academicJournals

Vol. 7(35), pp. 4443-4450, 30 August, 2013
DOI: 10.5897/AJMR12.1659
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Two reverse-transcription-loop-mediated isothermal amplification systems for highly sensitive detection of enterovirus 71 and coxsackievirus A16

Guo-ping Zhao[1], Guang-ya Yin[1], Hong Zhao[2], Guo-liang Li[1], Bin Meng[1], Xiao Hu[1], Ya-xiao Li[1], Yi-zheng Wang[1] and Hua-bing Zhao[1]*

[1]Tianjin Key Laboratory for Biomarkers of Occupational and Environmental Hazard, Logistics University of Chinese People's Armed Police Forces, Tianjin 300309, People's Republic of China.
[2]Tianjin Entry-Exit Inspection and Quarantine Bureau, Tianjin 300456, People's Republic of China.

Accepted 24 January, 2013

Human enterovirus 71 and coxsackievirus A16 are two of the major causative agents of hand, foot, and mouth disease (HFMD). EV71 and CoxA16 infections in HFMD are clinically indistinguishable, but EV71 infection is more frequently associated with serious neurological complications and fatalities. In this study, two specific reverse-transcription-loop-mediated isothermal amplification (RT-LAMP) assays were developed for the detection of EV71 and CoxA16. High specificity systems for the EV71 and CoxA16 RT-LAMP systems have been confirmed by ruling out cross-reactivity with RNA of other common enteric viruses. The EV71 and CoxA16 RT-LAMP assays were found to be 10-fold and 100-fold more sensitive than conventional PCR, respectively, with detection limits of both 10^1 copies. To evaluate the application of the two RT-LAMP assays for clinical diagnosis, 60 stool specimens from suspect HFMD patients were detected and all samples were achieved in less than 60 min (even as little as 10 to 15 min). The positive rate of the EV71 RT-LAMP assay was higher (31/60=51.7%) than the RT-PCR assay (27/60=45%), while the positive rate of the CoxA16 RT-LAMP assay was equivalent to that of the RT-PCR assay (7/60=11.7%). These results indicate that the two RT-LAMP systems are potentially useful for rapid and sensitive diagnosis during HFMD outbreaks.

Key words: Enterovirus 71, coxsackievirus A16, reverse-transcription-loop-mediated isothermal amplification, hand foot, mouth disease.

INTRODUCTION

Hand, foot, and mouth disease (HFMD), a common illness in children, is mainly caused by two enteroviruses, human enterovirus 71 (EV71) and coxsackievirus A16 (CoxA16). Since the first recorded EV71 infection in California in 1969, HFMD outbreaks have occurred in many parts of the world, especially in Southeast Asia (Ahmad et al., 2000; Ang et al., 2009; Chang 2008; Ho et al., 1999; Schmidt et al., 1974; Tee et al., 2009; Yang et al., 2005). Smaller outbreaks have been described in Europe (Ortner et al., 2009; van der Sanden et al., 2009).

Recent findings suggest that the recombination of EV71 and CoxA16, and co-circulation of these two viruses may have contributed to the increase in HFMD cases over the past few years in China, Singapore and Malaysia (Ang et al., 2011; Li et al., 2005; Liu et al., 2011; Podin et al., 2006). Until now, no antiviral drugs or vaccines have been approved for clinical use against HFMD (Xu et al., 2010). Therefore, developing methods to detect EV71 and CoxA16 is a key point for the prevention and control of HFMD outbreaks.

EV71 and CoxA16 are both members of cluster A of human enteroviruses (HEVs) according to the classify-cation based on molecular properties (Hyypiä et al., 1997). It is incapable of distinguishing between the EV71

*Corresponding author. E-mail: huabingzhao@aliyun.com. Tel: +86 22 84876791. Fax: +86 22 84876791.

and the CoxA16 in clinical settings. Ma et al. (2011) reported that most outbreaks are caused by CoxA16, but the basic reproductive number (R0) for EV71 was higher than that of CoxA16. Furthermore, EV71 infection is more frequently associated with serious neurological complications and fatalities (Chang et al., 1999; McMinn et al., 2001; Shimizu et al., 1999; Wang et al., 2011; Zhang et al., 2009). For these reasons, differential detection of EV71 and CoxA16 is helpful in clinical diagnosis.

The classical diagnostic method for enteroviruses is propagation in cell culture followed by neutralization to confirm the serotype. This process is expensive, time-consuming, and labor-intensive. In addition, some enteroviruses replicate poorly in cell cultures (Oberste et al., 2000; Rigonan et al., 1998; Wong et al., 1999). Recently, a number of nucleic acid amplification (NAA) assays, including numerous PCR methods, have been developed for detecting EV71 and CoxA16, and they demonstrated high sensitivity and specificity (Tan et al., 2008). Nonetheless, a sophisticated thermal cycling instrument and skilled technicians are indispensable requirements of such tests, limiting their applicability.

Loop-mediated isothermal amplification (LAMP) is a relatively novel NAA method developed by Eiken Chemical Co., Ltd., Japan. It can potentially replace PCR in molecular diagnosis because of its simplicity, rapidity, specificity, and cost-effectiveness (Mori et al., 2001; Nagamine et al., 2002; Notomi et al., 2000). To date, molecular diagnosis by RT-LAMP has been used successfully to detect human pathogenic RNA viruses (Hong et al., 2004; Parida et al., 2004; Parida et al., 2005; Wang et al., 2012; Yaqing et al., 2012; Yoneyama et al., 2007).

In the present study, two RT-LAMP systems were standardized by targeting the VP1 region for rapid detection of EV71 and CoxA16 viruses. The applicability of the two systems for clinical diagnosis of HFMD patients is validated by evaluation with stool samples collected from the latest epidemic in Tianjin, which lasted from April to June in 2010.

MATERIALS AND METHODS

Standard viral strains and clinical stool specimens

Standard virus strains including EV71, CoxA16, CoxB3, CoxB5, and Echo30 were provided by the Chinese Center for Disease Control and Prevention, Beijing, China. PV and HAV were provided by Nankai University in Tianjin, China. Stool specimens (n=60) were collected from pediatric patients at the Children's Hospital, Tianjin, China, between April to June 2010. All the stool specimens were transported to our laboratory within 2 h and stored at -70°C until use.

RNA extraction

Stool specimens were mixed thoroughly with 5 to 10 volumes of phosphate-buffered saline (pH 7.4) to yield homogeneous suspensions and the mixtures were clarified by centrifugation at 5,000×g for 20 min. The genomic viral RNA was extracted from supernatants of stool suspensions using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The RNA was eluted from the QIAspin columns in a final volume of 80 µl of elution buffer and was stored at -80°C until use.

Primer design

The two RT-LAMP primer sets were designed using PrimerExplorer version 3.0 (Eiken Genome; http://primerexplorer.jp/) based on the alignments of the VP1 regions of EV71 and CoxA16, respectively, including almost all the available circulating strains in China responsible for recent epidemics. All the primers used in this study were synthesized by the Beijing Genomics Institute (BGI, Beijing, China).

RT-LAMP assay

We optimized the reaction system by adjusting the reaction temperature and the concentration of Mg^{2+}, betaine, and dNTPs. The EV71 RT-LAMP reaction was carried out in a total 25 µl reaction volume containing 50 pmol each of the forward inner primers (FIP) backward inner primer (BIP) and, 5 pmol each of the outer primers F3 and B3, 1.4 mM dNTPs, 0.6 M betaine, 0.1% Tween 20, 10 mM $(NH_4)_2SO_4$, 3.5 mM Mg^{2+}, 10 mMKCl, 20 mMTris-HCl, 8 U of Bst DNA polymerase (New England Biolabs, Hitchin, UK) , 10 U of AMV reverse transcriptase (Fermentas Inc., Ontario, Canada), and 2 µl of target RNA. The components of the CoxA16 system were equal with the EV71's except the betaine (1 M) and the primers. 30 to 50 µl of mineral oil was added to each tube to prevent evaporation. The reaction mixture of EV71 and CoxA16 was incubated at 62°C and 63°C, respectively, for 45 to 60 min followed by heating at 80°C for 2 min to terminate the reaction. Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Analysis of RT-LAMP products

Agarose gel analysis

5 µl of the RT-LAMP products were electrophoresed on a 2% agarose gel in Tris-acetate-EDTA buffer (0.04 M Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and visualization on a UV transilluminator (BioRad Laboratories, Berkeley, USA) at 302 nm.

Direct visual detection

The tubes were examined for white turbidity and for the presence of a white pellet after a pulse spin. Alternatively, 10 µl of 100×SYBR Green I dye (Invitrogen, Carlsbad, USA) was added to the reaction. In the case of positive amplification, the original orange color of the dye would change to a green color that was visible in natural light. When there was no amplification, the dye remained orange. The color change is permanent and the sample can be kept for archival purposes.

Restriction enzyme digestion of the RT-LAMP product

The specificity of the RT-LAMP-amplified product was detected by restriction digestion with *Nla*IV (New England Biolabs, Hitchin, UK) for EV71 and *Eco*RI (New England Biolabs, Hitchin, UK) for

A

2254 CCCGAGATGGAGTGTTTGACTACTACACCACAGGGTTAGTCAGTATATGGTATCAGACAA
F3 F2
2314 ATTACGTGGTTCCAATCCGGGCGCCCAATACAGCCTATATAATAGCACTAGCGGCAGCCC
*Nla*IV F1 *Nla*IV B1
2374 AAAAGAACTTCACTATGAAATTGTGCAAGGATGCTAGTGATATCCTGCAGACGGGCACCA
B2
2434 TCCAGGGAGACAGGGT
B3

Primer name	type	length	sequence(5' to 3')
F3	Forward Outer	20	CCCGAGATGGAGTGTTTGAC
B3	Reverse Outer	18	ACCCTGTCTCCCTGGATG
FIP	Forward Inner	39	CGCCCCGATTGGAACCACGA CACCACAGGGTTAGTCAGT
BIP	Reverse Outer	40	AATAGCACTAGCGGCAGCCC CCCGTCTGCAGGATATCACT

B

1679 GGCGGAAATGCGAGTTGTTTACCTACATGCGCTTTGATGCTGAATTCACATTTGTCGTAG
F3 F2 *Eco*RI
1739 CCAAACCCAATGGTGAGCTAGTCCCCCAATTACTGCAGTACATGTATGTCCCACCAGGGG
F1 B1
1799 CTCCGAAACCTACTTCCAGAGATTCGTTTGCTTGGCAGACTGCCACCAACCCATCTGTGT
B2
1859 TTGTGAAAATGACGGACCCA
B3

Primer name	type	length	sequence(5' to 3')
F3	Forward Outer	19	GGCGGAAATGCGAGTTGTT
B3	Reverse Outer	20	TGGGTCCGTCATTTTCACAA
FIP	Forward Inner	42	TGGGGGACTAGCTCACCATTGG ACATGCGCTTTGATGCTGAA
BIP	Reverse Outer	40	TGTATGTCCCACCAGGGGCTAG TCTGCCAAGCAAACGAAT

Figure 1. The genomic position of primers used in this assay. The sequence was obtained from GenBank with the accession number EU864507 for EV71 (A) and AY821798 for CoxA16 (B).

CoxA16.

Nucleotide sequence analysis of the RT-LAMP product

The fastest-migrating band of the RT-LAMP product was cut from the gel and sequenced by BGI with FIP and BIP primers.

Standard RNA strands preparation

In order to check the detection limits of RT-LAMP and RT-PCR assays, the standard RNA strands were prepared. Firstly, the targeted regions of the EV71 VP1 and the CoxA16 VP1 were amplified by PCR and the amplicons were cloned into the T-A vector system (TransGen Biotech, Inc. Beijing, China), respectively. Then, the recombinant plasmids were linearized and RNA fragments were synthesized by the Riboprobe System-T7 (Promega, Madison, WI, USA). Finally, the plasmid templates were removed by digestion with 1 U RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 15 min at 37 °C.

RT-PCR

For an assessment of the sensitivity and specificity of the RT-LAMP assay, RT-PCR was performed by employing the two outer primer pairs targeting the VP1 of EV71 and CoxA16, respectively. PCR amplification was carried out in a 25 µl total reaction volume containing 2 µl of target RNA, 10 U of AMV reverse transcriptase (Fermentas Inc., Ontario, Canada), 2 U of recombinant TaqDNA polymerase (Beijing TransGen Biotech Co., Ltd.), 50 pmol specific forward and reverse primers, 0.4 mM concentrations of dNTPs, 20 mM Tris-HCl (pH 8.4), 20 mM KCl, and 1.5 mM $MgCl_2$. The cycling conditions consisted of 30 min at 50°C, 5 min at 95°C, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, and then 72°C for 10 min. The PCR products were examined by agarose gel electrophoresis.

RESULTS

Primers

The primers were designed on the basis of the EV71 C4 subgenotype and CoxA16 C subgenotype. These are the predominant strains circulating in mainland China, responsible for recent HFMD epidemics. The nucleotide sequences of EV71 C4 strains and CoxA16 C strains were downloaded from the GenBank database and aligned using the GeneTool software. The highly conserved regions in the VP1 were selected as the target for primer design. The details of the each primer with regard to their positions in the genomic sequences are shown in Figure 1.

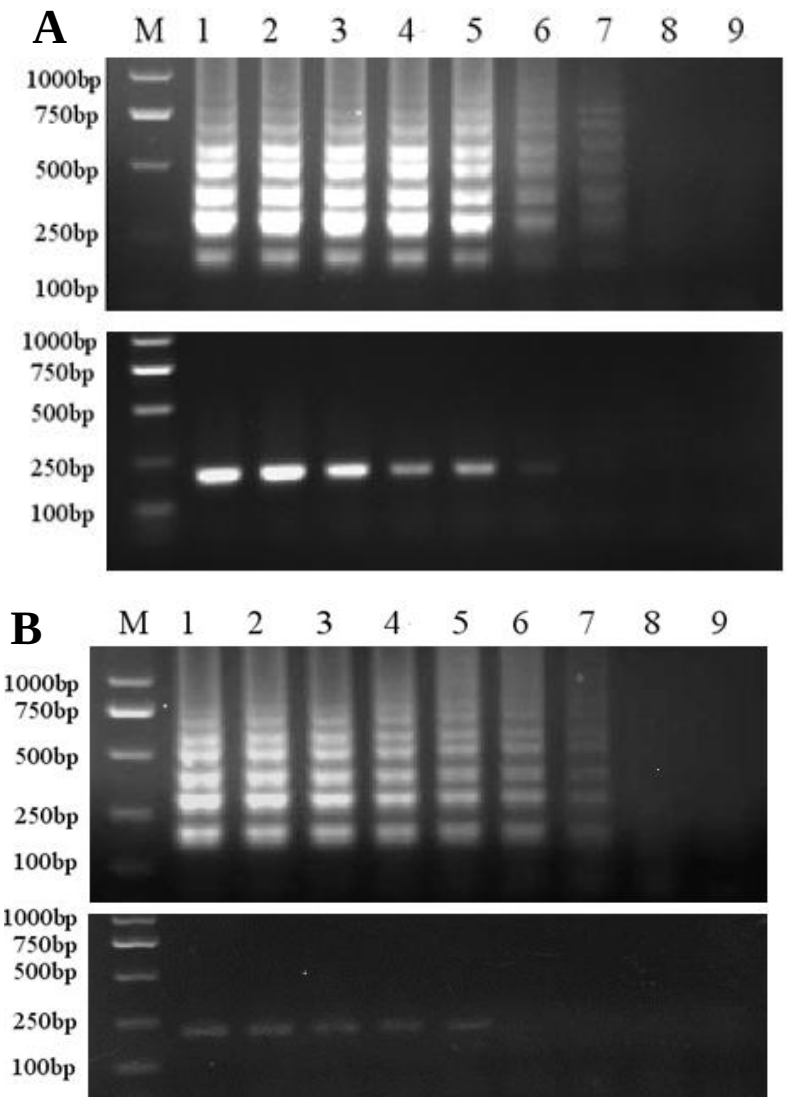

Figure 2. LAMP and PCR sensitivity for EV71 template (A) and CoxA16 template (B). Lane M, DNA marker; lanes 1 to 8, different concentrations of template ranging from 10^7 to 10^0 copies in a serial 10-fold dilution pattern. Lane 9, Negative control. Upper panel, LAMP; lower panel, PCR.

Sensitivity and specificity

The sensitivity of the RT-LAMP assays for the detection of EV71 and CoxA16 was determined by testing the corresponding standard RNA strands of 10-fold serial dilutions and comparing the results to those of conventional PCR. The EV71 RT-LAMP was found to be 10 times more sensitive than PCR, with a detection limit of 10^1 copies, against 10^2 copies for PCR (Figure 2A). The CoxA16 RT-LAMP was found to be 100 times more sensitive than PCR, with a detection limit of 10^1 copies, against 10^3 copies for PCR (Figure 2B). Upon visual detection, the detection limits of the two RT-LAMP assays were also validated by the observation of color change with the naked eye (upper panel of A and B) (Figure 3) or visualized under UV light transillumination (lower panel of A and B) (Figure 3) after employing a SYBR Green I. The specificity of the RT-LAMP primer sets for the EV71 and CoxA16 was established by ruling out cross-reactivities with HAV, PV, CoxB3, CoxB5, Echo30, and CoxA16/EV71. The EV71 and CoxA16 specific RT-LAMP primer sets both demonstrated high degrees of specificity by amplifying EV71 and CoxA16 only but yielded negative results for all other viruses tested (Figure 4). Furthermore, the specificity of the amplification was tested by restriction endonuclease digestion. The generated fragments were mainly about 131 and 57 bp after digestion with *Nla*IV for EV71 amplifi-

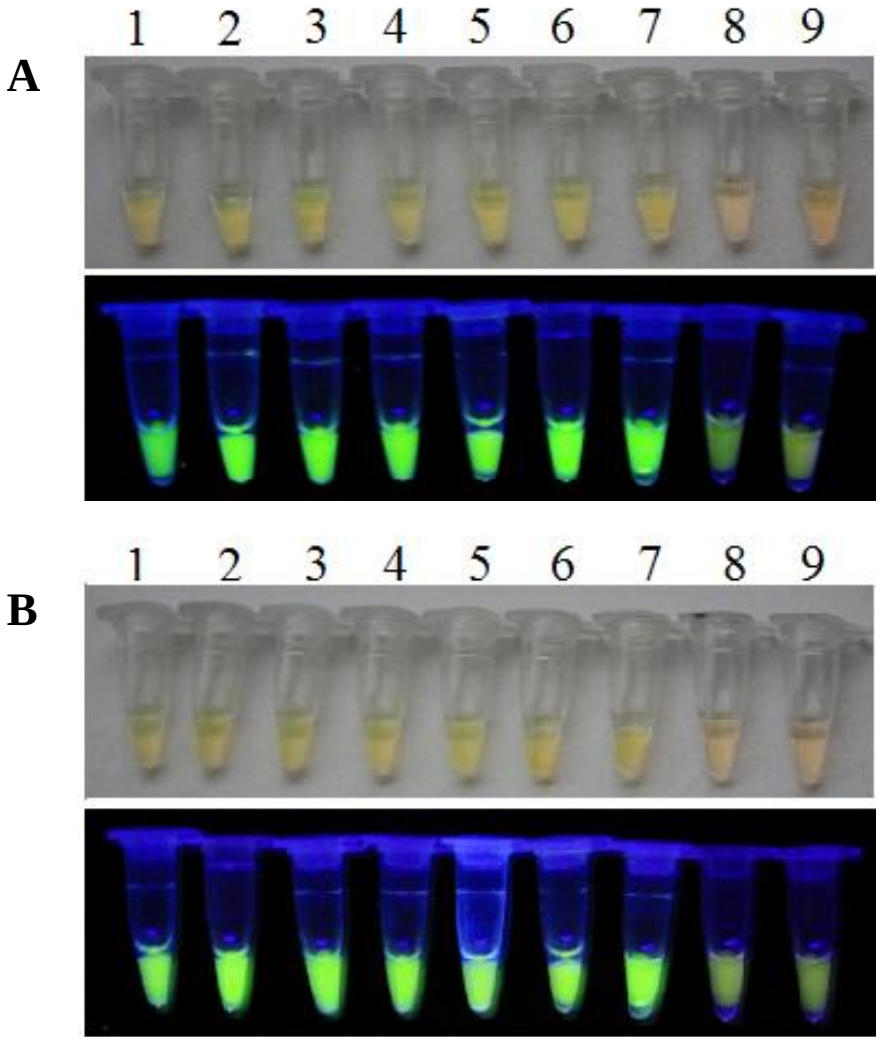

Figure 3. Visual detection of LAMP amplification products in 10-fold dilutions of EV71 template (A) and CoxA16 template (B). Tubes 1 to 8, different concentrations of template ranging from 10^7 to 10^0 copies in a serial 10-fold dilution pattern. Tube 9, DDW. Detection of the white precipitate (magnesium pyrophosphate) at the bottom of the tubes indicates a positive reaction, while the absence of precipitate indicates a negative reaction. After the addition of SYBR Green to the reaction mix, yellow fluorescence is detectable by the naked eye under natural light, while an orange color indicates a negative reaction (upper panel). Under UV irradiation, a positive reaction is indicated by a bright green fluorescent color (lower panel).

cation products and 190 and 200 bp after digestion with *Eco*RI for CoxA16 amplification products (Figure 4).

Clinical samples

The applicability of the two RT-LAMP assays to the clinical diagnosis of EV71 and CoxA16 was evaluated with 60 stool samples of suspected HFMD cases collected in Tianjin, China, 2010. These were compared to results generated using conventional PCR. Some stool samples containing high levels of virus could be detected as early as 10 to 15 min, and most of the RT-LAMP product was detected as early as 45 min, but the reaction time was still maintained for 60 min to ensure positive detection of lower concentration of viruses in the samples. We found 31 of the 60 samples to be EV71-positive and 7 of the 60 samples to be CoxA16-positive in the RT-LAMP assay. None of the samples was both EV71 and CoxA16 positive. In the PCR assay, 27 of the 60 samples were EV71-positive (Table 1) and 7 of the 60 samples were CoxA16-positive (Table 2). The positive rate of the EV71 RT-LAMP assay was 51.7%, higher than that of the RT-PCR assay (45%), while the positive rate of

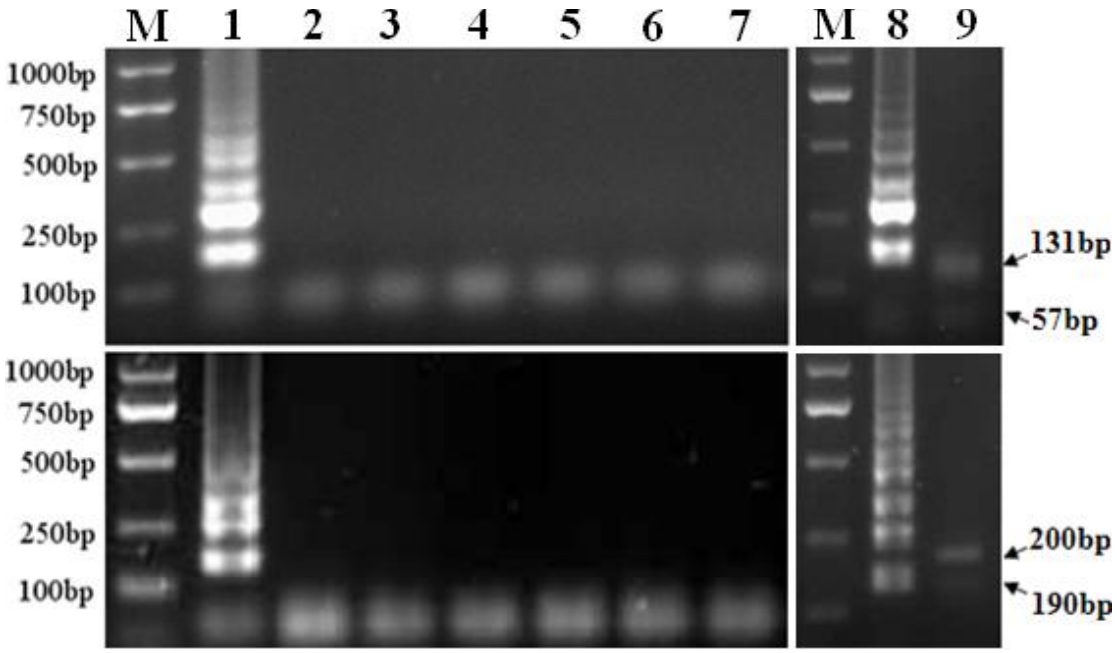

Figure 4. LAMP specificity for EV71 (upper panel) and CoxA16 (lower panel) templates, Lane M, DNA marker DL-2000 plus; lane 1, EV71 (upper panel)\CoxA16 (lower panel); lane 2, Cox A16 (upper panel)\EV71(lower panel); lane 3, Cox B3; lane 4, Cox B5; lane 5, Echo 30; lane 6. poliovirus; lane 7, HAV; Specificity of LAMP products, Lane 9, LAMP products were digested with *Nla*IV for EV71 (upper panel) and *Eco*RI for CoxA16 (lower panel), LAMP products (lane 8) were run simultaneously.

the CoxA16 RT-LAMP assay was equivalent to that of the RT-PCR assay (11.7%). None of the RT-PCR positive samples were missed by RT-LAMP, indicating that the RT-LAMP assay was more sensitive. Further confirmation of the RT-LAMP products was carried out by sequencing. The sequences obtained matched the expected viruses perfectly (data not shown).

DISCUSSION

EV71 and CoxA16 are the two major causative agents of HFMD, and the co-circulation of these two viruses may be responsible for the HFMD epidemics seen in China, Singapore, Malaysia (Ang et al., 2011; Li et al., 2005; Liu et al., 2011; Podin et al., 2006). EV71 and CoxA16 infections in HFMD are clinically indistinguishable, but EV71 can cause severe neurological disease with rapid progression and high mortality relative comparison to mild cases of CoxA16 infection. This is why there is a great demand for the rapid detection and differentiation of EV71 and CoxA16 infections, to provide timely, clinical treatment and disease control.

In China, the molecular diagnoses of EV71 and CoxA16 infections are currently dependent on RT-PCR and real-time PCR examination of clinical specimens (Chen et al., 2006; Xiao et al., 2009). Two obvious disadvantages of PCR methods are machine-dependence and the need for skilled technicians, which limit its application in communities, rural clinics, and field situations. Here, we reported two RT-LAMP assays that are simple to perform, react rapidly, and provide easy detection for the diagnosis of EV71 and CoxA16 infection from HFMD specimens.

As shown above, the sensitivities of the EV71 and CoxA16 RT-LAMP are both 10^1 copies per reaction mixture, which are 10 and 100 times higher than the corresponding RT-PCR assays. This is similar to the other EV71 RT-LAMP system described in previous studies, which had a detection limit of approximately 10 copies with a slightly longer reaction time at 1.5 h (Wang et al., 2012). Based on 60 stool specimens from suspected HFMD cases, the RT-LAMP assay also demonstrated higher sensitivity by correctly picking up four additional positive EV71 samples, which were missed by RT-PCR.

The higher specificity of the LAMP reaction is attributed to employing four primers that recognize six distinct regions of the target. This prevents cross-reactions very effectively (Notomi et al., 2000). Our data show that there was no cross-reactive amplification of EV71 and CoxA16 RT-LAMP assays with the RNA of other enteroviruses, such as HAV, PV, CoxB3, CoxB5, Echo30, or CoxA16/EV71. The high specificity of the two RT-LAMP assays was further validated in clinical samples by sequencing.

Table 1. Comparison of LAMP and PCR for detection of EV71 from stool samples.

PCR	LAMP		Total
	+	-	
+	27	0	27
-	4	29	33
Total	31	29	60

Table 2. Comparison of LAMP and PCR for detection of CoxA16 from stool samples.

PCR	LAMP		Total
	+	-	
+	7	0	7
-	0	53	53
Total	7	53	60

We found it difficult to design one set of primers suitable for the amplification of all the EV71/CoxA16 strains that originate in different regions. The EV71 strains detected from the Chinese mainland were closely related to each other and grouped into genotype C to form a new lineage, C4, distinct from the C1, C2, and C3 lineages (Li et al., 2005). There are three genetic CoxA16 lineages circulating in Asia; A, B, and C. In 1999, viruses of lineages B and C began to cocirculate on the Chinese mainland, with lineage C gradually becoming predominant (Li et al., 2005). We therefore designed primer sets based on the EV71 C4 lineages and CoxA16 C lineages by targeting the VP1 region. The two primer sets successfully amplified the target viral sequences from suspected HFMD patients in the Children's Hospital, Tianjin, China.

From a practical point of view, LAMP assay is more suitable as a routine diagnostic tool, especially in clinics and fields. First, the sensitivity of LAMP was less affected by inhibitory components in clinical samples (Kaneko et al., 2007). Second, LAMP products could be directly observed by turbidity or color change after the addition of dyes. Third, the LAMP reaction only needs a laboratory water bath or heating block rather than complicated equipment such as thermal cycling machines or electrophoresis apparatuses. However, due to the high amplification rate, any trace of LAMP DNA concatemers will be easily reamplified, leading to false-positive results (Paris et al., 2007; Peyrefitte et al., 2008). Thus, at the endpoint of amplification, turbidity or fluorescence reading should be preferred to agarose gel analysis. Mineral oil is also very useful in preventing contamination.

In summary, we have established a simple, rapid, highly sensitive, and highly specific RT-LAMP method. It has considerable potential to detect the EV71 and CoxA16 infections of HFMD patients in the primary health care institutions and field environments. When combined with nucleotide sequencing of the LAMP products, RT-LAMP may also be useful for studying the molecular epidemiology and evolution of HFMD.

ACKNOWLEDGEMENTS

We would like to thank Dr. Yongsheng Xu and Dr. Shuxiang Lin of Tianjin Children's Hospital, Prof. Wenbo Xu of the Chinese Center for Disease Control and Prevention for providing the virus samples and stool specimens. This study was supported by Tianjin Research Program of Application Foundation and Advanced Technology (No.09JCYBJC08400) and Open Fund of Tianjin Key Laboratory for Biomarkers of Occupational and Environmental Hazard (WZK201001).

REFERENCES

Ahmad K (2000). Hand, foot and mouth disease outbreak reported in Singapore. Lancet, 356:1338.

Ang LW, Koh BK, Chan KP, Chua LT, James L, Goh KT (2009). Epidemiology and control of hand, foot and mouth disease in Singapore, 2001-2007. Ann. Acad. Med. Singapore. 38:106-112.

Ang LW, Phoon MC, Wu Y, Cutter J, James L, Chow V (2011). The changing seroepidemiology of enterovirus 71 infection among children and adolescents in Singapore. BMC Infect. Dis. 11:270-277.

Chang LY (2008). Enterovirus 71 in Taiwan. Pediatr. Neonatol. 49:103-112.

Chang LY, Lin TY, Huang YC, Tsao KC, Shih SR, Kuo ML, Ning HC, Chung PW, Kang CM (1999). Comparison of enterovirus 71 and coxsackie-virus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. Pediatr. Infect. Dis. J. 18:1092-1096.

Chen TC, Chen GW, Hsiung CA, Yang JY, Shih SR, Lai YK, Juang JL (2006). Combining multiplex reverse transcription-PCR and a diagnostic microarray to detect and differentiate enterovirus 71 and coxsackievirus A16. J. Clin. Microbiol. 44:2212-2219.

Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, Wang JR, Shih SR (1999). An epidemic of enterovirus 71 infection in Taiwan. N. Engl. J. Med. 341:929-935.

Hong TC, Mai QL, Cuong DV, Parida M, Minekawa H, Notomi T, Hasebe F, Morita K (2004). Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol., 42:1956-1961.

Hyypiä T, Hovi T, Knowles NJ, Stanway G (1997). Classification of enteroviruses based on molecular and biological properties. J. Gen. Virol. 78:1-11.

Kaneko H, Kawana T, Fukushima E, Suzutani T (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J. Biochem. Biophys. Methods 70:499-501.

Li L, He Y, Yang H, Zhu J, Xu X, Dong J, Zhu Y, Jin Q (2005). Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. J. Clin. Microbiol. 43:3835-3839.

Liu Q, Ku Z, Cai Y, Sun B, Leng Q, Huang Z (2011). Detection, characterization and quantitation of Coxsackievirus A16 using polyclonal antibodies against recombinant capsid subunit proteins. J. Virol. Methods 173:115-120.

Ma E, Fung C, Yip SH, Wong C, Chuang SK, Tsang T (2011). Estimation of the basic reproduction number of enterovirus 71 and coxsackievirus A16 in hand, foot, and mouth disease outbreaks. Pediatr. Infect. Dis. J. 30:675-679.

McMinn P, Stratov I, Nagarajan L, Davis S (2001). Neurologica lmanifestations of enterovirus 71 infection in children during an outbreak of hand, foot, and mouth disease in Western Australia. Clin. Infect. Dis. 32:236-242.

Mori Y, Nagamine K, Tomita N, Notomi T (2001). Detection of loop mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289:150-154.

Nagamine K, Hase T, Notomi T (2002). Accelerated reaction by loop mediated isothermal amplification using loop primers. Mol. Cell. Probes. 16:223-229.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28:e63.

Oberste MS, Maher K, Flemister MR, Marchetti G, Kilpatrick DR, Pallansch MA (2000). Comparison of classic and molecular approaches for the identification of untypeableenteroviruses. J. Clin. Microbiol. 38:1170-1174.

Ortner B, Huang CW, Schmid D, Mutz I, Wewalka G, Allerberger F, Yang JY, Huemer HP (2009). Epidemiology of enterovirus types causing neurological disease in Austria 1999-2007: detection of clusters of echovirus 30 and enterovirus 71 and analysis of prevalent genotypes. J. Med. Virol. 81:317-324.

Parida M, Horioke K, Ishida H, Dash PK, Saxena P, Jana AM, Islam MA, Inoue S, Hosaka N, Morita K (2005). Rapid detection and differentiation of dengue virus serotypes by real-time reverse transcription-loop-mediated isothermal amplification assay. J. Clin. Microbiol. 43:2895-2903.

Parida M, Posadas G, Inoue S, Hasebe F (2004). Real-time reverse transcriptionloop-mediated isothermal amplification for rapid detection of West Nile virus. J. Clin. Microbiol. 42:257-263.

Paris DH, Imwong M, Faiz AM, Hasan M, Yunus EB, Silamut K, Lee SJ, Day NP, Dondorp AM (2007). Loop-mediated isothermal PCR (LAMP) for the diagnosis of falciparum malaria. Am. J. Trop. Med. Hyg. 77:972-976.

Peyrefitte CN, Boubis L, Coudrier D, Bouloy M, Grandadam M, Tolou HJ, Plumet S (2008). Real-time reverse-transcription loop-mediated isothermal amplification for rapid detection of rift valley Fever virus. J. Clin. Microbiol., 46:3653-3659.

Podin Y, Gias EL, Ong F, Leong YW, Yee SF, Yusof MA, Perera D, Teo B, Wee TY, Yao SC, Yao SK, Kiyu A, Arif MT, Cardosa MJ (2006). Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. BMC Public Health 6:180-190.

Rigonan AS, Mann L, Chonmaitree T (1998). Use of monoclonal antibodies to identify serotypes of enterovirus isolates. J. Clin. Microbiol. 36:1877-1881.

Schmidt NJ, Lennette EH, Ho HH (1974). An apparently new enterovirus isolated from patients with disease of the central nervous system. J. Infect. Dis. 129:3042-3049.

Shimizu H, Utama A, Yoshii K, Yoshida H, Yoneyama T, Sinniah M, Yusof MA, Okuno Y, Okabe N, Shih SR, Chen HY, Wang GR, Kao CL, Chang KS, Miyamura T, Hagiwara A (1999). Enterovirus 71 fromfatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. Jpn. J. Infect. Dis. 52:12-15.

Tan EL, Chow VT, Quak SH, Yeo WC, Poh CL (2008). Development of multiplex real-time hybridization probe reverse transcriptase polymerase chain reaction for specific detection and differentiation of Enterovirus 71 and Coxsackievirus A16. Diagn. Microbiol. Infect. Dis. 61:294-301.

Tee KK, Takebe Y, Kamarulzaman A (2009). Emerging and re-emergingviruses in Malaysia, 1997-2007. Int. J. Infect. Dis. 13:307-318.

van der Sanden S, Koopmans M, Uslu G, van der Avoort H; Dutch Working Group for Clinical Virology (2009). Epidemiology of enterovirus 71 in the Netherlands, 1963-2008. J. Clin. Microbiol. 47:2826-2833.

Wang X, Zhu JP, Zhang Q, Xu ZG, Zhang F, Zhao ZH, Zheng WZ, Zheng LS (2012). Detection of enterovirus 71 using reverse transcription loop-mediated isothermal amplification (RT-LAMP). J. Virol. Methods 179:330-334.

Wang Y, Feng Z, Yang Y, Self S, Gao Y, Longini IM, Wakefield J, Zhang J, Wang L, Chen X, Yao L, Stanaway JD, Wang Z, Yang W (2011). Hand, foot, and mouth disease in China: patterns of spread and transmissibility. Epidemiol. 22:781-792.

Wong KT, Chua KB, Lam SK (1999). Immunohistochemical detection of infected neurons as a rapid diagnosis of enterovirus 71 encephalomyelitis. Ann. Neurol. 45:271-272.

Xiao XL, He YQ, Yu YG, Yang H, Chen G, Li HF, Zhang JW, Liu DM, Li XF, Yang XQ, Wu H (2009). Simultaneous detection of human enterovirus 71 and coxsackievirus A16 in clinical specimens by multiplex real-time PCR with an internal amplification control. Arch. Virol. 154:121-125.

Xu J, Qian Y, Wang S, Serrano JM, Li W, Huang Z, Lu S (2010). EV71: an emerging infectious disease vaccine target in the Far East? Vaccine 28: 3516-3521.

Yang ZH, Zhu QR, Li XZ, Wang XH, Wang JS, Hu JY, Tang W, Cui AL (2005). Detection of enterovirus 71 and coxsackievirus A16 from children with hand, foot and mouth disease in Shanghai, 2002. Zhong hua Er Ke Za Zhi, 43:648-652 (In Chinese).

Yaqing H, Wenping Z, Zhiyi Y, Xionghu W, Shouyi Y, Hong Y, Yingchun D, Guifang H (2012) Detection of human Enterovirus 71 reverse transcription loop-mediated isothermal amplification (RT-LAMP). Lett. Appl. Microbiol. 54:233-239.

Yoneyama T, Kiyohara T, Shimasaki N, Kobayashi G, Ota Y, Notomi T, Totsuka A, Wakita T (2007). Rapid and real-time detection of hepatitis A virus by reverse transcription loop-mediated isothermal amplification assay. J. Virol. Methods, 145:162-168.

Zhang Y, Nan LJ, Wu GS, Tan XJ, Xu DD, Gu SY, Zhu SL, Yan DM, An HQ, Xu WB (2009). The epidemiologic and virological analysis of an outbreak of hand, foot, mouth disease in Inner Mongolia in 2007. Bing Du Xue Bao, 25:159-165 (In Chinese).