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DEVELOPMENT OF A NEW METHOD FOR DIAGNOSIS OF *COXSACKIE B5* VIRUSES BY REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

K.JAIANAND*[1], P.GUNASEKARAN[1], M.RAJKUMAR[1]AND A.K.SHERIFF[2]

[1]Department of Virology, King Institute of Preventive Medicine, Chennai-32, India.
[2]Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA.

*_Corresponding Author_ jaianand.k@lifecellinternational.com

ABSTRACT

We developed a one-step, single-tube genogroup-specific reverse transcription–loop-mediated isothermal amplification (RT-LAMP) assay for the detection of group B *Coxsackie* genomes targeting 5' UTR region. The amplification can be obtained in less than 1 h by incubating all of the reagents in a single tube with reverse transcriptase and *Bst* DNA polymerase at 63℃. Detection of gene amplification could be accomplished by agarose gel electrophoresis, as well as , the monitoring of gene amplification can also be visualized with the naked eye by using SYBR green I fluorescent dye. A total of 8 samples comprised of 6 positive samples and 2 negative samples were used in this study for comparative evaluation with RT-PCR. Thus, due to easy operation without a requirement of sophisticated equipment and skilled personnel, the RT-LAMP assay reported here is extremely rapid, cost-effective, highly sensitive, and specific and has potential usefulness for rapid detection of NPEV.

KEYWORDS

Coxsackie B5 viruses (Cox B5), Non-Polio Enterovirus (NPEV), 5'-UnTranslated Region (5'-UTR), Reverse Transcription – Polymerase chain Reaction (RT – PCR), Reverse Transcription–Loop Mediated Isothermal Amplification (RT-LAMP).

INTRODUCTION

The group B *Coxsackie* viruses consist of six serotypes (1-6), classified within the enterovirus genus of the family Picornaviridae. *Coxsackie* B viruses are the etiological agents of a wide spectrum of human disease, including mild respiratory infection, aseptic meningitis, and fatal myocarditis. Outbreaks of *Coxsackie* B viruses' infection occur annually throughout the world[1]. The major clinical presentations of *Coxsackievirus* B5 infections are similar to those observed for other group B *Coxsackieviruses* and include myopericarditis

and encephalomyocarditis syndrome, aseptic meningitis, meningoencephalitis, and acute flaccid paralysis. Hand, foot, and mouth disease and herpangina also were reported, and a potential association with development of type 1 diabetes has been suggested[2, 3]. Approximately half of all *Coxsackievirus* B5 detections came from young infants, and CSF was the most common source. The summer-fall seasonality in *Coxsackievirus* B5 detections was more prominent than for most other serotypes[4].

The routine diagnostic methods for *Coxsackievirus* by virus cell culture are followed by serum neutralization test, the gold standard for Enterovirus typing[5]. This method is generally reliable but also labor-intensive, time-consuming, and costly. Furthermore, the supply of antisera is limited and the problem of "untypeable" Enteroviruses is frequently encountered. The comparatively slow procedures of in vivo amplification of *Coxsackie* viruses in the cell culture may be replaced by rapid in vitro amplification of viral RNA sequences by the Loop mediated isothermal Amplification (LAMP).

More recently, several investigators have reported on fully automatic Real-Time Polymerase Chain Reaction (RT-PCR) assays for the detection of Enteroviruses. Moreover PCR-based technology holds more promise. As yet, however, PCR has not established a routine foothold in clinical laboratories because it is a time-consuming complex and needs a high-precision thermal cycler. Reaction equipment that is much simpler and amenable for use in hospital laboratories is required. By contrast, LAMP assay reported here is advantageous owing to its simple operation, rapid reaction, and easy detection. LAMP operates under isothermal conditions at 63℃ for 1 h. Therefore, no time is lost as a result of changes in temperature, as is the case with thermal cycling with PCR. Moreover, LAMP requires only simple reaction equipment; it can be performed using a regular laboratory bath or heat block that provides a constant temperature of 63℃[6]. It is even possible to determine the reaction directly with the naked eye, without electrophoretic analysis, unlike PCR. Since the LAMP assay is simple and relatively easy to perform, even a clinical microbiological laboratory that has not performed PCR or molecular testing could introduce this technology.

MATERIALS AND METHODS

Clinical samples: A total of 25 CSF received from patients with a clinical diagnosis of meningitis, acute flaccid paralyses were used for evaluation in this study. The samples were collected during the period between days 1 and 7 after the onset of symptoms. All the samples were stored at -80℃ until further investigation. In addition, a panel of 5 CSF collected from healthy individuals was also included as negative controls. Prior to RT-LAMP assay, out of these 25 samples, 6 samples were investigated for the presence of *Coxsackie* B5 specific RNA by RT-PCR and RT-LAMP.

Virus strains: Prototype strains of *Coxsackie B5 viruses (CVB5-Faulkner)* were propagated in HEp2 cell line. After complete cytopathic effect, the culture was harvested after three cycles of freezing and thawing. Initial clarification was carried out by centrifugation at 1000Xg for 15 min at 4°C and supernatant are stored at -80°C.

Cell Culture:HEp-2 cell line, RD cells-derived from human rhabdomyosarcoma, L20B-genetically engineered mouse cell line expressing the human poliovirus receptor was maintained at 37℃ under 5% CO_2 by regular sub culturing at periodic intervals of 4 to 5 days in Eagle's Minimal Essential medium (PAN Biotech, Aidenbach, Germany).

Viral RNA extraction: The genomic viral RNA was extracted from 100µl of standard viral 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 80µl of elution buffer and was stored at -70℃ until use.

Reverse transcription and PCR:cDNA was synthesized in a 20µl reaction mixture containing 75mM Tris-HCl (pH 8.3) (Sigma,St.Louis(MO), USA), 3mM $MgCl_2$ (Sigma,St.Louis(MO), USA), 10mM dTT (Roche Diagnostic,Penzberg, Germany), 0.2mM each dNTPs (Roche Diagnostic,Penzberg, Germany), 50pmol of primer "B" **5'-ATTGTCACCATA AGCAGCCA-3'** (Roche Diagnostic,Penzberg, Germany) positions 580-599 refer to the *Coxsackie* B-1 sequence, 10U of avian myoblastosis virus reverse transcriptase (Roche Diagnostic,Penzberg, Germany), and 5µl of RNA isolated from clinical samples and the reference viruses. After incubation at 37°C for 60 min, 1µl of cDNA was added to PCR master mix containing 50mM KCl (Sigma,St.Louis(MO), USA), 10mM tris HCl (pH-8.9) (Sigma,St.Louis(MO), USA), 3.6mM $MgCl_2$ (Sigma,St.Louis(MO), USA), 10mM dTT, 0.2 mM each dNTPs (Roche Disgnostic, USA), 50 pmol of each primer B and primer "A1" **5'-CAAGCACTTCTGTTTCCCCGG-3'** (Roche Diagnostic,Penzberg, Germany) positions 160-180 and 2.5U of *Taq* DNA polymerase (Roche Diagnostic,Penzberg, Germany).

Sensitivity of RT-PCR: The electrophoresis of amplified PCR product was carried out in 1.5% low melting Agarose gel (Sigma,St.Louis(MO), USA). Desired amplified product was separated and the sensitivity of RT-PCR for the detection of the *Coxsackie* B 5'UTR gene as observed by agarose gel analysis.

LAMP: The oligonucleotide primers used for RT-LAMP amplification of *Coxsackie* B5 were designed from the 5'UTR portion, to identify the conserved regions using DNASIS software. The potential target region was selected from the aligned sequences, and RT-LAMP primers were designed. A set of six primers comprising two outer, two inner, and two loop primers that recognize eight distinct regions on the target sequence was designed by employing the LAMP primer-designing support software program (Primer Explorer4, Eiken, Japan). The primers were selected based on criteria described previously[6]. The details of the each primer with regard to their positions in the genomic sequences are shown in Figure 1.

General location of the LAMP primer set in relation to previously defined regions of the target DNA.

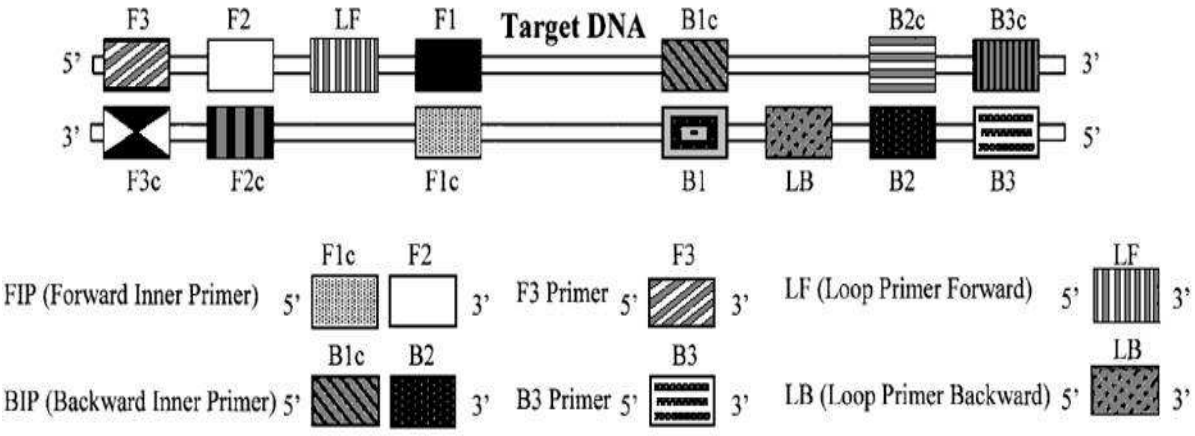

Figure 1: *Forward (F3) and backward (B3) outer primers and forward (FIP) and backward (BIP) inner primers are indicated. The specially designed inner primers, FIP and BIP, contain two distinct sequences (F1c plus F2 and B1c plus B2, respectively) corresponding to sense and antisense segments of the target DNA, one for priming in the first stage and the other for self-priming in a subsequent amplification reaction stage*

Strategies of the Amplification Reaction:

The RT-LAMP reaction was carried out in a total 25µl reaction volume using the Loopamp RNA amplification kit (Invitrogen, California, US) containing 50pmol each of the primers FIP and BIP (Sigma,St.Louis(MO), USA), 5pmol each of the outer primers F3 and B3 (Sigma,St.Louis(MO), USA), 1.4mM Deoxynucleoside Triphosphates(Fermentas, Burlington, Canada), 0.8M betaine (Sigma,St.Louis(MO), USA), 0.1% Tween 20 (Sigma,St.Louis(MO), USA), 10mM $(NH_4)_2SO_4$ (Sigma, St.Louis(MO),USA), 8mM $MgSO_4$(Sigma,St.Louis(MO), USA), 10mM KCl (Sigma, St.Louis(MO), USA), 20mM Tris-HCl (pH 8.8) (Sigma,St.Louis(MO), USA), 8 units of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 0.625 units of AMV reverse transcriptase (Invitrogen, California, US), and 2µl of RNA template. Negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Analysis of RT-LAMP product:Following incubation at 63℃ for 60 min, 10µl aliquots of RT-LAMP products were electrophoresed on a 1.5% agarose gel in Tris-borate buffer (Sigma,St.Louis(MO), USA) followed by staining with Ethidium bromide (Sigma,St.Louis(MO), USA) along with 100bp DNA ladder (Fermentas, Burlington, Canada) and visualization on a UV transilluminator at 302 nm.

Real-time monitoring:The real-time monitoring of the RT-LAMP amplification for NPEV was observed through spectrophotometric analysis by recording the OD at 400 nm every 5 min with the help of a Loopamp real-time turbidimeter (LA-200; Teramecs). The cutoff value for positivity by real-time RT-LAMP assay was determined by taking into account the PFU at which the turbidity increases above the threshold value fixed at 0.1.

Visualization by the naked eye: In order to facilitate the field application of the RT-LAMP assay, the monitoring of RT-LAMP amplification was also carried out with inspection by the naked eye. Following amplification, the tubes were inspected for white turbidity using the naked eye after a pulse spin to deposit the precipitate in the bottom of the tube. The inspection for amplification was also performed through observations of color change following the addition of 1 µl of SYBR Green I dye (Invitrogen, California, US) to the tube. In the case of positive amplification, the original orange color of the dye would change into green that can be judged under natural light as well as under UV light (302 nm) with the help of a hand-held UV torch lamp. In case there is no amplification, the original orange color of the dye would be retained. This change of color is permanent (Figure 4) and thus can be kept for record purposes.

RESULTS

A one-step, single-tube, accelerated, quantitative RT-LAMP assay was standardized for the rapid detection of *Coxsackie* B5 by targeting the highly conserved regions of the 5'-UTR gene based on multiple sequence alignments of all the circulating strains. The details of the each primer with regard to their positions in the genomic sequences are shown in Table 1.

Table 1
Sense and antisense Primer positions in the Genomic sequences

Primers	Sequence	Length of oligo nucleotides
Forward outer (F3)	GTAGGCGCTCACGAGAAC	18
Backward outer (B3)	AAAGTGAGTTGCAGCACTCT	20
Forward inner primer (FIP)	CGCCGCATTACTCGCGGAATC-CGAGCCTACGGTGGTTCT	39
Backward inner primer (BIP)	TCACCGAGCCACTAAAGGACGT-ACACGCTTCCACATTTGGT	41

Sensitivity of RT-LAMP assay: The sensitivity of the RT-LAMP assay for the detection of NPEV RNA was determined by testing serial 10-fold dilutions of virus that had previously been quantified by plaque assay and compared with that of RT-PCR. The detection of gene amplification is accomplished by real time monitoring of turbidity at 63℃. The result indicated that the minimum time required for the initiation of amplification was 16 min with viral RNA preparations. It was also observed that there is continuous amplification of the target sequence as revealed through increased turbidity compared to the negative control having no template, wherein the turbidity got fixed around 0.1, well below the threshold value. None of the positive samples tested over multiple times showed positivity in terms of increased turbidity after 60 min. Therefore, a sample with a Tp value of ≤60 min and turbidity above the threshold value of ≥0.1 was considered positive. The accelerated RT-LAMP assay was able to amplify, with a detection limit of 0.1 PFU of virus within 35 min (Figure.2), The comparative sensitivity of RT-LAMP and RT-PCR revealed that RT-LAMP was 10-fold more sensitive than RT-PCR, which has a detection limit of 1.0 PFU of virus (Figure.3), as indicated by the presence of a 175-bp amplicon.

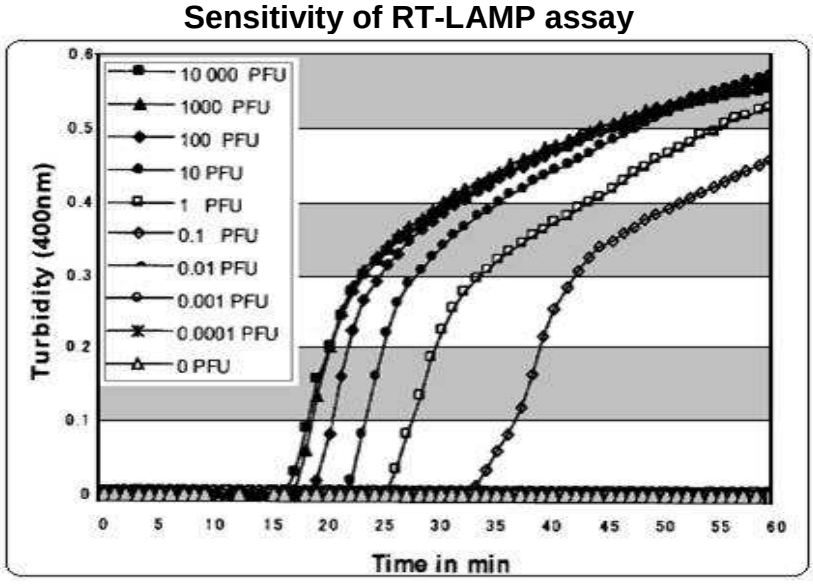

Figure 2: *Sensitivity of RT-LAMP for detection of NPEV RNA as monitored by real-time measurement of turbidity; Serial 10-fold dilutions of NPEV ranging from 10,000 PFU to 0.0001 PFU*

Gel analysis of RT-PCR amplified *Coxsackie* B5 RNA at different concentrations of virus dilution pattern

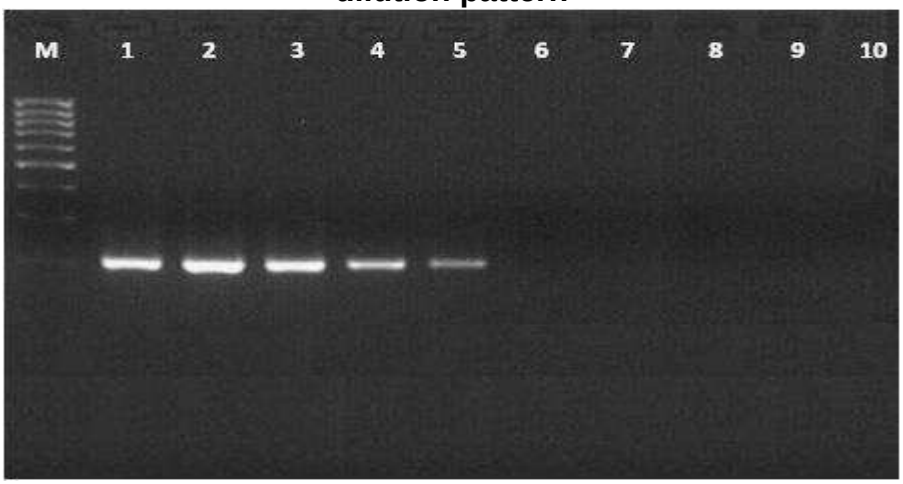

Figure 3: *Gel analysis of amplified product. Lanes: M, 100bp DNA ladder; 1, 10,000 PFU; 2, 1000 PFU; 3, 100 PFU; 4, 10 PFU; 5, 1 PFU; 6, 0.1 PFU; 7, 0.01 PFU; 8, 0.001 PFU; 9, 0.0001 PFU; 10, 0 PFU (Negative control without target RNA*).

Evaluation and comparison of RT-LAMP and RT-PCR: The applicability of the RT-LAMP assay for the clinical diagnosis of *Coxsackie* B5 was validated with CSF. The results were compared with those from RT-PCR. From a total of 8 samples comprised of 6 positive samples and 2 negative samples are randomly selected from the above-described 25 samples used in this study for comparative evaluation; None of the RT-PCR-positive samples (Figure 4) were missed by RT-LAMP (Figure 5), thereby indicating a higher sensitivity of the RT-LAMP assay. All healthy serum samples were also negative for both the tests, thereby ruling out the possibility of false positivity and thus establishing the specificity of the selected primer sets for *Coxsackie* B5 RT-LAMP assay. The RT-LAMP assay also picked up more positive samples than RT-PCR, Virus isolation, and Neutralization test.

Sensitivity of RT-PCR for the detection of *Coxsackie* B by Gel analysis

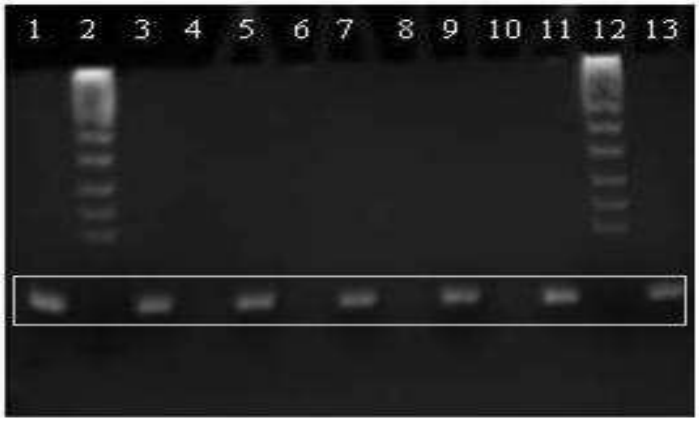

Figure 4 :*Electrophoresis analysis of CoxB5 PCR amplified products. Lane 1 – Positive control; Lane 3,5,7,9,11&13 – Positive isolates; Lane 2 & 12 – 100bp Marker; Lane 4 – Reaction control; Lane 6- Cell control; Lane 8 & 10 – Negative control respectively.*

Sensitivities of Electrophoretic analysis of *Cox* B5 LAMP amplified products.

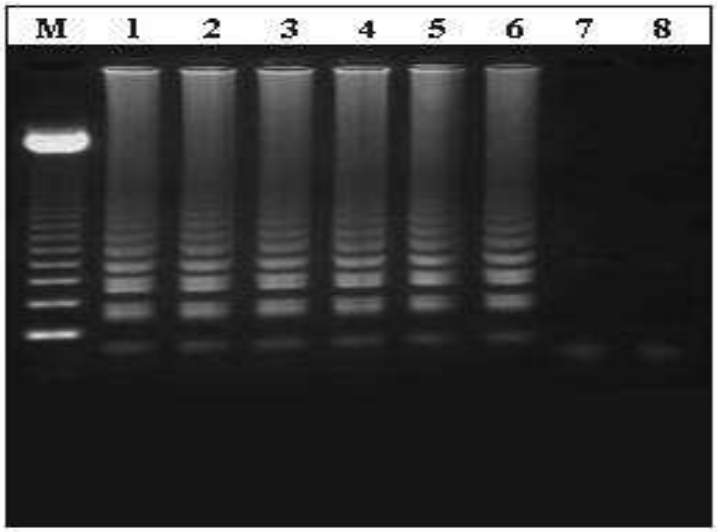

Figure 5: *Lane 1 to 6 is positive samples isolated from CSF; 7 and 8 are samples isolated from healthy individual and Lane M-100bp marker.*

The field applicability of the RT-LAMP assay was also validated by employing a SYBR Green I-mediated naked-eye visualization test. Following incubation at 63℃ for 30 min in a water bath, the monitoring of RT-LAMP amplification was accomplished through visualization by the naked eye with the addition Of 1 µl of SYBR Green I (1:1,000) dye to the amplified products (Figure 6). The comparative evaluation of this field-based, SYBR Green I-based RT-LAMP assay with 6 clinical samples randomly selected from the above-described 25 samples revealed a very good concordance with RT-PCR.

Visual detection of LAMP product under UV light

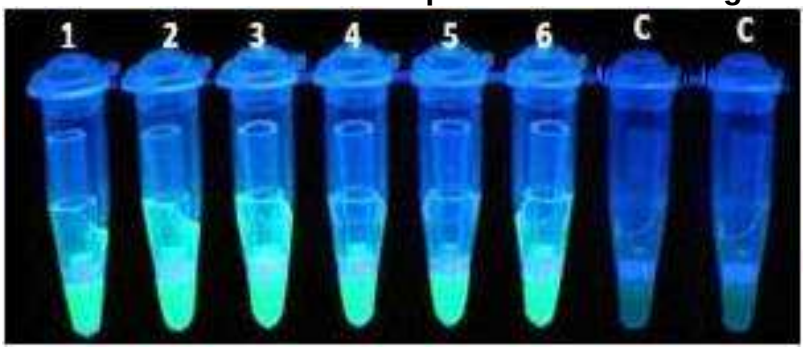

Figure 6: *Visualization by the naked eye; Tubes 1-6 are positive samples and tube C is negative control.*

DISCUSSIONS

The *Coxsackie* B virus genome is a single-stranded RNA molecule, approximately 7500 nuleotides long, of positive polarity. An approximately 750- nucleotide 5'-untranslated region (5'-UTR) is followed by a long open reading frame coding for an approximately 2100-aminoacid polyprotein. This is followed by a short 3'-untranslated region (3'-UTR) and a poly (A) tail. Enteroviruses use an error prone RNA dependent RNA polymerase enzyme for their replication; hence, the mutation rate is very high. The 5'-UTR seems to be extremely conserved among enteroviruses because the secondary structures in this region, the cloverleaf and internal ribosome entry site, are required for efficient replication and translation of the viral

RNA[7]. Therefore, the 5'-UTR has been used extensively in diagnostic RT-PCR assays for enterovirus infection and several important functions related to it, so this region is used in this study.

The type specific diagnosis of enterovirus infection still relies on neutralization assays using pools of type specific polyclonal antisera followed by confirmation with monospecific antisera but the results are often difficult to interpret[8, 9]. Recent developments in molecular biology have enabled the detection of *Coxsackie* B virus genomes directly in clinical samples. These tests do not however, allow typing of the virus strains, which would be very useful for both epidemiological and clinical purposes[10]. It is therefore, valuable to obtain a type specific enterovirus molecular method for diagnosis focusing on the regions that remain relatively conserved during viral replication. The high sensitivity and specificity of the RT-PCR followed by RT-LAMP assay proved to be very useful for diagnosis of *Coxsackie* B viruses.

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[11]. 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[12, 13]. In the present study, a one-step, single-tube, real-time, accelerated RT-LAMP assay was standardized by targeting 5'- UTR gene for rapid and real-time detection of group B5 *Coxsackie viruses*.

CONCLUSION

We investigated the applicability of a novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP) for detection of group B5 *Coxsackie* viruses. This method also exhibits extremely high amplification efficiency, specificity, owing, in part, to its isothermal nature; no time is lost as a result of changes in temperature, and the reaction can be conducted at the optimal temperature for enzyme function. The goal was to establish a highly sensitive and specific LAMP-based *Coxsackievirus* B5 amplification method and to examine its reliability in discriminating among species.

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