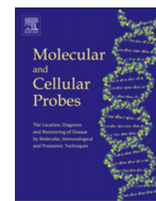
Molecular and Cellular Probes 25 (2011) 114–120

Contents lists available at ScienceDirect

Molecular and Cellular Probes

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



Single tube multiplex real-time PCR for the rapid detection of herpesvirus infections of the central nervous system

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ARTICLE INFO

Article history:
Received 31 July 2009
Received in revised form
6 February 2010
Accepted 16 March 2011
Available online 3 April 2011

Keywords:
PCR
Multiplex real-time PCR
Herpesvirus
CNS

ABSTRACT

Human herpesvirus infection of immunocompromised hosts may lead to central nervous system (CNS) infection and diseases. In this study, a single tube multiplex real-time PCR was developed for the detection of five herpesviruses (HSV-1, HSV-2, VZV, EBV and CMV) in clinical cerebrospinal fluid (CSF) specimens. Two primer pairs specific for the herpesvirus polymerase gene and five hybridization probe pairs for the specific identification of the herpesvirus types were used in a LightCycler multiplex real-time PCR. A singleplex real-time PCR was first optimized and then applied to the multiplex real-time PCR. The singleplex and multiplex real-time PCRs showed no cross-reactivity. The sensitivity of the singleplex real-time PCR was 1 copy per reaction for each herpesvirus, while that of the multiplex real-time PCR was 1 copy per reaction for HSV-1 and VZV and 10 copies per reaction for HSV-2, EBV and CMV. Intra and inter-assay variations of the single tube multiplex assay were in the range of 0.02%–3.67% and 0.79%–4.35%, respectively. The assay was evaluated by testing 62 clinical CSF samples and was found to have equivalent sensitivity, specificity and agreement as the routine real-time PCR, but reducing time, cost and amount of used sample.

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1. Introduction

Human herpesvirus infection can result in acute or chronic diseases. Primary as well as recurrent herpesvirus infections may lead to central nervous system infections and diseases like aseptic meningitis or encephalitis with a high mortality [1]. Therefore, early detection is important to decrease morbidity and mortality and to minimize persistent neurological damage [2]. Diagnosis based on clinical symptoms is often unreliable, mainly due to the fact that a wide variety of viruses must be considered as etiological agents of CNS infection [3,4]. Thus, a rapid and reliable laboratory diagnosis for CNS infection is important. The conventional laboratory diagnosis of CNS infections caused by these viruses has been found to be relatively unproductive [5,6]. HSV CSF cultures are positive in only about 4% of adult patients although the virus may be isolated in 25–40% of cultures obtained from infants with neonatal herpes, depending upon disease classification [6–8], whereas VZV isolation from CSF of patients with VZV-associated neurological syndromes are largely negative [9]. In cases CMV encephalitis, CSF cultures are rarely positive [10,11]. In addition, these diagnostic methods have limitations, as, for example, virus isolation is time-consuming, expensive and requires special facilities and skilled personal as well as fresh specimens with viable virus [12,13], whereas serological methods are often limited by antigenic cross-reactivity and may be unhelpful in the acute stage of disease or when applied to immunocompromised patients [13]. Due to these disadvantages, PCR has become an important tool for the detection of human herpesviruses in the cerebrospinal fluid (CSF) as it is highly sensitive and specific, rapid with a turnaround time of less than 6 h, and inexpensive. Moreover, multiplex PCR with the ability to detect simultaneously different herpesviruses in CSF has been developed and used in patients with various virus-related neurological diseases [1,3,14–16].

With the advent of real-time PCR, it has become a potential tool in biomedical research and clinical diagnostic applications [17]. Its principles are similar to that of conventional PCR, particularly in the main steps, i.e., denaturation, annealing, and extension. However, in the last step, the detection of PCR products, there are two

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doi:10.1016/j.mcp.2011.03.004

differences. The detection of amplified DNA by conventional PCR commonly uses gel electrophoresis and visualizes the results under ultraviolet light, whereas in real-time PCR, fluorogenic DNA-binding dyes or fluorochrome-labeled probes are used as the means for visualization of the results. This technique reduces detection time and the risk of amplicon contamination [18,19]. Up to now, multiplex real-time PCR has been described to detect two or three types of herpesviruses but it has not yet been developed to detect up to five herpesvirus types [29,30].

The aim of this study was to develop a single tube multiplex real-time PCR for the detection of herpesviruses, including herpes simplex virus (HSV) type 1 and 2, varicella–zoster virus (VZV), Epstein–Barr virus (EBV) and cytomegalovirus (CMV) in the CSF of patients with neurotropic infections. The results were compared with those obtained by routine singleplex real-time PCR.

2. Materials and methods

2.1. Viruses

The herpesviruses used in this study are herpes simplex virus type 1 strain KOS, herpes simplex virus type 2 strains Baylor 186, Epstein–Barr virus derived from the B95-8 cell line, the cytomegalovirus strain AD 169 and the varicella–zoster virus DNA strain Rod (Advanced Biotechnologies Inc., Columbia, MD).

2.2. Clinical specimens

Sixty-two CSF samples submitted to the Unit of Virology and Molecular Microbiology, Department of Pathology, Ramathibodi Hospital were extracted using the High Pure Viral Nucleic Acid Kit (Roche Applied Science, Germany) and subjected to herpesvirus detection by routine singleplex real-time PCR and our singleplex and multiplex real-time PCRs.

2.3. Primers and hybridization probes

In this study, two sets of primers and five sets of hybridization probes were designed by the LightCycler probe design software 2.0 with the use of the reference sequences of the polymerase gene of HSV-1, HSV-2, VZV, EBV, CMV retrieved from Genbank accession number NC_001806, NC_001798, NC_001348, NC_001345 and NC_001347, respectively. The sequences and concentrations of the primers and probes are detailed in Table 1. The quality of the primers and hybridization probes was primarily analyzed for specificity by Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [20] and BioEdit software [21] and for oligonucleotide interaction by the Oligoanalyzer software (http://www.idtdna.com/analyzer/applications/oligoanalyzer).

2.4. Singleplex and multiplex real-time PCR

Both singleplex and multiplex real-time PCR were done on a LightCycler 2.0 instrument (Roche Applied Science, Germany). For the singleplex real-time PCR, the 20 μl reaction contained 3 mM $MgCl_2$, 2 μl of 10x LightCycler FastStart Reaction Mix Hybridization Probe (Roche Applied Science, Germany), and 5 μl of DNA template. The primers and probes were added as outlined in Table 1. Cycling conditions were as follows: pre-incubation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 63 °C for 15 s and 72 °C for 25 s each. Melting analysis used 95 °C for 10 s and 63 °C for 30 s followed by increasing the temperature from 63 °C to 85 °C (0.1 °C/s) and continuous fluorescence recording. For the multiplex real-time PCR, the 20 μl reaction contained similar components to those of the singleplex real-time PCR except for the concentration of the primers and probes as detailed in Table 1. The probes and DNA templates used in the multiplex real-time PCR contained all five probes and five types of herpesvirus DNA, respectively. Cycling conditions were as follows: pre-incubation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 25 s each. The melting analysis was similar to that of the singleplex real-time PCR.

Table 1
Primers and probes used in the singleplex and multiplex real-time PCRs specific to the polymerase gene of five herpesviruses (HSV-1, HSV-2, CMV, EBV and VZV).

	Sequence (5′–3′)	Concentration in singleplex/multiplex real-time PCR (μM)
Primers:		
M4F	5′-tgK tBg act ttg cca gcc tgt acc-3′	0.5/1
M4R	5′–ABS SWg tcc gtg tcc ccg tag atg-3′	0.5/1
VR-TF	5′-tac ttg atc atc tgg acc aca atc acc-3′	0.5/1
VR-TR	5′-cag cga cga agg att aat tat tac ctg tag-3′	0.5/1
Probes		
HSV-1.1	5′-att ccc cag agc agc ccc gag ga–FL	0.15/0.15
HSV-1.2	LC Red 610– ccg tgc tcc tgg aca agc agc ag–P	0.3/0.3
HSV-2.1	5′-tct ccc tgc ggc ccg ag–FL	0.2/0.2
HSV-2.2	LC Red 610– cgt cgc gca cct gga gg–P	0.6/0.6
VZV 1	5′-cgt ccc tcc gtt gcc gcg agt a–FL	0.3/0.3
VZV 2	LC Red 640– acc att ctc ggc cca gtc caa tta tac t–P	0.3/0.3
EBV 1	5′-tct tta caa agt ggt aga cgc ccc ccg tg–FL	0.3/0.3
EBV 2	LC Red 670–cct gaa gga ctc ata gtc ttc tcc cgg gc–P	0.3/0.3
CMV–1	5′-gct ctg aaa agt tgt ctt tga tga acc gcg c–FL	0.3/0.6
CMV-2	LC Red 705–tgc gct cta gca tgt cgc gac cga t–P	0.3/0.6

2.5. Specificity, sensitivity and reproducibility

To determine the specificity of the assays, both singleplex and multiplex real-time PCR were performed against its corresponding type of herpesviruses, the other types of herpesviruses and other microorganisms frequently involved in CNS infection, e.g. *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Listeria monocytogenes*, *Mycobacterium tuberculosis* and adenovirus.

For the determination of sensitivity and reproducibility, five recombinant plasmids each containing a different type of herpesvirus polymerase sequence were constructed by amplifying the polymerase gene of the five types of the herpesvirus and the PCR products were ligated into pGEM-T Easy vector (Promega, U.S.A.). The sequences of all five plasmids were confirmed by sequencing. The sensitivity of both singleplex and multiplex real-time PCR were performed against a 10-fold serial dilution of plasmid DNA, ranging from 1 to 10,000 copies.

The intra-assay variation was determined by performing the PCR reaction in triplicate against five serial dilutions of plasmid DNA with 1, 10, 100, 1000 and 10,000 copies. For inter-assay variation determination, the PCR reaction was performed similar to that of the intra-assay variation for three consecutive days. The intra and inter-assay variations were calculated from the percentage of the coefficiency of variance (%CV) of the crossing point (Cp) values.

2.6. Data analysis

The reproducibility of the multiplex real-time PCR was determined by intra-assay and inter-assay variations. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the methods were determined according to Parikh et al. [22]. The agreement between the multiplex real-time PCR, singleplex real-time PCR and a routine singleplex assay was analyzed by κ analysis [23].

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