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MOLECULAR DETECTION OF CHIKUNGUNYA VIRUS TARGETING THE IMMUNODOMINANT ENVELOPE (E1) GENE: CURRENT STATUS AND FUTURE APPLICATIONS

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ABSTRACT

A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for detecting the E1 gene of Chikungunya virus (CHIKV). The RT-LAMP assay is a novel method of gene amplification that amplifies nucleic acid with high specificity, efficiency, and rapidity under isothermal conditions with a set of six specially designed primers that recognize eight distinct sequences of the target. The whole procedure is very simple and rapid, and amplification can be obtained in less than 1 h by incubating all of the reagents in a single tube with reverse transcriptase and *Bst* DNA polymerase at 63°C. Detection of gene amplification could be accomplished by agarose gel electrophoresis; it was found that the RT-LAMP assay demonstrated 10-fold higher sensitivity compared to RT-PCR, with a detection limit of 0.1 PFU of virus. Thus, due to easy operation without a requirement of sophisticated equipment and skilled personnel, the RT-LAMP assay reported here is a valuable tool for the rapid and real-time detection of CHIKV.

KEYWORDS

Chikungunya virus (CHIKV), Reverse transcription loop-mediated isothermal amplification (RT-LAMP), Reverse transcription Polymerase chain reaction (RT-PCR), *Bacillus stearothermophilus (Bst)*, Plaque Forming Units (PFU).

INTRODUCTION

The resurgence of Chikungunya (CHIK) in the Indian Ocean Islands and India has drawn worldwide attention due to its explosive nature, high morbidity and complex clinic pathological manifestations. The disease, caused by the chikungunya virus (CHIKV) has already involved in many outbreaks in Africa and Asia ever since its discovery in 1952 [1]. CHIK produces a dengue-like illness in humans, characterized by fever, rash, and severe arthralgia persisting for a few weeks to several months. The most significant characteristic of CHIK is the prolonged arthralgic syndrome that primarily affects the peripheral small joints associated with excruciating pain [2]. The disease is generally non-fatal and the acute phase resolves within 3–4 days leaving the arthralgic syndrome persisting for some more time. Man gets

infection through the bite of infected *Aedes* mosquitoes mainly *Aedes aegypti*, the incriminated vector of CHIKV [3]. Comparing the earlier outbreaks, the recent episode was massive, spread at a fast pace to wider areas causing serious economic and social impact. Symptoms and complications uncharacteristic of CHIK including deaths were reported [4].

Currently, the detection of most alphaviruses is dependent on virus isolation from the blood of viremic patients, infected tissues, or blood-feeding arthropods, which is time-consuming. The alphavirus species can be characterized by hemagglutination inhibition, enzyme-linked immunosorbent assay (ELISA), complement fixation, and neutralization of viral infectivity using reference sera [5]. Although CHIKV could be considered a reemerging threat, a few specific serological or molecular diagnosis tools are available. To date, only conventional reverse transcription (RT)-PCR methods have been suggested for the study of CHIKV replication in supernatants and clinical samples or for epidemiological survey [6]. These assays have proven to be sensitive and specific but, unfortunately, are often in a (semi-) nested format or require specific detection methods, which poses serious hazards for amplification product carryover. In addition, they still require time-consuming sample handling and post-PCR handling and are therefore often difficult to implement in a routine diagnostic setting.

The RT-LAMP assay is a novel approach to nucleic acid amplification and is based on the principle of a strand displacement reaction and stem loop structure that amplifies the target with high degrees of specificity and selectivity and with rapidity under isothermal conditions, thereby obviating the need for the use of a thermal cycler [7, 8]. The present study describes the standardization and evaluation of RT-LAMP assay for rapid detection of CHIKV in clinical specimens by targeting the E1gene. Thus, the RT-LAMP assay allows rapid detection of CHIKV in acute-phase serum samples without requiring sophisticated equipment and has potential usefulness for clinical diagnosis and surveillance of CHIKV in developing countries.

MATERIALS AND METHODS

Cell culture and Viruses

C6/36 mosquito cell line was maintained at 37°C under 5% CO_2 by regular sub culturing at periodic intervals of 4 to 5 days in Mitsuhashi and Maramorosch's medium. CHIKV are grown in <48 hours old suckling mice and also in C6/36 mosquito cell culture was used as viral antigen positive standard in the assay systems employed in the present study. Briefly, the monolayer of C6/36 cells (Fig 1.a) grown in 25-cm^2 culture flask was adsorbed with 0.5 ml of the inoculum at 37°C for 2 h. Following adsorption, the inoculum was replenished with 10 ml of maintenance medium supplemented with 2% fetal bovine serum. Suitable mock-infected cell controls were also kept. The cells were then incubated at 37°C and observed daily for cytopathic effects (Fig 1.b). Upon observation of 80 to 100% cytopathic effects, the infected culture supernatant was clarified by light centrifugation at 2,000 rpm for 10 min and further purified by sucrose density gradient ultracentrifugation according to standard protocols. The brain suspensions from mice infected with the virus were the source of antigen (Fig 2). Approval for use of mice for antigen preparation was obtained from the institutional ethical committee according to national guidelines.

Microscopic observation of C6/36 cell line

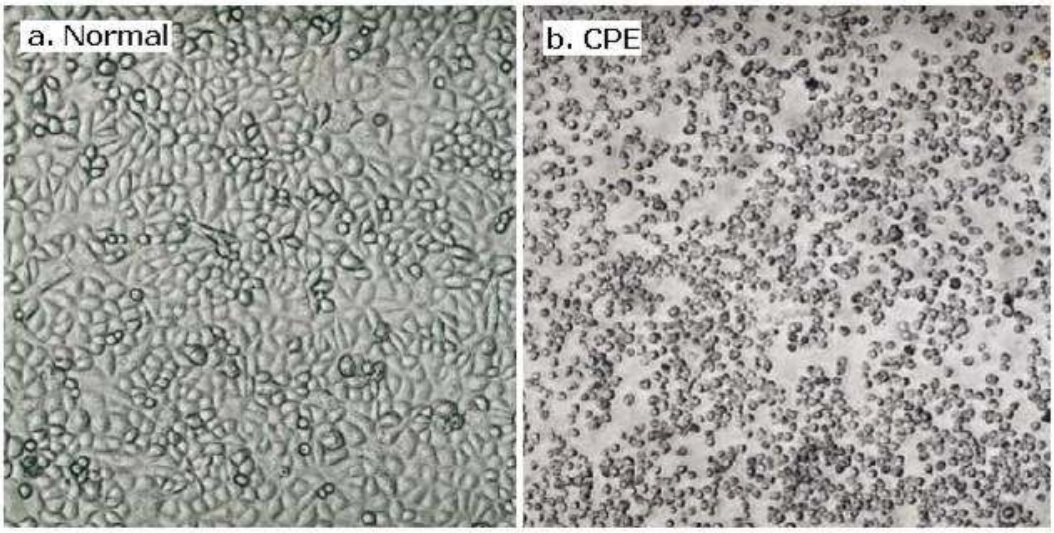

Figure 1 a
Normal healthy appearance of C6/36 control,

1 b
Cyto Pathic Effect on C6/36 cell control

Figure 2
Inoculation of viral antigen into the mice brain

Clinical Specimens

A total of 40 acute-phase serum samples received from patients with a clinical diagnosis of Chikungunya fever were used for evaluation in this study. The acute-phase samples were collected during the period between 1 and 7 days after the onset of symptoms. All the samples were stored at -80°C until further investigation. In addition, a panel of 10 serum samples collected from healthy individuals was also included as negative controls.

Virus titration and isolation

The haemagglutination activity of the arbovirus is pH dependent. Therefore it is necessary to titrate the antigen at different pH. This is useful to determine the pH for the antigen dilution to be used [9]. The antigen was vortexed and 100µl added in the first well as marked in the plate. Two-fold dilution was made by transferring 50µl from the first well of each column. The final 50µl is then discarded. 50µl of 0.4% Red Blood Cells (RBC) suspension prepared in the respective VAD to the corresponding well was added. First add to the control wells and then to the antigen containing well. Manually it was mixed by agitating the plates thoroughly. It was incubated at 37^0C for 1 h.

RNA extraction and RT-PCR

The genomic viral RNA was extracted from 100 µl of patient serum samples by using the QIA amp Viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's protocol. The RNA was eluted from the QIA spin columns in a final volume of 80 µl of elution buffer and was stored at -70°C until use. In order to compare the sensitivity and specificity of the RT-LAMP assay, one-step RT-PCR was performed by employing the two outer primer pairs targeting the E1 gene of CHIKV (CHIK-F3 [ACGCAATTGAGCGAAGCAC] [genome positions 10294 to 10312] and CHIK-B3 [CTGAAGACATTGGCCCCAC] [genome positions 10498 to 10480]).

Amplification reaction

The amplification was carried out in a 50µl total reaction volume by using an Ambion RT-PCR kit with 50pmol of forward and reverse primers and 2 µl of RNA according to the manufacturer's protocol. The thermal profile of RT-PCR was 48°C for 45 min and 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s and a final extension cycle at 72°C for 10 min.

Analysis of PCR amplified products

Reference strains (10 µl) of the amplification reactions were mixed with 2 µl electrophoresis sample buffer (0.25% xylene cyanol FF, 40% sucrose) and loaded onto non denaturing polyacrylamide gels [20 X 20 X 0.15 cm; 12.5% polyacrylamide (30:1 acrylamide : bis-acrylamide), 1 X TBE, pH 8.2]. After electrophoresis (10V/cm, 2 h), gels were soaked in 5 µg/ml Ethidium bromide (5 min) and rinsed twice in dH_2O, and the fluorescent DNA bands were visualized on a transilluminator (λ=310nm).

CHIKV reverse transcription-LAMP

The oligonucleotide primers used for RT-LAMP amplification of CHIKV were designed from the structural (E1) gene. The potential target region of 205 bp corresponding to the genome positions 10294 to 10498 was selected from the aligned sequences, and RT-LAMP primers were designed [10]. A set of six primers comprising two outer, two inner, and two loop primers that recognize eight distinct regions on the target sequence (Table 1) was designed by employing the LAMP primer-designing support software program (Primer Explorer 4, Eiken, Japan).

Table 1
Primer positions in the Genomic sequences

PRIMERS	PRIMER SEQUENCE	LENGTH OF BASE PAIRS
Forward outer (F3)	ACGCAATTGAGCGAAGCAC	19
Backward outer (B3)	CTGAAGACATTGGCCCCAC	19
Forward inner primer (FIP)	CGGATGCGGTATGAGCCCTGTA-TGGAGAAGTCCGAATCATGC	42
Backward inner primer (BIP)	TCCGCGTCCTTTACCAAGGAAAT-TTGGCGTCCTTAACTGTGAC	43
Forward loop primer (FLP)	GCTGATGCAAATTCTGT	17
Backward loop primer (BLP)	CCTATGCAAACGGCGAC	17

RT-LAMP

RT-LAMP was carried out in a final reaction volume of 25µl using the Loopamp RNA Amplification kit (Eiken Chemical Co., Ltd. Tokyo, Japan) with 5 pmol each of the primers F3 and B3, 20 pmol each of the primers Loop-F and Loop-B, 40 pmol each of the primers Forward Inner Primer (FIP) and Backward Inner Primer (BIP). 5µl of the extracted RNA was used as template per reaction. For real-time monitoring, the RT-LAMP reactions were incubated at 61 ºC for 60 min on a LA-200 Loopamp Real-time Turbidimeter (Teramecs, Japan) and inactivated at 80 ºC for 5 min. Negative controls were included in each run of the assay.

Agarose gel analysis

5µl of the RT-LAMP products were electrophoresed on a 1.5% molecular grade agarose gel prepared in 0.5 X Tris-borate EDTA buffer stained with 0.5 µg/ml ethidium bromide. The amplification products were visualized using a transilluminator with ultraviolet light at 302 nm.

Naked eye visualization

In order to facilitate the field application of the RT-LAMP assay, the monitoring of RT-LAMP amplification was also carried out with inspection by the naked eye. Following amplification, the tubes were inspected for white turbidity using the naked eye after a pulse spin to deposit the precipitate in the bottom of the tube. The inspection for amplification was also performed through observations of color change following the addition of 1 µl of SYBR Green I dye to the tube. In the case of positive amplification, the original orange color of the dye would change into green that can be judged under natural light as well as under UV light (302 nm) with the help of a hand-held UV torch lamp. In case there is no amplification, the original orange color of the dye would be retained. This change of color is permanent and thus can be kept for record purposes.

RESULTS

Chikungunya virus has been responsible for causing a major outbreak which left many in a debilitated state though not fatal. It is extremely important to identify this virus which can be used in epidemiological studies. The detection of this virus is usually done by ELISA or RT-PCR from the serum sample. Conventional RT-PCR methods have been suggested for the study of CHIKV replication in supernatants and serum samples for general sero surveillance, during an outbreak or epidemiological surveys. Though this produces a high amplification this requires very precise and expensive

equipments and an elaborate method for amplification. Moreover this cannot be used in rural areas due to the unavailability of a thermocycler to carry out the test. Thus designing of a diagnostic method that is feasible in any locations is a necessity and being cost effective and less cumbersome would be an added advantage to this method.

***Hemagglutination test for Antigen obtained from mice brain*:** Haemagglutination was performed after every passage of mice inoculation. The titre obtained after the first passage showed a titre of 1:2 indicating that the viral load during inoculation had been less than 1 HA unit as the virus used was freeze dried and preserved for long. The titre from the next passage showed an increase in titre indicating the active multiplication of the virus its adaptability to the cell line. The third passage showed the highest concentration of the antigen an indication that the virus had been well adapted to the mouse brain culture and had been harvested at the right time and antigen was extracted properly (Fig 3.a). Though the titre obtained was high the protocol was cumbersome, first to say that daily mice observation was must and sometimes the onset of symptoms was rapid and the mother eats off the sick mice. Sometimes it was seen that the mice had died and the antigen extracted from their brains did not yield good titre.

***Haemagglutination test for virus from Tissue culture*:** As the protocol of antigen extraction from mouse brain was cumbersome and had many drawbacks including that of the low final volume of antigen it was attempted to raise the virus in cell lines. The passages in cell lines yielded fewer antigens in the beginning but as the numbers of passages were increased the titre of antigen obtained was also high. After 5 passages in the cell line we were able to procure 15 ml of virus suspension of 1:32 concentration but though the 3 passage in mouse brain yielded 1:32 concentration the volume obtained was only 2 ml (Fig 3.b). Therefore the virus can be raised in cell lines itself as it is less cumbersome and yields more quantity of antigen which will be of much use during the outbreaks, when there is a spurt of cases and number of tests to be performed increases manifolds.

The haemagglutination plate showing the titre value of Chikungunya virus

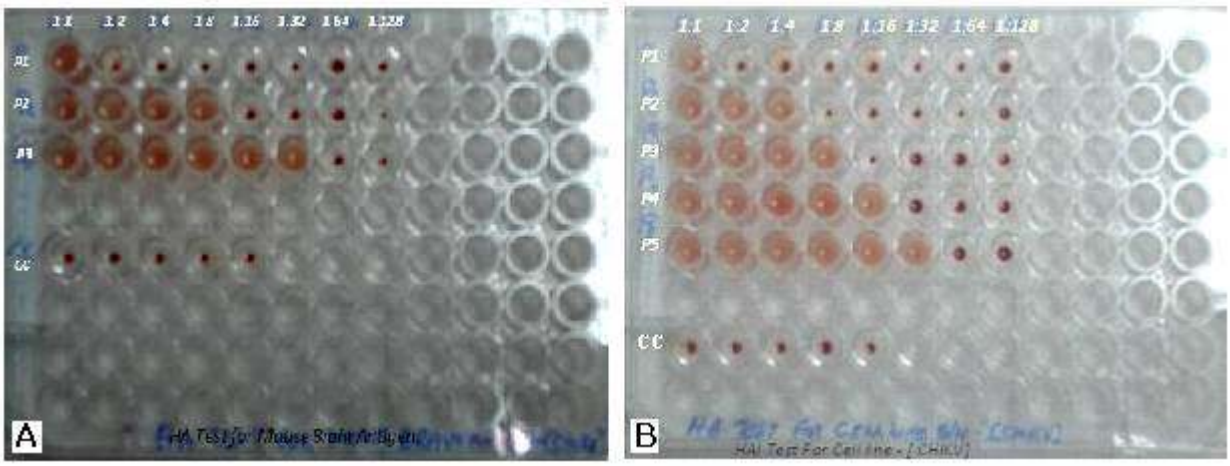

Figure 3 a
Chikungunya virus obtained during each passage in tissue culture;
Figure 3 b
Chikungunya antigen obtained during each passage in mice inoculation.

CHIKV from various sources: CHIKV are grown in Mice brain and also in Cell cultures. The presence of virus in the serum in the early stages of illness helps us in the early detection of the disease by RT-PCR. Therefore it is necessary to have standardized protocols for the detection of the viruses from various samples.

RT-PCR for Mouse Brain: RNA was extracted from the sick mouse brain and different concentration of the template was used to standardize the RT-PCR. The results showed that the concentration of 25ng of RNA was picked up efficiently and reproducibly by RT-PCR. The lower concentrations showed faint band and higher concentrations showed smudgy or more than one band. The presence of high viral content is evidenced by the effect that just 25ng of the nucleic acid is picked up by RT-PCR (Fig 4.a).

***RT-PCR for Cell Culture*:** The titre in the cell culture was equal to the mouse brain antigen in the fifth passage, but even though RT-PCR was able to pick up the nucleic acid only at the 50ng level. The reason attributing to this may be the increased amount of RNA from the cell source contaminating the viral RNA (Fig 4.b).

RT-PCR for Serum: 50ng of RNA in the serum sample was detected by RT-PCR, once again here an increased concentration of the nucleic acid is required when compared with the mouse brain this may be due to the presence of some interfering agents as seen in the cell line extract. Moreover the mouse brain extract when was tried as such did not yield results at all but when it was filtered and the purified form was extracted we obtained results, similarly centrifuging the cell culture fluid and filtering the serum will help us to get a more purified form of CHIKV RNA (Fig 4.c).

Agarose gel shows different concentration of RNA from mice brain, cell culture and serum

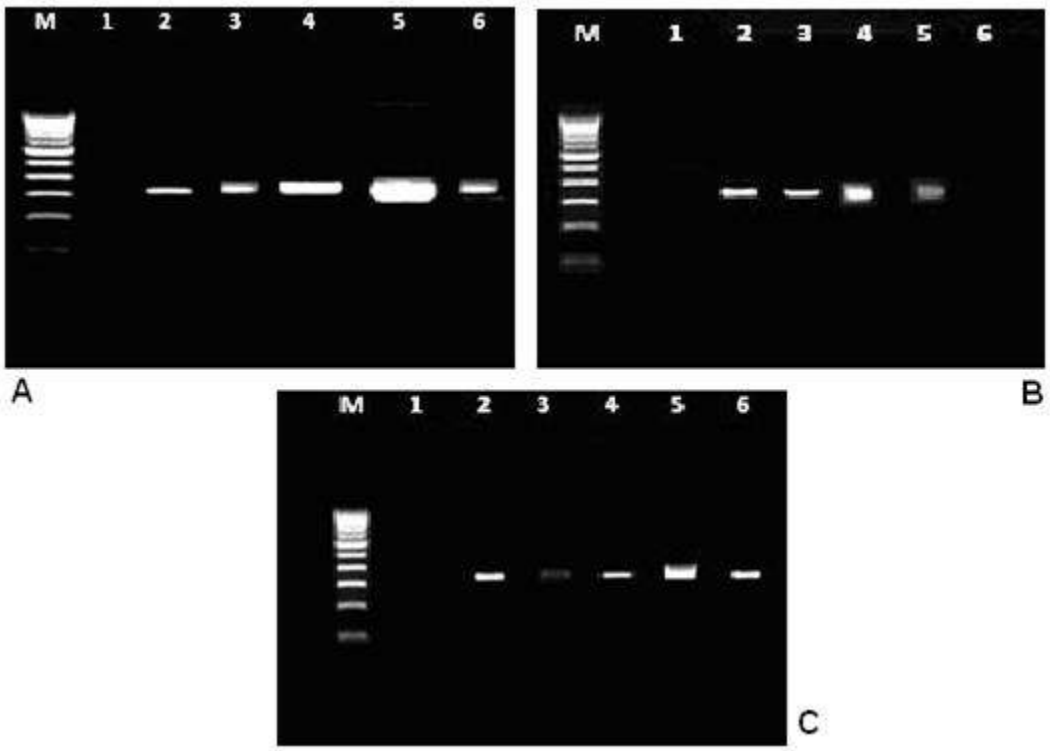

Figure 4

Lane M- Marker; Lane 1 – Negative control and lanes 2 to 6 are different concentration of RNA obtained from mice brain, cell culture and serum respectively.

RT-LAMP assay was standardized for the rapid detection of CHIKV by targeting the highly conserved regions of the E1 gene based on multiple sequence alignments of all the circulating strains. The details of the each primer with regard to their positions in the genomic sequences are shown in Table 1. The applicability of the RT-LAMP assay for the clinical diagnosis of CHIKV was validated with acute-phase serum samples from the ongoing epidemic of CHIKV in India. The results were compared with those from RT-PCR. A total of 50 samples comprised of 40 acute phase serum samples and 10 negative serum samples were used in this study for comparative evaluation. None of the RT-PCR-positive samples were missed by RT-LAMP, thereby indicating a higher sensitivity of the RT-LAMP assay. All 10 healthy serum samples were also negative by both the tests, thereby ruling out the possibility of false positivity and thus establishing the specificity of the selected primer sets for the CHIKV RT-LAMP assay. The RT-LAMP assay also picked up more positive samples than RT-PCR, virus isolation, and haemagglutination test (Fig 5).

CHIKV RT-LAMP assay as monitored by agarose gel analysis from different sources

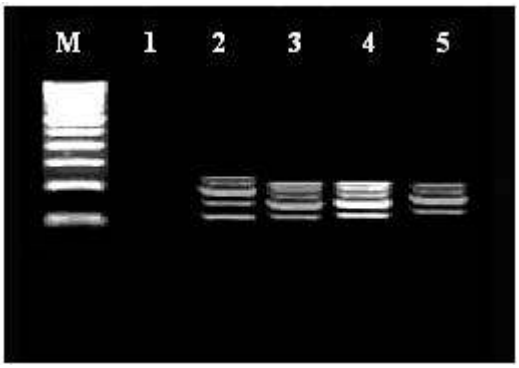

Figure 5
Lane 1-negative control; lane 2-5 from serum; mice brain; viral antigen and cell culture respectively.

The field applicability of the RT-LAMP assay was also validated by employing a SYBR Green I-mediated naked-eye visualization test. Following incubation at 63°C for 30 min in a water bath, the monitoring of RT-LAMP amplification was accomplished through visualization by the naked eye with the addition of 1 µl of SYBR Green I (1:1,000) dye to the amplified products (Fig 6). The comparative evaluation of this field-based, SYBR Green I-based RT-LAMP assay with 25 clinical samples randomly selected from the above-described 50 samples revealed good concordance with RT-PCR.

Naked eye visualization of SYBR Green

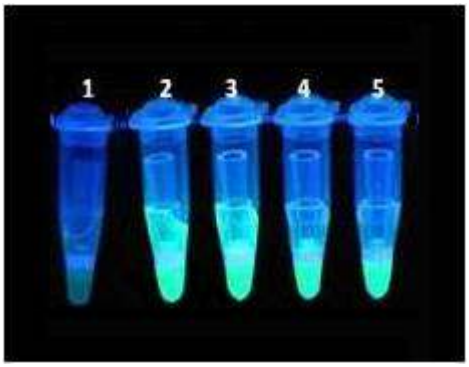

Figure 6
Tubes 2 to 5 are positive samples and tube 1 is negative control having no template.

DISCUSSION

Chikungunya (CHIK), a mosquito borne debilitating disease, is caused by CHIK virus, an alphavirus belonging to the family Togaviridae. The sudden onset of very high fever along with rash, and severe arthralgia especially in the small joints of hands and toes are the characteristics of the disease. It was first reported from Tanzania in 1952-53 and spread subsequently to sub-Saharan Africa, South East Asia and Pacific causing large epidemics. The virus exists in three genotypes, the Asian, West African and East Central South African that are responsible for outbreaks in the respective areas. The first outbreak in Asia was in Bangkok in 1958 followed by other Asian countries. India experienced massive outbreaks of CHIK in the 1960s and early 70s mainly in cities. After a gap of 32 years an explosive outbreak of CHIK devastated the country affecting more than 1.4 million people in 13 states. The epidemic also witnessed many unusual clinic-pathological complications including CHIK associated deaths and mother to child transmission. High morbidity with severe arthralgia persisted for several months made the people mentally and physically weak.

CHIKV produces an illness in humans that is often characterized by a sudden onset of fever, headache, fatigue, nausea, vomiting, rash, myalgia, and severe arthralgia. Polyarthralgia, the typical clinical sign of the disease, is very painful. Symptoms are generally self-limiting and last 1 to 10 days. However, arthralgia may persist for months or years. In some patients, minor hemorrhagic signs such as epistaxis or gingivorrhagia have also been described [11]. These clinical symptoms mimic those of dengue fever, and therefore, many cases of Chikungunya fever are misdiagnosed as dengue virus infections.

Since no effective vaccines or therapeutics are available, early detection and proper diagnosis plays the key role in the effective control of the infection. Infant mice inoculation and serological techniques [Haemagglutination, Haemagglutination Inhibition assay, complement fixation and neutralization test (NT)] were used effectively in the identification and characterization of viruses [9, 12]. The development of immunoglobulin M antibody (IgM) capture enzyme linked immunosorbent assay (MAC-ELISA) has been a major achievement in serology as it provided a rapid and reliable technique for the diagnosis of arboviruses [13]. Indirect immunofluorescent antibody technique is another reliable technique for detection and identification of viral antigens from clinical samples [14, 15]. In the event of a viral outbreak, the situation warrants rapid detection and identification of the etiological agent. The molecular devices therefore become handy for the detection and characterization. Reverse

transcription polymerase chain reaction (RT-PCR) using primers designed for structural and non-structural domains has been found useful in the rapid diagnosis of CHIKV [16]. The combination of RT-PCR/nested PCR has proved efficient for specific detection and genotyping of CHIKV [6, 17]. Recently, real time RT-PCR, has revolutionized the field with its unique advantages i.e. rapidity, sensitivity, reproducibility and reduced risk of contamination and is being routinely used for detection and quantitation of viruses [13].

The real-time PCR assay has many advantages over conventional RT-PCR methods, including rapidity, the ability to obtain quantitative measurements, a lower contamination rate, a higher sensitivity, a higher specificity, and easy standardization. Thus, nucleic acid-based assays or real-time quantitative assays might eventually replace virus isolation and conventional RT-PCR as the new gold standard for the rapid diagnosis of virus infection in acute-phase serum samples. However, all these nucleic acid amplification methods have several intrinsic disadvantages, in that they require either a high-precision instrument for amplification or an elaborate, complicated method for detection of amplified products. The high costs of the instruments required to perform the real-time assays restricted their use to laboratories with good financial resources. The aim of this study was to develop a rapid, sensitive, and specific real-time method to detect and quantify CHIKV in acute-phase patient serum samples.

The recent invention of Loop-mediated isothermal Amplification (LAMP) provides a new alternative for RT-PCR diagnosis. LAMP amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem structure. In subsequent LAMP cycling one inner primer hybridizes to the product and initiates displacement DNA synthesis, yielding the original stem DNA and a new stem-loop DNA with a stem twice as long.

The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeat of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity [18, 19].

The amplification efficiency of the RT-LAMP method is extremely high due to continuous amplification under isothermal conditions, which results in the production of a large amount of target DNA as well as a large amount of the by-product magnesium pyrophosphate, which leads to turbidity [20]. Therefore, quantitative detection of gene amplification is possible by real-time monitoring of the turbidity in an inexpensive photometer. In addition, the higher amplification efficiency of the RT-LAMP method enables simple visual observation of amplification with the naked eye under a UV lamp in the presence of an intercalating dye, such as SYBR Green I or ethidium bromide. Thus, the RT-LAMP assay has emerged as a powerful gene amplification technique for rapid identification of microbial infections [21, 22, 23]. In the present study, the RT-LAMP assay was standardized by targeting the immunodominant envelope (E1) gene for rapid detection of CHIK virus.

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