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ORIGINAL ARTICLE

Development of a reverse transcription recombinase-aided amplification assay for the detection of coxsackievirus A10 and coxsackievirus A6 RNA

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Abstract
Hand, foot and mouth disease (HFMD) is a serious public health problem, and coxsackievirus A6 (CVA6) and coxsackievirus A10 (CVA10) are two of the major causative pathogens, in addition to enterovirus 71 (EV71) and coxsackievirus A16 (CVA16). A simple and rapid reverse transcription recombinase-aided amplification assay (RT-RAA) was developed for the detection of CVA10 and CVA6 in this study. The analytical sensitivity for detection of CVA10 and CVA6 at 95% probability by probit regression analysis was 35 copies per reaction and 38 copies per reaction, respectively, with 100% specificity. Compared with commercial RT-qPCR assays, when testing 455 fecal specimens, the kappa value of the RT-RAA assay for CVA10 and CVA6 was 0.920 ($p < 0.001$) and 0.952 ($p < 0.001$), respectively. Moreover, four samples that were positive for CVA10 and five that were positive for CVA6 by RT-RAA but negative by RT-qPCR were further determined to be true positives. These results demonstrate that the proposed RT-RAA assays are very valuable tools for the detection of CVA10 and CVA6 and have potential for use in resource-limited settings.

Introduction

Hand, foot and mouth disease (HFMD) is a common infectious disease that often affects children under five years of age. In most cases, the disease clears up of its own accord one week after onset, but complications, usually characterized by fever and small sores and/or ulcers on the hands and feet and in the mouth, and even death, have occurred in a few cases [1, 2]. Since its first discovery in 1957, HFMD has been a major health problem and has caught the attention of the whole world [3–5]. Serious HFMD epidemics also occur in mainland China, for example, there were 488,955 cases of HFMD in 2008, including 126 fatal cases [6].

Human enteroviruses (HEVs) consist of four groups, A, B, C and D [7, 8], and are the aetiological agents involved in

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most cases of HFMD. Enterovirus 71 (EV71) and coxsackievirus A16 (CVA 16) belonging to HEVA are responsible for most HFMD cases worldwide [9, 10]. As other enteroviruses are gradually emerging, CVA6 and CVA10 are becoming increasingly prevalent causes of HFMD. In 2008, the proportion of HFMD in Singapore caused by CVA10 and CVA6 was 35.5% [11]. Studies in France, Finland, Spain and China have indicated that CVA10 and CVA6 have become the main pathogens causing HFMD in these areas, and measures must be taken to resolve this problem [12–15]. A powerful method to detect CVA10 and CVA6 is therefore in high demand.

Traditional detection methods for HEVs depend on cell culture [16] and neutralization tests [17, 18], but these methods call for complex procedures and are time-consuming and burdensome. Molecular diagnostics include a variety of PCR assays and isothermal nucleic acid amplification assays. RT-PCR is a well-developed method for CVA10 and CVA6 detection [19, 20], but it requires expensive instruments and professional operators. Loop-mediated isothermal amplification (LAMP) is very popular, but this method requires four or more primers, and primer design is complex [21, 22].

Recombinase-aided amplification (RAA) is a new isothermal amplification technology that does not require a classical thermostable enzyme. In the reaction, the recombinase UvsX, which is usually obtained from *Escherichia coli*, and single strand DNA-binding protein (SSB) are used in a polymerase chain reaction (PCR) to form single-stranded DNA without the need for heating. DNA polymerase Klenow fragment (3'→5' exo$^-$) is then used to carry out elongation. RAA is fast and simple and can be completed at about 39 °C in 30 min. RAA has already been successfully applied by other research groups to detect bacterial pathogens [23, 24] and in our laboratory to detect viral pathogens [25].

In this study, we attempt to develop RT-RAA assays for the detection of CVA10 and CVA6. The analytical specificity and sensitivity of the assays were evaluated. Clinical samples were also tested, and the results were compared with those obtained using commercial RT-qPCR assays.

Materials and methods

Clinical samples

A total of 455 clinical samples from clinically diagnosed HFMD patients were collected between 2016 and 2017 from hospitals in Hebei, Shandong and Hunan. The ages of the patients ranged from 7 months to 8 years, and 94.51% (430/455) were under 5 years old. Of those, 44.84% (204/455) were female and 55.16% (251/455) were male.

The study was approved by the Institutional Review Boards of the National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China. Caregivers of the children were informed, and informed-consent documents were signed.

Reference strains

Field isolates of human enteroviruses known to be associated with HFMD were obtained from the National Laboratory for Poliomyelitis, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, and stored in our laboratory [26, 27]. These isolates were used as control viruses to evaluate the specificity of the RT-RAA assay for CVA10 and CVA6 and were verified by pan-enterovirus nested RT-PCR, followed by sequencing of PCR products [28]. The control viruses included EV71, coxsackievirus group A serotypes (CVA2, 3, 4, 5, 9, 12, 14, 16, 24), coxsackievirus group B serotypes (CVB1, 2, 3, 4, 5) and Echoviruses (E3, 6, 9, 11, 18, 25, 30).

RNA extraction

Viral RNA was extracted using a Viral RNA/DNA Isolation Kit (Tianlong, Suzhou, China) according to the manufacturer's instructions. Purified RNA was eluted in 70 µl of elution buffer and was stored at -80 °C until used.

Construction of recombinant plasmids

Recombinant plasmids containing a 994-bp fragment of CVA10 (nt 2375–3368, GenBank accession no. KY272010) and a 990-bp fragment of CVA6 (nt 2321–3310, GenBank accession no. KY126092) were prepared. The primers used to construct the CVA10 plasmid were adopted from a previous report [29], and the primers for CVA6 were designed in this study (Table 1). The RT-PCR products of CVA6 and CVA10 were each cloned into the pClone007 vector. A Mini Plasmid DNA Extraction Kit (Beijing ComWin Biotech Co., Ltd., China) was used for extraction of recombinant plasmids. The plasmid DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Dreieich, Germany). The DNA copy number was calculated using the following formula: DNA copy number (copy number / µL) $=\{[6.02 \times 10^{14} \times \text{plasmid concentration (ng/µL)} \times 10^{-9}]\}$ / [DNA in length × 660]. The extracted recombinant plasmids were verified by sequencing and stored at – 20 °C until used.

Primer and probe design

The VP1 sequence is widely chosen as the target region for the detection of HEVs [30, 31]. All the VP1 sequences available for CVA10 were obtained from the National Center for Biotechnology Information (NCBI) database. Conserved regions of CVA10 VP1 sequences identified using BioEdit

Table 1 Sequences of primers for construction of CVA6 and CVA10 plasmids

Primer	Sequence (5' to 3')	Genomic position	Product size (bp)
CVA10-F	ACTGATGAGGTGACGCAACA	2375-2394	994
CVA10-R	CCAGGTGCCTATTGACCACT	3349-3368	
CVA6-F	CGTAGTGCCACCAGATACCC	2321-2340	990
CVA6-R	GTGGTTATGCTTGAACGGTC	3291-3310	

Sequence Alignment Editor were used to design the primers and probe. Primer candidates were further validated using Primer-Blast. The primers and probe for the RT-RAA assay for CVA6 were from a reverse transcription recombinase polymerase amplification (RT-RPA) assay for detection of CVA6 [32].

Primers and probes for detection of CVA6 and CVA10 were synthesized by Sangon Biotech (Shanghai, China). Detailed information about the primers and probes for the RT-RAA assays for CVA10 (GenBank accession no. KY272010) and CVA6 (GenBank accession no. KY126092) is given in Table 2.

RT-RAA assay

The RT-RAA assay was performed in a 50-µL volume using an RT-RAA exo kit (Qitian, Jiangsu, China). The reaction mixtures contained 2 µl of RNA template, 25 µl of buffer, 15.7 µl of DNase-free water, 2.1 µl of primer F (10 µM), 2.1 µl of primer R (10 µM), 0.6 µl of probe (10 µM), and 2.5 µl of 280 mM magnesium acetate. The reaction mixture was added to a tube containing the RT-RAA enzyme mix (SSB, 800 ng/µl; UvsX, 120 ng/µl; DNA polymerase, 30 ng/µl) in a lyophilized form. The tube lids were carefully closed, and the tubes were mixed well. The tubes were then transferred to the detection equipment QT-F7200-0001 (Qitian, Jiangsu, China) at 39.0 °C for 30 min. Nuclease-free water was used as a negative control in every run.

RT-qPCR assay

Commercial detection kits for CVA6 and CVA10 RNA (PCR-Fluorescence Probing) were purchased from Daan Gene (Guangzhou, China). The detection kits contained specific primers and probes, Tris-HCl buffer, Hot Start Taq enzyme, c-MMLV RT enzyme, and dNTPs. The cycling profile was as follows: 50 °C for 15 min and 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s and annealing/ extension at 55 °C for 15 s. There was no difference in the RT-qPCR conditions for CVA10 and CVA6 except for the primers and probes. RT-qPCR reactions were performed according to the manufacturer's instructions using a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA).

Analytical specificity and sensitivity of RT-RAA

The specificity of the RT-RAA assays for CVA10 and CVA6 was evaluated by amplifying RNA extracted from virus samples containing EV71, coxsackievirus group A serotypes (CVA10, 6, 2, 3, 4, 5, 9, 12, 14, 16, 24), coxsackievirus group B serotypes (CVB1, 2, 3, 4, 5) and Echoviruses (E3,

Table 2 Sequences of primers and probes for RT-RAA assays for CVA6 and CVA10

Primer /probe	Sequence (5' to 3')	Genomic position	Product size (bp)
CVA10-F	GCCACAGATGAGAACATGATTGAGACCCGT TGTGT	2579-2613 2718-2751	173
CVA10-R			
CVA10-P[a]	AAGCCCATAATGTCTATGTCCCATGTRGCA TACC AAATGGRGTRTTGGAAACCACTATCAACCA C[FAM-DT] T[THF][BHQ-DT]TCTCCCGCT CTGGATTA[C3-spacer]	2623-2647	
CVA6-F	GGCTAGTAGGAGTTGTGGAGGTGAAGG ACTC	2693-2723	267
CVA6-R		2927-2959	
CVA6-P[a]	ACGGGTTGGTAGCAGTCTGCCATTGGTATG ATT CAACATACATGCGYTTTGATGCYG AGT[FAM-DT] C[THF]C[BHQ-DT]TTTGTG TCCMACCTCA[C3-spacer]	2804-2851	

[a]Probe modifications: FAM, 6-carboxyfluorescein; HEX, 5-hexachlorofluorescein; THF, tetrahydrofuran; BHQ, black hole quencher; C_3-spacer, 3′ phosphate blocker

6, 9, 11, 18, 25, 30). Water was used as a negative control in the assays.

To determine the sensitivity of the assay, the recombinant plasmids containing CVA6 and CVA10 sequences were serially diluted from 10^4 to 10 copies per reaction and subjected to RT-RAA detection. Eight replicates were performed for each dilution.

Evaluation of the RT-RAA assay using clinical samples

To evaluate the performance of the RT-RAA assays, 455 clinical samples were tested. The performance of the RT-RAA assays was compared to that of commercial RT-qPCR assays for CVA10 and CVA6.

Statistical analysis

A probit analysis for the detection limit of RT-RAA was done at a 95% probability level. The kappa and p values of RT-qPCR and RT-RAA were calculated. All statistical analysis was done using SPSS 21.0 (IBM, USA).

Results

Analytical specificity of RT-RAA

The RT-RAA assays for CVA10 or CVA6 were both positive for the corresponding virus strain. All negative controls and control virus strains were negative in the RT-RAA assays (data not shown). Thus the RT-RAA assays for detection of CVA6 and CVA10 demonstrated high specificity for their corresponding targets.

Analytical sensitivity of RT-RAA

The sensitivity of the RT-RAA assays for CVA6 and CVA10 was determined using a panel of serially diluted recombinant plasmids. All of the dilutions of recombinant plasmids of CVA10 and CVA6 from 10^4 to 10^2 copies per reaction were positive in the assays. Four of the eight replicates with 10 copies per reaction tested positive in the RT-RAA assay for CVA10, and three of the eight were positive for CVA6 (Table 3). The detection limits of the RT-RAA assay at 95% probability were 35 and 38 copies per reaction for CVA10 and CVA6, respectively (probit analysis, $p \leq 0.05$). T the sensitivity of the RT-RAA assay for CVA6 and CVA10 is shown in Fig. 1A and B, respectively.

Table 3 Assay data used for probit analysis to calculate the detection limit of RT-RPA for CVA10 and CVA6

Copies per reaction	No. of positive samples /no. of samples tested by the RT-RAA assays for detection of CVA10 and CVA6[a]	
	CVA10	CVA6
10^4	8/8	8/8
10^3	8/8	8/8
10^2	8/8	8/8
10^1	4/8	3/8

[a]Each dilution was tested in a total of 8 replicates

Evaluation of the RT-RAA assay using clinical samples and comparison with RT-qPCR

A total of 455 clinical samples were used for evaluation of the RT-RAA assays. Compared with the RT-qPCR results, the kappa value of the RT-RAA assay for CVA10 and CVa6 was 0.920 ($p < 0.001$) and 0.952 ($p < 0.001$), respectively. No significant differences between the detection results of RT-RPA and RT-qPCR were observed. Four samples that were positive for CVA10 and five samples that were positive for CVA6 by RT-RAA were negative by RT-qPCR. Two samples were positive for CVA10 by RT-qPCR but negative for RT-RAA. These 11 samples were further determined to be true positives by pan-enterovirus nested RT-PCR followed by sequencing of the PCR products [28]. A detailed comparison of the results of the RT-RAA assay and RT-qPCR is shown in Table 4.

Discussion

In this study, we estimated the sensitivity and specificity of the RT-RAA assays for CVA10 and CVA6 and tested their clinical performance in parallel with commercial RT-qPCR assays using clinical samples. Both RT-RAA assays were sensitive and specific. The analytical sensitivity of the assay for CVA10 and CVA6 was 35 and 38 copies per reaction, respectively, while the sensitivity of the commercial RT-qPCR assays for CVA10 and CVA6 is only 1000 copies per reaction according to the manufacturer. The results obtained with clinical samples containing CVA6 and CVA10 by RT-RAA and RT-qPCR showed no significant difference. However, nine samples were positive for CVA6 or CVA10 by RT-RAA but were negative by RT-qPCR, suggesting that the proposed RT-RAA assays are more sensitive than the corresponding RT-qPCR assays in our study. Two samples were

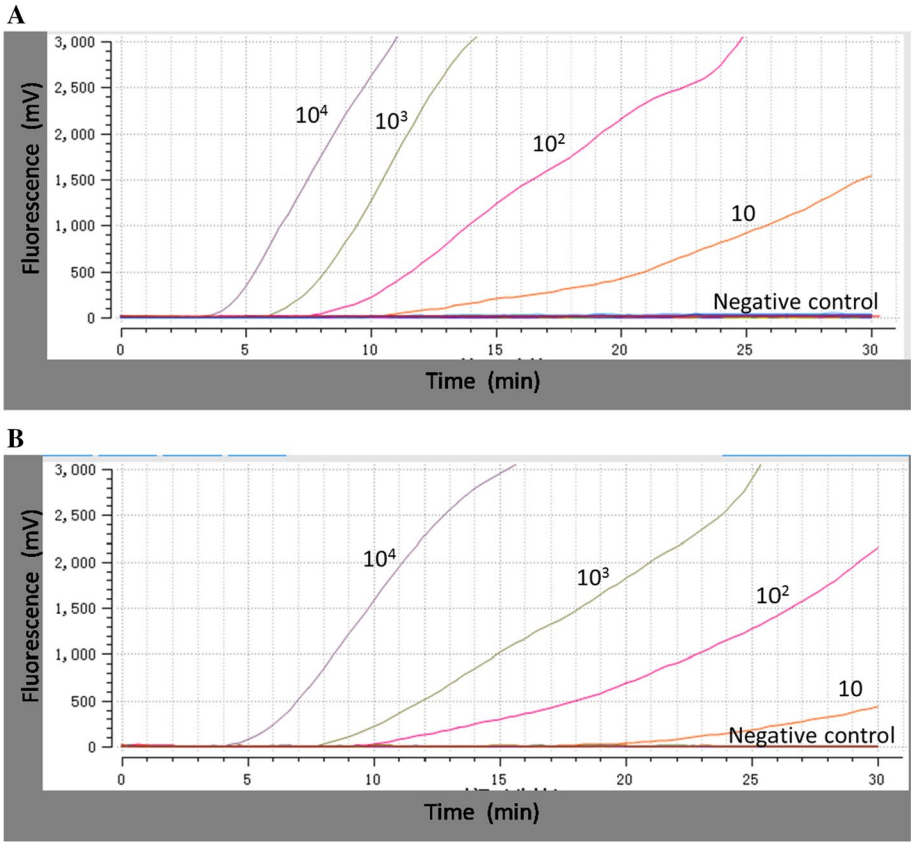

Fig. 1 Sensitivity of the RT-RAA assay for CVA10 and CVA6. A panel of serially diluted recombinant plasmids containing CVA10 and CVA6 sequences, from 10^4 to 10^1 copies per reaction, was used to determine the detection limit of the CVA10 (A) and CVA6 (B) RT-RAA assay, respectively

Table 4 Detection of CVA10 and CVA6 in clinical samples

Virus	RT-qPCR	RT-RAA		Total	Kappa	*P*-value of kappa
		Positive	Negative			
CVA10	Positive	38	2	40	0.920	0.000
	Negative	4	411	415		
	Total	42	413	455		
CVA6	Positive	58	0	58	0.952	0.000
	Negative	5	392	397		
	Total	63	392	455		

positive for CVA10 by RT-qPCR but negative by RT-RAA. The amount of extracted RNA from these two samples was insufficient to repeat the experiment. However, we assume that the false-negative results might be attributable to an operation error in the mixing or vortexing step (discussed in the following section).

In a study by Kaifeng Wang and colleagues [32], a rapid reverse transcription recombinase polymerase amplification (RT-RPA) assay was developed for detection of CVA6. The sensitivity of their assay was 202 copies per reaction, with 100% specificity, and the kappa value of the RT-RPA and RT-qPCR was 0.93 ($p < 0.001$). In this study, we established an RT-RAA assay for detection of CVA6 with the same primers and probe. The sensitivity was 38 copies per reaction, with 100% specificity, and the kappa value was 0.952 ($p < 0.001$). Both the sensitivity and the kappa value of the RT-TAA for CVA6 were superior to those of the RT-RPA. This might be due to a difference in the probit analysis to calculate the detection limit or the sample sources or RT-qPCR kit used in the evaluation of the RT-RAA or RT-RPA assay.

RAA is of great interest for molecular diagnostics, as it eliminates the need for thermocycling. In PCR, thermocycling is required to facilitate the separation of double-stranded DNA to enable amplification. The RT-RAA in this study could produce a positive signal in as little as 4 min and be completed in 30 min, while RT-qPCR requires 40 cycles and about 3 hours. The cost of the RT-RAA assay is half of that of RT-qPCR, and it can be performed in a portable device without any complicated process,

while the instrument for RT-qPCR is much more expensive than the one for RT-RAA. Moreover, the RT-RAA kit containing all enzymes in a lyophilized form is very convenient for preservation and transport. When compared to other isothermal amplification techniques such as nucleic acid sequence–based amplification (NASBA) [33], transcription-mediated amplification (TMA) [34], rolling-circle amplification (RCA) [35], loop-mediated isothermal amplification (LAMP) [21, 22], isothermal multiple self-matching-initiated amplification (IMSA) [36] and single-primer isothermal amplification (SPIA) [37], NASBA, TMA and RCA cannot be considered truly isothermal, as they require an initial heating step to denature the target nucleic acid prior to amplification. In addition, the primer design for RAA is simpler than LAMP, IMSA and SPIA. Therefore, RAA is more suitable for the detection of CVA10 and CVA6 and might be more conveniently integrated into point-of-care systems.

However, the RAA assay still has some limitations to overcome. Xie and colleagues have developed a multiplex real-time RT-PCR assay for simultaneous detection of coxsackievirus A6 and coxsackievirus A10 [38]. However, multiplex amplification of RAA is currently difficult because the primer for RAA requires more than 30 bp and the probe requires about 50 bp, which limits the development of a multiplex RAA assay. In addition, before being transferred to the detection equipment, the reaction mixtures in the tube must be mixed well. The mixing process has a great influence on the results. It would therefore be of great help if there were a specially designed instrument that could be used to vortex and briefly centrifuge samples in a standard manner.

The RT-RAA assay described here was demonstrated to be a sensitive and specific method for rapid detection of CVA10 and CVA6. In a future study, we will attempt to combine RAA with a lateral flow dipstick (LFD) assay for visual detection and with a gene chip for multiplex detection. As more effort is put into development and optimization of RAA assays, RAA will become a powerful and valuable tool for the detection of pathogens and will have the potential for use in resource-limited settings.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All operations in the experiments were approved by the Institutional Review Boards of the National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China. Caregivers of the children with HFMD were informed, and informed consent documents were signed.

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