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A diagnostic kit for the enteroviruses Coxsackie A6 and A10

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ABSTRACT. Recently, there has been an upward trend in the occurrence of hand-foot-mouth disease, which is correlated with Coxsackie A6 and A10 infections. Although two separate diagnostic reagents are available for these two viral strains, the protocol and diagnosis efficiency still need to be improved. More importantly, as co-infection with these viruses is common, the development of a single test kit that can diagnose both viruses would be most beneficial for clinical practice. In our study, specific primers targeting viral nucleic acids were designed and modified. Viral nucleic acids were extracted from fecal or throat swab samples by ultrasonic rupture and silicon membrane purification. The consistency, specificity, and sensitivity of the tests were further optimized by adjusting the polymerase chain

reaction (PCR) conditions. The efficiency of viral nucleic acid extraction was significantly enhanced by the ultrasonic rupture and silicon membrane elution approach. Specific amplifications of both viral nucleic acids were achieved using modified primers. The optimal conditions for PCR were also determined (60°C for 30 min and 95°C for 2 min, followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and elongation for 50 s at 72°C). Amplified products were confirmed as viral specific nucleotides by agarose gel electrophoresis and sequencing. The minimal nucleic acid concentration required for detection was 0.2 ng/L, which was adequate to yield satisfactory specificity and consistency. This novel diagnostic method has many advantages, including rapid protocols and accurate results, and can be promoted for large-scale clinical trials.

Keywords: Hand-food-mouth disease; Enterovirus coxsackie A6; Enterovirus coxsackie A10; PCR

INTRODUCTION

Hand-foot-mouth disease (HFMD) is a commonly occurring infectious disease in children and is caused by enterovirus. The most common pathogens for this disease include enterovirus 71 (EV71) and Coxsackie virus (CV) A16. A recent survey in Wuhan, China, found that more than half of the disease-causing viral strains belonged to CVA6 and CVA10 (Yang et al., 2015). CV, which is an RNA virus with a single positive strand, can be classified into two subtypes: A and B. It is transmitted via gastrointestinal and respiratory pathways and can cause flu-like symptoms, including fever, sneezing, and cough (Fechner et al., 2011). In the type A subgroup of CV, various viral strains such as A4, A5, A6, A9, and A10 have been confirmed to cause HFMD (Li et al., 2015). Recent outbreaks of HFMD in places such as Singapore (Ang et al., 2015) and China (Liu et al., 2015) further indicated the importance of rapid viral detection and classification for improving both genetic research and clinical treatments. The viral strains CVA6 and CVA9 have been suggested to be related to recent outbreaks of HFMD (Blomqvist et al., 2010; Lu et al., 2012; Mirand et al., 2012; He et al., 2013). The detection of viral nucleic acids of these two viral strains, however, still requires two separate test kits with limited efficiencies. Due to the high incidence of co-transfection between those two viruses, it is necessary to design a novel test kit that offers rapid detection of both CVA6 and CVA10 with high efficiency. Therefore, in this study, a diagnostic kit was developed to target viral nucleic acids of CVA6 and CVA10 based on the modification of reagents used in next-generation PCR systems. To improve the recognition ability and binding specificity of primers, a new extraction tool was created using a silicon membrane conjugated with a syringe. In addition, chemical modification of substrates utilized in primer synthesis was also performed.

MATERIAL AND METHODS

Sample collection and nucleic acid extraction

Fecal and throat swab samples were collected from HFMD patients who were admitted to our hospital between January 2015 and June 2015. The extraction system was developed, as

shown in Figure 1. In brief, samples were mixed with 1 mL lysis buffer via ultrasonic rupture (30 s sonication for 4 times, with iced bath between each ultrasonic pulse). The silicon membrane-containing syringe was used to separate nucleic acids from fecal samples.

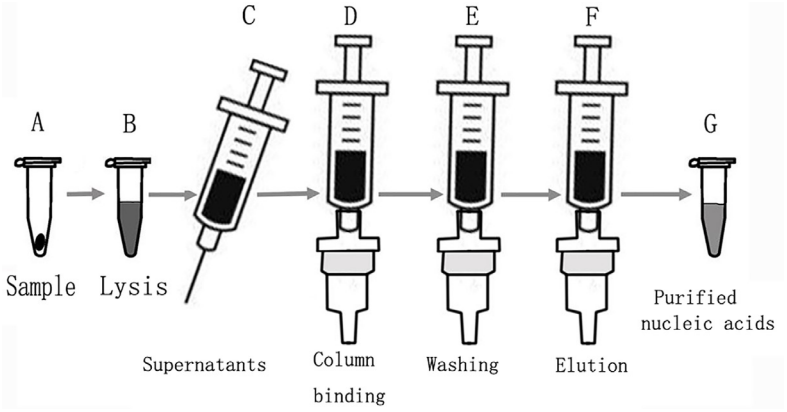

Figure 1. Illustration of the viral nucleic acid extraction system.

The extraction system mainly consisted of five components: 1) The lysis buffer, which used guanidinium thiocyanate as the main active lysis reagent and EDTA in Tris-buffered solvents, was used as a chelating agent to inhibit RNase activity. 2) Wash buffer 1 containing guanidine hydrochloride and Triton X-100 in Tris buffer was used to clear the cell debris on the membrane. 3) Wash buffer 2 comprising Triton X-100 in Tris buffer was used to further clean salts and small aqueous molecules on the membrane. 4) Elution buffer, which was a low-salt high-pH Tris buffer, was used to separate nucleic acids from the silicon membrane. 5) A syringe with silicon membrane was used for extraction.

Primer design and modification

The full-length genomic sequences of CVA6, CVA10 and other enteroviruses were obtained from the NCBI nucleic acid sequence databank. With the help of sequence alignment and literature reviews, CVA6 specific primers (forward, 5'-CAAGC TGCAG AAACG GGAG-3'; reverse, 5'-GCTCC ACACT CGCCT CATT-3') were designed based on common conserved regions in the VP1 gene (Zhang et al., 2012). CVA10 primers (forward, 5'-GGTAA CTTCC ACCAC CACC-3'; reverse, 5'-CCTCC GGCCC CTGAA TGCG GCTAA T-3') were designed based on the VP4 gene fragment (Ji et al., 2012; Yang et al., 2015). In order to improve the binding specificity of purine and pyrimidine molecules, the nucleotides A, C, and G used during primer synthesis were modified by the addition of phenyl groups (provided by Baosheng Biotech, China).

RT-PCR amplification of viral strains

Primary viral strains of CVA6 and CVA10 (Standard pathogen collection, USA) were used to infect RD cells as previously described (Blomqvist et al., 2010). After the confirmation of virus inoculation, viral nucleic acids were amplified by PCR in the four groups: blank control, CVA6-

positive, CVA10-positive, and mixture group. The PCR mixture (50 μL) contained RNA polymerase (2 μL), buffer (25 μL), primers (1 μL each), RNA template (10 μL), and RNase-free ddH_2O (11 μL). The amplification parameters were as follows: 60°C for 30 min and 95°C for 2 min, followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and elongation for 50 s at 72°C. Amplified products were separated subjected to gel electrophoresis on a 3% agarose gel, and were visualized using SYBR Green (Sigma, China) staining. Target DNA bands were isolated and purified from the gel bands and sequenced for confirmation.

Optimization of PCR condition

To maximize the efficiency and sensitivity of the PCR system, we tested and compared PCRs at different annealing temperatures (50°, 55°, and 60°C), template concentrations (0.5, 1.0, and 2.0 μL), and polymerase concentrations (2, 4, and 8 μL). Other parameters remained similar, and were as described in the previous section. After 40 cycles of RT-PCR, ΔCt values were obtained and compared between all groups to determine the optimal condition.

Characterization of diagnostic methods

The sensitivity of the test kit was determined by serially diluting viral nucleic acid mixtures (2 μg CVA6 and 2 μg CVA10 were used as the primary concentrations). The minimal concentration of the standard viral nucleic acid solution was used as a reflection of kit sensitivity.

The specificity of the test kit was tested on dozens of throat swab samples that were positive for enterovirus infection, including 10 cases of CVA16, 15 cases of EV71, 10 cases of CVA6, and 6 cases of CVA10 infection. The positive rate was calculated to determine the test specificity.

The consistency of this assay was determined using technical replicates; each analysis was carried out in triplicates from the same batch of single and mixed viral samples.

Diagnostic kit packaging and clinical trial

The established test reagents were packaged into a test kit, which included the following: 1) an extraction syringe with silicon membrane; 2) the lysis buffer; 3) wash buffers 1 and 2; 4) the elution buffer; 5) RT-PCR polymerase; 6) buffer solution; 7) pre-mixed primers; and 8) RNase-free ddH_2O. Both negative and positive controls were also included in this kit. We further obtained 62 clinical samples to test the efficiency of this diagnostic kit by comparing it to other clinical diagnostic methods.

RESULTS

Infection of RD cells

We used primary strains of CVA6 and CVA10 viruses to infect RD cells, which were monitored daily under an inverted microscope for pathological features of enterovirus infection (Song et al., 2014). Those characteristics included rounding of the cell, increase in intensity of light reflection, and detachment from the cultured flask (Figure 2).

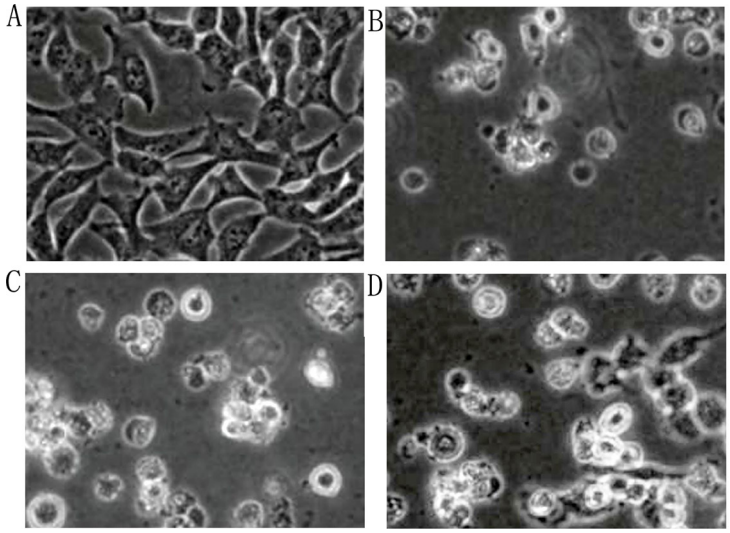

Figure 2. Morphological alterations in the RD cells following CV infection. **A.** normal RD cells; **B.** RD cells with CVA6 infection; **C.** RD cells with CVA10 infection; **D.** Mixed infection with CVA6 and CVA 10 viruses.

RT-PCR and identification of products

The optimal conditions for PCR were: an annealing temperature of 60°C and a primer concentration of 0.4 μM (1 μL). Following gel electrophoresis, the amplified products were 106 and 407 bp for CVA6 and CVA10, respectively, with no significant non-specific fragments (Figure 3). Further confirmation of amplicon identity was obtained by DNA extraction from the gel, followed by DNA sequencing. Those products were found to be bands specific for viruses; thus, we have demonstrated that this system can effectively amplify nucleic acids of both viruses.

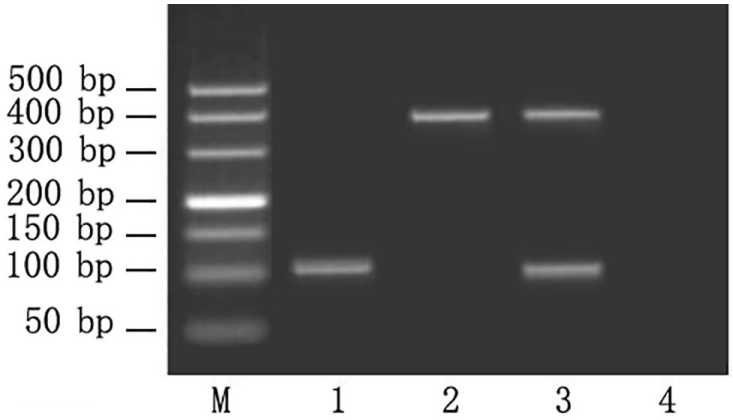

Figure 3. RT-PCR products of viral nucleic acids. *Lane M* = DL500 DNA size marker (Baosheng Biotech, China); *lane 1* = sample for nucleic acid extraction of CVA6; *lane 2* = sample for nucleic acid extraction of CVA10; *lane 3* = sample for nucleic acid extraction of both virus mixtures; *lane 4* = negative control.

Characterization of diagnostic kits

The sensitivity of this test kit was determined to be 0.2 ng/L. Under this concentration, PCR amplification still provided satisfactory results without any nonspecific binding (Figure 4). The specificity of this method was determined using different templates including cells that were inoculated with CVA6, CVA10, EV71, and CVA16. Following RT-PCR amplification, positive viral bands were observed for cells inoculated with CVA6 or CVA10, but not EV71 or CVA16 (Figure 5).

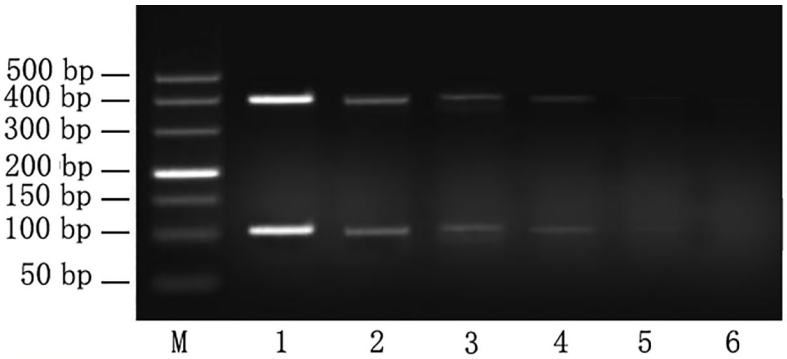

Figure 4. Sensitivity assay of the test kit, as assessed by RT-PCR. *Lane M* = DL500 DNA size marker; *lanes 1-6* = serially diluted standard samples of viral nucleic acid mixtures. Primary concentration: 2 µg CVA6 and 2 µg CVA10; Dilution gradient: 10^{-1}-10^{-6}.

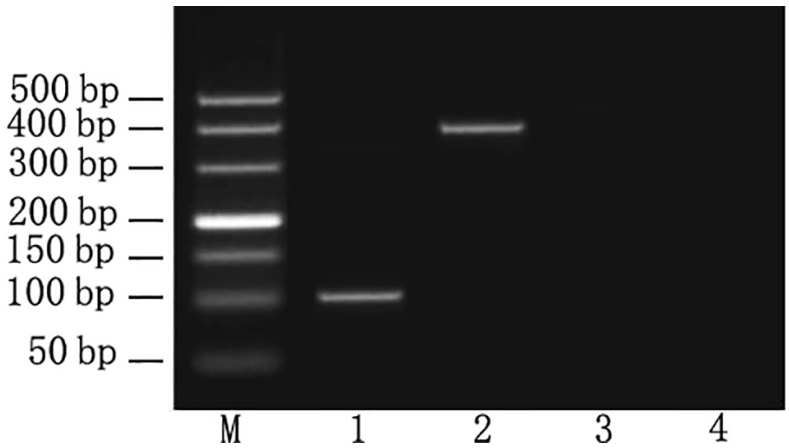

Figure 5. Sensitivity assay of the test kit, as assessed by RT-PCR. *Lane M* = DL500 DNA size marker; *lanes 1-6* = serially diluted standard samples of viral nucleic acid mixtures. Primary concentration: 2 µg CVA6 and 2 µg CVA10; Dilution gradient: 10^{-1}-10^{-6}.

The consistency of this method was tested by performing the PCR amplification in triplicates on the same CVA6, CVA10, or mixed sample. Consistent results were obtained under all circumstances, confirming the reliability of this test kit.

Application to clinical samples

This novel test kit was then used to test a total of 62 clinical samples, and results indicated that 21 samples were positive for CVA6 (33.87%), 12 samples were positive for CVA10 (19.35%), and 9 samples were positive for both (14.52%). All these results agreed with previous laboratory conclusions, further supporting the sensitivity and specificity of this detection method.

DISCUSSION

As one of the most common viral transmitted diseases in children worldwide, HFMD mainly presents as skin lesions, including roseola and blister on the skin of the hands, feet, and mouth (Nassef et al., 2015). In China, repeated outbreaks of HFMD since the year 2004 have made this disease a public health issue (Han et al., 2012). Under normal circumstances, HFMD is caused by EV71 or CVA16 infections, and little is known about other potential enterovirus pathogens responsible for this disease (Zhang et al., 2010). A recent epidemiology survey showed that there is an increasing number of CVA6 and CVA10 infections in HFMD outbreaks (Hu et al., 2015). However, rapid and easy diagnostics for these HFMD subtypes are still lacking.

Currently, semi-quantitative or real-time quantitative PCRs using primer pairs for a single viral nucleic acid are used to detect the presence of enterovirus via amplification of targeting gene fragments (Chen et al., 2012). Therefore, in order to detect two viral strains, at least two independent reactions are required, which increases both the time and expenditure. The development of a novel method that can simultaneous detect the presence of two viruses is thus of great importance. Rapid PCR is achieved mainly by increasing the concentration of enzymes in the system, thereby shortening the amplification time (Sakamoto et al., 2015). This is usually achieved with the new function module on the PCR cycler (such as the FAST module in qPCR cyclers by ABI Biosystems). Only a few manufacturers (for example, Kapa Biosystems) supply modified DNA polymerases that can increase amplification efficiency without any changes to the primers. No patents have been registered regarding the improvement in current amplification technology. In our rapid PCR method, which has been described in this article, currently used PCR cyclers and reaction tubes do not need to be changed; this method can be promoted as a technological platform that can be used for multi-PCR and real-time PCR without sacrificing the reliability of the assay.

In the process of nucleic acid extraction and purification, traditional methods, such as phenol/chloroform, silicon filters, and magnetic particle absorption, involve relatively complex protocols and require various heavy equipment. As a result, they cannot satisfy the requirements of on-site extractions and identifications (Frickmann et al., 2015). We have thus developed a novel method based on silicon membrane-coupled syringe and ultrasonic rupture technology, as described above. Syringes were used instead of ultracentrifugation, which shortens the total extraction time to 5-10 min. Ultrasonic rupture releases nucleic acid (viral RNA) for direct recruitment to the PCR system (Frostegård et al., 1999). This method remarkably simplifies the extraction and purification of nucleic acids, which greatly benefits multi-target assays and/or replicate studies. Furthermore, it only requires a single extraction process with an extraction efficiency and purity comparable to those obtained with magnetic bead absorption.

We have also improved the amplification of nucleic acids by developing a next-generation rapid PCR technology, which modified the properties of the PCR substrates. By using the One-step RT-PCR kit (Takara, Japan), sample contamination was minimized, as a closed reaction system

was used between cDNA synthesis and amplification. More importantly, we modified the bases A, C, and G by adding a phenol group, thus increasing the binding specificity of primers by enhancing the binding capacity of purine/pyrimidine pairs. Two separate primer pairs were used in to amplify nucleic acid fragments from both viruses, which allowed us to effectively probe for two pathogens in a single experiment. This should significantly improve identification of viral infection in clinical laboratories. The minimal test limit of this dual-viral method is 0.2 ng/L, which is more sensitive than any other methods used in clinical practice. All these results confirmed the high sensitivity, specificity, and consistency of this novel diagnostic method, which can be a promising diagnostic tool in the future.

In summary, the novel viral nucleic acid extraction and primer synthesis method used in this diagnostic kit offers several advantages, including a rapid and easy protocol and high accuracy, and can thus be promoted for large-scale clinical trials. Furthermore, the minimal equipment requirements for this kit further emphasizes its ease of use. This method can be applied in a wide array of clinical uses, however, more implementation need to be carried out to optimize the method for clinical trials.

Conflicts of interest

The authors declare no conflict of interest.

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