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Published Ahead of Print 29 November 2006.

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Rapid and Real-Time Detection of Chikungunya Virus by Reverse Transcription Loop-Mediated Isothermal Amplification Assay


Division of Virology, Defence Research and Development Establishment, Gwalior 474002 M. P., India; Department of Microbiology, Nizam’s Institute of Medical Sciences, Hyderabad 500 082 A. P., India; and Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Received 22 August 2006/Returned for modification 23 October 2006/Accepted 8 November 2006

Chikungunya fever is an acute arthropod-borne viral illness reported in many parts of Africa, Southeast Asia, the Western Pacific, and India. The causative agent, Chikungunya virus (CHIKV), is a single-stranded positive RNA-enveloped virus that is a member of the genus Alphavirus of the Togaviridae family and is transmitted from primates to humans generally by Aedes aegypti (6, 8). CHIKV is classified serologically as a member of the Semliki Forest antigenic complex, closely related to O’nyong-nyong virus because of its cross-reactivity (7, 22). Because humans act as very efficient reservoirs for the virus, Chikungunya virus is most prevalent in urban areas, and epidemics are sustained by the human-mosquito-human transmission cycle.

The first recognized outbreak of Chikungunya virus occurred in East Africa (Tanzania and Uganda) in 1952 and 1953. Soon thereafter, the virus has been observed in Austral Africa (Zimbabwe and South Africa), in West Africa (Senegal and Nigeria), and in Central Africa (Central African Republic and Democratic Republic of the Congo) (6, 11, 19). Since the first documented Asian outbreak in 1958 in Bangkok, outbreaks in Thailand, Cambodia, Vietnam, Laos, Myanmar, Malaysia, the Philippines, and Indonesia have been documented. The most recent epidemic reemergence was documented in 2001 to 2003 in Java, after 20 years (6, 9). In both Africa and Asia, the reemergence was unpredictable, with intervals of 7 to 8 years to 20 years between consecutive epidemics. Since 2003, there have been outbreaks in the islands of the Pacific Ocean including Madagascar, Comoros, Mauritius, and Reunion Island, with a surge in the number of cases after the tsunami of December 2004. Starting in December 2005, the rainy season gave rise to a renewed epidemic circulation of the virus. Since January 2006, several thousand cases each were reported in Mayotte, Mauritius, and the Seychelles Islands (24). More recently, circulation of the virus has also been documented in Madagascar and India.

In India, CHIKV was first reported in 1963 in Kolkata, and subsequently, the virus was isolated from numerous well-documented outbreaks from 1963 to 1973. Although no major outbreak was reported in India since the 1973 outbreak in Barsi, Maharashtra, however, the virus was isolated from sporadic cases in humans and mosquitoes during random surveillance and dengue virus outbreaks (13, 21). The recent outbreak of Chikungunya virus infection of an unprecedented magnitude in many parts of Southern India is a point of major concern. This is the largest and most severe epidemic, affecting more than 1,000,000 persons in the Andhra Pradesh, Maharashtra, and Karnataka states of India, and is spreading to several new areas, causing huge public health and administrative concerns for management of the situation (23).

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 gingival hemorrhage have also been described (6, 11). These clinical symptoms mimic those of dengue fever, and therefore, many cases of Chikungunya fever are misdiagnosed as dengue virus infections. At present, there is no vaccine or antiviral therapy available against Chikungunya infection.

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 (4, 6). 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 (2, 5). 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.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method developed by Eiken Chemical Co., Ltd., Japan, and has the potential to replace PCR because of its simplicity, rapidity, specificity, and cost-effectiveness (12, 14, 15). The RT-LAMP assay has emerged as a powerful gene amplification tool for the rapid identification of microbial infections and is being increasingly used by various investigators for rapid detection and typing of emerging viruses, viz, West Nile virus, severe acute respiratory syndrome, dengue virus, Japanese encephalitis (JE) virus, etc. (3, 16, 17). The LAMP method is cost-effective, as it requires only one type of DNA polymerase with strand displacement activity. In the present study, a one-step, single-tube, real-time, accelerated RT-LAMP assay was standardized by targeting the immunodominant envelope (E1) gene for rapid and real-time detection of CHIK virus. The data on the sensitivity and specificity of the method are discussed, and the applicability of the technology for clinical diagnosis of CHIK patients is validated by evaluation with acute-phase serum samples collected from the ongoing epidemic in Southern India.

### MATERIALS AND METHODS

#### Design of CHIK virus-specific RT-LAMP primers.

The oligonucleotide primers used for RT-LAMP amplification of CHIKV were designed from the structural (E1) gene. The nucleotide sequence of the E1 gene of the S-27 African CHIKV prototype strain was retrieved from GenBank (accession no. AF369024) and was aligned with the available E1 gene sequences of other strains, including the circulating strains in India responsible for recent epidemics, to identify the conserved regions using DNASIS software. 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. A set of six primers comprising two outer, two inner, and two loop primers that recognize eight distinct regions on the target sequence was designed by employing the LAMP primer-designing support software program (Net Laboratory, Japan [http://venus.netlaboratory.com]).

The primers were selected based on criteria described previously by Notomi et al. (12). The two outer primers were described as being forward outer primer (F3) and backward outer primer (B3). The inner primers were described as being forward inner primer (FIP) and backward inner primer (BIP). Furthermore, two loop primers, viz, forward loop primer (FLP) and backward loop primer (BLP), were designed to accelerate the amplification reaction. FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a complementary sequence of B1 and a sense sequence of B2. FIP and BIP were high-performance liquid chromatography-purified primers. The FLP and BLP primers were composed of the sequences that are complementary to the sequence between the F1 and F2 and B1 and B2 regions, respectively. The details of the each primer with regard to their positions in the genomic sequences are shown in Table 1.

#### Clinical samples.

A total of 45 acute-phase serum samples collected from patients with a clinical diagnosis of Chikungunya fever who were admitted to Nizam’s Institute of Medical Sciences, Hyderabad, AP, District Hospital, Chittoor, AP, and Government Hospital, Hiriyur, Karnataka, were used for evaluation in this study. The acute-phase samples were collected during the period between days 1 and 7 after the onset of symptoms. All the samples were transported to the laboratory under conditions of a strict cold chain and stored at −80°C until further investigation. In order to check the cross-reaction, 24 confirmed dengue virus patient sera were also included due to similar clinical signs and symptoms. In addition, a panel of 20 serum samples collected from healthy individuals was also included as negative controls. Prior to RT-LAMP assay, all these 89 samples were also investigated for the presence of CHIKV-specific RNA by RT-PCR and immunoglobulin M (IgM) and IgG antibodies by using an in-house dipstick ELISA (2, 18).

#### Cell culture.

BHK-21 cells obtained from the National Center for Cell Science, Pune, was maintained in the laboratory at 37°C under 5% CO2 by regular subculturing at periodic intervals of 3 to 4 days in Eagle’s minimum essential medium (Sigma) supplemented with 2% tryptose phosphate broth (Difco), 10% fetal bovine serum (Sigma), 3% t-glutamine (Sigma), and gentamicin (80 mg/liter) (Nicholas Piramal, India).

**Virus.** CHIKV African prototype strain S27, obtained from the National Institute of Virology, Pune, India, was used as viral antigen/positive standard in the assay systems employed in the present study. Briefly, the monolayer of BHK-21 cells grown in 25-cm2 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 incu-

### TABLE 1. RT-LAMP E1 gene primer sets designed for rapid and real-time detection of Chikungunya virus

<table>
<thead>
<tr>
<th>Primer</th>
<th>Genome positiona</th>
<th>Length of oligonucleotide (bp)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward outer (F3)</td>
<td>10294–10312</td>
<td>19</td>
<td>ACGCAAATTGGAGCCGAC</td>
</tr>
<tr>
<td>Backward outer (B3)</td>
<td>10498–10480</td>
<td>19</td>
<td>CTGAAAGACATTTGACCCCAC</td>
</tr>
<tr>
<td>Forward inner primer (F1c + TTTT + F2)</td>
<td>10378–10357 (F1c)</td>
<td>22</td>
<td>CCGATGCGGTATGAGCCCTGTA</td>
</tr>
<tr>
<td>Backward inner primer (B1 + TTTT + B2c)</td>
<td>10316–10335 (F2)</td>
<td>20</td>
<td>TGGAGAAGTCCGAATCATGC</td>
</tr>
<tr>
<td>Forward loop primer (FLP)</td>
<td>10391–10413 (B1)</td>
<td>23</td>
<td>TCCCGTCCTTACCAAGGAAT</td>
</tr>
<tr>
<td>Backward loop primer (BLP)</td>
<td>10472–10453 (B2c)</td>
<td>20</td>
<td>TTGGCGTCCTTAACTGTTGAC</td>
</tr>
</tbody>
</table>

* a S27 (African prototype strain) (GenBank accession no. AF369024).

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bated at 37°C and observed daily for cytopathic effects. 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 (1).

RNA extraction. The genomic viral RNA was extracted from 100 μl of patient serum samples by using the QIAamp Viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's protocol. The RNA was eluted from the QIAamp columns in a final volume of 50 μl of elution buffer and was stored at −70°C until use.

RT-PCR. 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 [ACGGCAATTTAGCGAAGCAC] [genome positions 10294 to 10312] and CHIK-B3 [CTGAAGACATTGGCCAC] [genome positions 10498 to 10480]). The amplification was carried out in a 50-μl total reaction volume by using a Promega Access Quick One-Step RT-PCR kit with 50 pmol 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.

Plasmid construction. In order to check the detection limit of RT-LAMP and RT-PCR assays, the targeted region of the E1 gene was amplified by RT-PCR using forward outer (F3) and backward outer (B3) primers as described above. The amplicon was then purified using a QIAQuick gel extraction kit and cloned into the pCR4-TOPO vector system (Invitrogen) according to the manufacturer's specifications. The standard control plasmid with insert was confirmed by sequencing. The sequence-confirmed plasmid was linearized using a vector-specific restriction enzyme (PstI) and then quantified by Quantity One software (Bio-Rad). The concentration of the plasmid preparation was determined by measuring the optical density (OD) at 260 nm. Conversion to genome equivalents was calculated with 1 OD at 260 nm equaling 50 ng/ml and 1 bp equaling 660 ng/ml, resulting in a molecular weight for the plasmid (4,156 bp) of 2.74 × 10^6 pg/μl. The copy numbers/μl were determined by using the following formula: copies/μl = concentration of plasmid in pg per μl (plasmid length 660) × 6.022 × 10^23. A further serial 10-fold dilution of the linearized plasmid was used for the detection of sensitivity and for the construction of a standard curve using the cycle threshold values obtained against the known concentration of the cloned target plasmid constructs.

RT-LAMP. The RT-LAMP reaction was carried out in a total 25-μl reaction volume using the Loopamp RNA amplification kit (Eiken Chemical Co., Ltd., Japan) containing 50 pmol each of the primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 25 pmol each of loop primers F and B, 1.4 mM deoxyribonucleoside triphosphates, 0.5 M betaine, 0.1% Tween 20, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 8 units of Bst DNA polymerase (New England Biolabs), 0.625 units of AMV reverse transcriptase (Invitrogen), and 2 μl of RNA template. The real-time monitoring of the RT-LAMP assay was accomplished by incubating the reaction mixture at 63°C for 10 min in a Loopamp real-time turbidimeter (LA-200; Teramecs, Japan). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Real-time monitoring of RT-LAMP amplification. (i) Real-time monitoring. The real-time monitoring of the RT-LAMP amplification of the CHIKV template was observed through spectrophotometric analysis by recording the OD at 400 nm every 6 s with the help of a Loopamp real-time turbidimeter (LA-200; Teramecs, Japan). The cutoff value for positivity by real-time RT-LAMP assay was determined by taking into account the positivity threshold (Tp) at which the turbidity increases above the threshold value fixed at 0.1, which is two times more than average turbidity value of the negative controls of several replicates.

(ii) Agarose gel analysis. Following incubation at 63°C for 60 min, 10-μl aliquots of RT-LAMP products were electrophoresed on a 3% NuSieve 3:1 agarose gel (BMA, Rockland, ME) in Tris-borate buffer followed by staining with ethidium bromide and visualization on a UV transilluminator at 302 nm.

(iii) Visualization by the naked eye. 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
A one-step, single-tube, real-time, accelerated, quantitative 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 detection of gene amplification is accomplished by real-time monitoring of turbidity at 63°C. The result indicated that the minimum time required for the initiation of amplification was 10 min with viral RNA preparations. It was also observed that there is continuous amplification of the target sequence as revealed through increased turbidity compared to the negative control having no template, wherein the turbidity got fixed around 0.01, well below the threshold value. None of the positive samples tested over multiple times showed positivity in terms of increased turbidity after 45 min. Therefore, a sample with a Tp value of ≤45 min and turbidity above the threshold value of ≥0.1 was considered positive.

Sensitivity and specificity of RT-LAMP. The sensitivity of the RT-LAMP assay for the detection of CHIKV RNA was determined by testing serial 10-fold dilutions of a cloned target that had previously been quantified through copy number determinations and compared with that of conventional RT-PCR. A linear relationship between the amount of input template copy number and Tp (in minutes) over a range of 2 × 10^6 to 2 × 10^7 copy numbers was obtained. The RT-LAMP was found to be 10-fold more sensitive than RT-PCR, with a detection limit of 20 copy numbers, against 200 copy numbers for RT-PCR, indicated by the presence of a 205-bp amplicon (Fig. 1A and B). A standard curve was generated for CHIKV RT-LAMP by plotting the graph between different concentrations of virus ranging from 2 × 10^6 to 2 × 10^7 copy numbers to time of positivity through real-time monitoring of the amplification (Fig. 2A). The quantification of the virus load in the positive samples was extrapolated on the basis of their time of positivity by employing the standard curve. Most of the patient serum samples were found to have very high concentrations of virus, within the range of 2 × 10^6 to 2 × 10^7 copy numbers (Fig. 2B).

The specificity of the RT-LAMP primers for the E1 gene of CHIKV was established by ruling out cross-reactivity with members of flavivirus group with similar clinical symptoms, such as dengue viruses 1 to 4, JE virus, West Nile virus (Env-101), and St. Louis Encephalitis virus (Paront strain) as well as with healthy human serum samples. The CHIKV-specific RT-LAMP primers demonstrated a high degree of specificity for CHIKV by amplifying CHIKV only but yielded negative results for all other viruses tested. Further confirmation of the structures of the amplified products was also carried out by sequencing wherein the sequences obtained perfectly matched the expected nucleotide sequences (data not shown).

Evaluation of CHIKV RT-LAMP assay with clinical samples. 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 Southern India. The results were compared with those from conven-
tional RT-PCR. A total of 89 samples comprised of 69 acute-phase serum samples and 20 negative serum samples were used in this study for comparative evaluation. The RT-LAMP assay demonstrated exceptionally higher sensitivity than conventional RT-PCR by picking up 21 additional positive cases \(P < 0.0001\) (Table 2). None of the RT-PCR-positive samples were missed by RT-LAMP, thereby indicating a higher sensitivity of the RT-LAMP assay. All 20 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 serology (IgM ELISA) (Table 2).

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. The comparative evaluation of this field-based, SYBR Green I-based RT-LAMP assay with 40 clinical samples randomly selected from the above-described 89 samples revealed a very good concordance of 93% with RT-PCR.

DISCUSSION

CHIKV, a member of the Alphavirus genus, is of considerable public health importance in Southeast Asian and African

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FIG. 1. Comparative sensitivity of RT-LAMP versus RT-PCR for detection of the CHIKV E1 gene. (A) Sensitivity of the RT-LAMP assay as monitored by real-time measurement of turbidity. Shown from left to right are the curves of decreasing concentrations of virus from \(2 \times 10^8\) to \(2 \times 10^{-1}\) copy numbers of the template in a serial 10-fold dilution. The detection limit for the assay was 20 copy numbers. (B) Sensitivity of RT-PCR for the detection of the CHIKV E1 gene as observed by using a 205-bp amplicon by agarose gel analysis with a detection limit of 200 copy numbers. Lane M, 100-bp DNA ladder (Sigma); lanes 1 to 11, different concentrations of virus ranging from \(2 \times 10^8\) to \(2 \times 10^{-1}\) copy numbers in a serial 10-fold dilution pattern.
countries. Chikungunya virus infections are rarely fatal and generally do not require admission to a hospital (6, 11). However, it is important to identify and quantify this infection for epidemiological studies. In fact, viral load should be a useful marker of disease progression and a measure of the efficiency of antiviral compounds. CHIKV diagnosis is based essentially on virus isolation, but RT-PCR and ELISA for the specific detection of CHIKV in mosquitoes or clinical samples have already been described (2, 4). Despite the fact that CHIKV resurgence is associated with an epidemic of unprecedented magnitude, a few specific serological or molecular diagnosis tools are available. Conventional RT-PCR methods have been suggested for the study of CHIKV replication in supernatants and clinical samples or for epidemiological surveys. Despite the high magnitude of amplification, these PCR-based methods require either high-precision instruments for the amplification or elaborate methods for the detection of the amplified products. In addition, these methods are often cumbersome to adapt for routine clinical use, especially in peripheral health care settings and private clinics. Therefore, a rapid, specific, and sensitive test is necessary for effective surveillance of new circulating CHIKV strains.

More recently, Pastorino et al. reported a fully automatic TaqMan real-time RT-PCR assay for the detection of CHIK virus in acute-phase serum samples (20). The real-time PCR assay has many advantages over conventional RT-PCR methods, including rapidity, quantitative measurement, a lower contamination rate, higher sensitivity, higher specificity, and easy standardization (10). Recently, the real-time RT-PCR technique has been used extensively to detect an amplicon that is amplified during PCR cycling in real time. The development of a fluorogenic PCR utilizing the 5'-3' nuclease activity of Taq DNA polymerase made it possible to eliminate post-PCR processing such as visualization in agarose gel. 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, including requiring either a high-precision instrument for amplification or an elaborate, complicated method for the detection of the amplified products. These rapid molecular tests might not be the method of choice in basic clinical settings in developing countries or in field situations because of the requirement for sophisticated instrumentation and expensive reagents. It is therefore critical to develop simple and economical molecular tests for the above-described scenarios.

In this regard, the RT-LAMP assay reported in this study is advantageous due to its simple operation, rapid reaction, and easy detection. The present study describes the standardization

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**TABLE 2. Comparative analysis of suspected CHIKV acute-phase serum samples for positivity by RT-LAMP, RT-PCR, virus isolation, and IgM antibody detection**

<table>
<thead>
<tr>
<th>Result</th>
<th>No. of positive or negative samples (% positive or negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-LAMP</td>
</tr>
<tr>
<td>Positive</td>
<td>38 (43)</td>
</tr>
<tr>
<td>Negative</td>
<td>51 (57)</td>
</tr>
</tbody>
</table>

* The numbers in parentheses indicate the percent positivity and negativity out of the total 89 samples analyzed in this study.
and evaluation of a one-step, single-tube, accelerated RT-LAMP assay for rapid and real-time detection of CHIKV in clinical specimens by targeting the immunodominant E1 gene. As shown above in Results, the RT-LAMP assay has demonstrated higher sensitivity by correctly picking up additional positive samples with low levels of virus that were missed by RT-PCR. Further retrospective analysis of these RT-LAMP-positive samples revealed that these are true-positive samples, indicated by the presence of virus-specific IgM antibodies and/or successful virus isolation, so these may be the borderline cases with very low levels of viremia collected during the window period of the disease, when viremia is in decline (undetectable by RT-PCR) and IgM antibody started appearing to a detectable limit.

The RT-LAMP assay is a simple diagnostic tool in which the reaction is carried out in a single tube by mixing the buffer, primers, reverse transcriptase, and DNA polymerase and incubating the mixture at 63°C for 30 min. Compared to RT-PCR and real-time PCR, RT-LAMP has the advantages of reaction simplicity and detection sensitivity. The higher sensitivity and higher specificity of the RT-LAMP reaction are attributed to continuous amplification under isothermal conditions by employing six primers that recognize eight distinct regions of the target (11, 12). Also, the higher amplification efficiency of the RT-LAMP reaction yields a large amount of by-product, pyrophosphate ion, leading to a white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in turbidity of the reaction mixture according to the production of the precipitate correlates with the amount of DNA synthesized, real-time monitoring of the RT-LAMP reaction can be achieved by real-time measurements of turbidity (9). The quantification of the virus load in patient serum samples can also be determined by employing the CHIKV RT-LAMP standard curve. It was observed that the amount of virus load in acute-phase serum samples was quite high, which is quite significant compared to those of other arbovirus infections like dengue virus and JE virus. This higher amount of virus may be attributed to the explosive nature of the unprecedented CHIKV epidemic, with the occurrence of multiple cases in a single hut.

As discussed above, the execution of the RT-LAMP reaction and the measurement of its turbidity are extremely simple compared to those of the existing real-time TaqMan RT-PCR and nucleic acid sequence-based amplification assays that require fluorogenic primers and probes as well as expensive detection equipment. One of the most attractive features of the RT-LAMP assay is that it has a great advantage in terms of monitoring of amplification that can be accomplished by SYBR Green I dye-mediated naked-eye visualization and by real-time monitoring by using an inexpensive turbidimeter according to the situation. Of particular importance is the substantial reduction in time required for the confirmation of results by the RT-LAMP assay, 30 min, compared to 3 to 4 h in case of RT-PCR. Thus, the RT-LAMP assay reported in this study allows rapid, real-time detection as well as quantification 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.

ACKNOWLEDGMENTS

We are thankful to K. Sekhar, Director, Defense Research and Development Establishment (DRDE), Ministry of Defense, Government of India, for his support and constant inspiration and providing necessary facilities and Director, Nizam’s Institute of Medical Sciences (NIMDS), Hyderabad, for providing clinical samples for this study.

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