Rapid Detection of Foot-and-Mouth Disease Virus by Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP)

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KEY WORDS: Foot and mouth disease, transcription loop-medicated isothermal one-step single-tube method of method of reverse transcription loop-mediated isothermal polymerase amplification; RNA polymerase $3D^{pol}$

ABSTRACT

The aim of this study was to produce a sensitive, simple and rapid diagnostic method for the detection of foot-and-mouth disease virus (FMDV) in suspected cases of FMD, a one-step single-tube method of reverse transcription loop-mediated isothermal polymerase amplification (RT-LAMP). A set of six common primers was designed to target the highly conserved region of the RNA polymerase 3Dpol gene of the seven FMDV serotypes. The sensitivity and specificity of RT-LAMP were evaluated by detection of 10-fold serial dilutions of the standard plasmids, and samples from experimental infection and suspected cases of FMD. The results showed that the target nucleic acid of four serotypes of FMDV (A, O, Asia1 and C) can be amplified rapidly by LAMP in a water bath in less than an hour. At least 10 copies of the DNA could be detected by RT-LAMP, which showed the same sensitivity as real-time PCR and another technique, RT-LAMP-1, but 10 times higher than that of reverse transcription polymerase chain reaction (RT-PCR). All 104 samples were detected by RT-LAMP, RT-LAMP-1, RT-PCR and real-time PCR; the positive ratios were 98.31%, 86.44%, 93.22%, and 100%, respectively. The results indicate that RT-

LAMP is a rapid, cost-effective, efficient and simple method. Therefore it may be applied for the rapid detection of FMDV in the laboratory or under field conditions.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious viral disease that affects cloven-hoofed animals such as cattle, sheep, goat, and pigs. Recent outbreaks of FMD have occurred worldwide that have caused severe economic impact.[6, 15, 27] Early identification of FMD virus (FMDV) is, therefore, critical for the control of disease and to minimize losses to the livestock industry. Although virological diagnostic techniques, such as antigen capture ELISA and virus isolation, have been developed for the detection of FMDV, these assays cannot offer timely results because they are time consuming and require specialized laboratory facilities.[9] Several molecular techniques have been developed as rapid assays for the detection of FMDV.[1, 2, 11, 23, 24] However, the methods cannot be used extensively on site or in developing countries because of their complexity and requirement for expensive equipment.

Recently, Notomi et al (2000) developed an alternative molecular technique, termed Loop-mediated isothermal amplification (LAMP) that has been used widely for the detection of viruses. According to the principles of LAMP, target DNA or RNA can be amplified rapidly in a heating block under isothermal conditions with a set of four specific primers.[10, 13, 18, 19, 21] It has been reported that loop primers (Loop primers hybridize to the stem-loops, except for the loops that are hybridized by the inner primer, and prime strand displacement DNA synthesis) can accelerate LAMP reactions and reduce amplification time markedly.20 In particular, the results can be visualized directly by naked-eye inspection using SYBR Green I.[14, 16, 25] The LAMP technique combined with a reverse transcriptase, termed RT-LAMP, has been used for the detection of RNA viruses such as West Nile virus.[22] Severe acute respiratory syndrome,[12] FMDV,[5]and Human immunodeficiency virus.[3] In a previous report, LAMP was used for the rapid detection of all seven serotypes of FMDV, but the specificity and sensitivity of the technique were not quite satisfactory. [5] In this study, to improve the sensitivity of LAMP and promote its use worldwide, a new alternative RT-LAMP was developed. The specificity and sensitivity of LAMP were determined by detection of FMDV in samples from experimental or suspect cases of FMD.

MATERIALS AND METHODS

Virus Isolates

Representatives of four serotypes of FMDV, namely O (China/99), Asia1 (JS/China/05), A, and C (donated from the former Soviet Union), from the National Foot-and-mouth Disease Reference Laboratory of PR China were used to develop the test. Types O and Asia1 were grown separately in BHK-21 cells, and types A, and C were propagated in 2-7-day old unweaned mice, respectively. Swine vesicular disease virus (SVDV, HK/70) was kindly donated by Dr S.H. Yin (Lanzhou Veterinary Research Institute, CAAS).

Preparation and Quantification of Standard

A fragment (1410 bp) of the 3D gene of the O/China/99 strain of FMDV was amplified and inserted into pGEM-T Easy vectors (Promega, Madison, WI). The recombinant E.coli cells carrying the recombinant plasmids were inoculated into a flask containing 10ml of Luria broth (LB) and incubated at 37°C overnight in a shaker. Plasmids were extracted from the cultures and verified by PCR and sequencing. The recombinants were quantified using a Unicom UV 300 (Themo Fisher Scientific, Madison, WI, USA), the copy number was then calculated and the standards were prepared by 10-fold serial dilutions of the plasmid DNA.

Samples from the Experimental and Suspected cases of FMD

A total of 104 samples from experimental and the suspected cases of FMD were collected by the National Foot-and-mouth Disease Reference Laboratory of PR China.

Table 1. *Field and experimental samples of FMDV used in this work*

Sample	Sero-type	Number	Virus	Location	Collection year
Field samples (n=40)	Asia1	14	JS/05 HK/05	Jiangsu, Shandong, Xinjiang, Beijing, Hubei, Hebei, Gansu, Ningxia, inner Mongolia, Hongkong	2005 2006
	O	12	China/99	Tibet, Yunnan, Hainan, Fujian	1999
	A	14	HB/09	Hubei, Shanghai, Jiangsu, Shandong	2009
Experimental samples (n=64)	Asia1	20	YN/58 JS/05		
	O	18	Akesu/58 China/99		
	A	16	Av88(L) HB/09		

Notes: Field samples from suspected cases in outbreaks of FMDV in China. The experimental samples from BHK-21 cells, muscle tissues of unweaned mice, oesophageal–pharyngeal (OP) samples and vesicular epithelium from cattle infected experimentally with corresponding FMDV. AV88(L) was donated from the former Soviet Union.

All the samples have been identified previously by virus isolation (Table 1).

Design of Primers for RT-LAMP

More than 100 sequences of the 3D gene of FMDV from GenBank were analyzed using a Clustal V method (DNASTAR, Madison, WI, USA). Of these sequences, 42 were chosen for sequence analysis using the Clustal V method (DNASTAR, Madison, WI, USA) (Figure1). A highly conserved region of the 3D gene was chosen and a set of common primers were designed according to the 3D gene of the virus isolate O/China/99 (GenBank accession number: AF506822). Two inner primers (forward inner primer (FIP) and backward inner primer (BIP)), two outer primers (F3 and B3) and two loop primers (F loop and B loop) were designed for RT-LAMP using the Primer Explorer version 4 software (http://primerexplorer.jp/elamp4.0.0/index. html). The primers F3 and B3 were also used for RT-PCR. The details of the primers and their locations are shown in Table 2. In addition, a set of primers against FMDV, as described by Dukes et al. (2006), were synthesized for the RT-LAMP procedure named RT-LAMP-1.

RNA Extraction

Viral RNA was extracted from all the virus isolates and samples described above using an RNeasy Mini kit (Qiagen, Germany). All processes were carried out according to the manufacturer's instructions in a biosafety laboratory class 3 (BSL-3). All RNA samples were stored at -70°C until use.

RT-LAMP

The reaction was carried out in a 25μl volume containing 12.5μl of LAMP buffer (20mM Tris-HCl (pH8.8), 10mM KCl, 8mM MgSO4, 10mM (NH4)2SO4, 0.1% Triton X-100, 0.8M betaine and 1.4mM each of the four deoxynucleoside triphosphates (dNTPs)), 1.0μl of primer mixture (40pmol each of FIP and BIP, 20pmol each of F loop and B loop, and 5pmol each of F3 and B3), 1.0μl (8U) of BstDNA polymerase (New England BioLabs, Beverly, MA, USA), 1.0μl (40U) of AMV reverse transcriptase (Takara, Japan), 2.0μl of template RNA, and 7.6μl of distilled water. The reaction mixture

Table 2. *Primers used for RT-LAMP and RT-PCR*

Primer name	Type	Length (bp)	Sequence position	Sequence (5´-3´)
F3	Forward outer	19	7870-7888	TGTGATGGCTTC-GAAGACC
B3	Reverse outer	16	8077-8062	TGCGTCACCGCA-CACG
FIP	Forward inner	46; F1C, 22	F1C, 7951-7930	TGCCACGGAGAT-CAACTTCTCC
	F1CTTTT+F2	F2, 20	F2, 7889-7908	TTTTCTCGAGGC-TATCCTCTCCTT
BIP	Reverse inner	46; B1C, 22	B1C, 8000-7979	GAGTACCGGC-GTCTCTTTGAGC
	B1C+TTTT+B2	B2, 20	B2, 8041-8060	TTTTC-GTTCACCCAACG-CAGGTAA
F Loop	Forward loop	17	7913-7929	TGTATGGTCCCACG-GCG
B Loop	Reverse loop	18	7995-8012	TTGAGCCTTTC-CAGGGCC

Note: Primer location refers to the O/China/99 strain of FMD virus (GenBank accession: AF506822).

was incubated at 63°C for 1 hr in a heating block. The reaction was terminated by heating at 80°C for 2 mn. Aliquots of 10.0 µl of the RT-LAMP products were separated in a 2.0% agarose gel by electrophoresis and visualized by staining with 0.5 µg/ml of ethidium bromide under UV light. Alternatively, the results could also be visualized by the naked eye using 1µl of a 10-fold dilution of SYBR Green I (1000× concentration) (Invitrogen, CA, USA). RT-LAMP-1 was also performed, as described by Dukes et al. (2006).

Real-Time PCR

Real-time PCR was also used for the detection of virus in all the samples mentioned above. The PCR reaction mixture contained 2×SYBR Green PCR Master Mix (Shanghai SOLOMON bio-sci&tech, Co.Ltd.), 20pmol of forward and reverse primers, and 2µl of extracted DNA. Both forward and reverse primers were synthesized as described elsewhere.8 The thermocycling program was 40 cycles of 95°C for 15s and 60°C for 1min, with an initial cycle of 95°C for 5 min. The data were analyzed using the ABI 7500 System SDS software (Applied Biosystems, Foster, CA, USA).

Identification of RT-LAMP Products

The purified LAMP amplicons were prepared with a DNA fragment purification kit (Takara, Japan), and then were digested with a restriction enzyme Hae III at 37°C for 2 hr. The target fragment was purified and cloned into the pMD18-T vector (Takara, Japan). The positive recombinants were validated by sequencing.

RT-PCR Reaction

The RT-PCR was performed according to the manufacture's instructions (Takara, Japan). Briefly, 25.0 µl of reaction mixture containing 2.5 µl of 10×one-step RNA PCR buffer, 5.0 µl of MgCl2 (25mM), 2.5 µl of dNTPs (10mM each), 1.0µl of RNase inhibitor (40U), 5.0U of AMV RTase XL, 5.0U of AMV-Optimized Taq and 1.0 µl of each primer (20 pmol), 2.0µl of RNA and 14.5µl of distilled water. The first strand cDNA was synthesized at 50°C for 30min, followed by incubation at 94°C for 5 min. The PCR was

then carried out for 30 cycles at 94°C for 1 min, 56°C for 30s and 72°C for 30s followed by a final extension for 8min at 72°C. The PCR products were electrophoresed in a 2% agarose gel.

RESULTS

Specificity of RT-LAMP

To determine the specificity of RT-LAMP, four serotypes of FMDV used as reference strains, namely types O (China/99), A, C, and Asia1 (JS/05), were tested in this study. Total RNA was extracted from SVDV, mock-infected cells and tissues used as the negative controls. Distilled water was used as a blank control. The results indicated that the four serotypes of FMDV could be amplified by RT-LAMP, and its products were shown as the typical ladder-like bands in 2% agarose gels (Figure 2). By contrast, none of the negative and blank controls was positive in this study. All positive results confirmed by gel electrophoresis were also positive by SYBR Green I staining. Briefly, the color changed from orange to green in a positive tube, whereas negative tubes remained orange (Figure 3). Furthermore, as shown in Figure 4, a fragment of 141 bp was obtained by digesting the LAMP amplicon with the restriction enzyme HaeIII, and the results were further confirmed by sequencing. The results indicated that RT-LAMP has a high specificity for FMDV.

Comparison of the Sensitivity Among Four Methods

To determine the sensitivity of detection, four methods, namely RT-LAMP, RT-LAMP-1, RT-PCR, and real-time PCR, were performed using 10-fold serial dilutions of template DNA (ranging from 10 to 1×106 copies). The results indicated that at least 10 copies of DNA could be detected by RT-LAMP (Figure 5), RT-LAMP-1 (Figure 6) and real-time PCR (Figure 7), while the sensitivity of detection by RT-PCR was 10 times lower than that of other three assays (Figure 8).

Evaluation of RT-LAMP in Field and Experimental Samples. All 104 samples, including type O, Asia1, and A, were tested by LAMP, LAMP-1, the conventional RT-PCR, and real-time RT-PCR. As shown in Table 3, RT-LAMP exhibited high concordance with the conventional RT-PCR and real-time PCR in the detection of FMDV samples from experimental and suspected cases of FMD. However, the virus in 8 out of 104 samples could not be detected by RT-LAMP-1 in this study.

DISCUSSION

The FMDV is a single-stranded plus-sense RNA virus. The genome is about 8.5 kb in length and encodes a large polyprotein, which is then cleaved into the structural proteins and nonstructural proteins. Like the genome of other RNA viruses, the FMDV genome has high variability in its structural proteins. However, the gene for a nonstructural protein, 3Dpol RNA polymerase, is a highly conserved region among the different sero- and subtypes of FMDV.[7, 17, 26] Thus, a set of common primers against the RNA polymerase genes of all seven types of FMDV can be designed and used for the rapid detection of all seven serotypes.

In this study, six primers that recognized eight distinct regions of FMDV RNA were designed and used in LAMP. Thus the specificity and sensitivity of LAMP were

Table 3. *Detection results of three methods in the field or on experimental samples*

Serotype	Number	No. (%) of positive samples			
		RT-LAMP	RT-PCR	Real-time PCR	Virus isolation
O	40	24 (100)	22 (91.7)	24 (100)	24 (100)
Asial	34	19 (98.00)	19 (98.00)	20 (100)	20 (100)
A	30	15 (100)	14 (93.30)	15 (100)	15 (100)
Total	104	58 (98.31)	55 (93.22)	59 (100)	59 (100)

Figure 1. *The alignment results of 3D of FMDV and locations of primer for LAMP. Referenced sequence used in this study (GenBank accession number AF506822). Locations of primer binding sequences are indicated by arrows.*

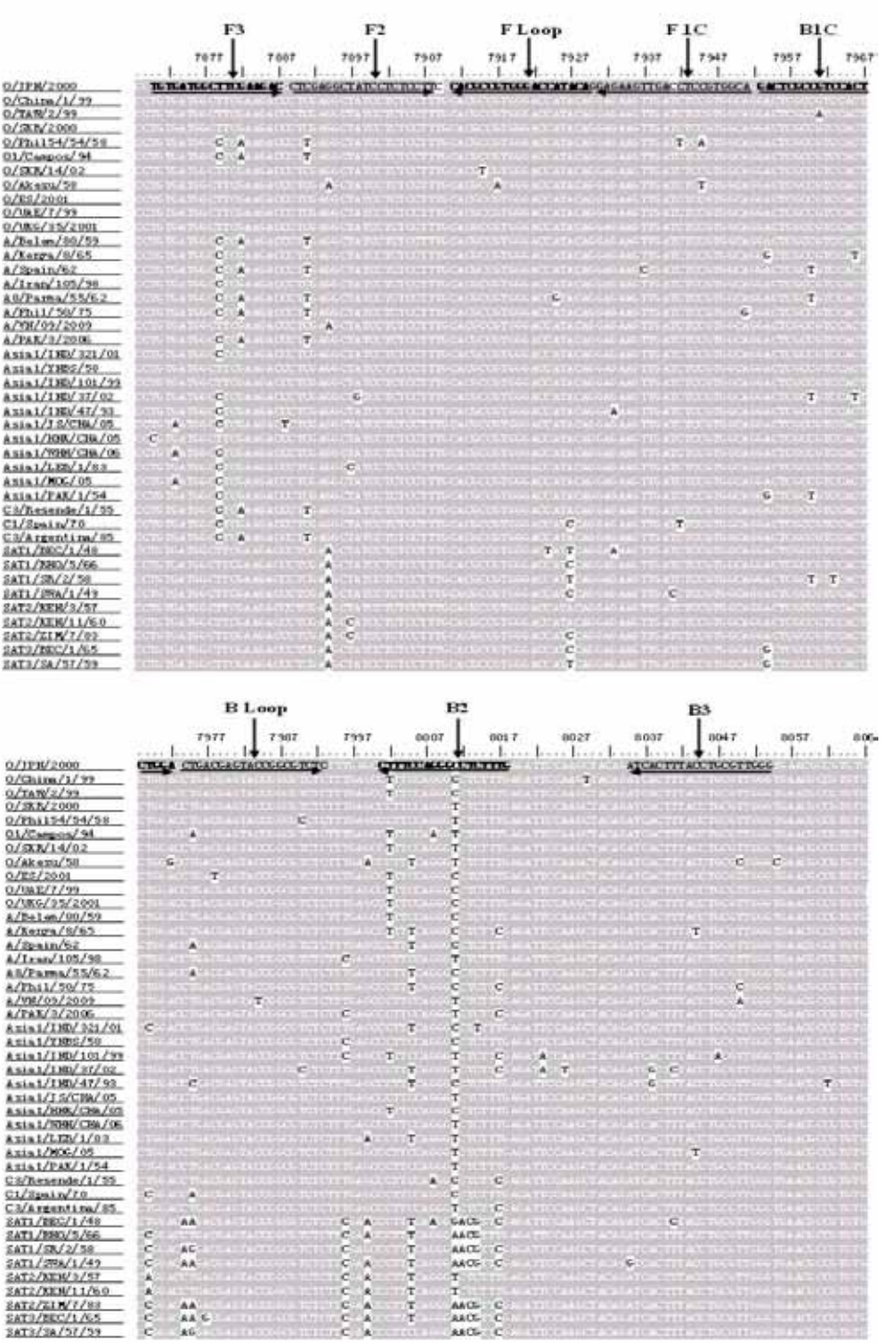
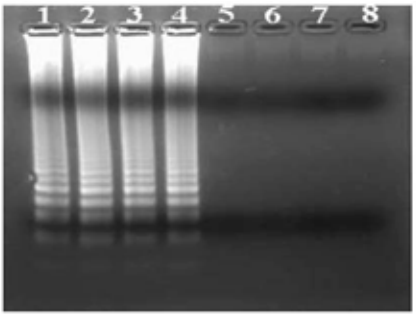
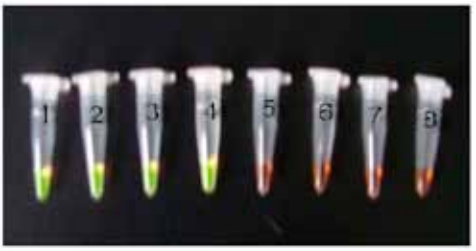


***Figure 2**. Agarose gel electrophoresis showing the specificity of RT-LAMP on amplification of FMDV serotype O, A, Asia1 and C.*



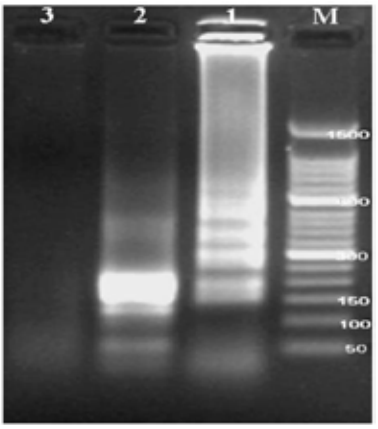
Lane 1–4: Amplicons of FMDV type O, A, Asia1 and C, respectively. Lane 5-8: SVDV, mocked-infected cells, negative tissue and distill water, respectively, were used as controls and no amplicons were visible.

***Figure 3**. Visual detection of RT-LAMP amplification products of the 4 FMDV serotypes using SYBR Green I stain.*



Tube 1-4: Color changes from orange to green were observed in the amplification products of type O, A, Asia1 and C FMDV mRNA, respectively. While no color change was visible in the amplification products from SVDV, mocked-infected cells, negative tissues and distilled water, respectively (from Tubes 5 to 8).

***Figure 4**. Identification of amplification product by Hae III digestion*

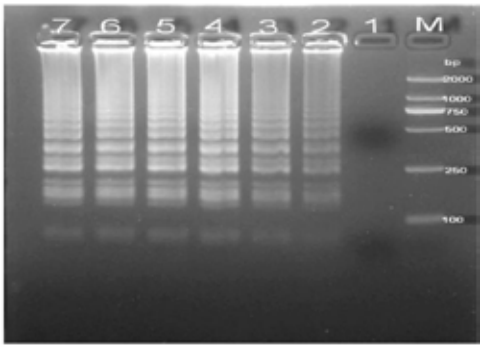


M, DNA Marker DL 50. 1, RT-LAMP amplification product; 2, RT-LAMP amplification product digested by Hae III; and 3, RT-LAMP without target RNA.

ensured and the number of false positive and negative reactions was minimized. The results demonstrated that the four serotypes of FMDV tested could be amplified specifically, unlike all negative and blank controls. In addition, three assays, RT-LAMP, LAMP-1, and real-time PCR, showed equivalent sensitivity and specificity.

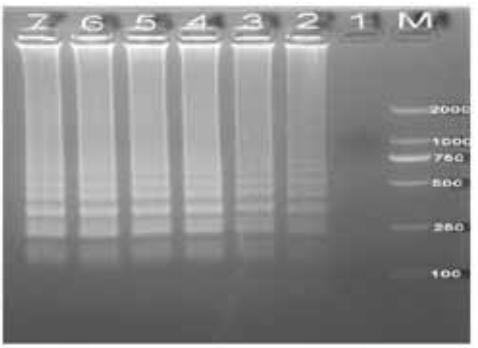
In contrast, the sensitivity of RT-PCR was lowest, at about 100 copies. These results were in agreement with previous reports.[20, 21] However, several samples from the experimental and suspected cases of FMDV could also not be detected by RT-LAMP-1 in this study. Dukes et al (2006) speculated that the failure of the RT-LAMP assay to detect certain isolates is most probably due to sequence changes at specific sites within the primers, which results in some mismatch between the primers and the specific target sequence. The sequence analysis indicated that some site mutations were present at the 3' end of B3 (T/C) and of F3 (T/C, G/A), and at the 5' end of B1C in

Figure 5. *The sensitivity of RT-LAMP was determined using 10-fold serial dilutions of the plasmid standard.*



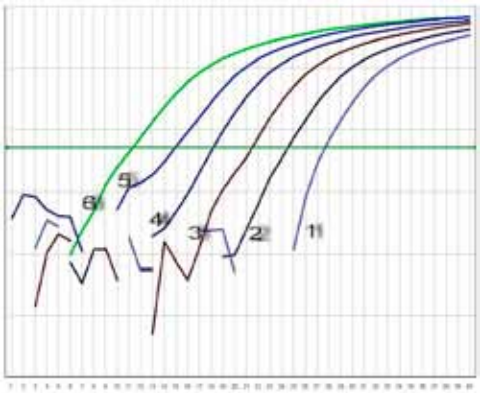
Lanes: 1, 0 copies; 2, 10 copies; 3, 1×102 copies; 4, 1×103copies; 5, 1×104 copies; 6, 1×105 copies; and 7, 1×106 copies. M, DL 2000 DNA Marker.

Figure 6*. The sensitivity of RT-LAMP-1 was determined using 10-fold serial dilutions of the plasmid standard.*



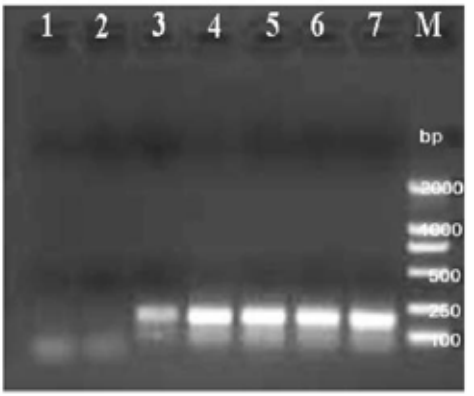
Lanes: 1, 0 copies; 2, 10 copies; 3, 1×102 copies; 4, 1×103copies; 5, 1×104 copies; 6, 1×105 copies; and 7, 1×106 copies. M, DL 2000 DNA Marker.

Figure 7. *The sensitivity of real-time RT-PCR was determined using 10-fold serial dilutions of the template DNA.*



Lanes: 1, 10 copies; 2, 1×102 copies; 3, 1×103copies; 4, 1×104copies; 5, 1×105 copies; 6, 1×106 copies.

Figure 8*. The sensitivity of RT-PCR was determined using 10-fold serial dilutions of template DNA.*



Lanes: 1, 0 copies; 2, 10 copies; 3, 1×102 copies; 4, 1×103copies; 5, 1×104 copies; 6, 1×105 copies; and 7, 1×106 copies. M, DL 2000 DNA Marker.

their primers (data not shown). Based on the principles of LAMP, these mutations were so very important that they markedly reduced the sensitivity of the primers. Therefore, we speculated that low detection ratio of RT-LAMP-1 may be attributed to mutations at the 3' end of the F3 and B3 primers.

Three serotypes of FMDV, SAT1, SAT2, and SAT3, were not involved in this paper because no samples were stored at any laboratory in China. However, the sequence analysis indicated that the target gene region used in the design of the primers was highly conserved among all seven serotypes of FMDV. There were a few mutations within the target sequences of the seven serotypes of FMDV, but the specificity of LAMP was barely affected when the common primers were used, because the mutations in the common primers were at the 5' end of

primers F3, B3, F2, and B2, and at the 3' end of primers F1C and B1C, or within F1C and B1C. Therefore, we speculate that RT-LAMP may also be used for the rapid detection of the other three types of FMDV. However, this conclusion needs to be further confirmed by future studies.

It has been reported that, unlike RT-PCR and real-time PCR, RT-LAMP is not affected by a high concentration of contamination from micro-organisms.[4, 28] The effects of contamination were not investigated in this study, but the effects of the contamination of animal samples will be considered in a future study. The results of RT-LAMP can be visualized directly by the naked eye without special equipment, which means that LAMP is simpler, more rapid, and safer than traditional gel electrophoresis. Moreover, LAMP can be performed in a water bath, which will facilitate its use on site or in developing countries for the rapid detection of FMDV. However, the disposable equipment must not be contaminated during sample preparation, and appropriate controls must be included to detect the presence of any cross-contamination during the entire experimental process.

It is concluded that a new version of LAMP that shows rapidity, sensitivity, efficiency and specificity has been developed in this study and this technique could be used for the detection of FMDV in the laboratory or under field conditions in future.

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