Journal of Virological Methods 159 (2009) 23–28

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Simultaneous detection of enterovirus 70 and coxsackievirus A24 variant by multiplex real-time RT-PCR using an internal control

Xing-Long Xiao[a,c], Hui Wu[a], Yi-Juan Li[b], Hui-Fang Li[c], Ya-Qing He[d], Gu Chen[a], Jing-Wei Zhang[c], Hong Yang[d], Xiao-Feng Li[a], Xiao-Quan Yang[a], Yi-Gang Yu[a,*]

[a] Institution of Food safety, Department of Food, College of Light Industry & Food, South China University of Technology, Guangzhou 510640, China
[b] Department of Pediatris, The First Affiliated Hospital of Zhongshan University, Guangzhou 510600, China
[c] Shenzhen Taitai Genomics, Inc., Shenzhen 518057, China
[d] Shenzhen Center for Disease Control and Prevention, Shenzhen 518000, China

Article history:
Received 3 November 2008
Received in revised form 17 February 2009
Accepted 17 February 2009
Available online 3 March 2009

Keywords:
Internal control
Multiplex real-time RT-PCR
Human enterovirus 70
Coxsackievirus A24
Detection

A B S T R A C T

Epidemics of acute hemorrhagic conjunctivitis are always explosive and extensive, and have been recognized as a serious international public health problem. Enterovirus 70 and coxsackievirus A24 variant have been identified as the major etiological agents in acute hemorrhagic conjunctivitis outbreaks worldwide. A novel multiplex real-time RT-PCR assay was developed for simultaneous detection, identification and quantitation of enterovirus 70 and coxsackievirus A24 variant. The specificity, sensitivity and reproducibility of the method were analyzed and 125 clinical samples were tested using this method. No cross-reactivity with other enteroviruses strains was detected. The detection limits achieved were 10 copies/tube of enterovirus 70 and 100 copies/tube of coxsackievirus A24 variant respectively, and the addition of the internal control does not compromise the sensitivity or specificity. One hundred and twenty five clinical samples were tested and the results were consistent with the results obtained by using virus isolation followed by neutralization and sequencing of VP1 region. Due to its high specificity, sensitivity and elimination of false negative results by the internal control, this assay is suitable for both research applications and rapid clinical diagnosis of enterovirus 70 and coxsackievirus A24 variant.

1. Introduction

Acute hemorrhagic conjunctivitis, also known as epidemic hemorrhagic conjunctivits is characterized by conjunctival hemorrhages, excessive lacrimation and foreign-body sensation in the eye (Hierholzer and Hatch, 1985). Acute hemorrhagic conjunctivitis first occurred during June 1969 in Accra, Ghana (Chatterjee et al., 1970), since then acute hemorrhagic conjunctivitis outbreaks have been reported from many parts of the world, infecting millions of people, and has gained recognition as a serious international public health problem. Acute hemorrhagic conjunctivitis can be caused by viruses including enterovirus 70 (EV70), coxsackievirus A24 variant (CV-A24V), and adenoviruses. Indeed, EV70 and CV-A24V have been identified as the major etiological agents in outbreaks acute hemorrhagic conjunctivitis worldwide. EV70 was involved in many outbreaks of acute hemorrhagic conjunctivitis in Ghana in 1970, in Singapore in 1980, in India and Bangladesh in 1981, in Saudi Arabia in 1990, in Japan in 1985 and 1994 (Chatterjee et al., 1970; Hossain et al., 1983; Manjunath et al., 1982; Ramia and Arif, 1990; Onorato et al., 1985; Uchio et al., 1999). CV-A24V was first isolated in 1970 during an epidemic in Singapore (Lim and Yin-Murphy, 1971). Since then, epidemics of acute hemorrhagic conjunctivitis caused by CV-A24V have been reported from Ghana, Japan, Thailand, India, Korea (Brandful et al., 1990; Christopher et al., 1977; Kosrirukvongs et al., 1996; Aoki et al., 1988; Oh et al., 2003). There are three genotypes of the variant of CV-A24V, designated I–III, identified by phylogenetic analysis of the 3C region. CV-A24V was not detected in genotypes I and II since related strains were isolated during the 1970s and disappeared since then, while genotype III comprises strains isolated between 1985 and 1995 in Japan, Taiwan, China, Hong Kong, Thailand, Singapore, Pakistan and Ghana. The detection of strains from genotype III circulating since 1985 and of the clustered strains related to Korean isolates which are implicated in all acute hemorrhagic conjunctivitis outbreaks reported since 2002 worldwide (Lévêque et al., 2007).

In China, the first acute hemorrhagic conjunctivitis epidemic occurred in 1970 and then outbreaks have been reported locally every few years. In the summer of 1984, an outbreak of acute hemorrhagic conjunctivitis occurred in Beijing and subsequently the infection spread to many parts of the country, infecting

* Corresponding author. Tel.: +86 20 87112853/13828797202; fax: +86 20 87112853.
E-mail address: redtreer@163.com (Y.-G. Yu).

0166-0934/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2009.02.022

Table 1
Primers and probes for detection of EV70, CV-A24V and internal control.

Target	Primer or probe[a]	Sequence (5′ → 3′)	Tm[d] (°C)	Size (bp)
EV70	EV70Pf	GCTACATCCAACACAGAACCAGA	57.9	23
	EV70Pr	ATACACACAAGTGCAGATCTGCC	57.9	23
	EV70Pb	FAM–TGCATGGTACTGCAGAGTGCCTGGTG–BHQ1	69.8	26
CV-A24[b]	CV-A24VPf	CCAACCACGGAGCAGGTGA	61.6	19
	CV-A24VPr	GAAACACGGACACCCAAAGTAGT	58.1	23
	CV-A24VPb	HEX–CAACCCAGCAACTAGCCTGTCGTAACGC–BHQ1	70.4	28
Internal control[c]	ICPb	CY5–ACTGGTGCAGAGTCTTGTGTGCAGGC–BHQ2	68.5	26

[a] Pf: forward primer; Pr: reverse primer; Pb: probe.
[b] Primers described by Lévêque et al. (2007).
[c] The internal control uses the same primers as the viral target of EV70.
[d] Melting temperature estimated by Primer Express 2.0 primer test document, dyes are not accounted for in the calculations.

millions of people. One decade later, the epidemic occurred again in 1994. Recently, the epidemic situation of acute hemorrhagic conjunctivitis in 2005, 2006, 2007 was evaluated using the data from the China Information System for Disease Control and Prevention. The results showed that the incidence of acute hemorrhagic conjunctivitis in 2007 has increased more than 5 times, as compared with 2006 (Wang and Ni, 2008). Since the recent and continuing acute hemorrhagic conjunctivitis in several provinces of China from 2005, greater attention should be paid to the prevention and control of epidemics of acute hemorrhagic conjunctivitis.

It is therefore required to develop a rapid and reliable method for the detection of the causative agent to limit the extension of the outbreak. Usually the identification of the serotype of the causative agent are based on virus isolation in cell culture, followed by neutralization assay, serological tests and sequence analysis of the VP1 region (Oberste et al., 2003). However, classical virus isolation and identification remains difficult and time-consuming. Detection of several individual viruses separately by RT-PCR is also laborious and expensive.

Recently, a TaqMan real-time RT-PCR method for CA-24V detection was developed, which is accurate and quantitative for routine diagnosis within 4 h. The one-step single tube test decreases the risks of laboratory contamination compared with the conventional RT-PCR assay (Lévêque et al., 2007). However, the drawback of this assay was the lack of an internal control. The use of multiplexed internal controls to monitor sample extraction and amplification inhibitors is necessary to prevent false negative results (Bustin and Nolan, 2004). In this study, a multiplex real-time RT-PCR assay with internal control for simultaneous detection of EV70 and CA-24V in the same reaction system was designed and evaluated in more than one hundred clinical specimens. The assay has not been described previously.

2. Materials and methods

2.1. Virus isolates and clinical samples

The EV70 viral isolates used in this study were obtained from the Shenzhen Prevention and Control Center during the acute hemorrhagic conjunctivitis epidemics in 1994, including three Beijing strains, two Shenzhen strains and five Guangzhou strains. Out of the 12 CV-A24V viral isolates used in this study, five were obtained from the Shenzhen Prevention and Control Center, from August to December 2003. The remaining seven CV-A24V viral isolates were obtained from the Guangzhou Prevention and Control Center, from May 2004 to February 2005. A total of 125 conjunctival swabs were collected from the patients in 2006 during an outbreak of acute hemorrhagic conjunctivitis in Shenzhen with 30,000 reported cases.

2.2. Design of primers and probes

The VP1 nucleotide sequences of 19 EV70 strains from the GenBank were analyzed. Using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) together with DNASTAR program, a highly conserved region of EV70 was defined. Primers and TaqMan probe were designed by Primer Express® Software (version2.0, PE Applied Biosystems, Foster City, CA, USA). Primers and probes for CV-A24V detection were essentially those described previously by Lévêque et al. (2007). The internal control uses the same primers as those of EV70, and a probe altered based on the probe of EV70. The three TaqMan probes were 5′-labeled with the fluorescent dye 6-carboxy-fluorescein (FAM), hexachloro-6-carboxy-fluorescein (HEX), indocarbocyanine (CY5) respectively, and 3′-labeled with the quenching dye black hole quencher 1 (BHQ1) or black hole quencher 2 (BHQ2). The sequences and details of primers and probes are shown in Table 1.

2.3. Design and preparation of internal control

The internal control nucleic acids (96 bp) contain primer binding regions identical to those of EV70 and a unique probe binding region, which had been altered for differentiation from the amplicon of EV70. The internal control was constructed according to the method described by Yoo et al. (2008) with slight modifications. Briefly, the internal control fragment was synthesized and then reamplified using primers of EV70Pf and EV70Pr. The amplicon were ligated directly into the pUCm-T vectors (Sangon, Shanghai, China) according to the instructions of the "T:A Cloning Kit". The pUCm-IC plasmid DNA was linearized with EcoRI restriction endonuclease and then used as the template for in vitro transcription by using RiboMAXTM Large Scale RNA Production System-T7 (Promega, Mannheim, Germany) following the manufacturer's instruction. After overnight incubation at 37 °C, the DNA template was removed from the transcribed RNAs by digestion with DNase. The transcribed RNAs were then purified by extraction with Trizol (Invitrogen, Madison, USA). After dilution, the concentration of the RNAs was calculated by measuring absorbance at 260 nm. The RNAs were then diluted serially 10-fold, ranging from 10 to 10^5 copies/μl, stored at −80 °C until use.

2.4. Preparation of RNA standards

In order to obtain a standard curve for the quantitative real-time PCR, serial dilutions of known copies of RNA standards were prepared. For EV70, PCR products amplified from EV70 templates were ligated directly into the pUCm-T vectors. Then in vitro transcription was performed following exactly the same procedure as described for the internal control. RNA concentration determination and storage procedure have been described previously. The RNA standards of CV-A24V were constructed and propagated in the same way.