# Detection and Typing of HSV-1, HSV-2, CMV and EBV by Quadruplex PCR

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The development of a multiplex polymerase chain reaction (PCR) method for rapid and accurate detection and typing of herpes simplex virus type 1 (HSV-1), and type-2 (HSV-2), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) is very important for clinical diagnosis to allow the deliver of therapy as early as possible. Large scale amplifications by multiplex PCR of viral DNA can lower the cost and time for viral diagnosis. In this study, therefore sensitive quadruplex PCR was achieved by optimizing parameters such as primers, and 1.5 mM magnesium and 200 uM dNTPs concentrations. The concentrations of HSV-1, HSV-2, CMV and EBV primers were 0.5, 0.3, 0.25 and 0.25 pmoles, respectively. Optimal annealing temperature was 54°C. Employing these conditions, we could detect 10 copies of reconstructed template plasmid DNA, which were cloned to vectors containing target sequences of viral DNA. PCR products of 271 bp for HSV-1, 231 bp for HSV-2, 368 bp for CMV, and 326 bp for EBV were separated on 5.0% polyacrylamide gel electrophoresis and confirmed by direct sequencing.

The present study showed that the quadruplex PCR assay described herein has potential application in clinical diagnosis, when rapid, accurate detection and typing of viruses HSV-1, HSV-2, CMV or EBV are necessary.

**Key Words:** Diagnosis, multiplex PCR, virus

## INTRODUCTION

Several human virus infections play an important role in human diseases. Herpes simplex virus type-1 (HSV-1), and type-2 (HSV-2), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are widespread human viruses frequently detected in specific tumors and immunosuppressed individuals. HSV-1 and HSV-2 cause meningitis, fatal encephalitis and other ocular diseases such as uveitis, keratitis, retinitis and acute retinal necrosis syndrome, and result in a significant mortality in neonates. Especially, CMV and HSV-2 have been demonstrated to display an oncogenic potential *in vitro*.[1-4] There have been numerous reports linking CMV and EBV to tumors, lymphoepithelial carcinoma, smooth muscle tumors and esophageal carcinoma, and suggesting that these viruses transactivate cellular oncogenes such as *fos, jun* and *myb*.[5-7] As they are also associated with many diseases, primary diagnosis of the latency period is important for therapy.

In the past, antibodies were developed for their diagnosis in many clinical pathology laboratories. Diagnosis of theses viruses is clinically difficult and usually depends on the isolation of the virus by tissue culture methods, by which some viruses grow slowly for one to several weeks.[8,9] Many authors have reported diagnostic methods to distinguish between HSV types which have a very similar DNA structure. HSV-1 and HSV-2 have been distinguished by various techniques including their different sensitivity to ($\varepsilon$)-5-(2-bromovinyl)-2'-deoxy-thymidine (BVDU), differential replication in chick embryo cells versus guinea pig embryo cells,[10] restriction endonuclease,[10-12] recognition by polyclonal or monoclonal antibodies[10,12,13] including immunofluorescence assay methods,[8] detection of viral nucleic acid by DNA hybridization,[14,15] and finally polymerase chain reaction (PCR). Serologic data, frequently used for diagnosis of

viral infections, represent only indirect markers of infection and are often unreliable in immunocompromised patients, whereas PCR can detect only the presence of viral genomes.[2,16-18] Because antiviral therapy is effective in the early stage of disease processing, the diagnosis of EBV, CMV, HSV-1 and HSV-2 (ECH12) has been improved by the (PCR) technology,[19,20] which allows rapid and sensitive detection of herpes viruses. The PCR method has replaced serological assays which are based on the detection of virus-specific IgM antibodies. Furthermore, multiplex PCR can save time and cost by simultaneously amplifying multiple sequences in a single reaction.[21] An efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize reaction conditions. In many cases, in order to obtain optimal multiplex PCR condition, an optimal single PCR condition has to be first established. In such a situation, the PCR primer pairs will retain target specificity. In the present study, we described the development of a quadruplex PCR method for the detection of HSV-1, HSV-2, EBV and CMV (ECH12) in a single step reaction.

## MATERIALS AND METHODS

### Samples

Infected samples of HSV-1, HSV-2 and CMV (ECH12) were obtained from Seoul Clinical Laboratory (SCL), and EBV was from the Department of Clinical Pathology, Wonkwang University Medical College. These samples were used as positive controls. Genomic DNA, used as negative control, was extracted from peripheral blood by Tris buffered phenol/chloroform and precipitated with ethanol.

### PCR amplification

The primers used for HSV-1 and HSV-2 PCR corresponded to the glycoprotein D coding sequence, which was the major target of vaccination studies for highly immunogenic and inducing neutralizing antibodies. The PCR primer sequences for detection of CMV and EBV were designed with the cording sequences of the structural proteins of virus, UL56 and late membrane

protein 1 (LMP-1), respectively. The HSV-1 primer sequences were 5'-CGTACCTGCGGCTCGTGAA GT-3' as the forward and 5'-AGCAGGGTGCTC GTGTATGGGC-3' as the reverse; 5'-TGGTATCG CATGGGAGACAAT-3' for the forward and 5'-CTCCGTCCAGTCGTTTATCTTG-3' for the reverse primer of HSV-2; 5'-ACGTGTTACTGGCG GAGTCG-3' for the forward and 5'-TTGAGTG TGGCCAGACTGAG-3' for the reverse primer of CMV; 5'-AGCACTGGCCAGCTCATATC-3' for the forward and 5'-TTGACGTCATGCCAAGGC AA-3' for the reverse of EBV. The expected sizes of the amplified sequences in the HSV-1, HSV-2, CMV and EBV genomes were 271, 231, 368 and 326 base pairs, respectively. Single PCR was undertaken in a final $20 \mu l$ volume containing 1.0 U *Taq* polymerase, 1X polymerase buffer [containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3)], 1.5 mM $MgCl_2$, 200 uM dNTPs and 0.5 pmole primers using a GeneAmp PCR System 2400 (Applied Biosystems, Foster, California, USA). PCR amplification conditions were, predenaturation step at 95℃ for 3 min., followed by denaturation at 95℃ for 30 sec., annealing at 54 ℃ for 30 sec. and primer extension at 72℃ for 30 sec. The final extension was performed for another 7 min, repeating 45 cycles of amplification.

### Detection of amplified products

The amplified PCR products were electrophoresed on 2.0% agarose gel and visualized with UV light after staining with ethidium bromide. Amplification products were also separated by electrophoresis through a 0.4 mm to 1.2 mm discontinuous gel and 5% T-5% C denaturing polyacrylamide gel containing 1 X TBE and 7M urea.

Five microliters of each amplified DNA sample were mixed with $3 \mu l$ of loading solution (2 mM EDTA, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95℃ for 3 min, and chilled on ice prior to loading. Electrophoresis was performed at 55 W for 140 min with the SA32 electrophoresis apparatus (Gibco BRL, Gaithersburg, Maryland, USA).

### Silver staining

After electrophoresis, the gel was washed with

triple distilled water for 1 min, and immersed in 1% nitric acid for 5 min. The oxidized gel was washed with triple distilled water for 1 min prior to treatment with 0.12 M silver nitrate solution for 20 min. The washed gel in distilled water was developed with 0.28 M anhydrous sodium carbonate and 0.19% formalin solution. The stained gel on 3 MM Whatman paper was dried at 80℃ for 1 hr with a vacuum gel dryer (Amersham Biosciences, Upssala, Sweden).

## Plasmid construction of ECH12 DNA

In order to calculate the amount of genomic DNA from PCR amplifications, PCR amplified products of 271, 231, 368 and 326 bps from the control strain were inserted into multicloning sites of 3015 bps pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) plasmid DNA. Plasmid DNA from cultured bacteria was extracted and purified as described by Sambrook et al.[22] The original number of each ECH12 copys was determined by optical density (OD) against the molecular weights of different dilutions of these plasmid DNAs, and the copy numbers of ECH12 DNA were calculated to be $2.724 \times 10^{11}$ molecules/$1.0\,\mu$g/uL, $2.712 \times 10^{11}$ molecules/$1.0\,\mu$g/uL, $2.772 \times 10^{11}$ molecules/$1.0\,\mu$g/uL and $2.802 \times 10^{11}$ molecules/$1.0\,\mu$g/uL, respectively.

## Sequencing of positive control DNA

For confirmation of sequences, fragments of each virus amplified through single PCR were resolved on agarose gel. After electrophoresis, the DNA band of interest was excised from the gel and eluted with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). About 50 ng of eluted amplified DNA were used as a template for direct sequencing. Sequencing was performed with ABI PRISM BigDye[TM] Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems) using an ABI PRISM[TM] 310 Genetic Analyzer (Applied Biosystems, Foster, California, USA).

## Multiplex PCR

Multiplex PCR was also performed with Gene Amp PCR System 2400 (Applied Biosystems,

Foster, California, USA). Preheating for 3 min. at 95℃, followed by 45 amplification cycles of 30 sec at 94℃, 10 sec at 54℃, and 30 sec at 72℃ were carried out in a final 20 uL volume containing 2 uL of GeneAmp 10X PCR Buffer (PE Biosystems, USA), 0.2 mM each of deoxynucleotide triphosphates (dNTP), 1 unit of *Taq* polymerase (Solgent, Korea) and 1.5 mM $MgCl_2$. The control contained 5 uL of DNA sample (positive control of ECH12 DNA or uninfected genomic DNA) which was added to the reaction mixture. After the last cycle, the samples were incubated for 7 min at 72℃ to complete the extension of the primers. To optimize the buffer concentration of multiplex PCR, we performed a series of reactions to determine the optimal primer and $Mg^{2+}$ concentrations and dNTP levels. Five microliters of each amplified products were analyzed on 2.0% agarose gel or 5% T-5% C denaturing acrylamide gel containing 7M urea.

## Specificity and sensitivity

The specificity of the PCR reaction was examined by varying the cycle numbers with 1 ng of mixed positive control viral DNA which had been inserted into the plasmid vector. Also, variable annealing temperatures and times for each single PCR and quadruplex PCR were examined. To evaluate the PCR sensitivity, a decreasing number of plasmid DNA copies, ranging from $10^8$ copies to 1, was amplified. The specificity and sensitivity were examined for each primer pair and also for the combined four primer pairs in the multiplex PCR.

## RESULTS

### Optimization of a single PCR

PCR was carried out under various conditions which affect amplification, such as cycle numbers, $MgCl_2$ and dNTPs concentrations, annealing temperatures, and time. The PCR reactions were carried out for various cycle numbers, ranging from 15 to 50 cycles, at increments of 5 cycles, and for various temperatures, ranging from 54℃ to 60 ℃, at increments of 2℃. EBV, CMV, HSV-1 and
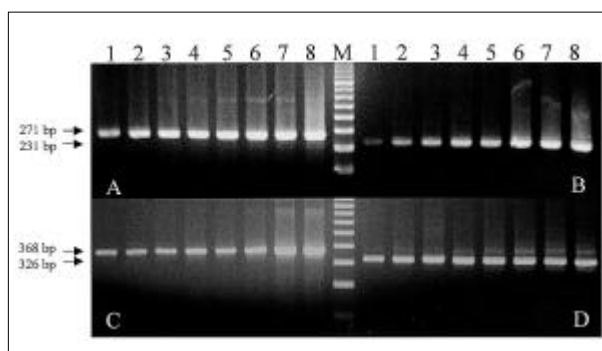
**Fig. 1.** Electrophoresis-analysis on agarose gel of single PCR products obtained with HSV-1 (A), HSV-2 (B), CMV (C) and EBV (D) primers. The upper panel shows amplified products which were 271 bp and 231 bp of HSV-1 and HSV-2, respectively. The lower panel shows 368 bp and 326 bp of CMV and EBV, respectively. Lane 1 was carried with 15 cycles, at increments of 5 cycles. M; 100 bp ladder.
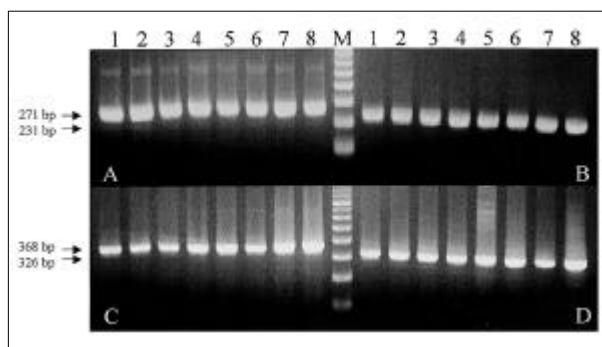


**Fig. 2.** Amplification as a function of annealing temperatures and time. PCR reactions of lanes 1 were performed at 54℃ for 30 sec. Annealing temperature was increased two degrees at each reaction and annealing times were 30 sec and 60 sec at a same degree. Upper panel; HSV-1 (A) and HSV-2 (B), Lower panel; CMV (C) and EBV (D), M; 100 bp ladder.

HSV-2 were amplified to the sizes of 326 bp, 368 bp, 271 bp and 231 bp, respectively (Fig. 1 and 2).

## Optimization of multiplex PCR

In order to obtain the optimal condition for multiplex PCR reactions, different dNTPs and $MgCl_2$ contractions were examined. Since $MgCl_2$ and dNTPs concentrations are dependent on each other and also on the primer concentration, multiplex PCR reactions were performed with template plasmids of $10^8$, $10^3$, $10^2$ and 10 copies, and in 1.5 mM, 2.0 mM and 2.5 mM $MgCl_2$ (Fig. 3). All of the amplified PCR products of ECH12 also included
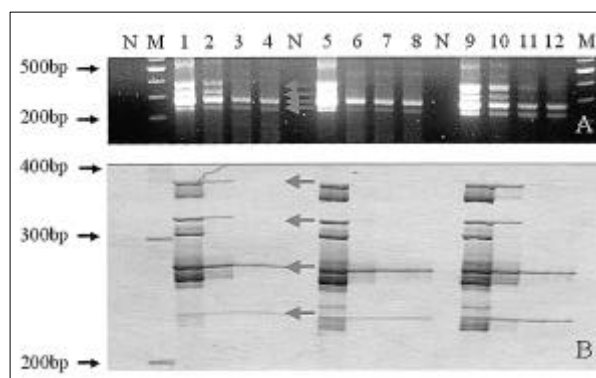


**Fig. 3.** Effect of $MgCl_2$ and template concentrations on multiplex PCR. The PCR products obtained with HSV-1, HSV-2, CMV and EBV primers were electrophoresed on 2.0% agarose gel (panel A) and polyacrylamide gel (panel B) under the various concentrations of $MgCl_2$ and template. N; negative control (10 ng of uninfected genomic DNA), lanes 1-4; 1.5 mM $MgCl_2$, lanes 5-8; 2.0 mM $MgCl_2$, lanes 9-12; 2.5 mM $MgCl_2$, lanes 1, 5. 9; $10^8$ copies of template DNA, lanes 2, 6, 10; $10^3$ copies of template DNA, lanes 3, 7, 11; $10^2$ copies of template DNA, lanes 4, 8, 12; 10 copies of template DNA. Arrows indicate amplified fragments, 231 bp, 271 bp, 326 bp and 368 bp. M; 100 bp ladder.

unexpected DNA under the condition of each $MgCl_2$ concentration, containing $10^8$ and $10^5$ copies of template DNA with silver stained gel (Fig. 3B, lanes 1, 2, 5, 6, 9, 10). With $10^3$ copies of template DNA at 1.5 mM and 2.5 mM $MgCl_2$, four kinds of amplified fragments of 231bp, 271 bp, 326 bp and 368 bp were detected (Fig. 3, lanes 2, 10), whereas 326 bp and 368 bp fragments were not detected with 2.0 mM $MgCl_2$ (Fig. 3, lanes 2, 6, 10). However, at all the concentrations of $MgCl_2$ and template plasmid tested, the fragments of HSV-1 and HSV-2 were amplified and detected (Fig. 3). When these PCR products were also electrophoresed on 5% polyacrylamide gel followed by silver staining, the results clearly indicated artifacts and a higher sensitivity than that performed on agarose gel (Fig. 3B). The best results were obtained with 1.5 mM $MgCl_2$ for the amplification of ECH12, therefore, this level was used to determine the optimal concentration of dNTPs. Thus, at the fixed concentration of 1.5 mM $MgCl_2$, multiplex PCR was performed with different concentrations of dNTPs (200 uM, 300 uM and 400 uM). As shown in Fig. 4, DNA patterns of amplified fragments were detected with $10^2$ copies of template plasmid. How-
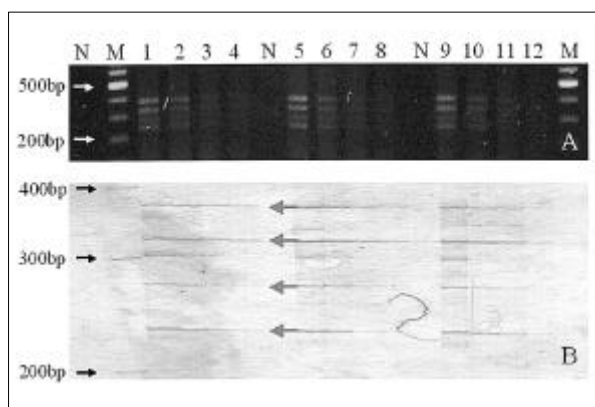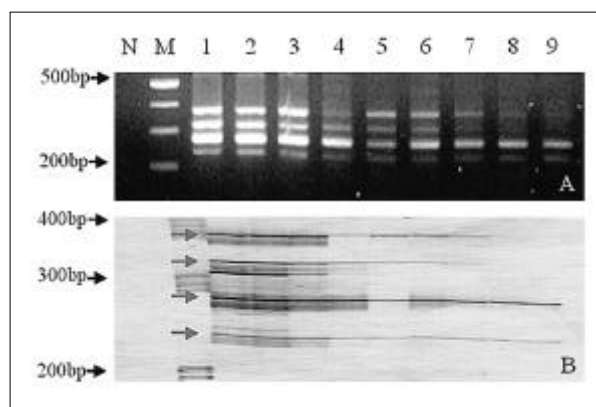
**Fig. 4.** Effect of dNTPs and template concentrations. Agarose gel (panel A) and acrylamide gel (panel B) analysis of multiplex PCR products obtained with HSV-1, HSV-2, CMV and EBV primers. N; negative control (10 ng of uninfected genomic DNA), lanes 1-4; 200 uM dNTPs, lanes 5-8; 300 uM dNTPs, lanes 9-12; 400 uM dNTPs, lanes 1, 5. 9; $10^8$ copies of template DNA, lanes 2, 6, 10; $10^3$ copies of template DNA, lanes 3, 7, 11; 102 copies of template DNA, lanes 4, 8, 12; 10 copies of template DNA. Arrows indicate amplified fragments, 231 bp, 271 bp, 326 bp and 368 bp. M; 100 bp ladder.



**Fig. 5.** Effect of copy numbers of template DNA on multiplex PCR. Multiplex PCR was performed with 1.5 mM $MgCl_2$ and 2.0 uM dNTPs. Agarose gel (panel A) and acrylamide gel (panel B) analysis of multiplex PCR products obtained with HSV-1, HSV-2, CMV and EBV primers. N; negative control (100 ng of uninfected genomic DNA), lanes 1-9; $10^8$-1 copies of template DNA. Arrows indicate amplified fragments, 231 bp, 271 bp, 326 bp and 368 bp. M; 100 bp ladder.

ever, different concentrations of dNTPs, including 200 uM, 300 uM and 400 uM, showed no difference. On the other hand, these fragments of 10-copy concentration were easily detectable on silver stained gel (Fig. 4B). These results, therefore, indicated that a 200 uM concentration of dNTPs was sufficient to amplify $10^8$ copies of template plasmid.

### Specificity and sensitivity

In order to establish the lowest possible detection limit for all four primer pairs of ECH12 in the multiplex amplification, amplification was performed with serial dilutions, ranging from $10^8$ to 1 copy, of template under the optimal $MgCl_2$ and dNTPs concentrations (Fig. 5). Also, 10 ng of the genomic DNA obtained from a human who was not infected with these viruses were used as a negative DNA control (Fig. 5, lane 1). Amplified PCR products of ECH12 were detectable with $10^2$ copies of template plasmid, and EBV could not be detected as a clear amplification band at a concentration lower than $10^2$. On the other hand, HSV-1 and HSV-2 were detected with this copy concentration, and artifact DNA bands were

observed with $10^5$ copy template plasmids (Fig. 5B). Indeed, PCR products of HSV-1 and HSV-2 were detected with 1 copy template plasmid on agarose gel. Four kinds of predicted fragments of ECH12 with 10 copies were detected on silver stained polyacrylamide gel (Fig. 5B, lane 8), but not on agarose gel (Fig. 5A, lane 8).

### DISCUSSION

Many molecular biological data suggest that certain types of some members of the *herpesviridae* family such as Epstein-Barr virus, human herpes simplex virus type 2 and cytomegalovirus may play an oncogenic role[1] and also induce neurological syndromes such as aseptic meningitis, polyradiculitis and acute encephalitis. Furthermore, these viruses are ubiquitously distributed, causing a wide range of acute central nerve system (CNS) diseases either during primary infection or in the course of virus reactivation or reinfection. Detection of viral DNA by PCR has been shown to be a sensitive and specific method for rapid diagnosis.[16]

We herein described a rapid and accurate multiplex PCR method for the detection and typing of HSV-1, HSV-2, CMV and EBV. In a multiplex

PCR, several parameters, which may affect the structure of primers and affinity to templates DNA, should be considered.[21] Multiplex PCR includes the problems of interference between the different primers, which can lead to nonspecific amplimers, and also coordinate amplification of the bands.[23] A sensitive assay condition was established by optimizing the concentrations of dNTPs and $MgCl_2$ that are necessary for elongation. In each single PCR, one-half unit of *Taq* polymerase was used and the amount of newly synthesized DNA fragments was found to be propositional to different cycling numbers. However, 50 PCR cycles were carried out in the present study (Fig. 1). The primer pairs of this study were found to be independent of annealing temperature and time. With different ranges of annealing temperature and time, PCR amplified fragments were visualized with ethidium bromide staining under UV light on 2.0% agarose gel. Nonspecific bands of about 550 bp were detected in HSV-1 reactions (Fig. 2). Nonspecific bands of the same size were also detected among the multiplex PCR fragments which were performed at higher template concentrations (Fig. 2 and 3). It was highly possible that these fragments might have been produced by primers of HSV-1, because these fragments were also detectable among the products of single PCR. And with multiplex PCR using a higher concentration of template DNA ($10^5$ copies), artifact bands at sizes of about 260 bp, 264 bp and 310 bp, which were not separated on agarose gel, were also detected by silver stained gel (Fig. 3B, 4B and 5B). These results strongly indicate that diagnosis using multiplex PCR methods should be carried out by polyacrylamide gel and silver staining, because rapid and accurate diagnosis is the most important factor for patients' therapy, and that their diagnosis by single PCR may be delayed. Our results indicated that multiplex PCR was successful with 10 copies of template and did not produce any amplified DNA fragments in the negative control (10 ng of genomic DNA), when agarose gel and silver stained polyacrylamide gel were used.

In the present study, optimal conditions of multiplex PCR were established, and amplified fragments were detected with high sensitivity by denaturing polyacrylamide gel. Quadruplex PCR was capable of clinically diagnosing HSV-1, HSV-2, CMV and EBV in one PCR reaction.

## ACKNOWLEDGEMENTS

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