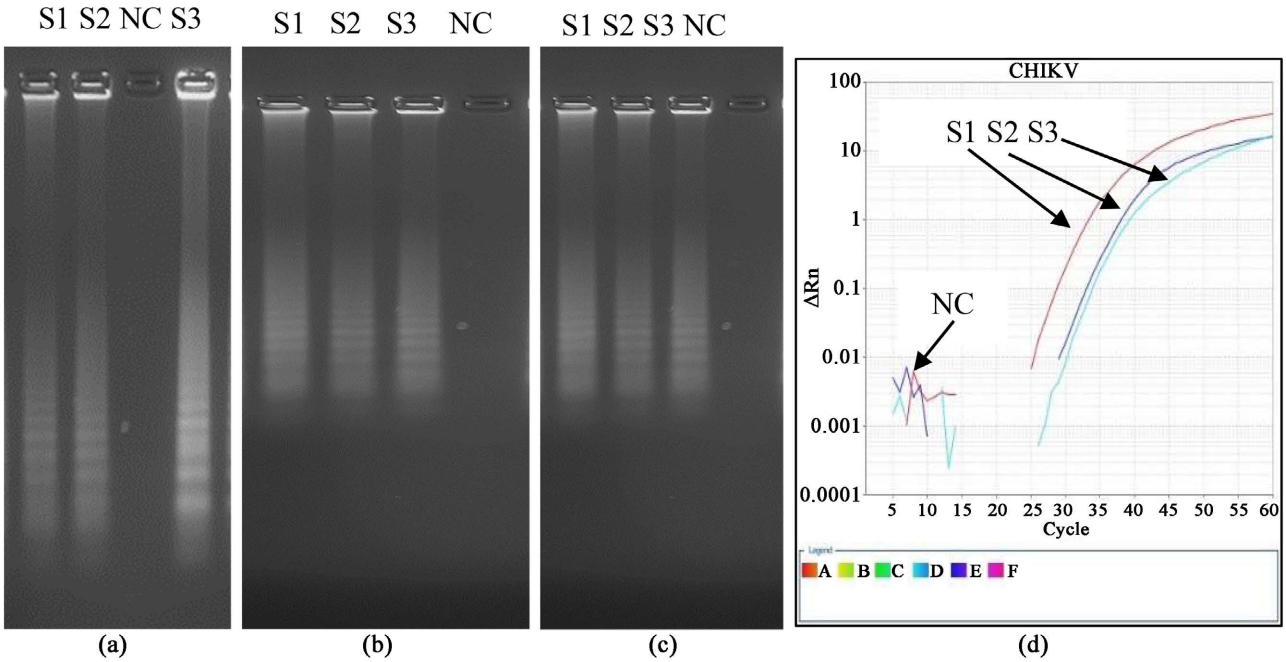

Figure 4. Demonestrates the comparison of heat-treated uncentrifuged samples in different instruments. (a) Water bath; (b) Heat block; (c) Conventional Thermal cycler; (d) Real-time thermal cycler.

In this present study, comparison between observation of amplified products in four detection methods of CHIKV RT-LAMP showed that all 70 CHIKV specific IgM positive serum samples were positive in four visualized techniques *i.e.* turbidity test, color change by HNB, step-ladder band pattern in agarose gel electrophoresis and amplification curve in real-time thermal cycler (**Table 8**). Corresponding **Figure 5**.

CHIKV RNA was detected by CHIKV qRT-PCR in IgM +ve samples and showed 67 (95.71%) samples were positive, in which all were positive for CHIKV

Table 8. Comparison of different detection methods of amplification products of CHIKV RNA by CHIKV RT-LAMP.

Serum samples	Number	Visualization of amplification products by			
		Turbidity	Color change of HNB dye	Amplification curve in real-time thermal cycler	Step-ladder pattern band in agarose gel electrophoresis
CHIKV IgM +ve	70	70 (100%)	70 (100%)	70 (100%)	70 (100%)
CHIKV IgM –ve and IgG +ve	20	00(00%)	00(00%)	00(00%)	00(00%)
CHIKV IgM and IgG both –ve	20	00(00%)	00(00%)	00(00%)	00(00%)
Healthy controls	10	00(00%)	00(00%)	00(00%)	00(00%)

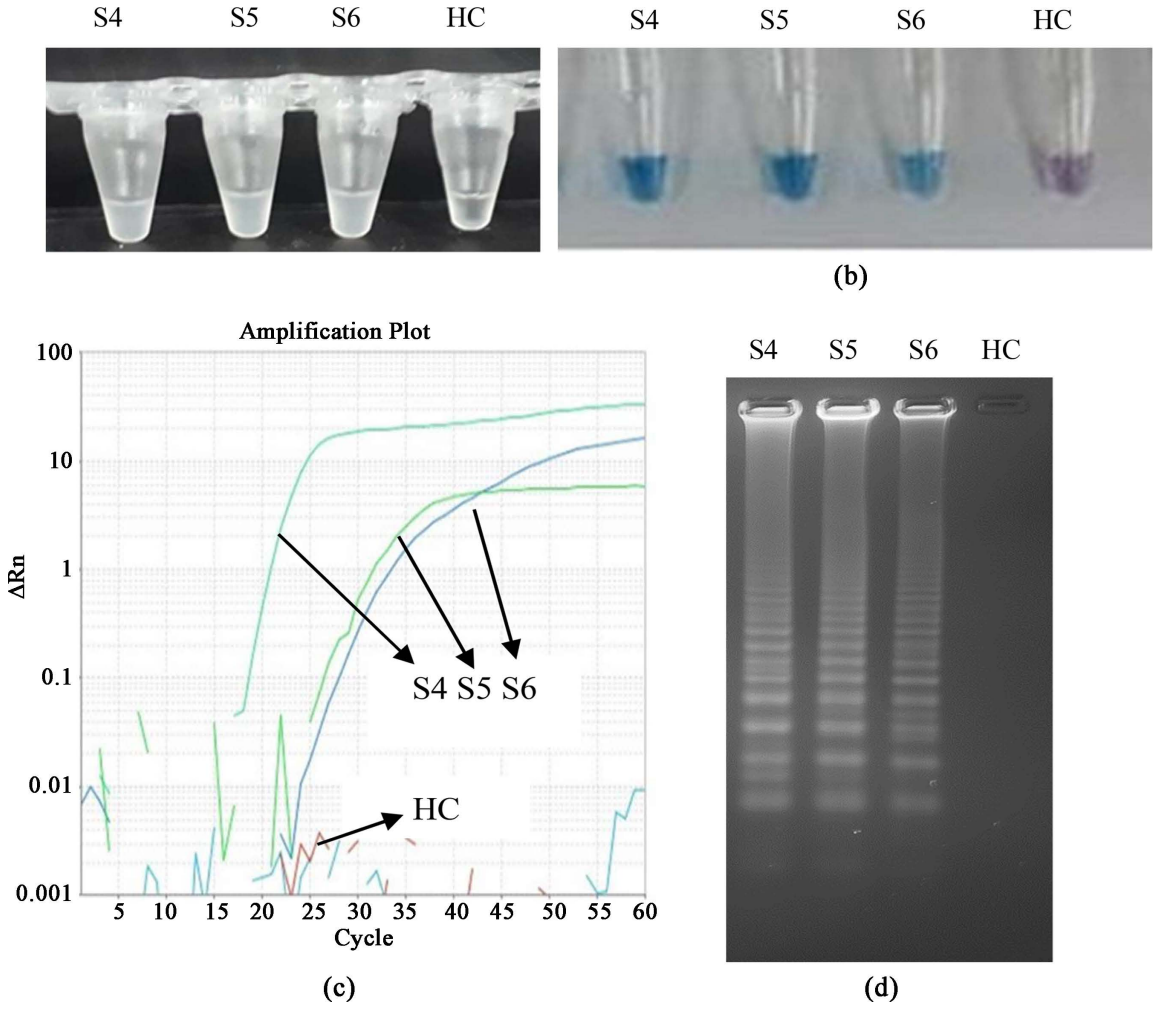

Figure 5. Demonestrates the comparison of different detection methods to observe the amplified products in CHIKV RT-LAMP. (a) Turbodity observed by naked eye; (b) Colour change by use of HNB; (c) Amplification curve in real-time thermal cycler; (d) Step-ladder band pattern band in agarose gel ekletrophoresis.

RNA by RT-LAMP (**Table 9** and **Figure 6**). It was observed that, there was a linear relationship between time of positivity (minutes) in CHIKV RT-LAMP and Ct value (cycle threshold) in CHIKV qRT-PCR. When the Ct value of RT-PCR were placed on X-axis and the time of positivity (minutes) of RT-LAMP were placed on Y-axis, there was a linear relationship and in 90% samples showed

Table 9. Detection ability of CHIKV RNA by CHIKV RT-LAMP and CHIKV qRT-PCR.

Serum samples	Number	Detection of CHIKV RNA by	
		CHIKV RT-LAMP	CHIKV qRT-PCR
CHIKV IgM +ve samples	70	70 (100%)	67 (95.71%)

Sensitivity-95.71%; Specitivity-100%; PPV-100%; NPV-86.95%.

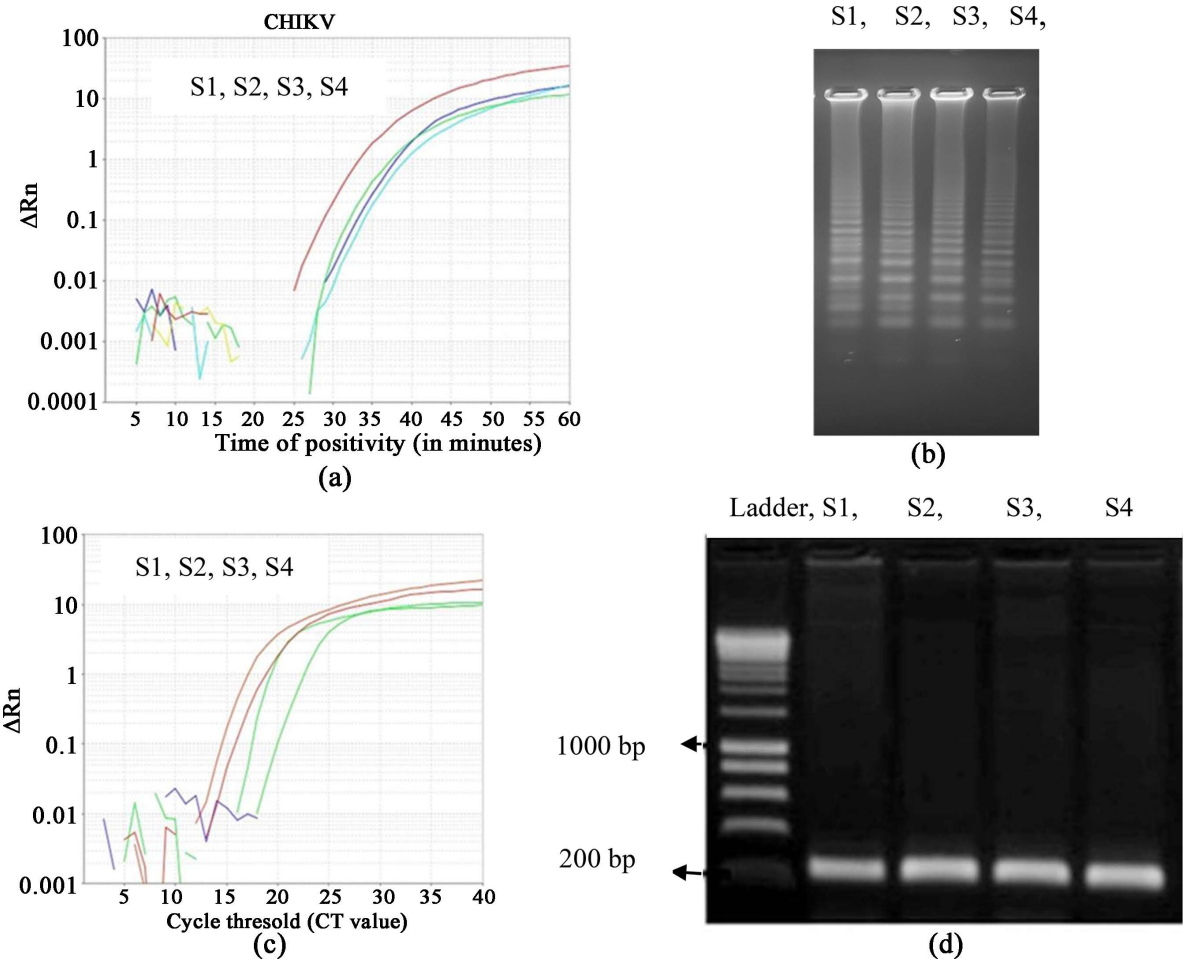

Figure 6. Shows detection of CHIKV RNA by CHIKV RT-LAMP and CHIKV qRT-PCR. (a) in real-time thermal cycler; and (b) agarose gel electrophoresis showes step-ladder pattern. (c) shows amplification curve in CHIKV qRT-PCR and (d) gel electrophoresis shows a band corresponding to 205 bp.

almost same results (Figure 7). Out of 70 CHIKV IgM positive serum samples, all were positive by CHIKV RT-LAMP in which 40 (57.14%) serum samples were positive within 20 cycles and remaining 30 (42.85%) serum samples were positive within 40 cycles of real-time CHIKV RT-LAMP assay. No samples were detected after 40 cycles of real-time CHIKV RT-LAMP assay.

In this study, 70 CHIKV IgM positive serum samples were identified by RT-LAMP assay (Heat treated centrifuged and uncentrifuged both were used) and the result of these positive samples were cross checked by gold standard method RT-PCR. The results that was came from using different instruments

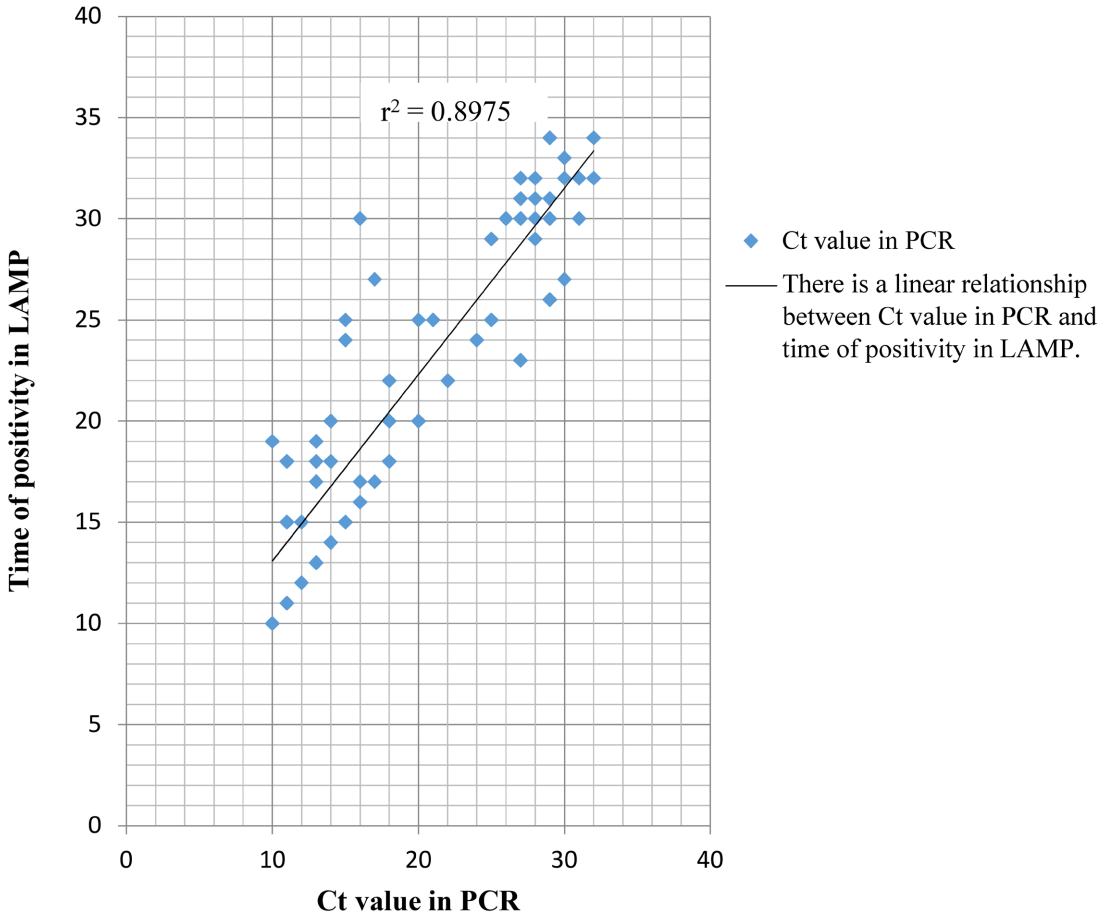

Figure 7. There is a linear relationship between Ct value in PCR and time of positivity in LAMP. Here, X-axis represents Ct value in qRT-PCR range from 0 - 40, and Y-axis represents the time of positivity in RT-LAMP range from 0 - 40. Every dot indicates one reaction.

like heat block, water bath, conventional thermal cycler and real-time thermal cycler all were compared with the result of RT-PCR. In CHIKV qRT-PCR, out of 70 samples, 67 (95.71%) samples were detected CHIKV RNA in which 20 (28.57%) serum samples were showed Ct value within 20 cycles and 47 (67.14%) serum samples showed Ct value within 35 cycles. Three serum samples (4.28%) were not detected CHIKV RNA by CHIKV qRT-PCR.

The sensitivity and specificity of CHIKV RT-LAMP was 95.71% and 100% respectively, considering qRT-PCR as a gold standard. The corresponding positive and negative predictive values were 100% and NPV was 86.95% respectively.

4. Discussion

Recently, CHIKV, a genre of the *Alphavirus* genus, is of important public health headache in Southeastern Asia and African countries. CHIKV infections are occasionally lethal and usually, patients do not need to admit to the hospital, but it is significant to identify for epidemiological studies. Expensive instruments, skilled personnel and sophisticated environment are needed in different molecular methods like PCR, Hybridization, etc. These extravagant, highly precious

instruments based test to amplify the DNA/RNA of the infectious organisms often cumbersome to adapt for routine clinical use, especially in the peripheral health care systems. Therefore, a promptly appointed and deligated test is necessary for fruitful surveillance of new moving CHIKV strains.

Nucleic Acid Amplification Tests (NAAT) is a worthy missile for the interpretation of infectious diseases. Highly precious & maintenance-based instruments are needed to amplify the DNA/RNA of the communicable diseases organism & also need handy personnel. In different studies, it has been suggested that using water bath or heat block, CHIKV RT-LAMP assay can be performed at a fixed temperature. This assay is more sensitive, simple operation, easy detection and faster to perform than other methods for detection of CHIKV [1] [10] [12]. This current assay was aimed to develop and optimize RT-LAMP assay for rapid identification of CHIKV RNA and to compare with the CHIKV qRT-qPCR.

In the current assay, the CHIKV RT-LAMP was performed using different instruments *i.e.*, heat block, water bath, conventional and real-time thermal cycler, these instruments can maintain isothermal temperature (62˚C) required for the CHIKV RT-LAMP assay. This was done to compare the effectiveness and to develop the simplest and easiest amplification and detection technique which could perform easily in resource-limited settings without the need of any expensive equipment. CHIKV RT-LAMP was performed by four different instruments in this study to observe the same efficacy. Further, heat-treated centrifuged and heat-treated uncentrifuged serum samples were compared in this study using the four different instruments of CHIKV RT-LAMP and both the forms yielded the same result.

There are several methods used for visualization of CHIKV RT-LAMP amplified products. To detect the amplified products of RT-LAMP, various colorimetric dyes (SYBR Green I, Eva Green and HNB) are used [13] [14].

In this study, four different methods were used for detection of amplified products of the RT-LAMP assay; these were 1) Naked eye observation of turbidity, 2) Watching the color change of HNB dye by open eye, 3) Observation of step-ladder band pattern in agarose gel electrophoresis and 4) Amplification curve in real-time thermal cycler. Within these four detection methods, observing the formation of turbidity due to white precipitates is a relatively simple approach to other detection methods followed by other researchers [15] [16] and [17]. This is a relatively less expensive method but it depends on visual capacity of investigators. Moreover, turbidity appears less in low-loaded samples. Besides this, interpretation power of turbidity of different people may vary. Hence, this detection method is not universally acceptable as an ideal method. Observation of color change of HNB by naked eye is a good detection technique as HNB dye is added before amplification, there is no chance of contamination and color change occurs in positive reaction from violet to blue. Gel electrophoresis had been used for detection of CHIKV RT-LAMP amplified products in ethidium bromide-stained 2% agarose gel by using gel do-

cumentation systems. Gel electrophoresis is a post-amplification method and chance of contamination is more during loading gel. In addition to this, it requires expertise, established laboratory setup and excess time for detection of amplified products. In this study, amplification was also observed in real-time thermal cycler by formation of amplification curves produced by each positive reaction; it is the most expensive amplification instrument among the four instruments that were used in RT-LAMP assay and requires expensive real-time thermal cycler and expertise. Thus, real-time LAMP is unsuitable for resource-limited settings. All CHIKV IgM +ve samples showed similar results in four detection methods.

To consider in this study, the newly discovered RT-LAMP assay is reported as it is serviceable due to its light procedure, simple reaction and quick detection. The current study elaborates the standardization and evaluation of a single step, individual tube RT-LAMP for quick and authentic time detection of CHIKV RNA in serum samples and compares with qRT-PCR. The most engaging feature of RT-LAMP is change of sample color after treating with HNB, spontaneously showed by naked eye. Another important feature is the undeniable reduction of time necessary for confirmation of results by RT-LAMP assay is only 45 minutes, compared to 3 - 4 hours in the case of RT-PCR. In this way, the CHIKV RT-LAMP assay indicated in this study allows a quick, real-time detection in symptomatic patient serum samples without expensive & sophisticated instruments and has dynamic utility for clinical diagnosis and genomic surveillance of CHIKV in rising countries.

In resources limited settings, a centrifuge machine is usually not available, therefore in present study only heat-treated uncentrifuged serum samples were used in LAMP technique which could detect all samples. It is less time-consuming, and has the same detection ability as centrifuged serum samples. This study showed that a water bath or a heat block is enough to amplify the CHIKV LAMP product. To notice the turbidity or color shift from violet to blue by use of HNB dye was a better option to visualize the CHIKV RT-LAMP amplified products. The laboratory setup for CHIKV LAMP is minimal and it can be performed by low-skilled technicians. There are limited nucleic acid amplification tests with enough plainness, vigor, and cheapest for common use at poorly resourced laboratories in flourishing countries. As observed above, LAMP is a ground-breaking gene amplification technique for utilization in resource-limited setup. This has been used in detection of other viral diseases *i.e.* Dengue serotyping, West Nile virus detection, Herpes simplex, HIV, HBV and Zika [18] [19]. It functions as a speedy and easy diagnostic tool for rapid detection and identification of CHIKV RNA, and simple to operate, ability to amplify nucleic acids under isothermal conditions without thermal cycler, and easily identified by open eye. Thus, CHIKV RT-LAMP assay may be appointed as the new diagnostic tool for punctual and proper diagnosis in clinical care, and genomic surveillance in resource-limited countries, such as Bangladesh.

5. Study Limitation

There was the limitation of the fund. As this was a thesis work, time limitation is an issue for this study.

6. Conclusion

This study was theory & evidence-based that can be used in the detection of abundant infectious diseases in narrow-resourced countries.

7. Recommendation

If there was an available fund, then sequencing is another option for confirming this test.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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