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Short Report

Development of a Sensitive Quantitative Competitive PCR Assay for Detection of *Human Cytomegalovirus* DNA

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ABSTRACT

Accurate and rapid diagnosis of human cytomegalovirus (HCMV) disease in immunocompromised patients has remained as a challenge. Quantitative competitive PCR (QC-PCR) methods for detection of HCMV in these individuals have improved the positive and negative predictive values of PCR for diagnosis of HCMV disease. In this study we used QC-PCR assay, using a co-amplified DNA standard, to quantitate the HCMV glycoprotein B (gB) gene in different samples. A DNA internal standard (IS) was designed by replacing HCMV primer binding site at 5' ends of primers that amplifies a 156-bp fragment of lambda genome, and a 200 bp amplicon was produced. Two DNA fragments of 257 bp wild type and 200-bp (IS) were co-amplified with the same oligonucleotide primer sets, analyzed by gel electrophoresis and used for construction of a standard curve. From this, the copy number of the gB gene present in different samples could be determined. Co-amplification with 1,000 copies of IS, allowed quantitation of 10-100,000 of HCMV DNA in a single PCR. This rapid assay avoids using radioactive components and other less efficient quantitative systems. It has the potential for early identification of patients at high risk of development of HCMV disease, and is useful for therapeutic monitoring. *Iran. Biomed. J. 9 (4): 187-191, 2005*

Keywords: Quantitative, PCR, Human cytomegalovirus (HCMV), Viral load, Immunocompromised patients

INTRODUCTION

Infection with human cytomegalovirus (HCMV) is an important cause of morbidity and mortality in immunocompromised individuals such as transplant recipients, AIDS patients and newborns [1, 2]. Similar to the other members of Herpesviridae family, HCMV establishes latent infection following primary infection, which occurs early in life in a majority of individuals [3]. Symptomatic HCMV infection is rare in immuno-competent people but is one of the most important opportunistic infections in immuno-compromised patients. In general, infection in the HCMV seropositive immunocompromised hosts can be classified into three forms: latent reactivated non-symptomatic and symptomatic infections. In the seronegative immunocompromised hosts, the primary infection follows a more severe course [1]. Differentiation between HCMV infection and HCMV disease is one of the most important problems in such patients. Since anti-HCMV chemotherapy is now commonly used, there is an essential need for fast, reliable and sensitive techniques to allow early HCMV diagnosis and to assess the efficacy of antiviral therapy [1, 4].

Various laboratory techniques are being used for HCMV diagnosis. These include virus culture and isolation, shell vial technique [3], direct immuno-staining of white blood cell (WBC) such as pp65 antigenemia assay [5, 6] and PCR [7, 8]. Some of these methods such as viral isolation lack desirable sensitivity. On the other hand, molecular techniques such as PCR assay have increased sensitivity in

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diagnosis of HCMV infection. The high sensitivity of PCR, which is associated with poor predictive value, has limited the value of this assay in differentiating HCMV disease from infection. However, more recent application of this technique, quantitative PCR, has been used to determine relative or absolute amounts of target HCMV DNA subjected to the amplification reaction. Quantitative competitive PCR (QC-PCR) can accurately and reproducibly determine the systemic and site-specific HCMV viral load [4].

Progress in the optimization of QC-PCR assays has developed into co-amplification of competitive internal standards (IS) The composition of these IS may be close to that of the target DNA. Alternatively, they may be fairly heterogeneous but importantly amplify with the same primers as those of the target DNA sequence [4, 9, 10]. Quantitation is achieved by initially providing a standard curve in which varying known amounts of target DNA are co-amplified with a fixed amount of IS, and the ratio of both target and IS amplicons are plotted against the input amount of target DNA. Unknown target DNA quantities can be determined by interpolation from this standard curve [4, 9-11].

In this study, a QC-PCR assay for HCMV with high sensitivity and specificity was developed in order to determine viral copy number in peripheral blood mononuclear cells and plasma of immunocompromised patients.

MATERIALS AND METHODS

Virus and tissue culture. A clinical isolate of HCMV was used in this study as the positive control. This strain had been previously approved by monoclonal antibody immunostaining and restriction enzyme analysis and kindly provided by S. Amini-bavil-olyaee (Virology Department, Tarbiat Modarres University, Tehran, Iran) [12]. Human diploid fetal foreskin fibroblast (HFFF, the Pasteur Institute of Iran, Tehran) cells were grown in DMEM supplemented with 10% fetal bovine serum (Gibco, England). These cells were used to propagate the virus.

Virus isolation. Urine samples from kidney transplant patients with suspected HCMV infection (supplied by Dr. Jamshid Roozbeh, Department of Transplantation, Namazi Hospital, Shiraz University of Medical Sciences, Shiraz) were inoculated into HFFF monolayer cells and examined daily until typical HCMV cytopathic effect of 30-40% was observed. The cell cultures were then frozen at -70^{o}C until their use for DNA extraction. Uninfected HFFF cells were used as negative controls.

Primers. Two 22-base primers: upstream primer, 82494-82515 nucleotide position (n.p.): 5′-3′ CGGTGGAGATACTGCTGAGGTC and downstream primer, 82729-82750 nucleotide position (n.p.): 5′-3′ CAAGGTGCTGCGTGATATGAAG) were designed by Generunner computer software that targeted glycoprotein B (gB) coding region of HCMV genome (Full HCMV genome sequence NCBI: NC-001347 associated number) and amplified a 257-bp fragment.

PCR Optimization. PCR optimization was performed using various amounts of $MgSO_4$, dNTPs and primers and at different annealing temperatures using several dilutions of template DNA in a matrix format. Optimization of different enhancers was also performed using enhancers in a matrix format. For the amplification of fragment in each sample, 5 µl (1 µg) extracted DNA or control DNA (extracted from anti-HCMV IgM negative newborn cord blood WBC) was added to a reaction mixture containing dNTPs, 200 µM; 25 pmol of each primer, $MgSO_4$, 2 mM; KCl, 10 mM; $(NH_4)_2S0_4$, 10 mM; Tris-HCl (pH 8), 20 mM; Triton X-100, 0.1%; BSA, 0.1 mg/ml and PFU, 1 U; (Fermentas, Lithuania) in a total reaction volume of 50 µl. The samples were then amplified for 38 cycles in an oil free automated thermal cycler, (Eppendorf master cycler, Germany) consisting of first denaturation at 95^{o}C for 6 min, followed by cycles of denaturation at 94^{o}C for 45 s, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 1 min, with a final incubation at 72^{o}C for 8 min. The PCR products were separated on 2% agarose gels and stained with 2 µg/ml ethidium bromide solution. The stained gel was photographed using gel documentation instrument with a digital imaging system (UV tech, Germany).

IS and wild type (wt) plasmid construction. In QC-PCR, co-amplification of a gene fragment with similar physical characteristics and primers binding site but different internal sequence from those of the target gene is necessary.

In this study, a 156-bp fragment of bacteriophage lambda genomes was chosen and amplified using a primer pair containing HCMV primer binding sites at 5' ends (Table 1).

Table 1. The sequence and position of oligonucleotides used in the production if IS sequence

Oligonucleotide		Sequence (5'-3') Position
Forward Primer	CGGTGGAGATACTGCTGAGGTCTGCGTGTAGGCGAATTTG	40954-40971
Reverse Primer	CAAGGTGCTGCGTGATATGAACCCACCGGAGAAACTAACGAC	40954-40971

The 200-bp amplicons were electrophoresed on 1.5% low melting point (LMP) agarose gel and stained with ethidium bromide. The visible bands were cut on a UV illuminator and placed in microfuge tubes containing 500 µl Tris buffer and then incubated at 60°C water bath for 10 min prior to purification of desired amplicons by standard phenol-chloroform method [13]. The chimeric 200-bp IS sequence was then cloned into PTZ57R plasmid at Eco 321 cut site with blunt–end cloning technique and constructed plasmid was transformed to competent *E. coli* X L1 Blue by heat shock and $CaCl_2$ [13]. Recombinant clones were identified on LB Agar with IPTG, XGAL plus Ampicillin (blue/white screening) and recombinant plasmids were purified by a Mini-preparation protocol and then further purified by LMP agarose [13] (Fig. 1). The DNA copy number (the plasmid copy number) was calculated from the molecular weight of the DNA construct and Avogadro number. The wt amplicons were purified, cloned and their copy numbers were calculated as mentioned above.

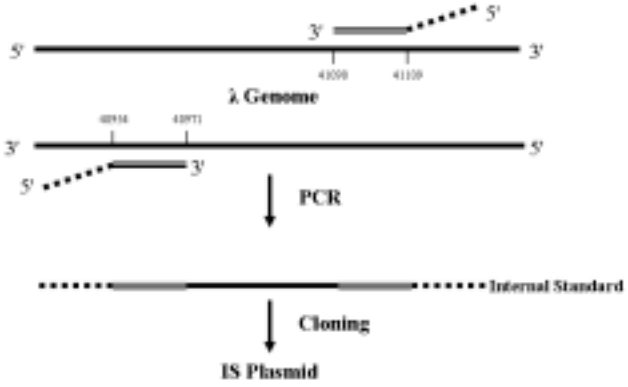

Fig. 1. Illustration of internal standard construction. The primers used to amplify the 200 bp are shown. The 3' ends of the primers correspond to bases 40954 to 40971 and 41090 to 41109 of the bacteriophage lambda genome. The 5' ends of primers have the same sequences as the primers that are used to amplify the HCMV gB.

QC-PCR optimization. Serial 10-fold dilutions of gB DNA fragments were co-amplified with known amounts of IS for determination of the standard curve. PCR products were separated on 2% agarose gel and stained with ethidium bromide and photographed. Images were analyzed using Scion image software as described previously [14]. Using this software, bands appeared on agarose gel can be quantified. Following optimization of the number of IS copies for co-amplification in QC-PCR, the standard curve was drawn by plotting the log ratio of wt/IS amplicons against the log of the gB copy number added to each PCR.

RESULTS

Virus isolates. All HCMV cell culture isolates that were approved to be positive by a commercial HCMV PCR kit (Cinagen, Tehran, Iran) were also positive using designated primers in this study.

QC- PCR studies. Our Experiments showed 1000 IS copies were the optimal number to be co-amplified with serial dilutions of target HCMV gB DNA fragments. This quantity of IS allowed sensitive detection of 10 gB DNA copies following co-amplification. Whereas, higher amounts of IS caused a decrease in the sensitivity of the PCR. Although sensitive detection of 10 gB DNA copies was also achieved with 100 IS copies, co-amplification with gB DNA number greater than 10,000 copies decreased amplification of the IS to below the limits of detection by the PCR. In addition, these studies showed when equal numbers of IS and gB DNA fragments were co-amplified, equal amounts of amplicons were produced indicating no differences between the amplification efficiency of the two DNA fragments for detection in the PCR. Regarding sensitivity, our PCR assay could detect about 10 copies, and measured DNA quantitatively from 10 to 100,000 copies in the extracts (Fig. 2). The addition of 1 µg of HCMV seronegative human genomic DNA to PCR reaction did not cause a decrease in the sensitivity or changed in the above detection range. The specificity of the designed primer sets was verified by PCR analysis of DNA extracts of negative clinical specimens and those of HSV1, HSV2, varicella-zoster virus and EBV infected immortal B cells. There was not any detectable band after gel electrophoresis analysis of these samples.

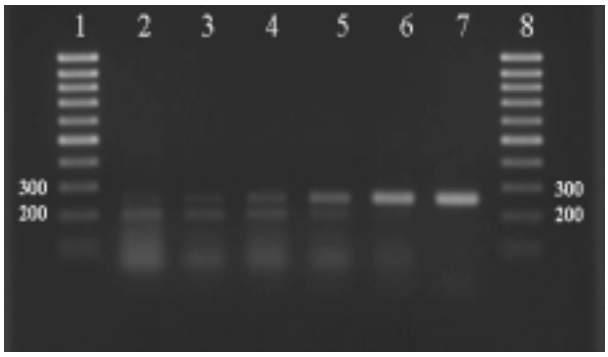

Fig. 2. Agarose gel electrophoresis of QC-PCR amplification products stained with ethidium bromide and visualized under UV illumination. Lanes 1 and 8, molecular weight markers; lanes 2-7, input number of 1000 copies of the IS plasmid and 10 fold increasing input number of the plasmid containing the wt DNA: lane 2, 10 copies; lane 3, 100 copies; lane 4, 1,000 copies; lane 5, 10,000 copies; lane 6, 100,000 copies; lane 7, 1,000,000 copies.

The standard curve. Serial gB DNA fragment dilutions were co-amplified with 1,000 IS copies in three separate experiments and the signals generated by amplicons were detected by photodensitometric analysis. The mean ratio of gB and IS amplicons was calculated for each input of gB DNA copies, and plotted against the number of gB DNA copies added to each PCR (Fig. 3).

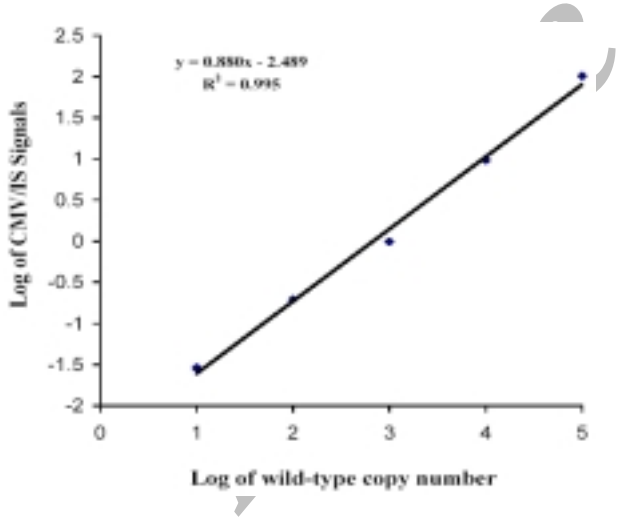

Fig. 3. Construction of a standard curve. Linear relation between logarithm of the wt/competitor PCR products ratio and logarithm of the copy number of input wt DNA amplified along with 1000 competitor copies. The best-fit line was determined using the Microsoft Excel software. Statistical analysis was performed using SPSS 11.5 software.

DISCUSSION

QC-PCR is considered as one of the useful diagnostic techniques for identification and prognosis of viral infections. In many conditions such as HCMV infection in immunocompromised patients, the qualitative PCR positively cannot be alone considered as an indicator of the disease [4, 15]. However, it has been shown that quantification of viral genome can give the physician a more accurate picture of the patient's situation. Various QC-PCR techniques for different viruses such as HCV [16], HIV [17], HCMV [18-20], HHV6 and HHV7 [14, 21] have been developed and are being used in clinical/research settings.

In this study, a QC-PCR with similar efficiency is developed to amplify both IS and target genomes. Using this technique, detection and quantification of the products become easier and faster and the results can be reported in less than 5 h. In similar studies, different strategies have been used for detection of the products. Fryer *et al.* [22] have used PAGE to evaluate their QC-PCR products. PAGE is necessary to distinguish bands with close molecular weights; however, the ease of work with agarose gel has encouraged researchers to use it. Kidds *et al.* [14] have introduced mutations in the target fragment in order to develop their IS. The resulting two products had very similar sizes and this forced them to use restriction endonucleases to cut the fragments in a manner which could be distinguished on the gel. It should be noted that a high level of similarities between the sequences in the designed IS and those of target makes it difficult to use more sensitive methods such as probe hybridization and ELISA for detection of both products in the next stages of development of QC-PCR. Chatellard *et al.* [23] have used radioactive probes to distinguish between target and IS upon blotting their products on the membrane. Then, they used photography and densitometric analysis to compare the bands.

The diagnosis and quantification of the products becomes easier and faster by using technique mentioned in this study. This procedure has the advantage of avoiding an enzymatic restriction digestion step, the use of simple agarose gel instead of PAGE and no use of radioactive materials. Using a single tube per sample, this approach allowed accurate determination of HCMV DNA copy number in samples over a range of 10-100,000 copies. This detection range can be adjusted by changing the amount of the competitor introduced in the reaction and the PCR cycle number for increased or decreased sensitivity. This study suggests that this technique could be useful in the monitoring of patients at risk of HCMV symptomatic infection, for the evaluation of the efficacy of antiviral treatment and the investigation

of the HCMV infection pathogenesis. Thus, this detection method facilitates the introduction of QC-PCR to a routine laboratory.

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