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A novel method of real-time reverse-transcription loop-mediated isothermal amplification developed for rapid and quantitative detection of human astrovirus

Haiyan Wei[a], Jing Zeng[a,*], Congliang Deng[a], Chengzhong Zheng[b], Ximeng Zhang[a], Dan Ma[a], Yong Yi[b]

[a] Food Safety Testing Center, Beijing Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China, Beijing 100026, China
[b] The 306th Hospital of PLA, Beijing 100101, China

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A B S T R A C T

A one-step, real-time reverse-transcription loop-mediated isothermal amplification (rRT-LAMP) method targeting the 5′ end of the capsid gene for rapid and quantitative detection of human astrovirus serotype 1 (HAstV 1) was developed. The assay is highly sensitive and comparable to real-time RT-PCR (rRT-PCR), with a detection limit of ~100 RNA copies per assay. The specificity of the method was validated by the absence of any cross-reaction with RNA samples of HAstV 2–8 and other gastroenteritis viruses, followed by nucleotide sequencing of the amplified product. Fecal specimens ($n = 120$) obtained from children under five years of age with gastroenteritis were tested by rRT-LAMP, rRT-PCR and transmission electron microscopy (TEM). Six (5%) of these samples were determined to be positive by both rRT-LAMP and rRT-PCR assay, and these two nucleic acid amplification methods resulted in a 200% increase in detection rates for HAstV infection compared with TEM alone. Furthermore, the rRT-LAMP assay is much more rapid than rRT-PCR and generates results in less than 20 min for positive samples. The quantitation of viral load in stool specimens was determined from the standard curve plot of time-of-positivity *versus* initial RNA concentration. Most viral loads were determined to be within the range of 10^5–10^8 copies. The results highlight the significance of the rapid rRT-LAMP method as a diagnostic and routine screening tool for the analysis of stool samples in hospital laboratories.

1. Introduction

Human astrovirus (HAstV) is a non-enveloped, small, round-structured, single-strand positive RNA virus (Morsy El-Senousy et al., 2007). It is a common cause of gastroenteritis primarily in young children under five years of age (Herrmann et al., 2001; Utagawa et al., 1994). The prevalence of HAstV ranges from 2 to 16% in children hospitalized with diarrhea and 5 to 17% in children with diarrhea as determined in community studies (Jeong et al., 2012). HAstV is classified into eight distinct antigenic serotypes, HAstV 1–8 (McIver et al., 2000), with serotype 1 predominating in most countries (De Grazia et al., 2004; Guix et al., 2002).

Transmission electron microscopy (TEM) has been the traditional diagnostic method used since the discovery of the virus in 1975 (Madeley and Cosgrove, 1975). However, the method is labor intensive and relatively insensitive, requiring up to 10^6 intact virus particles per ml in a stool sample (Madeley and Cosgrove, 1975). Molecular techniques for viral detection and evaluation, such as reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR (rRT-PCR) have provided new information regarding HAstV prevalence (Chapron et al., 2000; Dai et al., 2010; Grimm et al., 2004; Le Cann et al., 2004; Logan et al., 2007; Morsy El-Senousy et al., 2007; Zhang et al., 2006). However, these methods have the intrinsic disadvantage of requiring either a high-precision instrument and expensive reagents for amplification or a complex and elaborate method for the detection of amplified products. In addition, adaptation of these methods for routine clinical use is often cumbersome, especially in resource-limited situations. It is therefore critical to develop simple and economical molecular tests with high sensitivity and specificity for the rapid and accurate diagnosis of HAstV infection.

In this regard, the loop-mediated isothermal amplification (LAMP) developed by Notomi et al. in 2000 is advantageous due to its simple operation, rapid reaction, and easy detection. This technique relies on strand-displacing DNA synthesis performed using the Bst DNA polymerase large fragment under isothermal conditions, thereby obviating the need for a thermal cycler. The technique is highly specific for the target sequences, because at least six primer region sequences are required for recognition. Moreover, the LAMP method generates an increase in turbidity within positive samples, allowing detection by the naked-eye and

* Corresponding author at: Food Safety Testing Center, Beijing Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China, Tian Shui Yuan Street, No.6, Chao Yang District, 100026 Beijing, China. Tel.: +86 010 5861 9245; fax: +86 010 6585 3805.
E-mail address: zengj@bjciq.gov.cn (J. Zeng).

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by real-time monitoring of the turbidity of the reaction mixture. The method has also been rendered applicable to RNA genomes by combining it with reverse transcription reactions (RT-LAMP). The RT-LAMP assay has been applied successfully for the detection of many human pathogenic RNA viruses (Parida et al., 2004; Peyrefitte et al., 2008; Shirato et al., 2007; Suzuki et al., 2011; Lee et al., 2011), resulting in rapid, simple, and cost effective diagnostic measures.

In this study, a one-step, accelerated, real-time RT-LAMP (rRT-LAMP) assay was developed by targeting the 5′ end of the capsid gene for rapid and quantitative detection of HAstV 1. Data pertaining to the sensitivity and specificity of the method were discussed. The applicability of the technology for clinical diagnosis of HAstV infection was validated by testing 120 stool samples of children under five years of age with gastroenteritis. All the stool samples were collected from Beijing, China between 2008 and 2010.

2. Materials and methods

2.1. Human fecal samples

A total of 131 stool specimens were used in this study. Among them 120 samples were collected from the 306th Hospital of PLA and came from children under the age of five years with a clinical diagnosis of gastroenteritis. Pior to rRT-LAMP assays, all 120 samples were also investigated for the presence of HAstV particles by TEM. Another 10 stool samples were obtained from Beijing Children Hospital, identified previously to be HAstV-free, but individually positive for GI type norovirus (NV-GI) ($n = 1$), GII type norovirus (NV-GII) ($n = 4$), hepatitis A virus (HAV) ($n = 1$) or group A rotaviruses (HRV) ($n = 4$). A single fecal specimen derived from a child with gastroenteritis, which was tested previously to be positive for HAstV by RT-PCR, rRT-PCR, and TEM, was used as a positive control. All the stool samples were collected from the year 2008 to 2010.

Our experimental design and protocols adhered to the tenets of the Declaration of Helsinki and were approved by the institutional ethics committee of the 306th Hospital of PLA ([2011] Ethical Review [no.005]). All fecal samples were collected after written informed consent was obtained from each subject. All patients have the right to request the results of the tests conducted on their samples.

2.2. RNA extraction

The genomic viral RNA was extracted from 140 μl of patient stool sample by using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The RNA was eluted from the QIAspin columns in a final volume of 50 μl of elution buffer and was stored at −70 °C until use.

2.3. Design of astrovirus specific RT-LAMP assay primers

RT-LAMP primers were selected on the basis of a highly conserved region in the 5′ end of the capsid gene (orf2). Potential target sequences available in the GeneBank database (Fig. 1) were aligned using DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada). The maximum number of mismatched nucleotides between HAstV 1 and the other HAstV serotypes was incorporated to avoid cross-reactive amplification (Fig. 1). The published sequence of the Pune/063681/India strain (GenBank accession number JF327666) was used for the primer template. A set of six primers, comprising two outer (F3 and B3), two inner (FIP and BIP), and two loop (LF and LB) primers (where F indicates forward and B indicates backward) that recognize eight distinct regions on the target sequence, were designed using LAMP primer software PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html; Eiken Chemical, Tochigi, Japan), as described previously by Notomi et al. (2000). The sequences of RT-LAMP primers used for the amplification were shown in Table 1.

Table 1
Primers used for rRT-LAMP to detect the capsid gene of HAstV 1.

Primer name	Sequence (5′-3′)
Ast-F3	GCAGGTAACTGTTGAGGTCA
Ast-B3	GGTTTTGGTCCTGTGACACC
Ast-FIP (F1C + F2)	CTGCTCTGTCCCGCCCTCTAATGGCCGCAACAGGAGTA
Ast-BIP (B1C + B2)	AGGACTAGAAGACAGCCCGGATGACAATGTTACGGACACGT
Ast-LF	TTGTGAGCGGGCCCTTG
Ast-LB	CGCGGCAAACATCAATCTTCTCA

2.4. rRT-LAMP assay

RT-LAMP was carried out in a final reaction volume of 25 μl using a Loopamp RNA amplification kit (Eiken Chemical, Tochigi, Japan) with 5 pmol (each) of the primers F3 and B3, 40 pmol (each) of the primers FIP and BIP, and 20 pmol (each) of the primers LF and LB, 12.5 μl of reaction mix, 1.0 μl of an enzyme mixture consisting of 16 U of Bst DNA polymerase and 2 U of avian myeloblatosis virus (AMV) reverse transcriptase, and 5 μl of extracted RNA. For real-time monitoring, the RT-LAMP reactions were incubated at 63 °C for 60 min with a LA-200 Loopamp realtime turbidimeter (Teramecs, Kyoto, Japan) and inactivated at 80 °C for 5 min. The accumulation of magnesium pyrophosphate was monitored spectrophotometrically every 6 s at 400 nm. Tp values (time of positivity, *i.e.*, time [in minutes] at which the turbidity increases above the threshold value fixed at 0.1) for positive samples were also determined. Results were analyzed using the LA-200E software package (Teramecs, Kyoto, Japan). Positive and negative controls were included in each run of the assay.

2.5. rRT-PCR assay

A single-step HAstV rRT-PCR was performed with primers and probes designed to target the 5′ end of the capsid gene (orf2) of the serotype 1 Oxford strain described by Grimm et al. (2004). The reaction was carried out using the SuperScript III platinum one-step quantitative RT-PCR system with ROX (Invitrogen, Carlsbad, CA, USA). Reactions (25 μl) contained 0.4 μM (each) of the primers (AstU1, AstU2, AstU3, AstU4, AstL1, AstL2), 0.2 μM (each) of TaqMan® probes (AstP1, AstP2, AstP3, AstP4), 5 μl of RNA, as well as buffer and enzymes at concentrations recommended by the manufacturer. The rRT-PCR was performed on an ABI 7900 sequence detector (Applied Biosystems, Foster City, CA, USA) using the following thermal cycling conditions: 15 min at 50 °C, 2 min at 95 °C, and 40 cycles of 15 s at 95 °C and 30 s at 60 °C.

2.6. Astrovirus RNA standard construction

To obtain a quantitative RNA standard, the 218-bp PCR product was amplified by RT-PCR using AstU1 and AstL1 primers with HAstV RNA prepared from the positive control fecal sample and transcribed *in vitro*. The target PCR products were cloned into a vector (pCDNAII, Invitrogen, Carlsbad, CA, USA) that contained the T7 polymerase promoter. Plasmid DNA that contained the 218 bp insert was linearized with BamHI, purified using a Wizard DNA Clean Up system (Promega, Madison, WI, USA), and then *in vitro*-transcribed and DNase-digested using a T7 RiboMAX express large scale RNA production system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The RNA was purified according to the instructions of the RNeasy Protect kit (QIAGEN, Hilden, Germany). The quality of the RNA was evaluated by agarose gel

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