Rapid Detection and Differentiation of Dengue Virus Serotypes by a Real-Time Reverse Transcription–Loop-Mediated Isothermal Amplification Assay

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The development and validation of a one-step, real-time, and quantitative dengue virus serotype-specific reverse transcription–loop-mediated isothermal amplification (RT-LAMP) assay targeting the 3′ noncoding region for the rapid detection and differentiation of dengue virus serotypes are reported. The RT-LAMP assay is very simple and rapid, wherein the amplification can be obtained in 30 min under isothermal conditions at 63°C by employing a set of four serotype-specific primer mixtures through real-time monitoring in an inexpensive turbidimeter. The evaluation of the RT-LAMP assay for use for clinical diagnosis with a limited number of patient serum samples, confirmed to be infected with each serotype, revealed a higher sensitivity by picking up 100% samples as positive, whereas 87% and 81% of the samples were positive by reverse transcription-PCR and virus isolation, respectively. The sensitivity and specificity of the RT-LAMP assay for the detection of viral RNA in patient serum samples with reference to virus isolation were 100% and 93%, respectively. The optimal assay conditions with zero background and no cross-reaction with other closely related members of the *Flavivirus* family (Japanese encephalitis, West Nile, and St. Louis encephalitis viruses) as well as within the four serotypes of dengue virus were established. None of the serum samples from healthy individuals screened in this study showed any cross-reaction with the four dengue virus serotype-specific RT-LAMP assay primers. These findings demonstrate that RT-LAMP assay has the potential clinical application for detection and differentiation of dengue virus serotypes, especially in developing countries.

Dengue virus is a mosquito-borne flavivirus and the most widely prevalent arbovirus in tropical and subtropical regions of Asia, Africa, and Central and South America (4). There are four distinct serotypes, DEN-1, DEN-2, DEN-3, and DEN-4, which produce a spectrum of illness ranging from inapparent infection to moderate febrile illness and severe and fatal hemorrhagic disease. In recent years, dengue fever (DF) and its more serious forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), have emerged as a major public health problems, with expanded geographic distributions and increased epidemic activities (20).

Dengue virus infection induces a life-long protective immunity to the homologous serotype but confers only partial and transient protection against subsequent infection by the other three serotypes. Therefore, multiple and sequential infections with the four dengue virus serotypes would be expected for people living in a region where dengue is hyperendemic due to the lack of cross-protective neutralizing antibodies. Seroepidemiological studies have shown that secondary infection is a major risk factor for DHF and DSS through antibody-dependent enhancement (6, 16). Therefore, rapid detection and dif-

ferentiation between primary and secondary dengue virus infections and determination of the dengue virus serotypes of past and current infections are important for patient management as well as epidemiological investigations.

The three basic methods routinely practiced by most laboratories are virus isolation and characterization, detection of dengue virus-specific antibodies, and detection of genomic sequences by nucleic acid amplification techniques (5, 11, 26). Virus isolation through a mosquito cell line (C6/36) from acute-phase serum or plasma samples is the method of choice and remains the “gold standard,” although it has the disadvantage that more than 7 days is required to complete the process. The isolation of dengue virus in cell culture from clinical samples has generally been unsuccessful owing to the fastidious nature of the organism and the low level of transient viremia associated with the disease process.

Serologically, dengue virus infection can be inferred by immunoglobulin M (IgM)- and IgG-capture enzyme-linked immunosorbent assay (ELISA) (11). However, the confirmation of infection with the virus and typing of virus are based on the demonstration of a fourfold or more increase in the virus-specific neutralizing antibody titer by plaque reduction neutralization (PRNT) assay with several flaviviruses due to the existence of cross-reactive antibodies of cocirculating members of other closely related flaviviruses (5). Both virus isolation and PRNT assays are time-consuming and tedious and require

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more than a week for completion. Thus, virus isolation and antibody detection have less of an impact on patient management and the control measures exercised by medical and public health personnel. Therefore, there is a great demand for the rapid detection of dengue virus infection in the acute phase of illness in order to provide timely clinical treatment and etiologic investigation and disease control.

Molecular techniques based on genomic sequence detection by reverse transcription (RT)-PCR, nested PCR, nucleic acid sequence-based amplification (NASBA), and real-time PCR are therefore assumed to be significant for the rapid diagnosis and identification of dengue virus serotypes and have gradually been accepted as new standards over virus isolation for detection of dengue virus in acute-phase serum samples (3, 12, 13, 15, 18, 23, 25). Among these, the two-step nested RT-PCR approach is routinely practiced in almost all laboratories worldwide. The identification and typing of dengue virus serotypes have been achieved through RT-PCR, followed by nested PCR with complex- and serotype-specific primers, respectively (13). The two-step approach was later modified to a single-step multiplex RT-PCR system for the detection and typing of dengue viruses (7, 18, 23, 25). However, the existing RT-PCR test systems are less sensitive, and the assays are time-consuming (3 to 4 h) and much more complicated, with several steps of amplification (cDNA-PCR-nested PCR) that require the use of a high-precision thermal cycler. More sensitive and real-time-based assays are therefore needed to complement the existing PCR-based assay systems.

More recently, several investigators have reported on fully automatic real-time PCR assays for the detection of dengue virus in acute-phase serum samples (2, 9, 14, 24). 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 present study describes the development and evaluation of a simple, rapid, and cost-effective one-step, real-time, and quantitative reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for the rapid detection and differentiation of dengue virus serotypes. 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 (19, 21). 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

(17). 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 and has been applied to the identification of West Nile (WN) virus and sudden acute respiratory syndrome-associated coronavirus in our laboratory (8, 22). In the present study, the development and applicability of the RT-LAMP assay for the rapid detection and differentiation of dengue virus serotypes are described. Data on the sensitivity and specificity of the method are reported, and the feasibility of use of the technology for the clinical diagnosis of dengue virus infection is discussed.

MATERIALS AND METHODS

Viruses. Strains of the four dengue virus serotypes (DEN-1, Hawaii; DEN-2, ThNH7/93; DEN-3, PhMH-J1-97; and DEN-4, SLMC 318) were used in the present study. The viruses were propagated in *Aedes albopictus* clone C6/36 cells (10), and virus titers were determined by plaque assay in LLCMK2 cells, as per the standard protocol (1). Besides dengue virus serotypes, other viruses used for cross-reactivity studies were Japanese encephalitis (JE) virus (JaOArS982 strain), WN virus (strain NY99 [flamingo 382-99]), and St. Louis encephalitis (SLE) virus (Parton strain).

Human patient serum samples. The serum samples used in this study were collected from patients with confirmed and suspected dengue virus infections during epidemics in the Philippines (2000 to 2003), Bangladesh (2002), and India (2001 to 2004). A confirmed case of dengue virus infection was defined as a febrile illness associated with the isolation of dengue virus and positive RT-PCR and/or IgM ELISA results with a positive/negative ratio >2.0. The acute-phase serum samples collected during the period between days 1 and 7 after the onset of symptoms from cases of both DF and DHF caused by each serotype were used for evaluation. In addition, a panel of serum samples collected from volunteer healthy blood donors in the Philippines was also included as a negative control. All the serum samples were screened by IgM-capture ELISA as well as by RT-PCR with dengue virus group-specific consensus primer pairs for the presence of anti-dengue virus antibodies and dengue virus RNA, respectively.

RNA extraction. The genomic viral RNA was extracted from 140 μ l of infected culture supernatant with a known PFU of virus and 50 μ l of patient serum samples by using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. The RNA was eluted from the QIAspin columns in a final volume of 100 μ l of elution buffer and was stored at -70°C until it was used.

Design of dengue virus serotype-specific RT-LAMP assay primers. The serotype-specific oligonucleotide primers used for RT-LAMP assay amplification of dengue viruses were designed from the 3' noncoding region (NCR). The nucleotide sequences of the prototype strains of each dengue virus serotype were retrieved from GenBank (accession numbers are shown in parentheses), DEN-1 Western Pacific (U88535), DEN-2 New Guinea C (AF038403), DEN-3 H87 (M93130), and DEN-4 China Guangzhou B5 (AF289029), and were aligned with the available sequences of other strains of each serotype to identify the conserved regions by using DNASIS software (Hitachi, Japan). Potential target regions were selected from the aligned sequences, and RT-LAMP assay primers were designed for each serotype. 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 design support software program (Net Laboratory, Japan; <http://venus.netlaboratory.com>).

The primers were selected based on the criteria described by Notomi et al. (21). In addition to the general criteria of primer design, such as avoiding terminal dimer formation, 3' hairpins, self-complementarity, and 40 to 60% G+C contents, special care was taken to adjust the melting temperatures (T_m s) of the primers in such a way that the T_m s were in the following order: F1c and B1c > F2 and B2 > F3 and B3. The length of the loop (the distance between F2 plus F1 and B1c plus B2c) was adjusted to between 40 and 60 bp to achieve optimal results. The two outer primers were described as forward outer primer

TABLE 1. Details of RT-LAMP assay primer sets used for rapid detection and differentiation of dengue virus serotypes 1, 2, 3, and 4 targeting 3' NCR of the viral genome

Virus serotype ^a	Target (genome position)	Primer ^b	Sequence (5' → 3')
DEN-1	10469–10667 (199 bp)	F3 B3 FIP BIP FLP BLP	GAGGCTGCAAACCATGGAA CAGCAGGATCTCTGGTCTCT GCTGCGTTGTGTCTTGGGAGGTTTTCTGTACGCATGGGGTAGC CCCAACACCAGGGGAAGCTGTTTTTTTGTGTGTGCGGGGG CTCCTCTAACCCTAGTC GGTGGTAAGGACTAGAGG
DEN-2	10449–10659 (211 bp)	F3 B3 FIP BIP FLP BLP	TGGAAGCTGTACGCATGG GTGCCTGGAATGATGCTG TTGGGCCCCATTGTTGCTGTTTTAGTGGACTAGCGGTTAGAGG GGTTAGAGGAGACCCCCCAATTTTGAGACAGCAGGATCTCTGG GATCTGTAAGGGAGGGG GCATATTGACGCTGGGA
DEN-3	10289–10506 (218 bp)	F3 B3 FIP BIP FLP BLP	GCCACCTTAAGCCACAGTA GTTGTGTCATGGGAGGG TGGCTTTTGGGCCTGACTTCTTTTTTGAAGAAGCTGTGCAGCCTG CTGTAGCTCCGTGGTGGGGATTTTCTAGTCTGCTACACCGTGC CCTTGGACGGGGCT GGAGGCTGCAAACCGTG
DEN-4	10289–10517 (229 bp)	F3 B3 FIP BIP FLP BLP	CTATTGAAGTCAGGCCAC ACCTCTAGTCCTTCCACC TGGGAATTATAACGCCTCCCGTTTTTCCACGGCTTGAGCAAACC GGTTAGAGGAGACCCCTCCCTTTTAGCTTCCTCCTGGCTTCG GGCGGAGCTACAGGCAG TCACCAACAAAACGCAG

^a GenBank accession numbers: DEN-1 Western Pacific, U88535; DEN-2 New Guinea C, AF038403; DEN-3 H87, M93130; and DEN-4, China Guangzhou B5, AF289029.

^b F3, forward outer primer; B3, backward outer primer; FIP, forward inner primer; BIP, backward inner primer; FLP, forward loop primer; BLP, backward loop primer.

F3 and backward outer primer B3. The inner primers were described as forward inner primer FIP and backward inner primer BIP. Two additional 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 the B1 and B2 regions, respectively. The details of each primer with regard to its position in the genomic sequences are shown in Table 1, and a schematic representation of the RT-LAMP assay primer design is depicted for better understanding (Fig. 1).

RT-LAMP assay. The RT-LAMP reaction was carried out in a total 25- μ l reaction mixture by using a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Japan) containing 40 pmol each of primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 20 pmol each of loop primers F and B, 1.4 mM deoxynucleoside triphosphates, 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, 16 U of *Bst* DNA polymerase (New England Biolabs), 0.125 U of avian myeloblastosis virus reverse transcriptase (Invitrogen), and the specified amounts of target RNA, which were incubated at 63°C for 60 min in Loopamp real-time turbidimeter (LA-200; Teramecs, Japan). The analysis of each sample was performed in a set of four tubes, each of which had the primer mixture for a particular serotype. Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Monitoring of amplification by the RT-LAMP assay. (i) Real-time monitoring. The real-time monitoring of amplification of the dengue virus template by the RT-LAMP assay was observed through spectrophotometric analysis by recording the optical density at 400 nm every 6 s with the help of the 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), which was when the turbidity increases above the threshold value, fixed at 0.1, which is two times more than the average turbidity value of the negative controls of several replicates. None of the positive samples tested over multiple times showed

positivity in terms of increased turbidity after 30 min. Therefore, a sample with a T_p value ≤ 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- μ l aliquot of the RT-LAMP assay products was electrophoresed on 3% NuSieve 3:1 agarose gel (BioWhittaker Molecular Applications, Rockland, Maine) in Tris-borate buffer, followed by staining with ethidium bromide and visualization on a UV transilluminator at 302 nm.

(iii) Naked-eye visualization. In order to facilitate the field application of the RT-LAMP assay, the monitoring of amplification by the RT-LAMP assay was also carried out through naked-eye inspection. Following amplification, the tubes were inspected for white turbidity with 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 observation of a color change following addition of 1 μ l (1:1,000) of SYBR Green I dye to the tube. In the case of positive amplification, the original orange color of the dye changes to green, which 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 is retained. This change of color is permanent and, thus, can be kept for record purposes.

Specificity of RT-LAMP assay. The specificity of the RT-LAMP assay-amplified product was further validated by restriction digestion with a single restriction enzyme (RE) with a cutting site at one end of the target sequence of all four serotypes. Based on the RE site profile of the target sequences of each serotype, the *Ban*II enzyme was selected for restriction analysis of the products of DEN-1, -2, -3, and -4 amplified by the RT-LAMP assay. Following overnight digestion at 37°C, the digested products were analyzed by agarose gel electrophoresis as described above. In addition, the authenticities of the amplified products were also established by nucleotide sequencing of both digested and undigested products with two outer and two inner primers. Further cross-reaction studies within the four serotypes of dengue virus and with other closely related members of the *Flavivirus* family, viz., JE, WN, and SLE viruses, were also performed to ensure the specificity of each serotype-specific dengue RT-LAMP assay primer.

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

RT-PCR and nested PCR. In order to compare the sensitivity and specificity of the RT-LAMP assay, a one-step serotype-specific RT-PCR was performed with confirmed dengue virus-infected patient serum samples by employing the serotype-specific primers of Morita et al. (18). In the case of suspected dengue virus-infected samples, following the initial amplification by RT-PCR with dengue virus group-specific consensus primers, a second round of amplification was carried out by nested PCR with serotype-specific internal primers, according to the protocol described by Lanciotti et al. (13). The amplification was carried out in a 50- μ l total reaction volume with a TaKaRa LA Taq PCR kit (Takara Bio Inc., Japan) by using both Revertra RTAce (Toyobo, Japan) and LA Taq DNA polymerase (Takara) and 50 pmol of forward and reverse primers with 2 μ l of RNA, according to the manufacturer's protocol.

Evaluation of RT-LAMP assay. The applicability of the RT-LAMP assay for detection of dengue virus RNA in clinical specimens was validated by evaluating the assay system with a limited number of DF and DHF patient serum samples infected with each serotype. In addition, a panel of serum samples from healthy individuals comprising 20 volunteer blood donors from the Philippines was also included as a negative control to rule out the possibility of false-positive reactions. A total of 83 serum samples, comprising 25 samples from confirmed cases, 38 samples from suspected cases, and 20 samples from healthy individuals were processed for RNA extraction with the QIAamp viral RNA mini kit and were simultaneously screened by RT-LAMP, RT-PCR, and nested PCR for detection of viral RNA, as described above.

RESULTS

The success of RT-LAMP assay amplification relies on the specificities of the primer sets designed. The primers were selected on the basis of highly conserved regions of the dengue virus genome. Following nucleotide sequence alignment, the potential target regions were selected in the core and premembrane (C-prM), envelope (Env), and nonstructural 5' and 3' NCRs. Initial attempts to design dengue virus complex- and group-specific primers did not succeed owing to the lower percentage of homology (60 to 70%) among the four serotypes. Therefore, serotype-specific primers against the selected tar-

gets were designed, as mentioned above. To avoid primer dimer formation, a set of six primers for each serotype was designed after mismatches among the strains was taken into account. Upon screening of the several primer sets, four sets of

FIG. 2. Agarose gel electrophoresis and restriction analysis of dengue virus serotype-specific RT-LAMP assay products on a 3% agarose gel. Lane M, 100-bp DNA ladder (Sigma Genosys, Japan); lane 1, DEN-1 RT-LAMP assay amplification; lane 2, RE (BanII) digestion of DEN-1 RT-LAMP assay product, 109 bp; lane 3, DEN-2 RT-LAMP assay amplification; lane 4, RE (BanII) digestion of DEN2 RT-LAMP product, 132 bp; lane 5, DEN-3 RT-LAMP assay amplification; lane 6, RE (BanII) digestion of DEN-3 RT-LAMP assay product, 172 bp; lane 7, DEN-4 RT-LAMP assay amplification; lane 8, RE (BanII) digestion of DEN-4 RT-LAMP assay product, 186 bp; lane 9, negative control without target RNA.

FIG. 3. Real-time amplification of dengue virus by serotype-specific RT-LAMP assay depicting the kinetics of each serotype with regard to time of positivity. O.D., optical density

primers in the 3' NCR region were found to be serotype specific.

By using the primer sets selected, a four-tube one-step RT-LAMP assay system was standardized for rapid detection as well as serotyping of the dengue viruses. 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. 2). A chart showing the real-time amplification of each dengue virus serotype is depicted in Fig. 3, which shows different times of positivity, depending on the concentration of virus used. The times of positivity observed through real-time monitoring were found to be 14 min for DEN-1, 16 min for DEN-2, 12 min for DEN-3, and 15 min for DEN-4 (Fig. 3).

The monitoring of RT-LAMP assay amplification was also carried out by naked-eye inspection following addition of 1 μ l of SYBR Green I dye to the amplified products. In case of positive amplification, the color of the reaction mixture

changed to yellow by the addition of SYBR Green I, whereas in case of no amplification, the original orange color of the dye was retained, as observed with the negative controls with no RNA template (Fig. 4, left panel). The change of color in case of positive amplification was more appreciated as a bright apple green by using a UV light source (Fig. 4, right panel).

Sensitivity and specificity of serotype-specific dengue virus-specific RT-LAMP assay. In order to ascertain the detection limit of each of the dengue virus serotype-specific primers, serial 10-fold dilutions of each virus serotype that had been quantified by plaque assay were tested, and the results were compared with those of the one-step direct RT-PCR with serotype-specific primer pairs. The RT-LAMP assay demonstrated exceptionally higher sensitivity than the conventional RT-PCR. The RT-LAMP assay was found to be 10- to 100-fold more sensitive than RT-PCR, with a detection limit of 0.1 to 1 PFU of virus. The detection limits of the serotype-specific primers were determined to be 1 PFU for DEN-1 and 0.1 PFU for DEN-2, -3, and -4. By the real-time monitoring of the amplifica-

FIG. 4. SYBR Green I fluorescent dye-mediated monitoring of dengue virus serotype-specific RT-LAMP assay amplification. (Left) 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 for a negative control with no amplification, the original orange color is retained. (Right) Visual observation of green fluorescence of DNA binding SYBR Green I under UV light.

FIG. 5. Standard curves for four dengue virus serotype-specific RT-LAMP assays generated from the amplification plots between serial 10-fold dilutions of different virus concentrations (PFU) and the time of positivity by employing the serotype specific RT-LAMP assay.

tion of different concentrations of virus ranging from 10 to 10^6 PFU/ml, a standard curve depicting the linear relationship between the concentrations of virus to the time of positivity was generated for each serotype of dengue virus (Fig. 5).

The specificity of the serotype-specific dengue RT-LAMP assay was established through cross-reaction studies with the RNA templates of other closely related members of the *Flavivirus* group, as mentioned in Materials and Methods. It was demonstrated that all four serotype-specific primers were highly specific for the detection and differentiation of the appropriate serotypes. None of the serotype-specific primer sets amplified the JE, WN, or SLE viral RNA template, thereby indicating the specificity of the assay for each dengue virus serotype. The authenticity of the amplified product of each serotype was further established by digestion with a restriction enzyme (BanII) that has one cutting site at one end of the selected target of all the four serotypes. As depicted in Fig. 2, the size of the resultant digested product was in good agreement with the predicted size for each serotype, i.e., 109 bp for DEN-1, 132 bp for DEN-2, 172 bp for DEN-3, and 186 bp for DEN-4 (Fig. 2). Further confirmation of the structures of the amplified products was also carried out by sequencing, in which

the sequences obtained perfectly matched the expected nucleotide sequences (data not shown).

Evaluation of dengue virus RT-LAMP assay with patient serum samples. The applicability of the RT-LAMP assay for detection and differentiation of dengue virus serotypes in patient serum samples was validated by evaluating acute-phase serum samples from both DF and DHF patients infected with all the four serotypes, and the results were compared with those of conventional RT-PCR. A total of 25 acute-phase serum samples collected from patients confirmed to have dengue (DEN-1, $n = 5$; DEN-2, $n = 13$; DEN-3, $n = 6$; and DEN-4, $n = 1$), as confirmed by virus isolation and RT-PCR, were analyzed. In addition, 38 serum samples from suspected dengue patients from the ongoing dengue epidemic in India collected in 2004 were also screened for the presence of dengue virus RNA. As mentioned above, a panel of 20 healthy volunteer serum samples was also included as a negative control. Among the positive samples, the RT-LAMP assay could detect the dengue virus genome in all 31 positive samples (100%), whereas RT-PCR detected the virus genome in 87% (27 of 31) of the samples and virus isolation detected the virus

TABLE 2. Comparative evaluation of dengue virus serotype-specific RT-LAMP assay with RT-PCR, nested PCR, and virus isolation for detection of dengue virus in acute-phase patient serum samples^a

Type of case	Virus serotype	No. of samples	No. of samples positive by:			
			RT-LAMP ^d	RT-PCR	Nested PCR	Virus isolation
Confirmed ^b	DEN-1	5	5	5	NA ^e	4
	DEN-2	13	13	13	NA	12
	DEN-3	6	6	6	NA	4
	DEN-4	1	1	1	NA	1
Suspected ^c		38	6	2	4	4
Healthy		20	0	0	NA	NA
Total		83	31	27		25

^a Serum samples collected from days 1 to 7 after the onset of symptoms are referred to as acute-phase samples.

^b All serum samples were selected from patients with confirmed cases of dengue by a positive virus isolation result, a positive RT-PCR result, and/or a positive IgM ELISA result with a positive/negative ratio ≥ 2 .

^c Samples from clinically suspected patients referred by the Municipal Corporation of Delhi, Delhi, India, for screening are referred to as suspected samples.

^d In comparison with the results of the other assays, the RT-LAMP assay had a concordance of 100%, a sensitivity of 100%, and a specificity of 93%.

^e NA, not attempted.

genome in 81% (25 of 31) of the samples. The statistical analysis of the results suggests that the sensitivity of the RT-LAMP assay is higher than those of conventional RT-PCR and virus isolation ($P = 0.043$).

The sensitivity and specificity of the RT-LAMP assay for the detection of viral RNA in patient serum samples with reference to the results of virus isolation were 100% and 93%, respectively (Table 2). Among the suspected dengue patient serum samples, two samples were positive by both RT-LAMP and RT-PCR. However, the RT-LAMP assay could pick up four additional cases that were negative by RT-PCR (Table 2). Of those four samples, two were found to be positive by nested PCR and two were found to be positive by virus isolation, indicating the higher sensitivity of the RT-LAMP assay for the correct identification of samples with low levels of virus that were missed by RT-PCR and nested PCR. It was also observed that none of the four dengue virus serotype-specific RT-LAMP assay primer sets cross-reacted with any of the serum samples from healthy individuals analyzed in this study, thereby establishing the specificity of the dengue virus RT-LAMP assay. The quantification of the virus load in the positive samples was determined on the basis of their time of positivity by employing the standard curve for the corresponding serotype. Most of the samples had virus concentrations in the range of 10 to 10³ PFU/ml of serum, although in few samples the amount of virus was quite high, corresponding to 10⁶ PFU/ml (Fig. 6).

DISCUSSION

During the past decade, various nucleic acid amplification techniques, such as RT-PCR, nested PCR, Taqman real-time RT-PCR, SYBR Green I real-time RT-PCR, and NASBAs, have been developed to address the need for the rapid identification of dengue virus to the serotype level with more accuracy (12, 13, 15, 18, 23, 25). Even though the magnitude of amplification can be obtained, these PCR-based methods require either high-precision instruments for the amplification or elaborate methods for detection of the amplified products. In addition, these methods are often cumbersome to adapt to

routine clinical use, especially in peripheral health care settings and private clinics.

In this regard, the RT-LAMP assay reported in this study is advantageous due to its simple operation, rapid reaction, and easy detection. This is the first report on the application of the RT-LAMP assay technique for the rapid detection and differentiation of dengue virus serotypes. The one-step, four-tube, real-time RT-LAMP assay employs serotype-specific primers spanning the proximal half of the 3' NCR of the viral genome. Several group-specific and type-specific RT-PCR systems based on the 3' NCR of the dengue virus genome have been reported (9, 25). In the present study, the RT-LAMP primers were designed from the distal half of the 3' NCR, as the proximal half has major mismatches and missing sequences. In our experience with West Nile virus and the sudden acute respiratory syndrome-associated coronavirus, it was observed that the RT-LAMP assay primers are effective for strains with more than 90% homology with two to three internal mismatches. However, the RT-LAMP assay primers are not effective when the homology is less than 90% and when there are four to six mismatches. The 60 to 70% homology among the different serotypes was why we could not succeed in designing the dengue virus group-specific consensus RT-LAMP assay primers. Prior to designing the serotype-specific primers, we compared the sequence homologies of all the strains of each serotype available in GenBank, and more than 90% homology was observed among the different strains of each serotype. So, we hope that the dengue virus serotype-specific RT-LAMP assay primers designed in the present study will work for all the reported strains of each serotype.

The real-time RT-LAMP assay can also be useful in the quantitation of the virus load in a clinical sample, which in turn reflects the viremic status of the patient. Besides, the RT-LAMP assay demonstrated exceptionally higher sensitivity by correctly picking samples with low levels of virus that were missed by RT-PCR and nested PCR. The results for the RT-LAMP assay- and nested PCR-positive samples that were negative by virus isolation may be attributed to inactivation of the

FIG. 6. Quantification of virus titer in acute-phase dengue patient serum samples, as determined from the time of positivity based on the RT-LAMP assay standard curve for each dengue virus serotype.

virus. In addition, the RT-LAMP assay also demonstrated a high degree of specificity for dengue virus serotypes, with no false-positive results with any of the serologically related flaviviruses tested or with serum samples from healthy humans, indicating that it is highly specific for the target sequence.

The RT-LAMP assay is a simple diagnostic tool in which the reaction is carried out in a single tube by mixing of the buffer, primers, reverse transcriptase, and DNA polymerase and incubation of the mixture at 63°C for 1 h. Compared to RT-PCR and real-time PCR, the RT-LAMP assay has the advantages of reaction simplicity and detection sensitivity. The higher sensitivity and the higher specificity of the RT-LAMP assay reaction are attributed to continuous amplification under isothermal conditions by employing six primers that recognize eight distinct regions of the target. Besides, the higher amplification efficiency of the RT-LAMP reaction yields large amounts of a by-product, pyrophosphate ion, which leads to a white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in turbidity of the reaction mixture because of 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 measurement of turbidity (17, 19). The other isothermal amplification techniques, such as NASBAs and the self-sustained sequence reaction, 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 detection of the amplified products due to the poor specificity of target sequence selection (3, 15).

As discussed above, the execution of the RT-LAMP reaction and the measurement of its turbidity are extremely simple compared to the ease of performance of existing real-time Taqman RT-PCR assays and NASBAs, which require fluorogenic primers and probes as well as expensive detection equipment. One of the most attractive features of the RT-LAMP assay seems to be its great advantage in terms of monitoring of amplification, which can be accomplished by SYBR Green I dye-mediated naked-eye visualization and by real-time moni-

toring by using an expensive turbidimeter, according to the situation. The particular importance is the substantial reduction in time required for the confirmation of results by the RT-LAMP assay, which is less than 1 h (30 min), whereas RT-PCR requires 3 to 4 h.

In conclusion, the RT-LAMP assay developed in this study allows the rapid and accurate identification of dengue virus serotypes. Due to its simple operation without the need for sophisticated equipment, it will be a valuable tool for the rapid detection and differentiation of dengue virus serotypes in well-equipped laboratories, small-scale clinical laboratories, as well as field situations, like peripheral health care settings in developing countries.

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