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In situ reverse-transcription loop-mediated isothermal amplification (*in situ* RT-LAMP) for detection of Japanese encephalitis viral RNA in host cells

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ABSTRACT

Background: Clinical diagnosis of Japanese encephalitis is usually difficult due to non-specific signs at the early and acute stages of the infection. Virus isolation from peripheral blood is also not possible because of the short period and low level of transient viremia even in the acute stage of the disease. It is thus urgent to develop a feasible and convenient method for laboratory diagnosis of the infection.
Objectives: To establish a newly designed molecular approach that can be used to detect intracellular Japanese encephalitis viral RNA in host cells.
Study design: The method was firstly established and then was carried out to test its efficacy in cultured BHK-21 cells, subsequently in peripheral blood mononuclear cells (PBMCs) isolated from mice that have been inoculated with JE virus suspension.
Results: In this study, *in situ* reverse-transcription loop-mediated isothermal amplification (*in situ* RT-LAMP) was established; which combines merits of recently developed loop-mediated isothermal amplification (LAMP) and *in situ* reverse-transcriptase polymerase chain reaction (*in situ* RT-PCR).
Conclusions: The newly designed method can detect viral RNAs in peripheral blood mononuclear cells (PBMCs) in a short time with high sensitivity and efficiency.

1. Background

Japanese encephalitis (JE) is one of the very important mosquito-borne viral infectious diseases in the world, infecting approximately 10% of the susceptible population in Southeast Asian countries each year.[1] The etiological agent of JE belongs to the family Flaviviridae which also includes many other related viruses such as yellow fever virus, dengue viruses, and West Nile virus. The genome of the JE virus is composed of a positive single-stranded RNA of approximately ~10,000 bases in length.[2] Infection rates of JE virus in epidemic regions usually vary, ranging from a few cases to 20% of the population.[3] Clinically, at least 50,000 cases and 10,000 deaths occur each year, mostly among children in Asia,[4] in spite of mass vaccinations which have been implemented in many endemic countries.[5]

Diagnosis of JE by clinical symptoms is not feasible due to non-specific signs at the early and acute stages of the infection. Neither is it possible to isolate the JE virus from peripheral blood because of the short period and low level of transient viremia even in the acute stage of the disease.[6] Nowadays, the laboratory serodiagnosis of JE uses a versatile and convenient approach to detect immunoglobulin M (IgM) or IgG antibodies in serum or cerebrospinal fluid (CSF) by enzyme-linked immunosorbent assays (ELISAs).[7,8] More recently, molecular techniques, *e.g.*, the reverse-transcriptase polymerase chain reaction (RT-PCR), to amplify gene fragments of viral RNAs are increasingly being used to make diagnoses.[9,10] Nevertheless, the low copy number of viral RNA in the blood of JE patients makes it difficult to extract RNA from this source.[11] Utilization of the RT-PCR to detect viral RNAs in peripheral blood mononuclear cells (PBMCs), also called *in situ* RT-PCR, was thus developed as a molecular tool which does not require messenger (m)RNA extraction. The technique has been applied to diagnosing JE virus infection in PBMCs in a mouse model.[11]

The technique of loop-mediated isothermal amplification (LAMP) is based on the principle of a strand displacement reaction and the stem-loop structure that amplifies the target gene fragment under isothermal conditions.[12,13] Because the target is initially recognized by six distinct sequences, followed by another four distinct sequences, amplification of the target sequence is expected to be highly selective.[13] Furthermore, there is no need for any high-cost instrument (such as a thermocycler or gel imaging system), making the technique reliable, simple, rapid, and cost-effective for detecting and differentiating examined genomic nucleic acids.[12] Further, reverse-transcription LAMP (RT-LAMP) was designed; which is particularly useful for detecting RNA viruses.[14]

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2. Objectives

In this study, we aimed to establish a new approach, named as *in situ* reverse-transcription loop-mediated isothermal amplification (*in situ* RT-LAMP), in order to detect JE virus infection in cultured cells and in PBMCs isolated from infected mice.

3. Study design

Establishment of this approach is combined the techniques of RT-LAMP and *in situ* RT-PCR which was previously described by this laboratory.

3.1. Virus and cell culture

JE virus used in this study was the T1P1 strain that was previously isolated from field-caught *Armigeres subalbatus*.[15] The virus was propagated in C6/36 cells and titrated in BHK-21 cells based on previous reports. BHK-21 cells are being maintained in our laboratory at 37 °C with 5% CO_2 in minimum essential medium (GIBCO™ BRL Life Technologies, Grand Island, NY, USA) that contains 2% non-essential amino acids, 2.2 g/ml sodium bicarbonate ($NaHCO_3$), 0.4% antibiotic–antimycotic, and 10% fetal bovine serum. The plaque assay following our previous description was used for virus titration in this study.[15] The virus titer was calculated and expressed as plaque-forming units per milliliter (PFU/ml).

3.2. Primer design

The oligonucleotide primers used for RT-LAMP amplification used in this study were designed from the prM gene of the JE virus. The sequence of the T1P1 strain was retrieved from the GenBank database (accession no. AF254453). Sets of six primers comprising two outer (F3 and B3), two inner (FIP and BIP), and two loop primers (LF and LP) were selected based on the criteria shown in a previous description.[13] 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. The two loop primers were designed to accelerate the amplification reaction. The LF and LB primers were composed of sequences complementary to the sequences between the F1 and F2 and the B1 and B2 regions, respectively. Details of the primers with regard to their positions in the genomic sequence are shown in Table 1.

3.3. RNA extraction

Genomic viral RNA of the JE virus was extracted from infected culture supernatant using Viral Nucleic Acid Extraction Kit II (Geneaid Biotech, Taipei, Taiwan) in accordance with the manufacturer's protocol. For total RNA extraction from JE virus-infected BHK-21 cells, the standard Trizol (GIBCOBRL® Life Technologies)-chloroform-isopropyl-ethanol wash method was used as described previously.[15]

Table 1
List of primers used for RT-LAMP amplification of the Japanese encephalitis virus prM gene.

Primer name	Genome position	Sequence (5′–3′)
F3	497–516	GGGGAAGCTTTTGATGACCA
B3	663–682	C AC C AGC AATCC AC ATC CTC
FIP	F2: 526–544 Flc: 567–587	CCGGACCCAGCATCTGTTCTCCGGACATTGCAGACGTTAT
BIP	B2: 636–655 B2c: 591–612	ATCGACGTCGGCTACATGTGTGTTGCCCATGGTAAGCTTAGG
LF	545–566	CCTTTTGAGGTGGGAATCACG
LB	613–635	AGGACACTATCACGTACGAATGT

3.4. RT-PCR

In this study, the RT-PCR was used to compare with the RT-LAMP assay described below. The primer pair (10F and 877R) was used to amplify a specific genomic fragment of nt 10–877 of the JE virus, covering the 5′ non-coding region, C, and prM genes. The sequences of the 10F sense primer and the complementary 877R primer respectively were 5′-CTGTGTGAACTTCTTGGCTTAGTATCG-3′ and 5′-TCAGTTTTCATGAGATATCGTGTGTGGC-3′.

3.5. Two-step RT-LAMP assay

In this study, RT was performed with the BIP primer at 42 °C for 60 min. Then the LAMP reaction was carried out in a 25-μl total reaction mixture containing 1 μl cDNA mixture, 0.4 μM each of inner primers FIP and BIP, 0.05 μM each of outer primers F3 and B3, 0.2 μM each of loop primers LF and LB, Bst DNA polymerase (New England BioLabs, Ipswich, MA, USA), 10× buffer (New England BioLabs), 60 mM Betaine (Sigma, St. Louis, MO, USA), 0.4 mM dNTP, 0.2% Tween 20, and 1 μl of the target complementary (c)DNA. The mixture was incubated at 65 °C for 60 min.

3.6. In situ RT-LAMP assay

In this study, BHK-21 cells were infected with the T1P1 strain of the JE virus for 8 h at a multiplicity of infection (MOI) of 1 and then transferred from a flask to microtubes. Cells were fixed with 4% paraformaldehyde for 20 min, and then permeabilized with 0.1% Triton X-100 for 3 min at 4 °C. For the *in situ* reaction, after RT, cells were precipitated by centrifugation at 2000 × *g*. After removing the RT reaction mixture, cells were re-suspended with the LAMP reaction mixture, and the LAMP reaction was performed as mentioned above. In the following step, cells were treated with a blocking solution (5% bovine serum albumin; BSA) for 15 min. In order to visualize the target cDNA inside cells, we added 0.4 μM digoxigenin (DIG)-labeled primer (the 5′-end of the inner FIP primer labeled with DIG) or 0.01 mM DIG-labeled dCTP to the LAMP reaction. After cells were washed with phosphate-buffered saline (PBS), 50 μl of the diluted goat anti-DIG antibody conjugated with alkaline phosphatase (Roche, Mannheim, Germany) (1:500 in PBS) was added to the microtubes for a 1-h incubation. Subsequently, cells were washed twice with washing solution 1 which contained 100 mM maleic acid and 150 mM NaCl (pH 7.5) for 10 min followed by washing solution 2 (100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$; pH 9.5). Subsequently, the substrate nitroblue tetrazolium chloride/5-bromo-3-chloro-3-indolyl-phosphate (NBT/BCIP) was added to the microtubes and incubated for 10 min. Cells were washed again with PBS, removed from the tubes, and distributed onto 12-well slides.

3.7. Isolation of mouse peripheral blood mononuclear cells (PBMCs)

In this study, each female ICR mouse was intravenously injected with 100 μl of PBS (the control group) or 3×10^6 PFU of the JE virus suspension. PBMCs were then isolated from 1 to 2 ml of whole blood, taken at 1, 3, or 5 d post-inoculation, and pooled from seven or eight inoculated mice, with the Ficoll-Hypaque solution (GE Healthcare Biosciences, Uppsala, Sweden).

3.8. Southern blot

DIG-labeling of the primer or dCTP was used in this study to visualize amplified gene fragments in RT-LAMP; which was carried out by the method of Southern blotting. Briefly, a 20-μl aliquot of RT-LAMP products was first run by electrophoresis on a 2% agarose

gel in TAE buffer. The gel was treated with denaturing solution (1.5 M NaCl and 0.5 N NaOH) twice for 15 min each and then with neutralizing solution (0.5 M Tris–HCl and 3 M NaCl) for 30 min. After being transferred onto a nylon membrane, the membrane was exposed to UV for 3 min for cross-linking (Spectrolinker XL-1000 UV crosslinker). The membrane was then immersed in blocking solution (0.05% BSA, 100 mM maleic acid, 150 mM NaCl; at pH 7.5; then autoclaved) for 30 min, incubated with the anti-DIG-AP antibody (1:5000 in blocking solution) for 30 min, and ultimately in the solution containing the NBT/BCIP substrate until the color could be visualized.

4. Results

4.1. Detection of JE virus RNA with a two-step RT-LAMP method

The success of the RT-LAMP method relies on the specificity of the designed primer sets. The primers were selected to target the prM gene derived from the T1P1 strain of the JE virus. In order to validate the sensitivity and specificity of the primer set, 0.1 and 0.5 μg of total RNA extracted from JE virus- or mock-infected BHK-21 cells were separately subjected to the RT-LAMP reaction at 42 °C and 60 min for RT and subsequently at 65 °C and 60 min for the LAMP reaction. In the meantime, the RT-PCR was used in the same experiment as a positive control. The results indicated that the viral RNA could be detected from the extract of JE virus-infected BHK-21 cells, but not that from mock-infected cells, at both concentrations, shown as DNA ladder-like products (Fig. 1A). In the RT-PCR, viral RNA was also detected and showed a single band representing the 868-bp fragment (Fig. 1B). To examine the limit of the reaction time, LAMP was performed at 10-min intervals (20–60 min). While the viral gene was detected as early as 40 min, a time course of 60 min for amplification was shown to be the optimal condition (Fig. 2).

4.2. Optimization of the build-up of in situ RT-LAMP with DIG-labeling

In this study, DIG-labeling was used for better visualization after the reaction. When the *in situ* RT-LAMP was run with the DIG-labeled primer, amplified DNA ladder-like products were clearly detected in both groups using the unlabeled or Dig-labeled primer, although the signal when using the latter primer was relatively weak (Fig. 3A). However, no signal appeared on the nylon membrane on which amplified DNA fragments had been transferred, indicating that DIG molecules had not been incorporated into the amplified fragments (Fig. 3B).

On the other hand, using the *in situ* RT-LAMP with the DIG-labeled dCTP, DNA ladder-like amplified products were shown in the unlabeled group and in two groups labeled with dCTP (0.001 and 0.01 mM), but not in that from mock-infected BHK-21 cells

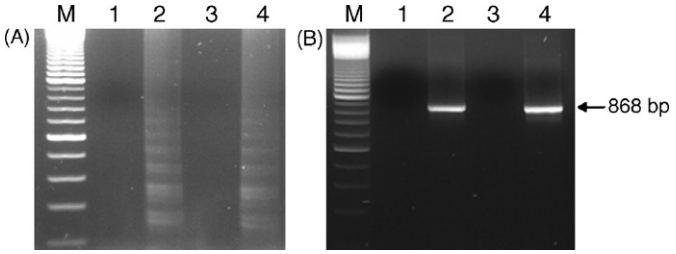

Fig. 1. Detection of Japanese encephalitis (JE) viral RNA extracted from infected BHK-21 cells at different RNA concentrations. (A) RT-LAMP was used for the detection. (B) RT-PCR was used for the detection. Lane M, 100-bp DNA ladder; lane 1: 0.1 μg RNA from mock-infected cells; lane 2: 0.1 μg RNA from JE virus-infected cells; lane 3: 0.5 μg RNA from mock-infected cells; lane 4: 0.5 μg RNA from JE virus-infected cells.

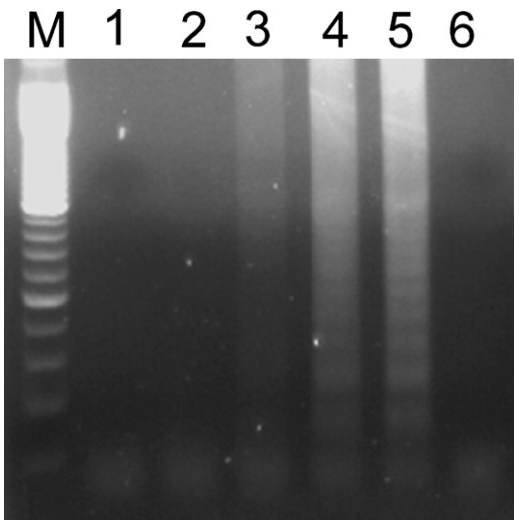

Fig. 2. Determination of time limits for efficiently performing the LAMP reaction to detect Japanese encephalitis (JE) virus. The viral gene appeared as early as 40 min, while the result with best resolution was shown at 60 min of amplification. Lane M, 100-bp DNA ladder; lanes 1–5: respective reaction times of 20, 30, 40, 50, and 60 min; lane 6: blank reaction which served as the negative control.

(Fig. 4A). When the RT-LAMP products were transferred onto nylon membranes and visualized with the relevant antibody and substrate, as mentioned above, DNA ladders were actually present on the membrane in a dose-dependent manner (Fig. 4B).

4.3. Detection of JE virus residing in BHK-21 cells using in situ RT-LAMP

When the *in situ* RT-LAMP was run with the DIG-labeled primer, no positive reaction was shown in either JE virus-infected (Fig. 5A) or uninfected (Fig. 5B) BHK-21 cells. However, a positive reaction

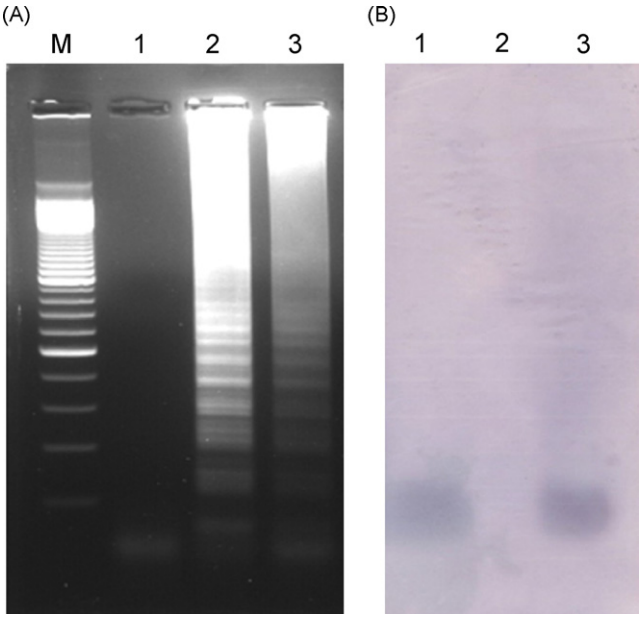

Fig. 3. *In situ* RT-LAMP performed with a Dig-labeled primer to detect Japanese encephalitis (JE) virus. (A) RNA extracted from mock-infected (lane 1) or JE virus-infected (lanes 2 and 3) BHK-21 cells were subjected to the RT-LAMP reaction with unlabeled (lane 2) or Dig-labeled (lanes 1 and 3) primer. (B) After the DNA products were transferred onto a nylon membrane, no signal was shown in any lane even when the reaction was performed with the unlabeled (lane 2) or Dig-labeled (lanes 1 and 3) primer.

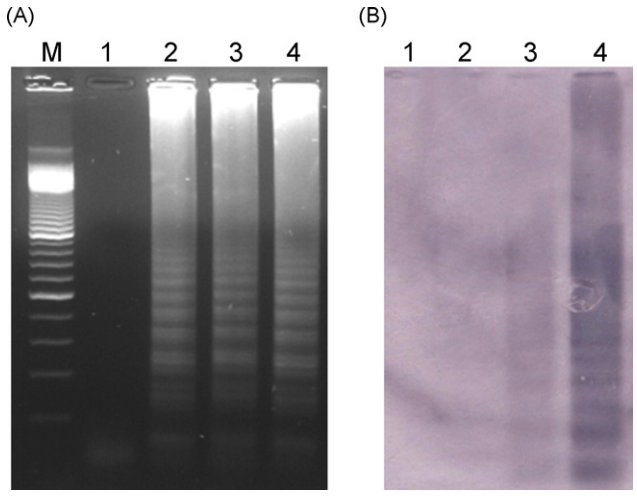

Fig. 4. *In situ* RT-LAMP performed with Dig-labeled dCTP to detect Japanese encephalitis (JE) virus. (A) RNA extracted from mock-infected (lane 1) or JE virus-infected (lanes 2–4) BHK-21 cells were subjected to the RT-LAMP reaction with unlabelled (lanes 1 and 2), or 0.001 (lane 3) or 0.01 mM (lane 4) Dig-labeled dCTP. (B) A nylon membrane carrying the RT-LAMP products derived from JE virus-infected cells (lanes 3 and 4) was positive after being treated with anti-DIG antibodies and the substrate NBT/BCIP.

with a deep-brown color was obvious in JE virus-infected BHK-21 cells (Fig. 5C), but not in uninfected cells (Fig. 5D), when the reaction was run with the Dig-labeled dCTP.

4.4. Detection of JE virus residing in isolated PBMCs

Using the DIG-labeled dCTP, results showed that the JE virus was detected in all PBMCs isolated from mice that had been inoculated with the virus suspension for 1, 3, and 5 d (Fig. 6A–C), but not from mock-inoculated mice (Fig. 6D). In addition, the results revealed that the highest infection rate of PBMCs occurred at 3 d post-inoculation, while a relatively low rate of PBMCs was shown at 5 d post-inoculation (Fig. 6B).

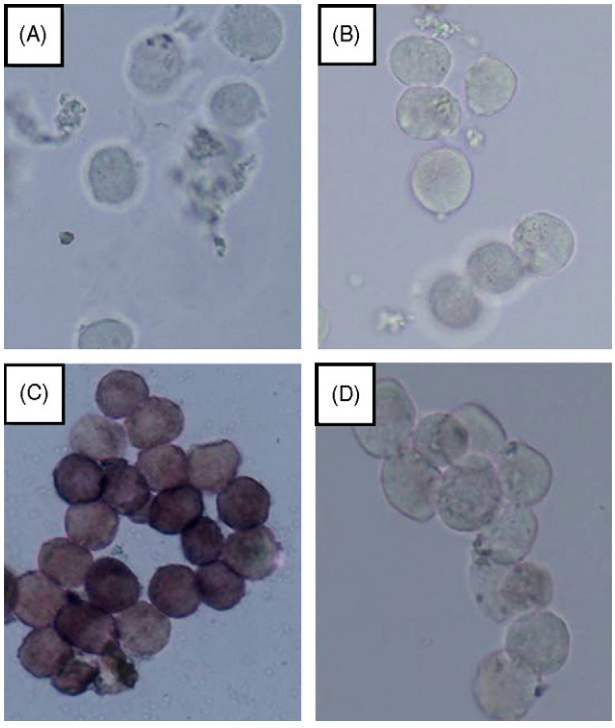

Fig. 5. Detection of Japanese encephalitis (JE) virus in BHK-21 cells using *in situ* RT-LAMP with a Dig-labeled primer or dCTP. Only infected cells detected with the Dig-labeled primer had a positive reaction. (A) Infected cells detected with the Dig-labeled primer. (B) Uninfected cells detected with the Dig-labeled primer. (C) Infected cells detected with Dig-labeled dCTP. (D) Uninfected cells detected with Dig-labeled dCTP (original magnification, 400×).

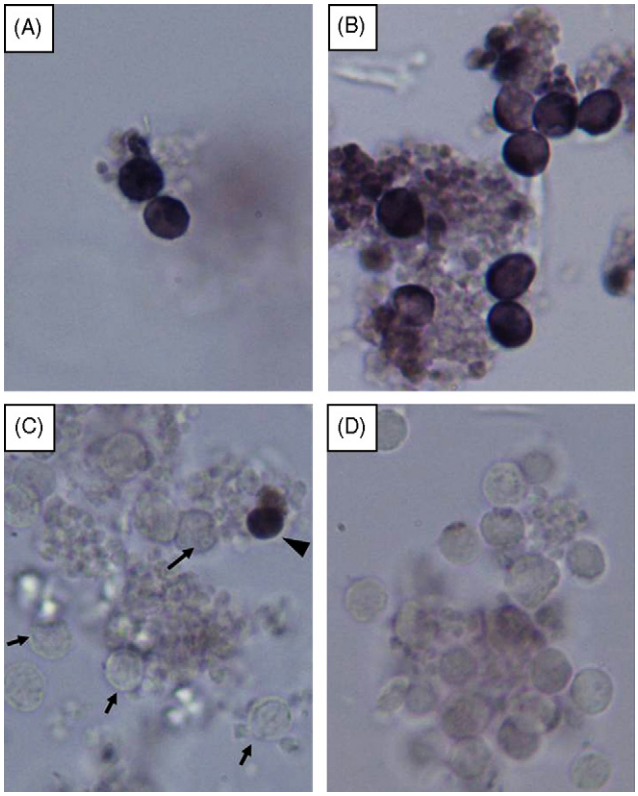

Fig. 6. Detection of Japanese encephalitis (JE) virus residing in isolated peripheral blood mononuclear cells (PBMCs). A positive reaction appeared in PBMCs isolated from a mouse blood pool (7–8 mice/pool). (A) PBMCs isolated from mice at 1 d post-inoculation; (B) PBMCs isolated from mice at 3 d post-inoculation; (C) PBMCs isolated from mice at 5 d post-inoculation; (D) PBMCs isolated from mock-infected mice as the negative control (original magnification, 400×).

5. Discussion

The known pathogenesis of JE reveals that the virus enters PBMCs including monocytes/macrophages as the primary site for replication in vertebrate hosts. Subsequently, the virus migrates to the central nervous system (CNS) where brain inflammation or encephalitis may occur.[16] JE can be fatal; fatality rates of the infection statistically differ in different localities from 10% to 50%.[17] Those who recover from the infection with clinical symptoms often display sequelae with serious neurological impairment and/or mental retardation.[18,19] As a result, rapid and efficient techniques for the early diagnosis of JE are urgently and critically needed. In past years, virus isolation from nerve tissues and antibody (IgM and IgG) detection from serum and/or CSF have extensively been used to detect JE virus in most laboratories. However, molecular techniques including RT-PCR and real-time RT-PCR assays are believed to be more useful due to their characteristics of rapidity, high sensitivity, and high specificity.[20,21] The techniques have been routinely used for identifying the JE virus in acute-phase serum or CSF samples from patients.[21] Drawbacks of these molecular techniques actually include requirements for delicate labor and experienced techniques as well as a relatively expensive instrument, resulting in restricted application in a number of laboratories in developing countries.

Various gene fragment-amplification techniques, such as RT-PCR, real-time RT-PCR (TaqMan or SYBR green), and nucleic acid sequence-based amplification (NASBA), have been developed during the past decade, with the goal of rapidly identifying the JE virus with greater accuracy.[22–24] Despite the high magnitude of amplification, these PCR-based methods require either high-precision instruments for amplification or elaborate methods for detection of the amplified products. In the meantime, these methods are often cumbersome to adapt for routine clinical use. After the development of the LAMP technique, a new era of the rapid and efficient identification of microbial infections has been initiated. It is particularly useful as its reaction can be monitored through real-time detection of changes in turbidity.[13,25] Moreover, the high sensitivity and specificity facilitates its potential use in the laboratory diagnosis of a number of infections, covering all kinds of organisms.[12] Thus far, the technique has been applied to detect or identify viruses,[26–29] fungi,[30] bacteria,[31] protozoa,[32–34] and even vertebrates.[35]

Recently, the technique of RT-LAMP was modified from the conventional LAMP; this is specifically useful in efficiently and rapidly detecting or identifying various RNA viruses, including flaviviruses,[28,36,37] togaviruses,[38] the severe acute respiratory syndrome (SARS) coronavirus,[26] and the H5N1 avian influenza virus.[27] Application of RT-LAMP to JE virus detection has been shown to be relatively rapid and allows for virus quantification.[14] It was demonstrated that the technique can be used for the rapid diagnosis of JE using acute-phase CSF samples during an epidemic.[37] In spite of RT-LAMP detection of the JE virus being rapid and quantitative, this approach requires a larger number of serum or CSF specimens to achieve a clinical diagnosis of JE patients. It was noted that the JE virus can persist in mice for 4–16 weeks following intraperitoneal inoculation and may later be reactivated.[39] In addition, the JE virus is also reported to persist in the nervous system of some patients.[40] As a matter of fact, flaviviruses including the JE virus and other flaviviruses have been identified to exist in PBMCs of convalescent blood samples, indicating the possibility of persistent viral infection in leukocytes.[41] Moreover, JE viral RNA fragment has ever been amplified from white blood cells during the acute phase of infection.[42] This suggests that detection of such viruses in leukocytes may help make a diagnosis even though the blood was collected in the acute viremia stage. In order to achieve this goal, we herein established a technique called *in situ* RT-LAMP, which was successfully applied to detect JE virus in both cultured cells and PBMCs isolated from infected mice.

The technique of *in situ* RT-LAMP can avoid the drawback from the potential inefficiency of RNA extraction that is a necessary step for conventional RT-PCR as well as RT-LAMP. Furthermore, one advantage is that the experiment can be run at a constant temperature. Thus there is no need for a thermocycler that is necessary when running this technique with a conventional RT-PCR. Although *in situ* RT-PCR can actually skip the process of RNA extraction, it still needs an expensive instrument, a thermocycler. In other words, *in situ* RT-LAMP actually is more convenient than both *in situ* RT-PCR and LAMP.

To build up a higher-efficiency protocol, we initially carried out DIG-labeling of the primers. However, no satisfactory signal was shown either in gel electrophoresis or on blotted NC membranes in the experiment using infected BHK-21 cells. This suggested that the large size of DIG molecules labeled on the outer primer FIP probably interrupted their binding to stem-loop DNA. Thus we shifted the DIG-labeling to dCTP. It turns out that the amplification of nucleic acids was successfully detected; it could clearly be differentiated from that in the control group (mock-infection). This indicated that Dig-labeling of dCTP is relatively efficient, compared to primer labeling, and is much more efficient, resulting in better visualization and easier differentiation from negative results. In addition to detecting the JE virus in cultured BHK-21 cells, the technique was also applied to detect the virus in PBMCs isolated from whole blood of infected mice. Eventually, the virus was detected at 1, 3, and 5 d post-inoculation, consistent with our previous work using *in situ* RT-PCR.[11] Nearly half of the inoculated mice dying of the disease (data not shown) suggested that most infected PBMCs may have migrated into brain tissues.[43]

Taken together, this newly designed technique of *in situ* RT-LAMP is a sensitive (a higher detection rate), rapid (no more than 1 h), efficient (under isothermal conditions), cheap (no need for expensive instrument), and convenient (not labor-intensive) method. This new diagnostic design can serve as a robust tool for diagnosing JE virus infection, especially in remote and developing countries. It is particularly useful to detect viruses in blood samples, usually the genomic copy number is low,[42] collected at the acute stage or which are persistently infected. Theoretically, this delicate technique can also be applied to diagnosing other viruses that normally infect leukocytes or PBMCs, *e.g.*, human immunodeficiency virus (HIV), with advantages of economy, convenience, and high efficiency.

Conflict of interest

We would like to declare that there is no financial or personal relationship with other people or organizations that could inappropriately influence our work during the submission process.

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