Development and Evaluation of Reverse Transcription–Loop-Mediated Isothermal Amplification Assay for Rapid and Real-Time Detection of Japanese Encephalitis Virus[▽]

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The standardization and validation of a one-step, single-tube accelerated quantitative reverse transcription–loop-mediated isothermal amplification (RT-LAMP) assay is reported for rapid and real-time detection of Japanese encephalitis virus (JEV). The RT-LAMP assay reported in this study is very simple and rapid; the amplification can be obtained in 30 min under isothermal conditions at 63°C by employing a set of six primers targeting the E gene of JEV. The RT-LAMP assay demonstrated exceptionally higher sensitivity compared to that of RT-PCR, with a detection limit of 0.1 PFU. The specificities of the selected primer sets were established by cross-reactivity studies with other closely related members of the JEV serocomplex as well as by evaluation of healthy human volunteers. The comparative evaluation of the RT-LAMP assay for clinical diagnosis with a limited number of patient cerebrospinal fluid samples revealed 85% concordance with conventional RT-PCR, with a sensitivity and a specificity of 100% and 86%, respectively. The concentration of virus in most of the clinical samples was 10² to 10⁵ PFU/ml, as determined from the standard curve based on the time of positivity in the samples. In addition, the monitoring of gene amplification can also be visualized with the naked eye by using SYBR green I fluorescent dye. 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 JEV not only by well-equipped laboratories but also by peripheral diagnostic laboratories with limited financial resources in developing countries.

Japanese encephalitis virus (JEV) is the most common cause of childhood viral encephalitis in the world, causing an estimated 50,000 infections and 10,000 deaths annually. Although Japan, South Korea, and Taiwan have nearly eliminated the disease in their human populations and China and Thailand have greatly reduced its incidence, epidemic Japanese encephalitis has expanded its distribution to countries such as Nepal and western India and become a substantial public health problem. The postmonsoon outbreak of JE is a regular feature, and outbreaks have been regularly reported from most parts of India (1, 9, 19).

JEV is a member of the genus *Flavivirus* (family *Flaviviridae*) that is transmitted between birds, pigs, and some domestic animals by *Culex* mosquitoes (12). The JEV genome, like that of all flaviviruses, is a positive-sense single-stranded RNA molecule of approximately 11 kb in length. It is capped at its 5' end and contains a single open reading frame (ORF) encoding a polyprotein. The viral structural proteins are encoded by the 5' one-third of the ORF and consist of the capsid (CA), membrane (M; formed by proteolytic cleavage of its precursor protein prM) and envelope (E) proteins. The nonstructural proteins (NS1 to NS5) are encoded in the remaining 3' region. The ORF is flanked by 5' and 3' untranslated regions approx-

imately 95 and 582 nucleotides long, respectively (22, 25). Humans are an incidental host, infected when living or passing in close proximity to this enzootic cycle. Hence, most infections of humans occur in rural tropical areas, where facilities for diagnosis are limited (20).

Laboratory diagnosis of JEV relies on virus isolation and characterization, the detection of virus-specific antibodies, and the detection of genomic sequences by nucleic acid amplification techniques. Even with the best laboratory facilities, JEV cannot usually be isolated from clinical specimens, probably because of low numbers of circulating virions and the rapid development of neutralizing antibodies. Therefore, the diagnosis is usually made serologically. Several serologic tests, such as the hemagglutination inhibition test, enzyme-linked immunosorbent assay (ELISA), serum neutralization techniques, and dot enzyme immunoassay, have been used for the detection of antibody for JEV infection (2, 21). For many years, the hemagglutination inhibition test has been employed but this has various practical limitations. Besides being cumbersome, it requires paired serum samples and therefore cannot give an early diagnosis.

The immunoglobulin M (IgM) capture ELISA for serum and cerebrospinal fluid (CSF) has become the accepted standard for the diagnosis of JEV. However, the confirmation and typing of virus are based on the demonstration of a fourfold or more increase in virus-specific neutralizing antibody titer by a plaque reduction neutralization assay with several flaviviruses due to the existence of cross-reactive antibodies of cocirculating members of other closely related flaviviruses. Both virus

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TABLE 1. Details of RT-LAMP E gene primer sets designed for rapid and real-time detection of JEV

Primer name and primer component	Genome position ^a	Length of oligonucleotide (bp)	Sequence
F3	990–1008	19	GGAATGGGCAATCGTGACT
B3	1224–1206	19	CGTTGTGAGCTTCTCCAGT
FIP (F1c+TTTT+F2)			
F1c	1109–1088	22	GCGGACGTCCAATGTTGGTTTG
F2	1029–1046	18	GCCACTGGGTGGACTTG
BIP (B1+TTTT+B2c)			
B1	1123–1144	22	AAGCTAGCCAACCTGCTGAGGT
B2c	1187–1168	20	CGTCGAGATGTCAGTGACTG
FLP	1087–1070	18	TCGTTTGCCATGATTGTC
BLP	1147–1166	20	GAAGTTACTGCTATCATGCT

^a Prototype strain JaOArS982 (accession no. M18370).

isolation and plaque reduction neutralization assays are time consuming and tedious, thereby requiring more than a week. Thus, virus isolation and antibody detection have less impact on the patient management and control measures exercised by medical and public health personnel. Therefore, in order to provide timely clinical treatment and etiologic investigation and disease control, there is a great demand for the rapid detection of JEV infection in the acute phase of the illness.

Molecular techniques based on genomic sequence detection, viz., RT-PCR and real-time PCR assays, have therefore assumed significance in the rapid diagnosis and identification of JEV. These assays have now been gradually accepted as a new standard over virus isolation for the detection of the viral genome in acute-phase serum samples. Among these, the RT-PCR approach is being routinely practiced in almost all laboratories worldwide (8, 10). However, the existing RT-PCR test system is less sensitive, time consuming, and labor intensive and has a very high risk of contamination. Besides, the high cost of the instruments required for performing the real-time RT-PCR assay restricts its use to laboratories with good financial resources.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method developed by Eiken Chemical Co., Ltd., Tokyo, Japan, and has the potential to replace PCR because of its simplicity, rapidity, specificity, and cost-effectiveness. It is based on the principle of strand displacement reaction and the stem-loop structure that amplifies the target with high specificity, selectivity, and rapidity under isothermal conditions, thereby obviating the need for using a thermal cycler (14, 15). The amplification efficiency of the LAMP method is extremely high due to continuous amplification under isothermal conditions producing a large amount of target DNA as well as a large amount of the by-product magnesium pyrophosphate, leading to turbidity. Therefore, quantitative detection of gene amplification is possible by real-time monitoring of turbidity in an inexpensive turbidimeter (13). In addition, the higher amplification efficiency of the LAMP method enables simple visual observation of amplification through the naked eye by using a UV lamp in the presence of an intercalating dye such as SYBR green I or ethidium bromide. Thus, the LAMP assay has emerged as a powerful gene amplification tool for the rapid identification of microbial infections and is increasingly being used by various investigators for the rapid detection and typing of emerging viruses, viz.,

West Nile virus, severe acute respiratory syndrome coronavirus, and Dengue virus (6, 17, 18). Recently, Toriniwa and Komiya also reported the standardization of RT-LAMP for the rapid detection and quantification of JEV by targeting the E gene, but the application of this novel gene amplification system for the clinical diagnosis of JE patients during epidemic situations needs to be established through evaluation with a large number of clinical samples (24). In the present study, we report the development as well as extensive evaluation of a one-step, single-tube real-time accelerated RT-LAMP assay with acute-phase CSF samples collected during the Gorakhpur epidemic in 2005. This is the first report about the usefulness of RT-LAMP as a rapid diagnostic tool to detect JEV from CSF, the most important diagnostic material for JE. The data on the sensitivity and specificity of the method is reported, and the applicability of the technology for clinical diagnosis of JE patients is discussed.

MATERIALS AND METHODS

Design of JEV-specific RT-LAMP primers. The oligonucleotide primers used for RT-LAMP amplification of JEVs were designed from the E gene. The nucleotide sequence of the E gene of the JEV prototype strain JaOArS982 was retrieved from GenBank (accession no. M18370) and aligned with the available E gene sequences of other strains, including those of the circulating strains in India responsible for recent epidemics, to identify the conserved regions by using DNASIS software. The potential target region of the 235 bp corresponding to genome positions 992 to 1225 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 employing the LAMP primer designing support software program (Net Laboratory, Japan; <http://venus.netlaboratory.com>).

The primers were selected based on the criteria described by Notomi et al. (15). 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, viz., the forward loop primer (FLP) and the 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 primers with regard to their positions in the genomic sequences are shown in Table 1.

Clinical samples. The total of 50 CSF samples evaluated in this study were collected with informed consent from patients admitted with clinical diagnoses of encephalitis in the pediatrics and medicine wards of BRD Medical College, Gorakhpur, Uttar Pradesh, India, during July to November 2005. The acute-phase samples were collected during the period between days 1 to 7 after the

FIG. 1. Schematic representation of the primer design for RT-LAMP assay. Construction of two inner primers (FIP and BIP) having both sense and antisense sequences that help in loop formation is depicted. F1C and B2C are the complementary sequences of F1 and B2, respectively.

onset of symptoms. All of the samples were transported to the laboratory under a strict cold chain and stored at -80°C until further investigation. In addition, a panel of 10 CSF samples collected from patients with other central nervous system complications and without clinical and laboratory evidence of viral infections were also included as negative controls.

These negative samples were thoroughly checked for JEV by serology and RT-PCR prior to analysis by RT-LAMP. In addition, these samples were also screened by flavivirus group-specific RT-PCR to rule out the involvement of any related viral hemorrhagic or encephalitic infections.

Cell culture. C6/36 cells (cloned cells of larvae of *Aedes albopictus* cells) (7), initially obtained from the National Center for Cell Science, Pune, India, were maintained in our laboratory at 28°C by regular subculturing at periodic intervals of 3 to 4 days in Eagle's minimum essential medium (Sigma, St. Louis, MO) supplemented with 10% tryptose phosphate broth (DIFCO, Detroit, MI), 10% fetal bovine serum (Sigma), 3% L-glutamine (Sigma), and gentamicin (80 mg/liter) (Nicholas Piramal, Lower Parel, Mumbai, India).

Virus. Japanese encephalitis virus (strain JaOArS982) was used as viral antigen/positive standard in the assay systems employed in the present study. Briefly, a monolayer of C6/36 cells grown in a 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. Suitable mock-infected cell controls were also kept alongside. The cells were then incubated at 37°C and observed daily for cytopathic effects. Upon an observation of 80 to 100% cytopathic effects, the infected culture supernatant was clarified by light centrifugation at 2,000 rpm for 10 min and stored in aliquots at -80°C until use. The quantification of the virus infectivity was carried out in Vero cells grown in 24-well tissue culture plates as per standard protocol (5). Resulting plaques were counted, and the virus titer was determined. In addition, the four dengue virus serotypes (DEN-1, Hawaii; DEN-2, ThNH7/93; DEN-3, PhMH-J1-97; and DEN-4, SLMC 318), West Nile virus strains (Eg 101), and St. Louis encephalitis virus (Parton strain) were also used in the present study for checking the cross-reactivity.

RNA extraction. The genomic viral RNA was extracted from 140 μl of infected culture supernatant with known PFU of virus and 140 μl of patient CSF samples by using the QIAamp viral RNA mini kit (QIAGEN, Germany) according to the manufacturer's protocol. The RNA was eluted from QIAspin columns in a final volume of 100 μl of elution buffer and stored at -70°C until used.

RT-PCR. In order to compare the sensitivity and specificity of the RT-LAMP assay, one-step RT-PCR was performed with JEV-specific primers JE-NS3-S, AGA GCG GGG AAA AAG GTC AT (position no. 5739 to 5758), and JE-NS3-C, TTT CAC GCT CTT TCT ACA GT (position no. 5900 to 5881), targeting 162 bp of the NS3 gene of the viral genome (8, 23). The amplification was carried out in a 50- μl total reaction volume by using the Promega access quick 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, 72°C for 30 s, and a final extension cycle of 72°C for 10 min.

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., Tokyo, 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 deoxynucleoside triphosphate, 0.8 M betaine, 0.1% Tween 20, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 8 mM MgSO_4 , 10 mM KCl, 20 mM Tris-HCl, pH 8.8, 8 U of Bst DNA polymerase (New England Biolabs), 0.625 U of the avian myeloblastosis virus reverse transcriptase (Invitrogen), and 2 μl of target RNA. The reaction

mixture was incubated at 63°C for 60 min in a heating block and followed by heating at 80°C for 2 min to terminate the reaction. For real-time monitoring of RT-LAMP assay, the reaction mixture was incubated at 63°C for 60 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.

Monitoring of RT-LAMP amplification. (i) **Real-time monitoring.** The real-time monitoring of the RT-LAMP amplification of the JEV template was observed through spectrophotometric analysis by recording the optical density at 400 nm at every 6 s with the help of a Loopamp real-time turbidimeter. The cutoff value for positivity by the real-time RT-LAMP assay was determined by taking into account the time of positivity (T_p ; in min), at which the turbidity increases above the threshold value fixed at 0.1 that is two times more than the average turbidity value of the negative controls of several replicates. None of the positive samples tested over multiple times showed positivity in terms of increased turbidity after 30 min. Therefore, a sample having T_p values of ≤ 30 min and turbidity above the threshold value of ≥ 0.1 was considered positive.

(ii) **Agarose gel analysis.** Following incubation at 63°C for 60 min, a 10- μl aliquot of RT-LAMP products was 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 an UV transilluminator at 302 nm.

(iii) **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 a naked-eye inspection. Following amplification, the tubes were inspected for white turbidity through 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 the observation of color change following the addition of 1 μl of SYBR green I dye to the tube. In the case of a positive amplification, the original orange color of the dye will change to a green that can be judged under natural light as well as under UV light (302 nm) with the help of a handheld UV torch lamp. In case there is no amplification, the original orange color of the dye will be retained. This change of color is permanent and can thus be kept for record purposes.

RESULTS

The success of RT-LAMP amplification relies on the specificity of designed primer sets. The primers were selected targeting the highly conserved regions of the E gene of JEV based on the multiple sequence alignment results of all of the circulating strains. The details of the primers with regard to their positions in the genomic sequences are shown in Table 1, and a schematic representation of the RT-LAMP primer design is depicted for better understanding (Fig. 1).

A one-step, single-tube, real-time RT-LAMP assay was standardized by employing the selected primer set as described in Materials and Methods. The detection of gene amplification is accomplished by real-time monitoring of turbidity at 63°C . The result indicated that the time required for the initiation of amplification was 16 to 17 min (Fig. 2). There is continuous amplification of the target sequence in a stepwise gradient

FIG. 2. Real-time kinetics of JE RT-LAMP amplification of the E gene showing the amplification curve with serial 10-fold dilutions of the virus ranging from 10^4 to 0.1 PFU. 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.

manner, as observed by increased turbidity compared to that of the negative control having no template, where the turbidity gets fixed around 0.01 below the threshold value.

As observed on agarose gel electrophoresis, the RT-LAMP assay could amplify the 235-bp target sequence of the E gene of JEV at 63°C in 30 min. The amplification was observed as a ladder-like pattern on the gel due to the formation of a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures, with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Fig. 3A).

Sensitivity and specificity of RT-LAMP. The sensitivity of the RT-LAMP assay for the detection of JEV RNA was determined by testing serial 10-fold dilutions of virus that had previously been quantified by plaque assay and compared with that of conventional RT-PCR. The accelerated RT-LAMP assay was able to amplify the 10^4 PFU of virus in 16 to 17 min, with a detection limit of 0.1 PFU of virus (Fig. 3A). The comparative sensitivity of RT-LAMP and RT-PCR revealed that RT-LAMP was 100-fold more sensitive than RT-PCR, which has a detection limit of 10 PFU of virus, as indicated by the presence of a 162-bp amplicon (Fig. 3B).

The specificity of the RT-LAMP primers for the E gene of JEV as shown in Table 1 was established by checking the cross-reactivity with that of other serologically related members of the flavivirus group, such as DEN-1 to -4, West Nile virus, and St. Louis encephalitis virus. The JEV-specific RT-LAMP primers demonstrated a high degree of specificity for JEV by amplifying the JEV only and yielding negative results for all other viruses tested. Further confirmation of the structures of the amplified products was also carried out by se-

quencing, wherein the sequences obtained perfectly matched with the expected nucleotide sequences (data not shown).

Evaluation of JE RT-LAMP assay with clinical samples. The applicability of the RT-LAMP assay for clinical diagnosis of JEV was validated with acute-phase CSF samples collected from the Gorakhpur epidemic in 2005, and the results were compared with those of conventional RT-PCR. A total of 50 clinical samples comprising acute-phase CSF samples and 10 negative CSF samples were used in this study for comparative evaluation.

The comparative evaluation of RT-LAMP vis-à-vis traditional RT-PCR with a limited number of clinical samples as described above revealed very good correlation in detecting viral RNA. The result indicated an overall 85% concordance between the two test systems (Table 2). The RT-LAMP assay demonstrated exceptionally higher sensitivity compared to that of conventional RT-PCR by detecting nine additional positive cases ($P < 0.001$). None of the samples positive by RT-PCR were missed by RT-LAMP, thereby indicating the superior sensitivity of RT-LAMP assay. All 10 negative CSF samples were negative by both tests, thereby ruling out the possibility of false positivity and thus establishing the specificity of the selected primer sets for the JE RT-LAMP assay. The overall sensitivity and specificity of the RT-LAMP assay relative to the conventional RT-PCR assay were found to be 100 and 86%, respectively (Table 2).

A standard curve depicting the linear relationship between the concentrations of virus (PFU) and the time of positivity was generated for JEV RT-LAMP through the real-time monitoring of the amplification of different concentrations of virus, ranging from 10^7 to 10^2 PFU/ml (Fig. 4). The quantification of

FIG. 3. Comparative sensitivity of RT-LAMP and RT-PCR for the detection of JEV. (A). Sensitivity of the JEV RT-LAMP assay as monitored by agarose gel analysis. Lane M, 100-bp DNA ladder (Sigma); lanes 1 to 6, different concentrations of virus, ranging from 10^4 to 0.1 PFU in a serial 10-fold dilution pattern; lane 7, negative control having no template. The detection limit for the RT-LAMP assay was 0.1 PFU. (B) Sensitivity of RT-PCR as observed by a agarose gel analysis revealing a 162-bp amplicon specific for the NS3 gene of JEV with a detection limit of 10 PFU.

the virus load in the positive samples was determined on the basis of their time of positivity by employing the standard curve. Most of the samples had a virus concentration in the range of 10^2 to 10^5 PFU/ml (Fig. 5).

The field applicability of the RT-LAMP assay was validated by employing SYBR green I-mediated, naked-eye visualization following incubation at 63°C for 30 min in a water bath. The monitoring of the RT-LAMP amplification was carried out through naked-eye visualization by the addition of 1 μl of SYBR green I (1:1,000) dye to the amplified products (Fig. 6).

DISCUSSION

During the past decade, various nucleic acid amplification techniques, such as RT-PCR, TaqMan real-time RT-PCR, SYBR green real-time RT-PCR, and nucleic acid sequence-based amplification (NASBA), have been developed to ad-

dress the need for the rapid identification of JEV with more accuracy (3, 4, 8, 10). 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.

More recently, several investigators have reported fully au-

TABLE 2. Comparative analysis of JEV-suspected acute-phase CSF samples for positivity by real-time RT-LAMP, RT-PCR, virus isolation and IgM antibody detection^a

Result	No. (%) of samples with indicated result by:			
	RT-LAMP	RT-PCR	IgM	Virus isolation
Positive	13 (22)	4 (7)	19 (32)	7 (12)
Negative	47 (78)	56 (93)	41 (68)	53 (82)

^a For RT-LAMP versus RT-PCR, concordance was 85%, sensitivity was 100%, and specificity was 86%.

FIG. 4. Standard curve for a JEV-specific RT-LAMP assay generated from the amplification plots between serial 10-fold dilutions of different virus concentrations (PFU) and Tp.

FIG. 5. Quantitative determination of virus concentration in clinical samples employing a standard curve generated for the JE RT-LAMP assay by plotting T_p with regard to various concentrations of virus in PFU based on their T_p values.

tomatic real-time PCR assays for the detection of JEV in acute-phase serum samples (4). The real-time PCR assay has many advantages over conventional RT-PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity, and easy standardization. Recently, the real-time RT-PCR technique has been used extensively to detect the amplicon that is amplified during PCR cycling in real time. The development of fluorogenic PCR utilizing 5' to 3' nuclease activity of *Taq* DNA polymerase made it possible to eliminate post-PCR processing, such as visualization in agarose (11). 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, such as requiring either a high-precision instrument for the amplification or an elaborate

complicated method for the detection of 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 of sophisticated instrumentation and expensive reagents. It is therefore critical to develop rapid, reliable, and simple molecular tests to complement the existing techniques.

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 and evaluation of the one-step, single-tube accelerated RT-LAMP assay for rapid and real-time detection of JEV in clinical specimens by targeting the immunodominant E gene.

The evaluation of the newly developed JEV-specific RT-LAMP has been undertaken with a panel of defined clinical samples collected from a recent epidemic after thorough investigation employing all the test systems, viz., virus isolation, serology (IgM and IgG antibody detection), RT-PCR, sequencing, etc. This epidemic of JEV was reported from July to November 2005 in Gorakhpur, Uttar Pradesh, India. This was the longest and most severe epidemic in three decades, spanning over seven districts of eastern Uttar Pradesh and affecting 5,737 persons with more than 1,344 deaths (18).

As already shown in Results, the RT-LAMP assay has demonstrated a higher sensitivity by correctly detecting nine additional positive samples with low levels of virus that were missed by RT-PCR. Further retrospective analysis of these samples positive by RT-LAMP revealed the presence of virus-specific IgM antibodies in corresponding CSF samples suggestive of confirmatory infection. The positive IgM capture ELISA of CSF has become the accepted standard for the diagnosis of Japanese encephalitis. The presence of virus-specific IgM in the CSF is excellent evidence of central nervous system infection as the IgM antibody cannot cross the blood-brain barrier under normal conditions. It is only when there is damage to the blood-brain barrier due to encephalitis or any other diseased condition that the IgM antibody crosses the blood-brain barrier and thus appears in the CSF. So these may be the samples collected during the

FIG. 6. SYBR green I fluorescent dye-mediated monitoring of JEV RT-LAMP amplification. (A) Naked eye inspection under normal light. The original orange color of the SYBR green I changed to yellow in the case of positive amplification, whereas in negative control having no amplification, the original orange color is retained. (B) Visual observation of green fluorescence of DNA binding SYBR green I under UV light.

window period of the disease, where the viremia is in decline (undetectable by RT-PCR) and the 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 a mixing of the buffer, primers, reverse transcriptase, and DNA polymerase, and incubating the mixture at 63°C for 60 min. Compared to RT-PCR and real-time PCR, the RT-LAMP has the advantages of reaction simplicity and detection sensitivity. The higher sensitivity and specificity of the RT-LAMP reaction are attributed to continuous amplification under isothermal conditions employing six primers that recognize eight distinct regions of the target. Besides, 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 turbidity (16, 17). The other isothermal amplification techniques, such as NASBA and self-sustained sequence reaction (3SR), are, however, reported to be less specific, owing to the low stringency (40°C), and thus require either a precision instrument or an elaborate method for the detection of the amplified products due to poor specificity of target sequence selection (3).

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 NASBA 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 seems to have great advantage in terms of the monitoring of the 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. The particular importance is the substantial reduction in time required for the confirmation of results by RT-LAMP assay (in 30 min compared to 3 to 4 h in the case of RT-PCR). Thus, the RT-LAMP assay reported in this study allows rapid, real-time detection as well as quantification of JEV in acute-phase CSF samples without requiring sophisticated equipment and has potential usefulness for clinical diagnosis and surveillance of JEV in developing countries.

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