Development of Loop-Mediated Isothermal Amplification (LAMP) for Universal Detection of Enteroviruses

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Abstract Enteroviruses are found in most environments and cause several diseases in humans. Loop-mediated isothermal amplification (LAMP) was adapted and evaluated for the rapid detection of enteroviruses. Based on the highly conserved 5' untranslated region (5'-UTR) of the human enteroviruses (HEVs), particularly human enterovirus A (HEV-A) and HEV-B, a set of universal primers was designed. The LAMP amplification was carried out under isothermal conditions at 61 °C, depending on the template concentration results were obtained within 45–90 min. The detection limits were found to be 10¹ copies of cloned enterovirus 71 fragments, more sensitive than conventional PCR. Nine water samples collected from drinking water sources during three seasons and 19 stool specimens collected from HFMD patients were analyzed. By using the LAMP assay, the majority of samples was tested positive, 9/9 (100 %) and 18/19 (94.7 %), respectively. LAMP is a practical method for the rapid detection of enteroviruses in environmental and clinical samples.

Keywords Detection · Enterovirus · LAMP ·
Drinking water source · HFMD

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Introduction

Enteroviruses, members of the *Picornaviridae* family, are among the most common human-infecting viruses worldwide [1]. They are associated with several human diseases that cause a wide range of clinical syndromes, such as the common cold; hand, foot, and mouth disease (HFMD); acute flaccid paralysis; acute hemorrhagic conjunctivitis; aseptic meningitis; and myocarditis [2, 3]. Infections are transmitted mainly through the fecal-oral and oral–oral route but also through direct contact with secretions from ophthalmic and dermal lesions [4]. Contact with water, food, and soil contaminated with infected feces may cause fecal-oral transmission [4, 5].

The traditional gold standard for the diagnosis of enteroviral infections is cell culture assay, followed by analysis with neutralizing antisera. However, method is time-consuming, expensive, and impractical for monitoring. Recently, several molecular-biology-based methods for the detection of enteroviruses have been developed. For example, a powerful version of polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), and real-time quantitative reverse transcriptase PCR (qRT-PCR) has been used for detection of enteroviruses in clinical and environmental specimens [6–8]. However, these methods have their own intrinsic disadvantages. For example, the equipment needed for amplification is expensive and the method for detection of amplification products is complex. Therefore, rapid, simple, and cost-effective molecular methods are needed.

Loop-mediated isothermal amplification (LAMP), a relatively novel nucleic acid-based single tube technique [9], maybe is an alternative approach. The method has been applied in several areas [10–12]. The LAMP reaction is based on strand displacement by a *Bst* DNA polymerase

under isothermal conditions that the temperature range is from 60 to 65 °C. A set of four primers that strictly recognize six distinct regions on the target DNA sequences ensures high specificity. The method generates a large amount of amplification products in positive samples, allowing some assessment of these products with the naked eye and fluorescent dye.

Here we established a LAMP assay whose primer set was specially designed for universal detection of human enteroviruses, particularly human enterovirus A (HEV-A) and HEV-B. This assay exhibited a high positive rate with significant accuracy in the detection of enteroviruses in stool specimens of HFMD patients and drinking water sources.

Materials and Methods

Design of LAMP Primers

The sequences of enteroviruses were downloaded from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Based on the highly conserved 5'-UTR sequence of the most prevalent HEVs genotypes, human enterovirus-specific LAMP primers (Fig. 1) were designed using software Primer Premier 5.0 (Premier Biosoft International Co., CA, US).

Plasmid Construction

For optimization of the LAMP assay conditions and evaluation of the detection limit, we generated a plasmid p5'UTR-EV71. For that purpose a 193 bp target DNA sequence at the 5'-UTR of the EV71 genome was amplified by PCR using the same outer primers (F3 and B3) as those used in the LAMP reaction. The amplified product was then cloned into pGM-T cloning vector (Tiangen Biotech (Beijing) Co., Ltd., China).

Fig. 1 Locations of the primer sequences (EV71 strain Fuyang-0805, GenBank accession no.FJ439769) on HEVs LAMP and the site of the restriction enzyme *NlaIV*, respectively. Primer FIP consists of F1 complementary sequence and F2 direct sequence. Primer BIP consists of B1 direct sequence and B2 complementary sequence. Locations of primer-binding sequences are *underlined*

LAMP Assay

The reaction was carried out in a 25 µl mixture containing 1.6 µM each of the inner primers FIP and BIP, 0.2 µM each of the outer primers F3 and B3, 2.5 µl of 10 × *Bst* DNA polymerase reaction buffer, 8 U *Bst* DNA polymerase (NEB, U.S.), 1.8 mM dNTPs, 0.8 M betaine, 2 mM MgCl₂ and 5 µl of target sample. The reaction mixture was incubated at 61 °C for 45 to 90 min and the reaction was terminated at 80 °C for 10 min.

Analysis of LAMP Products

The products obtained by LAMP were detected by (1) direct visual detection: the LAMP reaction causes turbidity proportional to the amount of amplified DNA. The turbidity visible with the naked eye was considered indicative of a successful LAMP procedure. The products were also detected visually using a color change after the addition of SYBR Green I dye (Invitrogen, US). The solution turned green in the presence of a LAMP amplicon, while it remained orange when no amplification had taken place; (2) electrophoresis: LAMP products were subjected to electrophoresis on a 2 % agarose gel, followed by visualization under UV light transillumination. And (3) by sequence analysis: LAMP products were also sequenced (BGI LifeTech Co., Ltd., Beijing, China) with the FIP or BIP primers (Fig. 1) by cutting the fastest migrating band of the amplification products.

Specificity of LAMP Assay

The specificity of the assay was tested using templates from standard virus strains. A panel of viral genomic RNA was provided by the Chinese Center for Disease Control and Prevention (Beijing, China), including coxsackievirus A16 (CV-A16), enterovirus 71 (EV-71), coxsackievirus B3 (CV-B3), coxsackievirus B5 (CV-B5), echovirus 30 (EV-30). Poliovirus (PV) and hepatitis A virus (HAV) were provided by Nankai University (Tianjin, China).

Sensitivity of LAMP and PCR Assay

The detection limit of the LAMP assay was evaluated by using a series of tenfold serial dilutions (10⁶ to 10⁰ copies·µl⁻¹) of plasmid p5'UTR-EV71, and 5 µl of each dilution was used as templates. In order to evaluate the sensitivity of the LAMP assay a PCR assay was performed by using the two outer LAMP primers, F3 and B3, as forward and reverse primers, respectively. The PCR reaction mixture contained 5 µl template of plasmid p5'UTR-EV71, 2.5 µl of 10 × EasyTaq Buffer, 1.25 U of EasyTaq DNA Polymerase (BeijingTransGen Biotech Co., Ltd.,

China), 0.2 mM dNTPs, and 1 µl of each forward and reverse primer (each 10 µM) in a reaction volume of 25 µl. The PCR conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and then 72 °C for 5 min.

Application of LAMP to Detect HEVs in Drinking Water Sources and Stool Samples

Between October 2009 and May 2010, 9 water samples from drinking water sources were collected in Tianjin, a metropolis in northern China. All sites were sampled using special buckets transported back to the laboratory within 2 h for immediate processing. Five hundred milliliters of source water samples were concentrated in batches using a Ultracel-100 k centrifugal filter device (Millipore, US), in the end, about 200 µl of concentrated sample was collected from the retention cup and frozen at −70 °C until use in LAMP analysis. Stool specimens ($n = 19$) were collected from pediatric patients at the Children's Hospital in Tianjin, China. The patients had clinical diagnoses of HFMD (between April to June 2010). All stool specimens were transported to our laboratory and stored at −70 °C until use.

Viral RNA was extracted from concentrated water samples and supernatants of stool suspensions with QIA-amp Viral RNA Mini Kit according to manufacturer's instructions (QIAGEN, Germany). Subsequently, RNA

genome was converted to cDNA using an Easy Script RT First-Strand cDNA Synthesis Super Mix (BeijingTransGen Biotech Co., Ltd., China).

Results

LAMP Primer Design

The 5'-UTR have been exploited for the development of primers used in detection of the enteroviruses because of their absolute (or near absolute) conservation among the enteroviruses. Based on the nucleotide sequence of 5'-UTR, all human enteroviruses can be clustered into two major groups. One cluster includes PV, HEV-C, and HEV-D species. HEV-A and HEV-B constitute the other cluster [13, 14].

It was difficult to design one set of primers with the same high levels of amplification efficiency and accuracy for detection of the both groups of enteroviruses. For this reason, we designed a set of LAMP primers (Fig. 1) perfectly matching the strictly conserved sequences in the 5'-UTR of the HEV genome, particularly HEV-A and HEV-B. This is notable because HEV-A and HEV-B remain a significant cause of morbidity and are responsible for a wide spectrum of clinical symptoms, such as HFMD [4]. Taking EV-71 (GenBank accession no.FJ439769) as an example, the location of each primer is shown in Fig. 1.

Table 1 Partial HEVs strains in GenBank identified by the primers

Serotype	Molecular classification	GenBank accession No.	Serotype	Molecular classification	GenBank accession No.
CV-A2	HEV-A	AY421760	E-15	HEV-B	AY302541
CV-A3	HEV-A	AY421761	E-17	HEV-B	AY302543
CV-A4	HEV-A	AY421762	E-20	HEV-B	AY302546
CV-A6	HEV-A	AY421764	E-29	HEV-B	AY302552
CV-A7	HEV-A	AY421765	E-30	HEV-B	DQ246620
CV-A8	HEV-A	AY421766	E-32	HEV-B	AY302555
CV-A9	HEV-B	D00627	E-33	HEV-B	AY302556
CV-A10	HEV-A	AY421767	EV-69	HEV-B	AY302560
CV-A12	HEV-A	AY421768	EV-71	HEV-A	FJ439769
CV-A14	HEV-A	AY421769	EV-75	HEV-B	AY556070
CV-A16	HEV-A	U05876	EV-77	HEV-B	AY843302
CV-B2	HEV-B	AF081485	EV-80	HEV-B	AY843298
CV-B3	HEV-B	M88483	EV-82	HEV-B	AY843300
CV-B4	HEV-B	AF328683	EV-84	HEV-B	DQ902712
CV-B5	HEV-B	AF114383	EV-85	HEV-B	AY843303
E-2	HEV-B	AY302545	EV-86	HEV-B	AY843304
E-3	HEV-B	AJ849942	EV-87	HEV-B	AY843305
E-4	HEV-B	FJ172447	EV-88	HEV-B	AY843306
E-5	HEV-B	AF083069	EV-101	HEV-B	AY843308
E-11	HEV-B	EF634316

Fig. 2 Optimization of LAMP conditions for detection of the HEVs. **a** Effect of concentration of MgCl_2 . Lane M: DNA marker; lane 1: 0 mM; lane 2: 1.0 mM; lane 3: 2.0 mM; lane 4: 3.0 mM; lane 5:

4.0 mM; lane 6: 5.0 mM. **b** Effect of concentration of betaine. Lane M: DNA marker; lane 1: 0 M; lane 2: 0.2 M; lane 3: 0.4 M; lane 4: 0.6 M; lane 5: 0.8 M; lane 6: 1.0 M; lane 7: 1.2 M

Fig. 3 The specificity of LAMP for detection of HEVs. Lane M: DNA marker; lane 1: EV71; lane 2: CV-A16; lane 3: CV-B3; lane 4: CV-B5; lane 5: EV-30; lane 6: PV; lane 7: HAV; lane 8: negative control

Other partial HEV strains which can be identified by this set of universal primers are shown in Table 1.

Optimization of LAMP

To determine the optimal reaction conditions including reaction temperature and length, concentrations of Mg^{2+} and betaine, LAMP was performed with a standard plasmid template (p5'UTR-EV71). The reaction temperature was optimized by incubating the mixture at 60, 61, 62, 63, 64, or 65 °C for a predetermined time (60 min) and the most obvious ladder-like pattern bands were detected at 61 °C. We then chose 61 °C as the optimal reaction temperature. The LAMP product was detected as early as 45 min at 61 °C, but the reaction time was still maintained for 60–90 min to ensure positive detection of lower concentration of template in the system. The optimal concentrations of Mg^{2+} and betaine were 2.0 mM and 0.8 M, respectively (Fig. 2), determined by evaluating different concentrations of MgCl_2 (0–5.0 mM) and betaine (0–1.2 M) in the reaction mixture.

Specificity of the LAMP Assay

The specificity of the method was evaluated by testing the assay on a battery of HEVs (i.e., CV-A16, CV-B3, CV-B5, EV71, EV-30, and PV). A related enteric virus, HAV, which is a member of *Hepatovirus* was included as well. CV-A16, CV-B3, CV-B5, EV71, and EV-30, were grouped into HEV-A or HEV-B, tested positive in the LAMP assay. The assay had little sensitivity for this serotype of PV. No DNA band was observed from HAV. By agarose gel electrophoresis a characteristic ladder of multiple bands was observed (Fig. 3). The specificity test indicated that the LAMP assay is specific for the detection of enteroviruses, such as HEV-A and HEV-B.

Sensitivity of the LAMP Assay

The sensitivity of the method was performed under optimal conditions at 61 °C for 90 min using the standard plasmid p5'UTR-EV71 as a template. As shown by gel electrophoresis (Fig. 4a), ladder-like DNA fragments were detected when different amounts of template were used, ranging from 10^6 to 10^1 copies μl^{-1} . But no signal was obtained at 10^0 copies μl^{-1} . The same observations were made by visual inspection (Fig. 5a) and under UV light transillumination (Fig. 5b). After the addition of SYBR Green I the solution changed color. In this way, the detection limit of the LAMP assay was 10^1 copies μl^{-1} . In contrast, the target band obtained by conventional PCR using 10^1 copies μl^{-1} templates was almost invisible (Fig. 4b). The LAMP assay showed more sensitivity than PCR in detection of HEVs.

Evaluation of Enteroviruses LAMP Assay with Drinking Water Sources and Stool Samples

All water samples (100 %) and 18 of the 19 stool specimens (94.7 %) tested positive for amplification, showing a

Fig. 4 Comparison of sensitivity between the LAMP reaction and PCR reaction for detection of the HEVs. Both **a** LAMP and **b** PCR reactions were carried out with tenfold serial dilutions of cloned EV71 plasmid (copies/ μ l). Lane **M**:DNA marker; lane 1: 10^6 , lane 2: 10^5 , lane 3: 10^4 , lane 4: 10^3 , lane 5: 10^2 , lane 6: 10^1 , lane 7: 10^0 copies/ μ l, respectively; lane 8:negative control

Fig. 5 Detection of LAMP products by observing color changes after addition of SYBR Green I. **a** shows color judged by naked eyes; **b** shows fluorescence visualized under UV light transillumination. 1–7, reaction was carried out with tenfold serial dilutions of cloned EV71 plasmid (copies/ μ l). Lane 1: 10^6 , lane 2: 10^5 , lane 3: 10^4 , lane 4: 10^3 , lane 5: 10^2 , lane 6: 10^1 , lane 7: 10^0 copies/ μ l, respectively; lane 8:negative control

specific ladder-like pattern, while the Milli-Q water tested negative.

The specificity of the amplification was confirmed by restriction endonuclease digestion with *Nla*IV. The obtained fragments were mainly about 87 and 103 bp in size, like the products obtained with the standard template p5'UTR-EV71 (data not show). Ten products were sequenced. The sequences obtained exhibited high homology (96–100 %) with those 5'-UTRs of the enteroviral genomes in the GenBank (Table 2). Two products were assigned to coxsackievirus A16, and 8 products were assigned to enterovirus 71.

Discussion

A wide range of clinical outcomes have been recognized as associated with enteroviruses. In most cases of enterovirus disease, an aetiological diagnosis based on clinical symptoms only is impossible and members of several other virus families have also to be considered in addition to the almost 70 different enteroviruses [13]. Therefore, it is

crucial to have universal enteroviruses detection methods that could be most useful in cutting time of screening tests, thereby improving the early warning system for future epidemics. Moreover, universal detection is also a useful tool in the diagnosis of disease caused by co-circulation viruses. For example, the co-circulation of EV71 and CV-A16 may be responsible for the HFMD epidemics seen in Asia in recent years. Besides the clinical samples, enteroviruses have been isolated from different kinds of aquatic environments such as sewage, seas, rivers, streams, ground water and even drinking water [4]. And enteroviral epidemics are predominantly waterborne [15]. Development of methods for universal detection of enteroviruses could be also helpful in reducing the risk of waterborne epidemic outbreak.

PCR assays are the most popular molecular methods and have been developed extensively for detection of enteroviruses, both in clinical and environmental trials [6–8]. But two obvious disadvantages of PCR methods, machine-dependence and the need for skilled technicians, limit its application in communities, rural clinics and field

Table 2 Sequence homology of the LAMP products amplified from environmental and clinical samples

Type of sample	BLAST identification		
	Max identity (%)	Serotype	GenBank accession No.
Water	100	EV71	HQ456308.1
Water	100	EV71	HQ456308.1
Water	97	EV71	HQ456308.1
Stool	100	EV71	JF894383.1
Stool	99	EV71	JF894383.1
Stool	99	EV71	HQ456308.1
Stool	99	EV71	HQ456308.1
Stool	98	CV-A16	JX986741.1
Stool	96	EV71	KC109780.1
Stool	96	CV-A16	KC117317.1

situations. In comparison with PCR, LAMP is a more promising method in diagnosis because of its simplicity, rapidity, specificity, and cost-effectiveness [9].

In the present study, we developed a universal LAMP assay to detect HEVs encoding 5'-UTR as a suitable target. The design of universal primers set should carry out to cover every known polymorphism in the genomes of viral subtypes. Unfortunately, the 5'-UTR of enteroviruses is classified into two major groups based on its primary structure, PV-like and CBV-like 5'-UTR. It was difficult to design one set of primers having the same high sensitivity for detection of all the enteroviruses [16, 17]. A RT-LAMP system for rapid and highly sensitive detection of HEV-C strains including PV, directly from stool samples of acute flaccid paralysis patients have been reported [18]. But this RT-LAMP system showed less sensitive to the prototype strains of HEV-A and HEV-B that might due to the designed specificity of RT-LAMP just for PV-like 5'UTR. During the process of our study, we have to face the same problem. We screened a set of universal LAMP primers perfectly matching the 5'-UTRs of most of the prevalent HEVs genotypes listed in NCBI, particularly HEV-A and HEV-B.

No cross-reaction was observed in the universal HEVs LAMP assay with related enteric viruses, such as HAV. And the detection limit is 10^1 copies of standard plasmid per reaction mixture, which is more sensitive than the traditional PCR assay and almost equal to the other LAMP system for enterovirus detection described in previous studies [11, 19]. In order to evaluate the reliability of the method, 9 water samples collected from drinking water sources and 19 fecal samples of HFMD patients were detected. As a result, the LAMP assay demonstrated higher sensitivity by correctly picking up 9 and 18 positive samples, respectively. And the specificity of the method was

not affected by the complexity of the RNA in the samples. Furthermore, some stool specimens could be detected as early as 10–15 min, and most of stool specimens could be detected in less than 45 min.

In addition to the accuracy and the speed of detection, the only equipment needed for the LAMP reaction is a regular laboratory water bath or a heat block. We also found that the LAMP-positive amplification could be identified easily by the white precipitate of magnesium pyrophosphate and the color changing from orange to green after adding SYBR Green I. Another great advantage of the LAMP assay is that the reaction is tolerant of substances contained in environmental and clinical samples, which might because the inhibitors have little impact on *Bst* DNA polymerase [20]. This property facilitates the application in field tests.

All these advantages would make such a diagnostic tool very useful in the early determination of HEVs. We believe that this LAMP assay will be suitable for the monitoring of enteroviruses and forewarning people the existence of this hazard.

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