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Short communication

Rapid detection of encephalomyocarditis virus by one-step reverse transcription loop-mediated isothermal amplification method

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ABSTRACT

The encephalomyocarditis virus (EMCV) can cause acute myocarditis in young pigs or reproductive failure in sows. In this study, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed to detect EMCV RNA. The RT-LAMP assay was highly sensitive and able to detect 2.2×10^{-5} ng of EMCV RNA, as no cross-reaction was observed with other viruses. The RT-LAMP assay was conducted in isothermal (62 °C) conditions within 50 min. The amplified products of EMCV could be detected as ladder-like bands using agarose gel electrophoresis. This is the first report to demonstrate the application of a one-step RT-LAMP assay for the detection of EMCV. The sensitive, specific and rapid RT-LAMP assay developed in this study can be applied widely in clinical diagnosis and field surveillance of EMCV.

Encephalomyocarditis virus (EMCV) belongs to the genus *Cardiovirus* of the family *Picornaviridae*, and has a worldwide distribution (Minor et al., 1995). Rodents are considered as reservoirs or natural hosts of the virus (Zimmermann et al., 1994). Ever since fatal disease of swine caused by EMCV was first described in 1958 (Murnane et al., 1960), the virus has been recognized worldwide as a pathogen that can infect several host species including pigs, rodents, cattle, elephants, raccoons, marsupiala, and primates such as baboons, monkey, chimpanzees, and even humans (Gelmetti et al., 2006; Krylova and Dzhikidze, 2005; Spyrou et al., 2004). EMCV infection has been confirmed in several pig farms in China by etiology and serology (Ge et al., 2007, 2010; Lin et al., 2012).

The current methods of detecting EMCV include virus isolation, immunofluorescence detection, electron microscopy (EM), and reverse transcription-polymerase chain reaction (RT-PCR) (Ge et al., 2007; Jia et al., 2008; Yuan et al., 2014). However, all of these techniques have some intrinsic disadvantages, such as requiring high-precision instrument or being time-consuming. In recent years, a rapid nucleic acid detection method, loop-mediated isothermal amplification (LAMP), was developed (Notomi et al., 2000). For the method, only a water bath is needed to amplify large amounts of nucleic acids in 1 h approximately. The LAMP and reverse transcription loop-mediated isothermal amplification (RT-LAMP) methods have been proven to be rapid, simple, sensitive, specific and inexpensive for the detection of different DNA and RNA viruses (Chowdry et al., 2014; Ge et al., 2013; Locher et al., 2010; Qiu et al., 2012; Xie et al., 2012; Yin et al., 2010; Zhang et al., 2011). In this study, a rapid and feasible one-step RT-LAMP method was developed for the detection of EMCV RNA.

A multiple sequence alignment was performed for 19 randomly selected EMCV isolates from GenBank database. The 3D of EMCV, which is sufficiently conserved among the randomly selected EMCV isolates, was chosen for primer design. A set of RT-LAMP primers were designed using Explored V4 Software (http://primerexplorer.jp/e/) based on highly conserved regions in the 3D gene of EMCV from BD2 strain (GenBank accession no. KF709977.1). All primers were synthesized by invitrogen (Beijing, China). The nucleotide sequences and locations of the primers for BD2 are shown in Table 1 and Fig. 1.

The optimization of the RT-LAMP reaction was performed by evaluating different volumes of 25 mM $MgCl_2$ (0.5–3.0 μL), 2.5 mM dNTP (1–6 μL) and 8 U/μL *Bst* DNA polymerase (0.5–3.0 μL). The optimal reaction system is shown in Table 2. The reaction temperatures (60–65 °C) and times (10, 20, 30, 40, 50, and 60 min) were also optimized. The RT-LAMP products were electrophoresed using a 2%

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Table 1
RT-LAMP primers designed for the detection of EMCV.

Primer name	Type	Sequence (5′–3′)	Position[a]
F3	Forward outer	GGCAGATCTTGGAGGAAGC	6100–6118
B3	Backward outer	AAGACTTGACGGGCAACG	6292–6309
FIP	Forward inner (F1c–F2)	GCCCGAATCATCTCCTGTGACA-CCTTGGCATCCATTCTGCTG	6177–6198; 6127–6146
BIP	Backward inner (B1c–B2)	AATGCCTTTGAGCCACAGGGTG-GGCGTAGTGCTGTTTTACGT	6206–6227; 6268–6287

[a] Numbers represent the nucleotide position within the genome of BD2 (GenBank accession number: KF709977.1).

Table 2
Optimized parameters for the one-step RT-LAMP reaction.

Component (concentration)	Volume (μL) per 25 μL reaction
Rnasin inhibitor	0.5
10 × *Bst* DNA Buffer	2.5
$MgCl_2$ (25 mM)	2.5
dNTPs (2.5 mM)	3
F3 primer (10 μm)	0.5
B3 primer (10 μm)	0.5
FIP primer (10 μm)	2.5
BIP primer (10 μm)	2.5
RNA template	1.0
AMV reverse transcriptase (200 U/μL)	1.0
Bst DNA polymerase (8 U/μL)	1.5
DEPC H_2O	7.0

agarose gel to determine the optimal parameters. The optimized RT-LAMP parameters were as follows: incubation temperature at 62 °C (Fig. 2A) and incubation time of 50 min (Fig. 2B). With these parameters (Table 2) the ladder-like bands from EMCV RNA could be amplified adequately by RT-LAMP.

To evaluate the specificity of the RT-LAMP assay, experiments were performed using the optimized reaction parameters (Table 2) on an EMCV strain BD2. In addition, classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), porcine circovirus type 2 (PCV2), porcine epidemic diarrhea virus (PEDV), and porcine reproductive and respiratory syndrome virus (PRRSV) were included. The specificity was further confirmed by agarose gel electrophoresis (data not shown). No cross-reactivity with other viruses was observed.

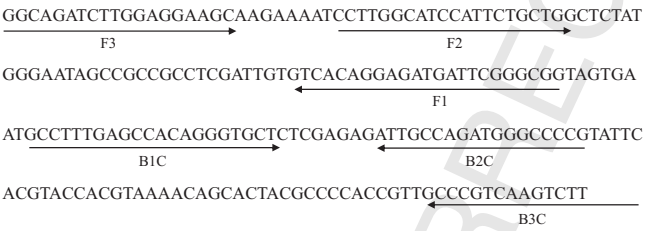

Fig. 1. Locations of the primers used in RT-LAMP. The GenBank accession number for BD2 strain is KF709977.1. The nucleotide sequences of primers are underlined.

To examine the sensitivity of RT-LAMP for EMCV amplification, RT-LAMP, RT-PCR, and real-time RT-PCR reactions were conducted using various concentrations of EMCV RNA as template. EMCV RNA was extracted from cell cultured supernatants of the infected cells by using QIAamp viral RNA kit (Qiagen, Valencia, CA). The RNA was quantified by NanoDrop 1000 (Thermo Scientific, USA) and was diluted serially 10-fold from 2.2 ng/μL to 2.2×10^{-6} μg/μL as template for three methods. RT-LAMP was performed using the optimized reaction parameters. After the reaction was completed, 3 μL of the reaction products was electrophoresed using 2% agarose gel. RT-PCR was performed using EMCV-specific primers (P1: 5′-CAG AGG CTG ATG TAG ATG AAG TGG C-3′; P2: 5′-CAG AAT GCA ATG CTC AAA TGG TGG A-3′) (data not shown). Briefly, RT-PCR was performed by using 1 μL of diluted RNA template, 0.5 μL Taq DNA Polymerase (Cwbiotech, Beijing, China), and 10 μmol of each primer in a 25 μL reaction volume by following the manufacturer's protocol with the following cycling times and temperatures: 94 °C for 3 min and 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 25 s. Three microliters of RT-PCR product was analyzed by agarose gel electrophoresis. The real-time RT-PCR assay was performed in a 25 μL reaction mixture containing 1 μL diluted RNA, 12.5 μL Fast-TaqMan Mixture (Cwbiotech, Beijing, China), 0.5 μL AMV reverse transcriptase (Transgen, Beijing, China) 400 nM each of forward and reverse primer, and 200 nM of probe (data not shown). Amplification and detection were performed with an Bio-Rad iQ5 real-time PCR detection system under the following conditions: RNA was reverse transcribed at 50 °C for 20 min, followed by PCR activation at 95 °C for 3 min and 40 cycles of amplification (5 s at 95 °C and 40 s at 60 °C). Analysis of each assay was conducted with iQ5 Standard Edition Optical System Software (version 2.1; Bio-Rad). The detection limit RT-LAMP and real-time RT-PCR were both 2.2×10^{-5} ng/μL, whereas that of RT-PCR was 2.2×10^{-2} ng/μL (Fig. 3). Comparisons between the RT-LAMP and RT-PCR amplification indicated that RT-LAMP is 1000-fold more sensitive than RT-PCR (Fig. 3A and C).

The sensitivity of the RT-LAMP method in detecting EMCV RNA from 95 clinical samples was compared with those of RT-PCR and real-time RT-PCR methods. Results are shown in Table 3. Eleven of 95 samples (11.6%) were positive by RT-PCR analysis, whereas

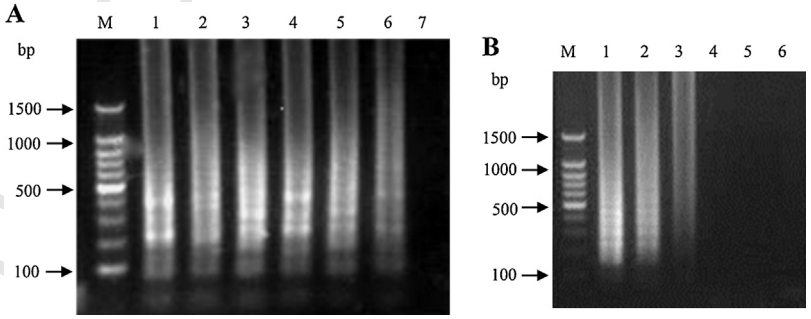

Fig. 2. Optimization of the RT-LAMP parameters for EMCV detection. (A) Optimization of reaction temperature. M: 100 bp DNA Marker. Lanes 1–6: RT-LAMP reaction temperature at 60–65 °C, respectively; Lane 7: RT-LAMP reaction without RNA template. (B) Optimization of RT-LAMP reaction time. M: 100 bp DNA Marker; Lanes 1–6: RT-LAMP reaction incubated for 60, 50, 40, 30, 20 and 10 min, respectively.

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