Letter to the Editor

Visual Detection of Murray Valley Encephalitis Virus by Reverse Transcription Loop-Mediated Isothermal Amplification*

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A sensitive reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for rapid visual detection of Murray valley encephalitis virus (MVEV) infection. The reaction was performed in one step in a single tube at 63 °C for 60 min with the addition of the hydroxynaphthol blue (HNB) dye prior to amplification. The detection limit of the RT-LAMP assay was 100 copies per reaction based on 10-fold dilutions of *in vitro* transcribed RNA derived from a synthetic MVEV DNA template. No cross-reaction was observed with other encephalitis-associated viruses. The assay was further evaluated using spiked cerebrospinal fluid sample with pseudotype virus containing the NS5 gene of MVEV.

Murray valley encephalitis virus (MVEV) is a mosquito-borne Flavivirus (Flaviviridae: Flavivirus) which is closely related to Japanese encephalitis virus, West Nile virus and St. Louis encephalitis virus. MVEV is the most serious of the endemic arboviruses in Australia[1]. In 2010-2011, there were 16 confirmed human cases of Murray Valley encephalitis acquired in Australia[1-2]. The activity of MVEV in Australia is monitored by detection of seroconversions in flocks of sentinel chickens. The availability of the RT-PCR assay for the detection of MVEV provides additional opportunities to confirm the presence of this virus in clinical samples[3]. However, these methods might not be suitable in local ports of Entry-Exit Inspection and Quarantine or for field use because the methods are time-consuming and require the sophisticated instrumentation. There is a growing demand for a simple, rapid and sensitive molecular test for the inspection and quarantine testing in the local ports in China. Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method firstly described in 2000[4]. It is a powerful gene amplification tool due to its simplicity, speed, specificity and cost-effectiveness and nowadays, this technique is being used increasingly for rapid detection and typing of emerging viruses[5-9]. In the present report, a sensitive reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for rapid visual detection of the MVEV infection. The NS5 gene of MVEV was used to distinguish the members of the genus Flavivirus and all the NS5 nucleotide sequences of MVEV available in GenBank were downloaded and compared. The most conserved segment within the NS5 gene of MVEV (corresponding to the nucleotide positions at 10504-10908, numbering based on the MVEV strain MVE-1-51, GenBank accession no. AF161266.1) was selected as the target. All primers were designed by a software program for LAMP primer design (Eiken Chemical Co. Ltd., Tokyo, Japan) and then subsequently validated by BLAST (http://www.ncbi.nlm.nih.gov/BLAST). All the primers were HPLC purified, as shown in Table 1.

Table 1. RT-LAMP Primers Designed for the Detection of the NS5 Gene Sequence of MVEV

Primer Name	Genome Position[a]	Sequences (5'-3')
MVEV-F3	10604-10621	CCCAGAACCGTCTCGGAA
MVEV-B3	10808-10790	CGCAGGGTCTCCTCTAACC
MVEV-FIP	(10667-10686)+ (10623-10640)	CTCGGCGAAGTGGCGCTTT CAGGAGTCCCTGCCAACAA
MVEV-BIP	(10691-10710)+ (10753-10770)	GCAATCTGTGAGGCCCCAG GTTGCATCACCTCCTCCCG

Note. [a]Genome position depending on MVEV strain MVE-1-51 (GenBank accession no. AF161266.1).

doi: 10.3967/bes2015.031

*This work was supported by the Scientific Research Project of General Administration of Quality Supervision, Inspection and Quarantine of P.R.C (2011IK132) and the China Mega-Project for Infectious Disease (2013ZX10004-001).

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RT-LAMP was performed in a total of 25 μL of reaction mix prepared as follows: 8 U Bst DNA polymerase (New England Biolabs, Ipswitch, MA, USA), 10 U avian myeloblastosis virus reverse transcriptase (Promega, Madison,USA), 120 μmol/L HNB (Lemongreen, Shanghai, China), 12.5 μL the 2×RNA amplification reaction mix (Deaou Biotechnology Co., Ltd., Guangzhou, China) and 1 μL of each primer of MVEV (F3 and B3: 5 μmol/L; BIP and FIP: 40 μmol/L) and 2 μL of template RNA. The reaction was incubated in a Loopamp turbidimeter (LA-320C; Teramecs, Japan) for real-time monitoring of the amplification at 63 °C for 60 min, followed by heating at 85 °C for 3 min to terminate the reaction. Positive and negative controls were included in each run, and positive reactions were defined as those samples having a threshold value of the turbidities curve greater than 0.2 or a color change from violet to sky blue, as described previously[6-7].

In order to determine the specificity and the sensitivity of the RT-LAMP method, the recombinant plasmid pBluescript II SK (pSK-MVEV-NS5) harboring the NS5 gene fragment of the MVEV was constructed. Synthetic RNA fragment of MVEV NS5 gene was made by *in vitro* RNA transcription under T7 promoter with a commercial Riboprobe combination system-SP6/T7 kit (Promega, Madison, USA) and stored at -80 °C and, then, was used as reference RNA. Field isolates of human encephalitis-associated viruses were preserved and obtained from Hubei Entry-Exit Inspection and Quarantine Bureau, China. These isolates were used as control viruses to evaluate the specificity of the RT-LAMP assay for MVEV. The control viruses included dengue virus type I strain GZ01/95 (Den 1, GenBank accession no. EF032590.1), dengue virus type II strain New Guinea C (NGC) (Den 2, GenBank accession no. AF038403.1), enterovirus 71 Strain FY17.08/AN/CHN/2008 (EV71, GenBank accession no. EU703812), and Japanese B encephalitis virus strains P3 (JEV P3, GenBank accession no. U47032.1), AT31 (JEV AT31, GenBank accession no. AB196926.1), SA14-14-2 (JEV SA14-14-2, GenBank accession no. AF315119.1). The RNA of hepatitis C virus (HCV), a member of the genus Flavivirus within the family Flaviviridae, was also included in this study as a control RNA. Viral RNA was extracted from 140 μL of various control viruses by using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was eluted from the QIAspin column in a final volume of 60 μL of RNase-free water and stored at -80 °C until use.

In order to establish the specificity of the RT-LAMP assays for MVEV, the extracted RNAs from various control viruses (Den 1, Den 2, EV71, JEV P3, JEV AT31, JEV SA14-14-2, HCV) and the RNA fragment of MVEV NS5 gene were used as templates, respectively. RT-LAMP assay was performed as described above and both positive and negative controls were included in each run. It was founded that the turbidity increase and a sky blue color was only observed in the prepared RNA o fragment of MVEV NS5 gene (Figure 1A).

In order to determine the detection limit of the RT-LAMP assay, a panel RNAs of MVEV NS5 gene with concentrations ranging from 10^1 to 10^8 copies/μL was prepared. RT-LAMP assay was performed as described in above reaction protocol and 1 μL template RNA was added in each reaction. Positive and negative controls were included in each run. The reaction at each template concentration was repeated three times and similar results were obtained. As shown in Figure 1B, the detection limit of the RT-LAMP assay was approximately 10^2 copies per reaction.

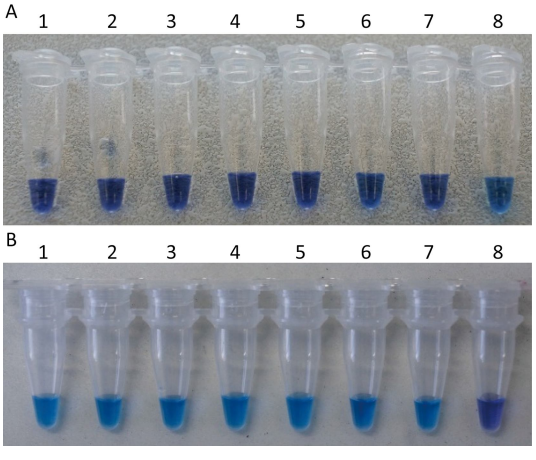

Figure 1. A: Specificity of the RT-LAMP assay for the detection of human MVEV. Positive reactions were only observed in reference RNA and none of the control viruses showed an increase in turbidity or color change. Tube 1: Den 1, Tube 2: Den 2, Tube 3: EV71, Tube 4: JEV P3, Tube 5: JEV AT31, Tube 6: JEV SA14-14-2, Tube 7: HCV, Tube 8: RNA fragment of MVEV NS5 gene. B: Sensitivity of the RT-LAMP assay for the detection of MVEV. RT-LAMP was carried out using different copies of *in vitro* RNA transcripts. The tubes 1-8 shown from left to right have decreasing concentrations of RNA from 10^8 to 10^1 copies/reaction, correspondingly. The detection sensitivity for MVEV was 10^2 copies per reaction.

The RT-LAMP Assay was further evaluated with armored RNA. Armored RNA (virus-like particles) is a noninfectious and quantifiable synthetic substitute for live or chemically inactivated RNA virus. The utility of armored RNA as an assay standard has been documented in clinical applications. In this study, armored RNA containing target sequence from the NS5 gene of MVEV was constructed based on the MS2 coliphage (ATCC 15597-B1) and designed to provide a means of evaluating all steps for the RT-ALMP assays, including RNA extraction and recovery, reverse transcription, primer binding, amplification, and detection.

The MS2 maturase and coat protein gene were obtained by RT-PCR from MS2. The PCR product was then digested with restriction enzymes Kpn I and BamH I and ligated with linearized pET-32a (Novogen, Darmstadt, Germany) to generate vector pET-32a-MS2. The MVEV NS5 gene fragment was obtained by PCR from pSK-MVEV-NS5. The PCR product was then digested with restriction enzymes Not I and Xho I and ligated with linearized pET-32a-MS2 to generate vector pET32a-MS2-MVEV-NS5 and the armored RNA was expressed and purified. Approximately 10^4 armored RNA spiked into 300 µL of cerebrospinal fluid (CSF) from healthy human was used as a surrogate to mimic MVEV-positive clinical CSF. Another aliquot of cerebrospinal fluid (CSF) was included as a control sample. The total RNA was extracted by using the QIAamp Viral RNA Mini Kit as described above and the RT-LAMP assay was performed. Positive and negative controls were included in each run. The turbidity increase or a sky blue color was only founded in the prepared RNA of spiked sample (data not shown).

In the present study, the RT-LAMP with HNB dye was shown to be a sensitive and easy assay for detection of human MVEV. The HNB dye-based assay has a remarkable advantage compared with other color-based assays[5,9-11] in that the HNB is mixed prior to amplification. The need to open the assayed samples to add the dye is thereby omitted, thus reducing the risk of cross-contamination. The RT-LAMP primers for the diagnosis of MVEV infections was shown to be specific for synthetic RNA and spiked sample but the further validation of this assay requires the availability of cell cultured virus and appropriate clinical samples from infected animal or humans. Though no internal control was included in this method, which is similar to previous publications using LAMP detection[6,12-15], the simplicity and ease of use of this method makes it an attractive assay for the rapid screening of human MVEV infections in the local ports for Entry-Exit Inspection and Quarantine in China.

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Received: June 17, 2014;

Accepted: December 17, 2014

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