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Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Method for Rapid Detection of Severe Acute Respiratory Syndrome Coronavirus

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The development and evaluation of a one-step single-tube accelerated real-time quantitative reverse transcription (RT) loop-mediated isothermal amplification (LAMP) assay is reported for rapid detection of the severe acute respiratory syndrome coronavirus (SARS-CoV) replicase gene. A total of 49 samples (15 throat washes, 13 throat swabs, and 21 combined throat and nasal swabs) collected from patients admitted to the Hanoi-French and Ninhbinh hospitals in Vietnam during the SARS epidemic were evaluated and compared to conventional RT-PCR. The RT-LAMP assay demonstrated 100-fold-greater sensitivity, with a detection limit of 0.01 PFU. The sensitivity and specificity of RT-LAMP assay for detecting viral RNA in clinical specimens with regard to RT-PCR were 100 and 87%, respectively. The specificity of the RT-LAMP assay was further validated by restriction analysis as well as nucleotide sequencing of the amplified product. The concentration of virus in most of the clinical samples was 0.1 PFU (0.1 to 10^2 PFU), as determined from the standard curve of SARS RT-LAMP and based on the time of positivity. The assay procedure is quite simple, wherein the amplification is carried out in a single tube under isothermal conditions at 63°C, and the result can be obtained in less than 1 h (as early as 11 min). Thus, the RT-LAMP assay reported here has the advantages of rapid amplification, simple operation, and easy detection and will be useful for rapid and reliable clinical diagnosis of SARS-CoV in developing countries.

Severe acute respiratory syndrome (SARS) is a recently emerged human disease associated with pneumonia. The fears of a SARS epidemic still loom throughout the world due to multicountry outbreak, rapid air travel, and spread into the wider community, especially among healthcare and laboratory workers. The outbreak is believed to have originated in November 2002 in the Guangdong province of China, with several hundred cases of severe atypical pneumonia (18, 21). Following the detection of similar cases in Hong Kong, Vietnam, and Canada, the World Health Organization (WHO) issued a global alert for the illness, designated SARS (7, 16, 20). The disease has since then spread rapidly around the world and caused 8,099 cases, with 774 deaths in 30 countries, spreading over five continents, with an economic loss estimated to be $50 billion to $100 billion worldwide (1, 4, 21).

The coordinated efforts of the WHO through international collaboration resolved the etiology of the SARS to be a novel coronavirus (CoV) with some unusual properties that had not previously been present in human populations (3, 6, 13). Unlike other human CoVs, SARS-CoV could be isolated in Vero cells. The genome of SARS-CoV is single-stranded, nonsegmented positive-sense RNA, which is 29,727 nucleotides in length with 11 open reading frames. The genome organization is similar to that of other CoVs with the characteristic gene order 5′-replicase (Rep)-spike-envelope-membrane-nucleocapsid-3′ and short untranslated regions at both termini (8, 18).

SARS is a type of viral pneumonia with an incubation period ranging from 2 to 7 days. The infection is usually characterized by fever, dry cough, dyspnea, headache, and hypoxemia (17). Death from progressive respiratory failure occurs in about 3% to nearly 10% of cases (14). The laboratory findings include lymphopenia and mildly elevated aminotransferase levels. Death may result from progressive respiratory failure due to alveolar damage in 3 to 6% cases (10, 16). The mechanism of transmission of the SARS virus is not yet fully established. The virus is believed to be spread by droplets produced by coughing and sneezing; however, other routes of transmission, such as fecal contamination, cannot be ruled out (2). Currently, there are no suitable antiviral drugs or an effective vaccine for SARS virus. Rapid laboratory confirmation of SARS-CoV infection is therefore important for managing patient care and for preventing nosocomial transmission. Thus, development of early, rapid, and reliable diagnostic assay systems for SARS-CoV is of top priority to strengthen the surveillance mechanisms for the prediction and prevention of large-scale epidemics in future.

The laboratory diagnosis of SARS-CoV is based on virus isolation, followed by electron microscopy studies to identify the virus on cell culture, which are technically very demanding. Serologically, the diagnosis is accomplished by looking for anti-

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CoV antibodies by employing indirect fluorescent antibody testing and enzyme-linked immunosorbent assays. Although some patients have detectable CoV antibody within 14 days of illness onset, definitive interpretation of negative CoV antibody tests is possible only for specimens obtained >21 days after the onset of fever (11, 14). While serological testing is reliable as a retrospective diagnostic method, diagnosis of the infection in the early phase of the illness is important for patient care. To address the need for early and rapid identification of SARS-CoV, a reverse transcription (RT)-PCR-based assay was advocated by the WHO and is being routinely used for detecting virus-specific RNA (15, 22, 23). The existing PCR tests are reported to be very specific but lack the sensitivity and are time-consuming. The testing of more than one respiratory specimen is advocated for improved sensitivity of the existing PCR assays (23). More-sensitive and real-time-based assays are therefore needed to complement the existing PCR-based assay systems.

The present report describes the development and evaluation of a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), for the rapid detection of SARS-CoV in clinical specimens in Vietnam. The LAMP is a novel approach for nucleic acid amplification that amplifies DNA with high specificity, selectivity, and rapidity under isothermal conditions, thereby obviating the need for a thermal cycler (10, 12). The amplification efficiency of the LAMP method is extremely high because there is no time loss for thermal change due to its isothermal reaction. Since the amplification of DNA is directly correlated with the production of magnesium pyrophosphate, leading to turbidity, real-time monitoring of the LAMP reaction is possible by real-time measurement of turbidity in an inexpensive photometer (9). Therefore, the LAMP assay has emerged as a powerful tool to facilitate point-of-care genetic testing at the bedside. We report the development of a one-step single-tube accelerated real-time quantitative RT-LAMP assay for rapid detection of SARS-CoV. Data on the sensitivity and specificity of the method are reported, and applicability of the technology for clinical diagnosis of SARS is discussed.

MATERIALS AND METHODS

Clinical specimens. A total of 49 specimens comprising 15 throat washes, 13 throat swabs, and 21 combined throat and nasal swabs samples were collected from 45 patients with clinically suspected SARS admitted to the Hanoi-French and Ninhbinh hospitals during the SARS epidemic in Vietnam between 4 February and 7 March 2003. Of 45 patients, 10 had confirmed cases of SARS, as reported by the Centers for Disease Control and Prevention, and 35 patients who had cases that fulfilled the modified WHO definition (22) of SARS were grouped as having suspected and probable cases. All of the samples were collected on day 3 after the onset of illness.

Virus. The SARS-CoV isolated from the throat wash sample of one patient was used in this study for standardization of the RT-LAMP assay. Briefly, the monolayers of Vero E6 cells grown in a 15-cm^2 culture flask were 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. The cells were then incubated at 37°C and observed daily for cytopathic effects. Upon observation of 80 to 100% cytopathic effect, the infected culture supernatant was clarified by light centrifugation at 700 × *g* for 10 min and stored in aliquots at −80°C until use. The quantification of virus infectivity was carried out in Vero E6 cells grown in 24-well tissue culture plates as per standard protocol (19). The resulting plaques were counted, and virus titer was determined.

Designing of primers. Oligonucleotide primers used for RT-LAMP amplification of SARS-CoV were designed from the open reading frame 1b Rep gene sequences obtained from GenBank (accession number NC-004718) after comparing the alignment of Rep gene sequences of CoVs from human, bovine, porcine, and avian origins. The sequences of the selected primers were conserved to other SARS-CoV Rep gene sequences. A set of six primers comprising two outer, two inner, and two loop primers that recognize eight distinct regions on the target sequence were designed by using the LAMP primer designing support software program (Net Laboratory, Kanagawa, Japan). The detailed sequences of primers used for amplification of SARS-CoV are shown in Fig. 1. The two outer primers are described as forward outer primer (F3) and backward outer primer (B3). The inner primers are described as forward inner primer (FIP) and backward inner primer (BIP). An additional two loop primers (loop F and loop B) were designed to accelerate the amplification reaction. FIP consists of a complementary sequence of F1 (Fig. 1) and a sense sequence of F2. BIP consists of a complementary sequence of B1 and a sense sequence of B2. The loop F and loop B primers are composed of the sequences that are complementary to the sequence between the F1 and F2 and B1 and B2 regions, respectively.

RNA extraction. The clinical specimens were suspended in viral transport medium. Following centrifugation at 6,500 × *g* for 1 min, the supernatant was collected. Viral RNA was extracted from 140 μl of the supernatant by using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). The initial processing of specimens was performed under biosafety level 3 containment facilities. After lysis of the sample by the lysis buffer, the mixture was applied to a spin column as described by the manufacturer. The extracted RNA was eluted in a total volume of 60 μl of elution buffer and was stored at −70°C until further use.

RT-LAMP assay. The RT-LAMP reaction was carried out in a 25-μl (total) reaction mixture by using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tochigi, Japan) containing 40 pmol (each) of the primers FIP and BIP, 5 pmol (each) of the outer primers F3 and B3, 20 pmol (each) of loop primers F and B, 1.4 mM concentrations of deoxynucleoside triphosphates, 0.8 M betaine, 0.1% Tween 20, 10 mM $(NH_4)_2SO_4$, 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 avian myeloblastosis virus reverse transcriptase (Invitrogen), and the specified amounts of target RNA was incubated at 63°C for 60 min in a heating block, followed by heating at 80°C for 2 min to terminate the reaction. For the real-time monitoring of RT-LAMP assay, the reaction mixture was incubated at 63°C for 60 min in the Loopamp real-time turbidimeter (LA-200; Teramecs, Kyoto, Japan). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Interpretation of RT-LAMP results. The real-time amplification by RT-LAMP assay was monitored through spectrophotometric analysis by recording the optical density at 400 nm every 6 s with the help of a Loopamp real-time turbidimeter (LA-200; Teramecs). The cutoff value for positivity by the real time RT-LAMP assay was determined by taking into account the real time to positivity (T_p, in minutes) at which 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 multiple times showed positivity in terms of increased turbidity after 60 min. Therefore, a sample having T_p values of ≤60 min and turbidity above the threshold value of ≥0.1 was considered positive. In addition, 10-μl aliquots of RT-LAMP products were electrophoresed on a 3% NuSieve 3:1 agarose gel (BMA, Rockland, Maine) for electrophoresis in Tris-borate buffer, followed by staining with ethidium bromide and visualization on a UV transilluminator at 302 nm. The specificity of the RT-LAMP amplified product was further validated by restriction digestion with HpaI enzyme as well as by nucleotide sequencing of both digested and undigested products with two outer and two internal primers.

RT-PCR. To compare the sensitivity and specificity of the RT-LAMP assay, RT-PCR was performed with the primer sets designed by the WHO SARS network laboratory at the Bernhard-Nocht Institute in Hamburg, Germany with slight modifications. The sequences of these primers were 5′-ATGAATTACCA AGTCAATGGTTAC (BNIout-sense) and 5′-CATAACCAGTCGGTACAG CTAC (BNIout-antisense). Following cDNA synthesis with the reverse primer (BNIout-antisense) at 42°C for 30 min, the PCR amplification was carried out with the LA *Taq* PCR kit (TaKaRa Bio Inc., Shiga, Japan) by using 1 μl of cDNA and 50 pmol of each primer in a 50-μl total reaction volume according to the manufacturer's protocol. The thermal profile for RT-PCR was 94°C for 2 min, followed by 34 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. The amplified products were then analyzed through a 3% NuSieve 3:1 agarose gel (BMA) by electrophoresis in Tris-borate buffer, and the target bands were visualized by staining with ethidium bromide.

Evaluation of RT-LAMP. The applicability of the RT-LAMP assay for detection of SARS-CoV RNA in clinical diagnosis was validated by evaluating the

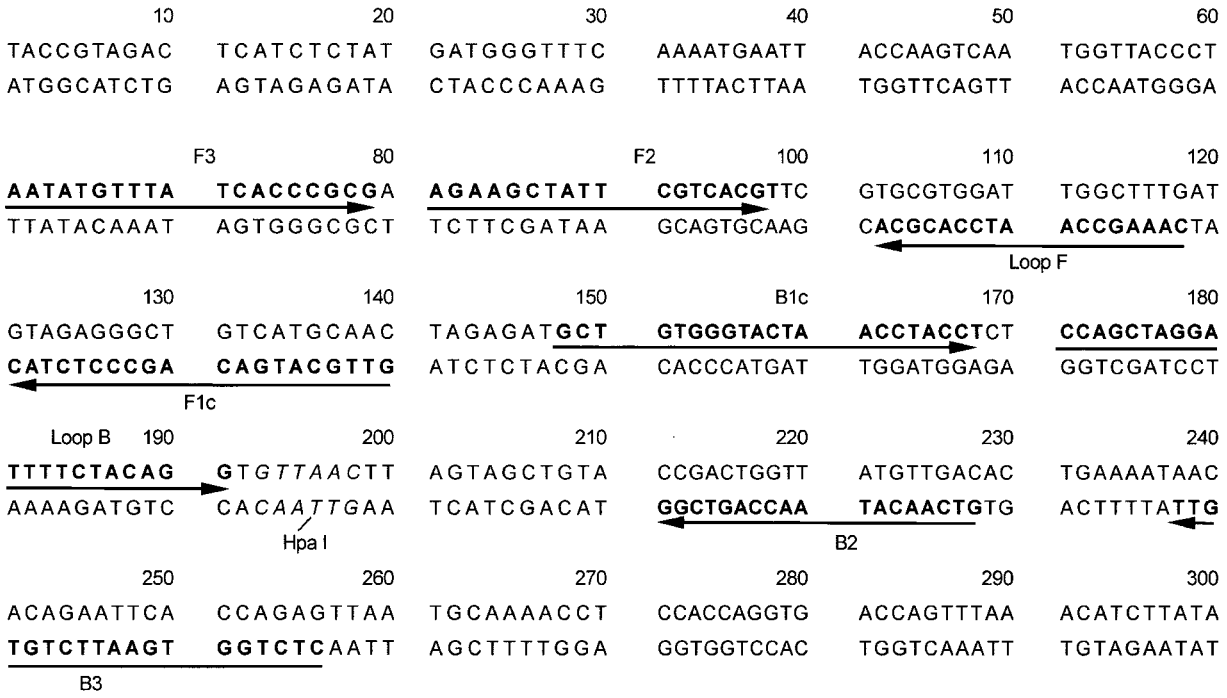

FIG. 1. Oligonucleotide primers used for RT-LAMP amplification of SARS-CoV (GenBank accession number NC-004718). The underlined letters indicate the sequences of primers.

assay system with the 49 samples (15 throat washes, 13 throat swabs, and 21 combined throat and nasal swabs) collected from patients admitted to hospitals as mentioned above. In addition, 10 throat wash samples collected from apparently healthy individuals were also included as negative controls. All 59 samples comprising 49 suspected SARS samples and 10 healthy samples were processed for RNA extraction by the QIAamp viral RNA mini kit and were screened by RT-LAMP and RT-PCR simultaneously for detection of viral RNA as described above.

RESULTS

The RT-LAMP assay was standardized with the SARS-CoV isolated from one of the patient throat wash samples. The identity of the isolated SARS-CoV was established by confirmation through RT-PCR with the specific primer sets described above. The RT-LAMP assay successfully amplified the 196-bp target sequence of the Rep gene of SARS-CoV at 63°C in 60 min, as observed by agarose gel electrophoresis. 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). The real-time kinetics of the RT-LAMP reaction revealed that the amplification signal with 10^4 PFU of virus could be detected as quickly as in 11 min, as indicated by the continuous increase in turbidity (Fig. 3), whereas the turbidity was fixed around 0.01, below the threshold value in the negative control which had no template.

Sensitivity and specificity of SARS RT-LAMP. To ascertain the detection limit of the RT-LAMP assay for the detection of SARS-CoV, serial 10-fold dilutions of virus that had been quantified by plaque assay were tested and compared with that of conventional RT-PCR. The detection limits for the RT-LAMP assay and RT-PCR were found to be 0.01 and 1 PFU, respectively (Fig. 4A and B, respectively). Thus, the comparative sensitivity of RT-LAMP and RT-PCR indicated that the RT-LAMP was 100-fold more sensitive than RT-PCR. The amplification by RT-LAMP showed a ladder-like pattern, whereas the RT-PCR showed a 195-bp amplicon (Fig. 4B). By

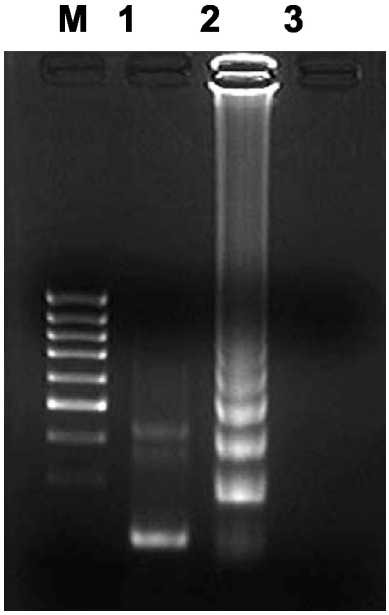

FIG. 2. Agarose gel electrophoresis and restriction analysis of RT-LAMP products of the Rep gene of SARS-CoV on a 3% agarose gel. Lane M, 100-bp DNA ladder (Sigma Genosys, Hokkaido, Japan); lane 1, RT-LAMP product of SARS-CoV digested with HpaI (135 bp); lane 2, RT-LAMP products of SARS-CoV; lane 3, RT-LAMP without target RNA.

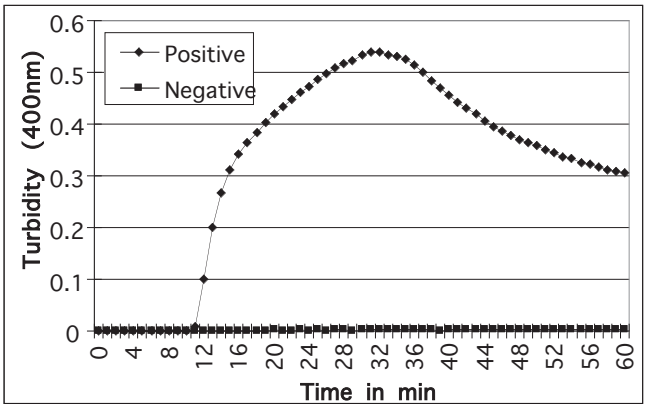

FIG. 3. Real-time kinetics of RT-LAMP amplification of SARS-CoV RNA, as monitored by real-time measurement of turbidity on a Loopamp real-time turbidimeter (LA-200; Teramecs).

real-time monitoring of the amplification of different concentrations of virus ranging from 10^4 to 0.01 PFU, a standard curve depicting the linear relationship between the concentration of virus (in PFU) to T_p was generated for SARS-CoV (Fig. 5A).

The specificity of the amplification was confirmed by digestion with restriction enzyme HpaI to ensure that the amplification product had the corresponding sequences of the selected target. The resultant digested product of 135 bp was in good agreement with the predicted size (Fig. 2). Further confirmation of the structures of the amplified products were also carried out by sequencing, wherein the sequences obtained perfectly matched with the expected nucleotide sequences (data not shown)

Evaluation of SARS RT-LAMP with clinical specimens. A total of 59 samples including 15 throat washes, 13 throat swabs, 21 combined throat and nasal swabs, and 10 healthy throat wash samples were screened by RT-LAMP and RT-PCR simultaneously. The RT-LAMP assay detected 13 positive samples and 46 negative samples; the RT-PCR assay detected 6 positive samples and 53 negative samples. A concordance of 88% (52 of 59) was observed between the two test systems. Of 59 samples, 6 were positive and 46 were negative by both tests. However, the RT-LAMP assay was able to detect SARS-CoV RNA in 7 additional specimens that were negative by RT-PCR. The sensitivity and specificity of the RT-LAMP assay with regard to RT-PCR were 100 and 87%, respectively. Of 15 throat wash samples, 67% (10 of 15) were positive by RT-LAMP compared to 27% positive by RT-PCR (Table 1). None of the throat swab samples were positive by any of the test systems. The concentration of the virus in different kinds of clinical samples was calculated from the standard curve based on their T_p values. Of 13 positive samples, 8 samples had a virus concentration of 0.1 PFU. The range of virus concentration among different kind of samples was 0.1 to 10^2 PFU (Fig. 5B). None of the healthy throat wash samples tested were positive, thereby indicating the specificity of the two assay systems.

DISCUSSION

SARS is a newly emerging life-threatening respiratory disease that has created international anxiety because of its nov-

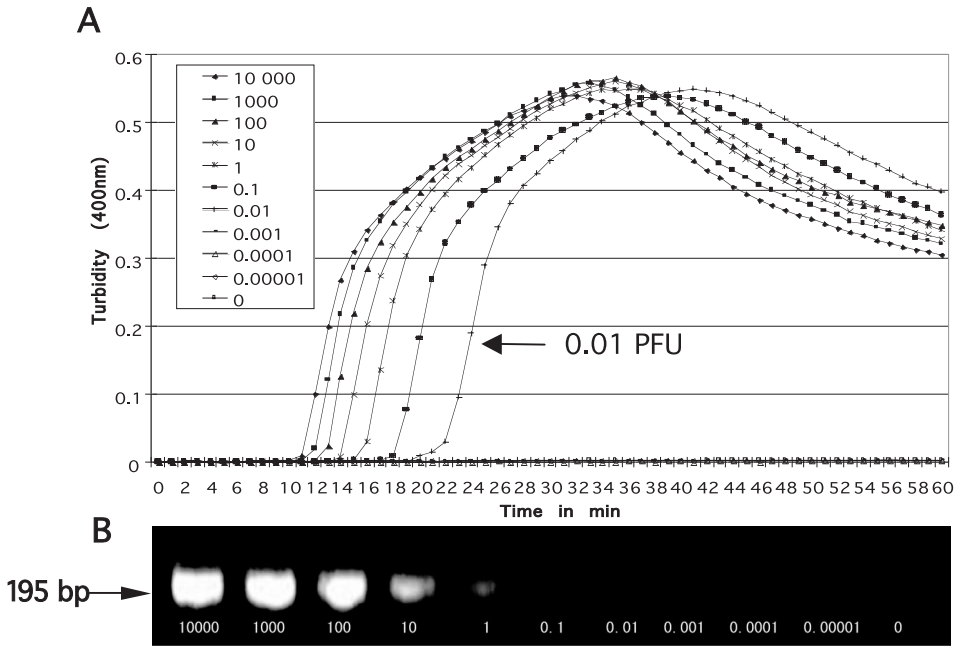

FIG. 4. Comparative sensitivity of RT-LAMP and RT-PCR for detection of SARS-CoV. The amplification by RT-LAMP (A) showed ladder-like pattern, whereas the RT-PCR (B) showed 195-bp amplification. (A) Sensitivity of SARS RT-LAMP assay as monitored by real-time measurement of turbidity (LA-200; Teramecs). Shown from left to right are the curves of decreasing concentrations of virus from 10,000 to 0.00001 PFU. The detection limit for the assay was 0.01 PFU. (B) Sensitivity of RT-PCR for the detection of SARS-CoV as observed by agarose gel analysis with a detection limit of 1 PFU.

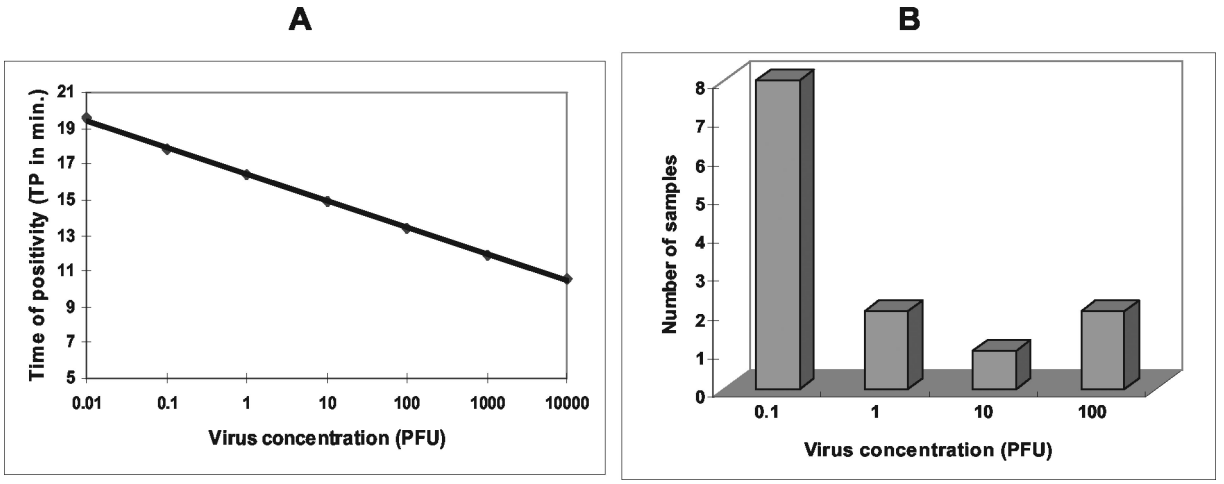

FIG. 5. Quantitative determination of virus concentration in clinical samples employing a standard curve of the SARS RT-LAMP assay. (A) Standard curve generated for SARS RT-LAMP assay by plotting T_p with regard to various concentrations of virus in PFU. (B) Determination of virus titer in clinical samples derived from the standard curve based on their T_p values.

elty, communicability, and rapid spread, leading to multicountry outbreak. The development of early, rapid, and reliable diagnostic assay systems are of high priority for early vigilance and timely implementation of control measures. The pace of SARS research has been astounding. The identification of the virus and subsequent availability of the SARS-CoV genome sequence is important from a public health perspective. This has led to the development of rapid and reliable PCR-based assays based on the novel sequence features helping in the discrimination of SARS from other circulating CoVs. SARS virus genome-based PCR assays formed an important part of a public health strategy to control the spread of this syndrome.

The prompt communication and exchange of information among the WHO collaborating laboratories facilitate the development of RT-PCR-based rapid diagnostic assays. This is now being routinely practiced all over the globe for rapid identification of SARS cases. In addition, quantitative PCR methods like a real-time PCR and *Taq*Man format assays to detect SARS-CoV in nasopharyngeal aspirate and stool samples have been developed (15). Despite the obtainable magnitude of amplification, these methods are time-consuming (e.g., RNA extraction, RT, and real-time PCR can be completed in 3 to 4 h) and more complicated and need a high-precision thermal cycler, and contamination of samples in laboratories may lead to false-positive results.

TABLE 1. Comparative evaluation of RT-LAMP assay and RT-PCR for detection of SARS-CoV RNA in clinical specimens

Type of specimen	No. of samples tested	% (no.) of positive samples for assay	
		RT-LAMP	RT-PCR
Throat wash	15	67 (10)	27 (4)
Throat swab	13	0	0
Throat and nasal swabs	21	14 (3)	10 (2)
Healthy throat wash	10	0	0
Total	59	22 (13)	10 (6)

On the contrary, the RT-LAMP assay reported here is advantageous due to its simple operation, rapid reaction, and easy detection. The RT-LAMP assay is a simple diagnosis 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 1 h in a regular laboratory water bath or heat block that provides a constant temperature of 63°C. The amplification can be performed in a shorter time than amplification by RT-PCR because there is no time loss due to thermal cycling. In addition, the specificity of the reaction is extremely high because it uses six primers recognizing eight distinct regions on the target DNA. The LAMP method was initially standardized for detection of hepatitis B virus DNA (12) and was recently applied for direct detection of the *Mycobacterium tuberculosis* complex, *Mycobacterium avium*, and *Mycobacterium intracellulare* in sputum samples (5). By using RNA as a template, the RT-LAMP assay has also been used for the detection of prostate-specific antigen mRNA in K562 cells (12).

We developed a one-step single-tube accelerated real-time quantitative RT-LAMP assay for the early and rapid diagnosis of SARS-CoV. The assay can detect SARS-CoV at an early stage of infection by using throat washes and combined throat and nasal swab specimens. The evaluation of the RT-LAMP assay for detection of viral RNA in clinical specimens has been validated with 59 samples, including 15 throat washes, 13 throat swabs, 21 combined throat and nasal swabs, and 10 healthy throat wash samples collected from the Hanoi-French and Ninhbinh hospitals during the SARS epidemic in Vietnam, and results were compared with those of conventional RT-PCR. Of 49 samples, 10 samples were confirmed to be SARS-CoV, as reported by the Centers for Disease Control and Prevention, by using virus isolation and/or nested RT-PCR methods, and 35 samples were grouped as probable or suspected cases based on the WHO case definition.

The data presented in this study suggested that the RT-LAMP assay is more sensitive than RT-PCR by picking up 7 additional cases that were negative by RT-PCR. It is pertinent

to note that all 7 of these samples were collected from confirmed cases of SARS. The RT-LAMP assay demonstrated exceptionally higher sensitivity than conventional RT-PCR. The RT-LAMP assay was found to be 100-fold more sensitive than RT-PCR, with a detection limit of 0.01 PFU in clinical samples. In addition, by using a real-time RT-LAMP assay, the quantitation of virus concentration in the clinical sample is possible, which will indicate the early stage of the virus infection as well as potential source transmitters.

Among the various types of clinical specimens overall, 22% of throat washes and 10% of combined throat and nasal swabs were positive by both RT-LAMP and RT-PCR assays employed in this study. None of the throat swab samples were positive by any of the test systems, indicating only that throat swab samples are not suitable for detection of SARS-CoV RNA. This is in agreement with those of previous studies (3, 6), wherein it was demonstrated that swab specimens yield less virus for detection by virus isolation. From a diagnostic point of view, it is important to note that nasal and throat swabs seem less suitable for diagnosis, since these materials contain considerably less viral RNA than throat washes, and the virus may escape detection if only these materials are tested. The throat wash samples were found to be the most suitable specimens for detection of SARS-CoV.

In conclusion, the one-step single-tube accelerated real-time quantitative RT-LAMP assay developed in this study is simple, rapid, and cost effective as well as highly sensitive and specific. This has potential usefulness for clinical diagnosis and surveillance of SARS virus in developing countries, as it does not require the use of sophisticated equipment or skilled personnel.

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