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Three-step method for the amplification of the coxsackievirus A10 genome

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ABSTRACT. The whole-genome sequencing of coxsackievirus (CV)-A10 does not follow a conventional experimental protocol. To fully understand the genetic variation and evolution of CV-A10, complete genome amplification is necessary. Most previous studies have concentrated on partial sequences of the CV-A10 genome, such as the VP1 gene. The few studies that have investigated CV-A10 at the genomic level have reported only two complete genome sequences to GenBank. The basic fault may be attributed to the regional nature of the genetics and evolution of CV-A10 and to the lack of laboratory procedures for obtaining the genomes. In this study, we present a robust "three-step" protocol performed with A105UF/A820, EVP4/A6141, and A4879/A1005R for the full-length genome amplification of CV-A10. The results revealed that the method is able to accurately and reproducibly amplify three fragments with overlaps of the full-length genome of eight CV-A10 strains. Compared with other methods, this assay is both quick and specific. In addition, the three-step

protocol could be capable of amplifying the full-length genomes of CV-A10 strains isolated from different countries and regions. The specific three-step protocol may be particularly useful for investigating samples co-infected with CV-A10 and other viruses.

Key words: CV-A10; Three-step protocol; Full-length genome amplification

INTRODUCTION

Hand, foot, and mouth disease (HFMD) is a common childhood disorder characterized by vesicular rashes on the palms, soles, and buttocks, and by oropharyngeal ulcers. In rare cases, patients also develop neurological complications, such as encephalomyelitis and aseptic meningitis (McMinn, 2002; Shah et al., 2003; Pérez-Vélez et al., 2007). Several large epidemics of HFMD have been reported in the Asia Pacific region, particularly in Southeast Asia, since 1997 (Lu et al., 2012). Previous studies have shown that outbreaks of HFMD are mainly caused by human enterovirus 71 (EV-71), CV-A6, and CV-A16 (Chang et al., 1999; Yan et al., 2001; Lin et al., 2003; Osterback et al., 2009; Zhang et al., 2009; Zhang et al., 2010). In addition, outbreaks or sporadic cases may also be caused by other human enteroviruses, such as CV-A10. In recent years, CV-A10-related HFMD has been associated with increasingly common but sporadic cases of HFMD, and by global outbreak events (Blomqvist et al., 2010; Wu et al., 2010; Yu and Song, 2010; Davia et al., 2011; Lu et al., 2012; Mirand et al., 2012; He et al., 2013). All of these studies strongly demonstrated that CV-A10 infections are an important cause of HFMD. Therefore, it is important to investigate the genetic variation and evolution of CV-A10 (Figure 1) that takes place during HFMD epidemics. Most previous studies have focused on partial sequences, such as the VP1 gene, owing to the absence of an efficient method for obtaining the full-length CV-A10 genome. The aim of this study was to develop a “three-step” amplification method for sequencing the CV-A10 genome.

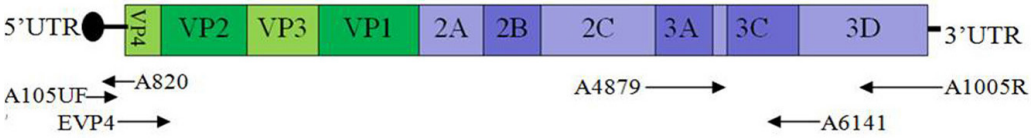

Figure 1. Schematic representation of the coxsackievirus A10 (CV-A10) genome. The genome comprises a poly-adenylated positive-strand RNA sequence that is covalently linked to a small VPg (viral protein genome-linked) at the 5' terminus. The single open-reading frame is flanked by two untranslated regions (5’UTR and 3’UTR). The position and direction of the primers used are indicated with bold arrows.

MATERIAL AND METHODS

Viruses

CV-A10 strains were isolated, identified, and transported to the Fuyang City Center for Disease Control and Prevention. All isolates were cultured using RD cells (a cell line derived from human rhabdomyosarcoma). According to the manufacturer instructions, the culture flasks were

freeze-thawed once before the culture liquid was collected. The cellular debris was removed by low-speed centrifugation (4000 *g*, 10 min). Subsequently, CV-A10 was concentrated using Amicon Ultra-15 (Millipore, Bedford, MA, USA) filtration tubes (100 kDa), and the virus-containing retentate was stored at -80°C.

Purification of viral RNA

The concentrated CV-A10 preparation was extracted and purified using an SV total RNA isolation kit (Promega, Beijing, China). The products were stored at -80°C until required.

Three-step reverse transcription polymerase chain reaction (RT-PCR) and six-step PCR

Reverse transcription was performed using SuperScript™ Reverse Transcriptase (Invitrogen, Shanghai, China), and the products were stored at -80°C until required. According to the manufacturer instructions, the complementary DNA (cDNA) was synthesized using oligo-dT at 42°C for 50 min. The cDNA was used directly for full-length PCR amplification or stored at -80°C.

Three-step PCR was successively performed with A105UF/A820, EVP4/A6141, and A4879/A1005R (Table 1) using PrimeSTAR GXL DNA Polymerase (TaKaRa, Dalian, China). The PCR regimen was as follows: 1) 35 cycles of 98°C for 10 s, 55°C for 15 s, and 68°C for 35 s; 2) 35 cycles of 98°C for 10 s, 63°C for 15 s, and 68°C for 4 min; and 3) 35 cycles of 98°C for 10 s, 55°C for 15 s, and 68°C for 1 min and 20 s. The products of eight CV-A10 strains were then subjected to 1.2% agarose gel electrophoresis (Figure 2). The A105UF, A1005R, and EVP4 primers were those described by Hu et al. (2011) and Ishiko et al. (2002). The A820, A6141, and A4879 primers were designed in reference to the Kowalik strain (AY421767) and the CV-A10/SD/CHN/09 strain (HQ728262), and synthesized by Invitrogen Co. (Invitrogen, Shanghai, China). The primers for the three-step protocol were used according to Figure 1.

Table 1. Primers for the amplification of coxsackievirus A10 (CV-A10) using the three-step method[a].

Primer	Sequence (5'→3')	Nucleotide position	Size of product (bp)
A105UF	TTAAAACAGCCTGTGGGTTG	1-20	
A820	TTGTAGATCCTCCAGTGGC	799-817 or 820-802	817
EVP4	CTACTTTGGGTGTCCGTGTT	548-567 or 551-570	
A6141	GTGGAGAACATTGCCCACAT	6141-6122 or 6144-6125	5594
A4879	AAGCTCTGCTCAGAGAACAACAC	4879-4901 or 4882-4904	
A1005R	GCTATTCTGGTTATAACAAATTTAC	7385-7409	2488

[a]5'UTR, EVP4, and A1005R refer to Ishiko et al. (2002) and Hu et al. (2011), and nucleotide positions were based on the genomes of the CV-A10-Kowalik strain (AY421767) or the CV-A10/SD/CHN/09 (HQ728262) strain.

The six-step PCR was carried out with six pairs of primers (Table 2) for the amplification of the whole-genome sequences of the CV-A10 strains (Hu et al., 2011). The six-step PCR was performed according to the following regimen: a total of 35 cycles of 30 s at 95°C, 30 s at 55°C, and 0.5 to 3 min at 72°C. The PCR products were visualized on 1.2% agarose gel (Figure 3) and compared with the gel shown in Figure 2.

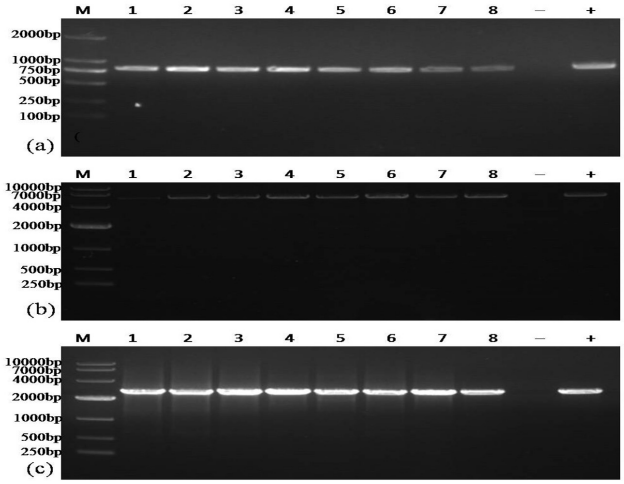

Figure 2. Agarose gel electrophoresis of the amplicons of A105UF/A820-PCR (**a**), EVP4/A6141-PCR (**b**), and A4879/A1005R-PCR (**c**) of eight different coxsackievirus A10 (CV-A10) strains (PCR is polymerase chain reaction). The FY01 to FY08 strains are represented in *lanes 1* to *8*, respectively, and the negative and positive controls are labeled with "-" and "+". The DL2000 DNA marker was used in **a** (TaKaRa, Dalian, China), and the DL10000 DNA Marker was used in **b** and **c** (TaKaRa, Dalian).

Table 2. Primers for the genome amplification of coxsackievirus A10 (CV-A10) using the six-step method[b].

Primer	Region	Position	Sequence (5'→3')	Size of product (bp)
A105UF	5'UTR	1-20	TTAAAACAGCCTGTGGGTTG	
A105UR	5'UTR	488-469	CAGTTAGGATTAGCCGCATT	488
A1001F	5'UTR	393-414	GGCAACCCATGGGACGCTCTAA	
A1001R	VP3	2063-2040	CTTTAACGAGCCTGACCATTGTGT	1671
A1002F	VP3	1732-1754	GAGCTCAGGCCCGGGACTAATCA	
A1002R	2B	3808-3794	TTGATATAGTCAGACACTC	2077
A1003F	2B	3720-3742	CCTCGTGGGGTTTGCTGATGTGA	
A1003F	3C	5428-5451	GTCGGTCTGCGCTTGTCTGATGTT	1732
A1004F	2A	5270-5293	TTGCGGTTGTGTCCCTTGTGTATG	
A1004R	3D	7056-7033	GGTTGCGTTCTCCCAGGTGACTTC	1787
A1005F	3D	6957-6974	CTAAACATGGTGGCCTACGGAGAT	
A1005R	3'UTR	7409-7385	GCTATTCTGGTTATAACAAATTTAC	453

[b]Table 2 refers to Hu et al. (2011), and nucleotide positions were based on the genome of the CV-A10/SD/CHN/09 (HQ728262) strain.

Verification of the three-step assay

To verify the results of the three-step protocol, all the amplicons produced using the A105UF/A820, EVP4/A6141, and A4879/A1005R primers were sequenced and assembled by Sangon Co. (Sangon, Shanghai, China). The near full-length genome sequences of eight CV-A10 strains were registered at GenBank.

RESULTS

Whole-genome amplification using the three-step protocol

The amplification of the whole-genome sequences of CV-A10 was conducted using primers

in accordance with Figure 1. Using the three-step protocol proposed, the complete genomes of eight CV-A10 strains were obtained using three fragments with overlaps. As shown in Figure 2, various primers, such as A105UF/A820 (820 bp), EVP4/A6141 (5594 bp), and A4879/A1005R (2488 bp), were used, as reported in Table 1. In addition, the PCR products were also visualized on an agarose gel to confirm that they were the correct size after amplification. The results revealed that this three-step method worked well for PCR assays.

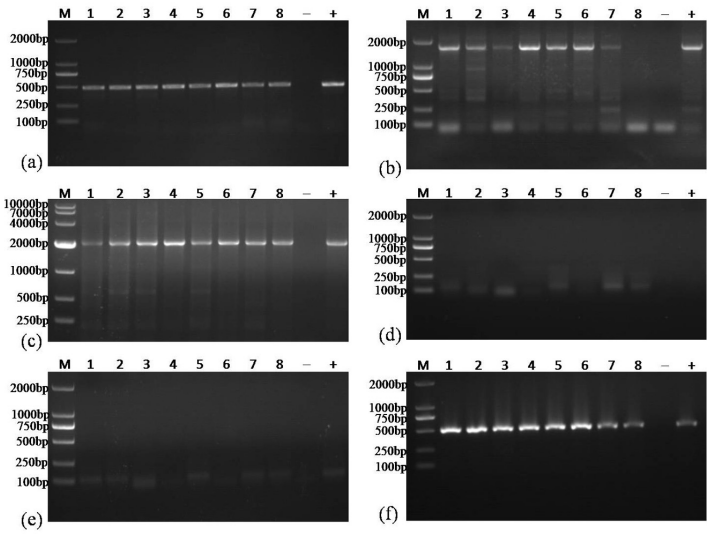

Figure 3. Agarose gel electrophoresis of the amplicons of A105UF/A105UR (**a**), A1001F/A1001R (**b**), A1002F/A1002R (**c**), A1003F/A1003R (**d**), A1004F/A1004R (**e**), and A1005F/A1005R (**f**) of eight different coxsackievirus A10 (CV-A10) strains. The FY01 to FY08 strains are represented in *lanes 1* to *8*, respectively, and the negative and positive controls are labeled with "-" and "+". The DL2000 DNA marker was used in **a**, **b**, **d**, **e**, and **f** (TaKaRa, Dalian, China), and the DL10000 DNA marker (Takara, Dalian) was used in **c**.

Optimization of PCR conditions

To optimize the long fragment PCR conditions, the annealing temperature, annealing time, and extension time were rigorously tested based on the PrimeSTAR GXL DNA Polymerase manufacturer instructions. First, the annealing temperature of EVP4/A6141 was tested in the range 55°-65°C, and the most suitable temperature was found to be 63°C. Second, the annealing time of EVP4/A6141 was examined from 0 to 30 s, and the best time was found to be 15 s. Third, the extension time of EVP4/A6141 was tested from 4 to 6 min, and the shortest possible time was found to be 4 min. Based on the PrimeSTAR GXL DNA Polymerase manufacturer instructions, the amount of cDNA for long fragment PCR should be between 250 and 750 ng. The viral suspension was concentrated by low-speed centrifugation according to the manufacturer instructions to produce the long fragment PCR product using EVP4/A6141.

GenBank accession Nos.

The near full-genome sequences of eight CV-A10 strains were BLASTed in GenBank, and the results revealed that they belonged to CV-A10 (GenBank accession No. KP009574-KP009581).

Whole-genome amplification using the six-step protocol

To assess the six-step protocol, the primers reported by Hu et al. (2011) were used for the amplification of eight CV-A10 strains and the results obtained were compared with those obtained using the three-step protocol. As shown in Figure 3, A105UF/A105UR (a), A1001F/A1001R (b), A1002F/A1002R (c), A1003F/A1003R (d), A1004F/A1004R (e), and A1005F/A1005R (f) (Hu et al., 2011) were amplified under various conditions. The A1003F/A1003R and A1004F/A1004R primers resulted in a negative reaction. Therefore, it was necessary to develop a laboratory procedure for the full-length genome amplification of CV-A10 isolated from different countries and regions, and without any limitations.

DISCUSSION AND CONCLUSION

Currently, only two full-length genomes of CV-A10 have been reported (Oberste et al., 2004; Hu et al., 2011), probably because of the regional nature of its distribution or because it is underemphasized (Hu et al., 2011; Lu et al., 2012; He et al., 2013). Thus, there is very little information about the genetics and evolution of CV-A10. In previous studies, a method using universal primers was established to amplify the complete genome of CV-A10 (Oberste et al., 2004). In addition, a six-step protocol with specific primers was used for the amplification of the full genome of CV-A10 (Hu et al., 2011). As shown in Figure 3, the six-step protocol was used to obtain the complete genomes of eight CV-A10 strains. However, at least two of the PCRs (d and e) were unsuccessful. Moreover, nonspecific reactions (b and c) also took place.

This study presents a robust three-step protocol for the full-length genome amplification of CV-A10 based on long fragment PCR. Virus concentration and reverse transcription referred to the method for full-length genome amplification of a poliovirus strain (Chumakov, 1996; Boot et al., 2004; Laassri et al., 2005). The long fragment PCR was performed using EVP4/A6141 and the product was 5594 bp. As shown in Figure 2, a, b, and c produced fragments of sizes 750 to 1000 bp, 4000 to 7000 bp, and 2000 to 4000 bp, respectively. The results showed that the three PCRs carried out using the primers shown in Figure 1 were specific, accurate, and reproducible. Compared with the six-step protocol, the three-step protocol can not only effectively improve the specificity of the amplification and the range of applications, but also reduce the probability of errors in the full-genome amplification of CV-A10 using a step-by-step process. Furthermore, the three-step protocol not only simplifies the process of the whole-genome amplification of CV-A10, but also avoids cloning to the pGEM-T vector for sequencing. Thus, the universal primers method was not carried out as a comparison to the three-step method in this study.

A viral stock is a collection of different genomes that are closely related, and is often referred to as a quasispecies. A full-length RT-PCR on CsCl-purified material of oral poliovirus vaccine poliovirus strains has shown that the quasispecies is preserved during the *in vitro* RNA-to-cDNA-to-RNA conversion cycle (Chumakov, 1996; Boot et al., 2004). Like the poliovirus strains, CV-A10 is also a quasispecies. For this reason, we developed a three-step method that comprises one long fragment PCR, which is aimed at preserving the quasispecies nature of the viral stock as much as possible. A rapid DNA polymerase with high fidelity (PrimeSTAR GXL, see also the third subsection in the MATERIAL AND METHODS section) was used for long fragment PCR, because we hoped to achieve genome amplification that was quick and accurate. Thus, we expect that the three-step method will have a significant impact on the surveillance of the genetics and evolution of CV-A10.

Conflicts of interest

The authors declare no conflict of interest.

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