Journal of Virological Methods 200 (2014) 29–34

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of a high resolution melting analysis for detection and differentiation of human astroviruses

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Article history:
Received 14 October 2013
Received in revised form 16 January 2014
Accepted 21 January 2014
Available online 6 February 2014

Keywords:
HRM
Human astroviruses
Phylogenetic analysis
RT-PCR

A B S T R A C T

Human astroviruses (AstVs), the common causes of viral gastroenteritis, consist of 8 different sero- or genotypes in which a variety of subtypes have been found. In the present study, a rapid and high-throughput method for detection and sequence-discrimination of AstVs by high resolution melting (HRM) analysis was developed. A newly designed primer set for the assay targeting ORF1b–ORF2 junction region of AstVs successfully reacted with all 8 serotypes of AstVs and allowed genotyping using their amplicons. The HRM assay consists of intercalating dye based real time quantitative PCR (qPCR) and melting curve analysis. The qPCR assay was sensitive enough to detect 1.0×10^1 copies/reaction of AstV serotypes. However, 1.0×10^3 copies/reaction of AstVs gene was required to obtain a sequence-specific difference curve, indicating that pre-amplification is necessary to apply the assay to samples containing low numbers of AstVs. AstVs in clinical specimens were subjected to the HRM assay after pre-amplification. The strains possessing same nucleotide sequences at the target region showed an identical difference curve and those possessing different nucleotide sequences showed a distinguishable difference curve. The newly developed HRM assay is an effective technique for screening of AstVs to quantify and discriminate the strains.

1. Introduction

Human astroviruses (AstVs), which belong to the family *Astroviridae*, are known as the common cause of infantile viral gastroenteritis worldwide (Mustafa et al., 2000; Nadan et al., 2003; Malasao et al., 2008; Guo et al., 2010; Verma et al., 2010). AstVs are excreted in feces by infected individuals (up to 10^{15} copies g stool^{-1}) (Zhang et al., 2006) and transmit via the fecal-oral route (Schwab, 2007). AstVs are small (28 nm in diameter), icosahedral shaped and non-enveloped viruses containing a 6.8 kb single stranded positive-sense RNA. Their viral genome consists of open reading frame (ORF) 1a, ORF1b and ORF2 encoding serine protease, RNA-dependent RNA polymerase and viral capsid, respectively (Madeley and Cosgrove, 1975; Matsui and Greenberg, 2001; Guix et al., 2005). Currently, AstVs are divided into 8 genetically and antigenically distinct types (AstV-1 to -8). Genotyping surveys have shown that AstV-1 is the most common type, followed by AstV-2, -3, -4 and -5, whereas AstV-6, -7, and -8 have been detected more rarely (Wang et al., 2001; Gabbay et al., 2007; De Grazia et al., 2011, 2013). It is known that genotype of AstVs correlates well with serotype (Noel et al., 1995) and currently, genotyping is applied more commonly than serotyping for characterization of AstV strains. Sequence analysis of short fragments at either the 5′- or 3′-end of ORF2 and RT-PCR genotyping protocols with type-specific primers have been used for genetic characterization of AstV-1 to -8 (Noel et al., 1995; Guix et al., 2005).

High resolution melting (HRM) analysis, an intercalating dye-based real-time quantitative PCR (qPCR) coupled with melting curve analysis, is a tool for screening nucleotide substitutions. A previous study demonstrated that the HRM assay is sensitive enough to detect single-nucleotide substitution (Lee et al., 2011). Compared to other techniques for molecular characterization such as Sanger sequencing and pyrosequencing, HRM is superior in respect to rapidity and simplicity (Lee et al., 2011). Several HRM assays have been developed successfully and applied for detection and genotyping of viruses such as polyomaviruses (Dumonceaux et al., 2008; Matsuda et al., 2011), noroviruses (Tajiri-Utagawa et al., 2009; Hara et al., 2010), influenza A viruses (Lin et al., 2008; Curd et al., 2011; Lee et al., 2011), and fowl adenovirus (Marek et al., 2010). However, HRM assay for AstVs has not been reported yet.

In the present study, we aimed to develop an HRM assay that would allow simultaneous detection and differentiation of AstVs.

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http://dx.doi.org/10.1016/j.jviromet.2014.01.023

Table 1
Primers used in this study.

Application	Primer	Sequence(5′–3′)[a]	Polarity	Location[b]
Pre-amplification	AHAstVF1	AATCACTCCATGGGAAGCTCCT	+	4139–4160
	AHAstVR1	CCTARCGCYTGCACDGG	–	4697–4713
HRM	AHAstVF2	CAGAAGAGCAACTCCATCGCAT	+	4280–4301
	AHAstVR2	GTRCTYCCWGTAGCRTCCTTAAC	–	4664–4686

[a] Mix bases in degenerated primers and probes are as follows: R stands for A or G; Y stands for C or T; D stands A, G or T; W stands for A or T.
[b] Corresponding nucleotide position of AstV-1 strain Oxford (accession number: L23513).

2. Materials and methods

2.1. AstVs and clinical specimens

Each serotype of isolated AstV strain was obtained from Centers for Disease Control and Prevention (CDC). The strains were propagated in CaCo-2 cell line. A total of 13 clinical specimens (F1–F13) were collected from gastroenteritis patients aged under 5 years diagnosed with diarrhea by pediatric clinics in Japan. Electron microscopy showed all the specimens were positive for AstVs. The AstV isolates and stool specimens were kept at −80 °C until use.

2.2. Nucleotide sequence alignment of AstVs

A total of 281 nucleotide sequences of AstVs (AstV-1: 17 sequences; -2: 67 sequences; -3: 43 sequences; -4: 63 sequences; -5: 24 sequences; -6: 14 sequences; -7: 8 sequences; -8: 45 sequences) taken from GenBank database were aligned with Clustal W version 1.83 software (http://clustalw.ddbj.nig.ac.jp/top-j.html). The accession numbers for the sequences used in the present study are listed in the supplemental material. Based on the obtained alignment, two sets of primer-pairs to amplify 575 bp and 407 bp fragments of ORF1b–ORF2 junction region of all types of AstVs were designed (Table 1).

2.3. RNA extraction and reverse-transcription (RT)

Viral RNA in 140 μL of the 10% stool suspension was extracted using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) to obtain a 60 μL of RNA extract, following the manufacture's protocol. The RNA extract was subjected to RT reaction using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan). Briefly, RNA (15 μL) was added to 15 μL of RT mixture containing RT buffer and 75 U of MultiScribe reverse transcriptase (Applied Biosystems). The RT reaction mixture was incubated at 25 °C for 10 min, then at 37 °C for 120 min, and then at 85 °C for 5 min to inactivate the enzyme.

2.4. Construction of the plasmid DNAs

To construct standard plasmid DNAs for each AstV type, 575 nucleotides (nts) encoding ORF1b–ORF2 junction of each type of the isolated AstV strains were amplified by PCR with KOD -Plus- Ver.2 DNA polymerase (TOYOBO, Osaka, Japan) and the primers for pre-amplification (AHAstVF1 and AHAstVR1) (Table 1). A 50 μL of PCR mixture containing 400 nM each of forward and reverse primer, 5 μL of 10× Buffer for KOD -Plus- Ver.2, 200 nM of dNTPs, 1.5 mM of $MgSO_4$, 1.0 U of KOD -Plus- and 5 μL of template cDNA was subjected to a reaction consists of initial denaturation of 2 min at 94 °C, 30 cycles of amplification of 10 s at 98 °C, 30 s at 54 °C and 30 s at 68 °C and final extension of 7 min at 68 °C. The PCR products were subjected to electrophoresis using 2% agarose gel and those of expected sizes were excised with a QIAquick Gel Extraction kit (Qiagen). The excised PCR products were cloned into a Zero Blunt TOPO pCR 2.1 vector (Invitrogen, Carlsbad, CA) and the plasmid was transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen), according to the manufacturer's protocol. Plasmid DNA was extracted from the transformed competent cells using a QIAprep spin mini prep kit (Qiagen). The concentration of the purified plasmid DNA was determined by measuring the optical density at 260 nm with NanoDrop ND-1000 instrument (LMS, Tokyo, Japan).

2.5. HRM assay

HRM assay, which consists of PCR amplification and melting curve acquisition and analysis, was performed on the LightCycler 480 instrument (Roche Diagnostics, Tokyo, Japan). Prior to applying the HRM assay to clinical specimens, pre-amplification was performed with KOD -Plus- Ver.2 DNA polymerase under the PCR condition described in Section 2.4. A 20 μL of mixture containing 10 μL of 2× LightCycler 480 HRM master mix (Roche Diagnostics), 2.5 mM of $MgCl_2$, 200 nM each of the forward and reverse primers for the HRM assay (AHAstVF2 and AHAstVR2) (Table 1) and 2 μL of PCR product or plasmid DNA was subjected to the reaction. The qPCR was performed as follows: initial denaturation at 95 °C for 10 min to activate DNA polymerase, 45 cycles of amplification with denaturation at 95 °C for 10 s and annealing at 62 °C to 58 °C in 0.5 °C cycle^{-1} increments for 10 s, and extension at 72 °C for 15 s, and 1 cycle of 95 °C for 60 s and 40 °C for 60 s. Subsequently, the high-resolution melting curve analysis was performed by raising the temperature from 60 °C to 97 °C, with an increment of 0.11 °C s^{-1} to obtain the melting curves. The melting-curve data was analyzed with the LightCycler 480 gene-scanning software module (Roche) to obtain the difference curves. Discrepancies among the difference curves of the samples were analyzed by the software.

2.6. Sequencing and phylogenetic analysis

The PCR products obtained from clinical specimens using pre-amplification primers with KOD -Plus- Ver.2 and plasmid DNAs were sequenced with the BigDye cycle sequencing kit, version 3.1, and the 3130 genetic analyzer (Applied Biosystems). The pre-amplification primers and the universal M13 primers were used for sequencing reaction for the PCR products and plasmid DNAs, respectively. Nucleotide sequences were assembled using the program Sequencher version 4.2.2 (Gene Codes Corporation, Ann Arbor, MI) and aligned with Clustal W version 1.83 with previously assigned sequences of AstV-1 (GenBank accession number: L23513), -2 (GenBank accession number: L13745), -3 (AF141381), -4 (DQ344027), -5 (DQ28633), -6 (GQ495608), -7 (AF248738) and -8 (AF292073) as referential AstV sequences. The distances were calculated using Kimura's two-parameter method (Kimura, 1980), and phylogenetic dendrogram from a bootstrap analysis with 1000 replicates were constructed by the neighbor-joining method.