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# Development of multiplex real-time hybridization probe reverse transcriptase polymerase chain reaction for specific detection and differentiation of Enterovirus 71 and Coxsackievirus A16

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**Abstract**

Large outbreaks of hand, foot, and mouth disease have been reported in the Asia Pacific region over the last few years and resulted in significant fatalities. The 2 main etiologic agents are Enterovirus 71 (EV71) and Coxsackievirus A16 (CA16). Both viruses are closely related genetically and show similar clinical symptoms. However, EV71 are associated with neurologic complications and can lead to fatalities. In this study, we developed a multiplex real-time hybridization probe reverse transcriptase polymerase chain reaction to detect and differentiate EV71 from CA16 using the LightCycler (Roche Molecular Biochemicals). Specific primers and hybridization probes were designed based on highly conserved VP1 region of EV71 or CA16. Our results showed high specificity and sensitivities in detecting EV71 or CA16 from 67 clinical specimens, and no other enterovirus serotype was detected. Rapid diagnosis to differentiate EV71 from CA16 in outbreak situations will enable pediatricians to identify and manage the patients more effectively.


*Keywords:* Hand, foot, and mouth disease (HFMD); Enterovirus 71; Coxsackievirus A16; Multiplex real-time RT-PCR; Rapid detection and differentiation

## 1. Introduction

The Asia Pacific region had experienced large epidemics of hand, foot, and mouth disease (HFMD) over the last few years, and the 2 main etiologic agents of HFMD were Enterovirus 71 (EV71) and Coxsackievirus A16 (CA16) (Brown et al., 1999; Chang et al., 1999). EV71 and CA16 are closely related genetically, sharing 77% nucleotide and 89% amino acid homologies (Brown et al., 1999). However, infection by EV71 is more often associated with severe neurologic diseases like aseptic meningitis, and brainstem and cerebellar encephalitis, which are not observed in HFMD cases caused by CA16 (AbuBakar et al., 1999; Lum et al., 1998). In the major HFMD outbreaks in Southeast Asia, EV71 is the main etiologic agent that caused 41 deaths in Malaysia in 1997 (Lum et al., 1998), 78 deaths in Taiwan in 1998 (Ho et al., 1999), and 4 deaths in Singapore in 2000 (Ahmad, 2000).

Because the clinical symptoms of EV71- and CA16-associated HFMD are similar, diagnosis depends largely on virus isolation and serotyping (Lim and Benyesh-Melnick, 1960). However, this requires 2 to 3 weeks of growth and neutralization of the viral isolates. However, antigenic typing could be hindered by nonneutralizable viruses because of aggregation, antigenic drifts, or the presence of multiple viruses in the specimen (Melnick, 1996).

The development of polymerase chain reaction (PCR) techniques has contributed significantly to laboratory diagnosis of viral infections in terms of sensitivity, specificity, and the rate of detection in comparison with the cell culture method. Using conventional reverse transcriptase PCR

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(RT-PCR) strategy, specific detection of EV71 or CA16 had been reported (Bendig et al., 2001; Brown et al., 2000; Nix et al., 2006; Singh et al., 2002; Tsao et al., 2002). However, the 2-step RT-PCR process is time consuming, and the risk of cross-contamination is increased by this approach. In addition, detection of the viruses based on conventional RT-PCR requires at least 6 to 8 h and could be a limiting factor during outbreak situations. In a recent study, a conventional multiplex RT-PCR in combination with the microarray method was developed to differentiate EV71 from CA16 using specific primers targeting each of the viral RNA. RT-PCR was 1st carried out to amplify EV71 and CA16 viral RNA. The amplicons were then labeled with fluorescent dyes and added to array slides, which were spotted with 60-mer degenerate oligonucleotide probes specific for EV71 or CA16. A diagnostic accuracy of 92% and 95.8% was achieved for specific detection of EV71 and CA16, respectively (Chen et al., 2006). In another study, Tsao et al. (2006) evaluated a microchip method developed by DR. Chip Biotechnology (Miao-Li, Taiwan). The detection was based on the biotinylated PCR products hybridizing to specific probes, and this approach achieved a sensitivity of 82% (Tsao et al., 2006). However, the detection of EV71 from clinical specimens using both approaches is laborious. Besides, the entire diagnostic process using both methods require at least 10 h which is too long in outbreak situations.

In recent years, real-time PCR has gained wider acceptance for viral diagnosis in laboratories because of higher sensitivity and specificity, and faster rate of detection, and it provides real-time monitoring of the amplification process through fluorescence emission (Mackay et al., 2002). Previous studies have shown that enteroviruses that cause HFMD could be differentiated from other viruses such as varicella-zoster virus, poliovirus, and Herpes simplex virus using real-time PCR SYBR Green I-based (Read et al., 2001) and TaqMan probe-based assays (Nijhuis et al., 2002; Petitjean et al., 2006). In all the 3 studies, the primers and probes were designed to target at the 5′-untranslated region (5′UTR) of the enterovirus genome, which is highly conserved among enteroviruses, and molecular epidemiologic studies have shown that the diversity in this region did not correlate well with enterovirus serotypes (Oberste et al., 1999). Thus, in these studies, different enterovirus serotypes could not be differentiated effectively from 1 another without DNA sequencing.

We have previously developed a real-time hybridization probe RT-PCR to detect EV71 specifically from clinical specimens (Tan et al., 2006). However, with increased concerns over the HFMD infections caused by EV71 and CA16, there is a need for a rapid and highly specific method to distinguish these 2 viruses from other enteroviruses in large outbreak situations. In this study, we developed a 1-step quantitative multiplex real-time hybridization probe-based RT-PCR to detect and differentiate EV71 specifically from CA16 directly from clinical specimens within 1 to 2 h. We also evaluated the efficacies of the real-time PCR assay in detecting EV71 or CA16 directly from clinical specimens such as sera, saliva, urine, stools, throat, and rectal swabs.

## 2. Materials and methods

### 2.1. Viral isolates

A CA16 strain (CA16-G-10) was kindly provided by Dr. M.A. Pallansch, CDC, Atlanta, GA. Three Japanese strains, namely, 1585-Yamagata-01 (genogroup C2), 75-Yamagata-03 (genogroup C4) and 2933-Yamagata-03 (genogroup B5) were kind gifts from Prof. K. Mizuta, Yamagata Prefectural Institute of Public Health, Yamagata, Japan. One Singapore strain, the fatal 5865/SIN/00009 strain (designated as strain 41), was isolated from patients during the outbreak in October 2000 and cultivated in tissue cultures. Other enterovirus isolates analyzed in this study included CA24, Coxsackieviruses B1 (CB1), CB2, CB3, and Echoviruses 6 and 7.

### 2.2. Sample processing and storage

A total of 67 clinical specimens were obtained from 40 pediatric patients who were admitted to the National University Hospital, Singapore, with suspected HFMD. The clinical specimens included 8 stools, 12 rectal swabs, 10 blood serum, 13 throat swabs, 11 saliva, and 13 urine specimens. The saliva and the urine specimens were processed for RNA extraction directly upon receiving them. The throat and rectal swabs were 1st suspended in 1% phosphate-buffered saline (PBS) before being processed for RNA extraction. A 10% stool suspension was made by adding 0.5 g of stool (0.5 mL for fluid stools) to 5 mL of 1% PBS. The suspension was then centrifuged at 12 000 × *g* for 10 min and filtered. The filtrate was then subsequently processed. Blood samples were allowed to stand in a vertical position for about 15 to 20 min. After centrifuging for 10 min at 12 000 × *g*, the serum was then aspirated and transferred to a clean 1.5-mL sterile Eppendorf tube.

### 2.3. RNA extraction

Viral RNA extraction was carried out using QIAamp Viral RNA Mini Kit according to the manufacturer's instruction (QIAGEN, Valencia, CA). Briefly, the specimens were lysed with a QIAGEN viral lysis (AVL) buffer, and the RNA released would be bound to the membrane. After washing twice with wash buffers to remove any contaminating proteins and lipids, the RNA was then eluted with an elution buffer (QIAGEN).

### 2.4. Design of primers and hybridization probes

The VP1 nucleotide sequences of EV71 strains and CA16 strains from the GenBank were analyzed. Using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and DNASTAR program, we defined a highly conserved VP1 region of EV71