Development of a Quadriplex Polymerase Chain Reaction for Human Cytomegalovirus Detection

Panayotis Markoulatos,¹ Vicky Samara,¹ Nikolaos Siafakas,¹ Elias Plakokefalos,¹ Niki Spyrou,¹ and Maurice L. J. Moncany^{2*}

¹Virology Department, Hellenic Pasteur Institute, Athens, Greece

²Laboratoire de Biologie Cellulaire et Moléculaire, Université de La Rochelle, La Rochelle, France

The development of a quadriplex PCR method with amplification of HCMV in a single-step procedure using primers taken from four different regions of the viral genome is described. Different concentrations of dNTPs and MgCl₂ were assayed in order to optimize the constitution of the buffer for the multiplex PCR. The specificity of the PCR was tested with 100ng, 10ng, and 1ng of genomic MRC-5 cell DNA infected with CMV in the presence of 10μg of uninfected MRC-5 cell DNA. The sensitivity of the PCR was evaluated by the amplification of various amounts (100ng, 10ng, 1ng, and 0.1ng) of genomic MRC-5 cell DNA infected with CMV. The specificity and sensitivity assays were performed for each pair of primers and for the combined four primer pairs in the multi-

plex PCR. CMV was consistently detected from 10ng of genomic MRC-5 cell DNA with each primer pair. When all four sets of primers were combined in a single reaction tube, the sensitivity of the assay was equivalent to 10ng of genomic MRC-5 cell DNA, whereas amplification from 1ng genomic MRC-5 cell DNA produced only a subset of the amplimers. By amplifying four target-sequences of HCMV simultaneously with minimum incubation time at each temperature, a quadriplex, highly sensitive PCR assay was performed. The use of four primer sets designed in different genomic regions of HCMV allowed the detection of variants and achieved maximal sensitivity and specificity which are essential for a diagnostic utilization. *J. Clin. Lab. Anal.* 13:99–105, 1999. © 1999 Wiley-Liss, Inc.

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INTRODUCTION

Human cytomegalovirus (HCMV) infections play an important role in human diseases. The virus is a major pathogen in immunosuppressed individuals (1,2) and a common infectious cause of congenital abnormalities (3). Intra-uterine infection with CMV is the most common of all the infections. It can have a variable outcome and may result from either primary infection acquired during gestation or a recurrent maternal infection (reactivation or reinfection) in seropositive women (4). Only 40% of pregnant women who develop primary CMV infection will transmit it to their fetuses and 5%–10% of these infants will exhibit symptoms at birth (5). The most severely affected are those with cytomegalic inclusion disease (CID) manifested by hepatosplenomegaly, jaundice, chorioretinitis, cerebral calcification, and microcephaly. These children constituted the major long-term public health problem associated with CMV. Ninety percent of congenitally infected infants are symptomatic at birth, while 10% of these will develop manifestations of infection later in childhood, usually hearing loss or poor intellectual performance (6). CMV is a ubiquitous virus that infects most persons at some time during their life. Acquisition appears to require

close or intimate contact with persons who are excreting CMV in their saliva, urine, or other secretions. Infants and children acquire CMV infection either from other infected children or from their mothers in utero, at birth, or during the perinatal period (7). CMV can also be transmitted via blood transfusions, breast milk, and transplanted organs (8–10). Infection in normal children and adults is usually asymptomatic, although in immunocompromised hosts CMV may be an opportunistic pathogen, which causes serious illness with high morbidity and mortality (11).

Diagnosis of HCMV infection is clinically difficult and usually depends on the isolation of virus by tissue culture methods where HCMV grows slowly (one to several weeks), and which are work intensive and time consuming. Detection of specific antibody response can only give indirect evidence of HCMV infection. Since antiviral therapy is available for severe HCMV infections, rapid methods to identify early-

*Correspondence to: Maurice L. J. Moncany, Laboratoire de Biologie Cellulaire et Moléculaire, Université de La Rochelle, 17042 La Rochelle, Cedex, France.

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infected patients are needed to institute and monitor therapy (12). Alternative rapid and reliable methods have been developed which include detection of viral antigens by enzyme-linked immunosorbent assay (ELISA) (13,14), shell vial assay coupled to immunofluorescence assay (15,16), and detection of viral nucleic acid by DNA hybridization (17,18). These methods have proven to be effective for the detection of HCMV but sometimes lose in specificity and sensitivity. A number of studies stressing the potential speed and sensitivity of PCR for the diagnoses of HCMV infection have been reported (19–26). It was demonstrated to be a very sensitive method for viral detection in peripheral blood polymorphonuclear leukocytes, yet presence of viral DNA did not always correlate with viremia (27), antigenemia (28), or clinical symptoms (29), complicating the interpretation of its clinical significance. The detection of HCMV DNA by PCR alone was never associated with the presence of clinical symptoms; however, the persistence of viral DNA in blood represented a risk of relapse of viral infection and HCMV disease (29).

It is well known that a single primer pair is not sufficient to detect all clinically isolated HCMV. Although the sensitivity of the PCR is nearly 100%, the sequence variability of clinically isolated HCMV (30) could raise a problem resulting in false negative results. Therefore, it is very important to use more than one primer set directed against different regions of the genome in order to achieve maximal sensitivity and specificity. Considerable time and effort can be saved by simultaneously amplifying multiple sequences in a single reaction, a process referred to as multiplex PCR (31–33). Multiplex PCR requires that primers lead to amplification of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions. In addition, methods must be available for the analysis of each individual amplification product from within the mixture of all the products. The development of an efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize reaction conditions. In many cases PCR primer pairs retain their target specificity when combined into a single reaction tube using the amplification conditions established for the individual pairs. The present report describes the development of a quadriplex PCR method with amplification of HCMV in a single step procedure, using primers chosen from four different regions of viral genome and allowing a direct specification of the detected viral DNA.

MATERIALS AND METHODS

Cell Cultures, Viruses

Human lung diploid cells MRC-5 (American Type Culture Collection, Rockville, MD) were grown in MEM-D medium supplemented with 2% fetal calf serum. Human cytomegalovirus (HCMV), strain AD169 (American Type Culture Collection, Rockville, MD) were inoculated into MRC-5 cells and examined daily until the observation of typical cytopathic

effect (CPE) of 75%–80%. The cell cultures were then frozen at -70°C until their use in DNA extraction procedure. Uninfected MRC-5 cells were used as negative control.

DNA Extraction

MRC-5 cells uninfected or infected with CMV were harvested by centrifugation at 550g for 5 min. The supernatant was discarded and the cell pellet subjected to DNA extraction using Xtreme Genomic DNA purification kit II (Pierce, Rockford, IL).

Primers

The primer pairs could enable identical amplification efficiencies for their amplicon in a multiplex reaction. Primers with nearly identical optimum annealing temperatures worked under fairly similar conditions as they anneal with single copy sequences. Similar annealing temperatures were achieved using primers with a G/C base composition near 50% with a fairly even distribution of G/C bases versus A/T bases. Primer concentration is a critical parameter for successful multiplex PCR. If all the primers in a reaction anneal with equal efficiencies they can generally be used at the same concentration.

The PCR primers were synthesized with an Applied Biosystems oligonucleotide synthesizer and their sequences are reported on Table 1. They were shared between primers allowing the detection of several herpesviruses and HCMV specific ones. Primers P-1/P-2 published by Rozenberg and Lebon (21) were directed against the HSV 1, HSV 2, HCMV, and Epstein-Barr viruses. HCMV specific primers were: MIE-4/MIE-5 and LA-1/LA-6, published by Demmler et al. (19), and we designed CP-15/CM-3 from DNA sequence of the human cytomegalovirus strain AD 169 complete genome listed in Gene Bank (accession number X17403). They were located at position 8868 to 8887 for CP-15 and 9105 to 9124 for CM-3, and chosen by use of the Primer 3 method, Whitehead Institute, elaborated by Steve Rozen and Helen J. Skaletsky (1996, 1997). The code is available at <http://www-genome.wi.mit.edu/genomesoftware/other/>.

TABLE 1. Amplification primers

Primers	Sequences (5' → 3')	Length of amplicons ^a
P-1	CGACTTTGCCAGCCTGTACC	589
P-2	AGTCCGTGTCCCCGTAGATG	
MIE-4	CCAGCGGCCTCTGATAACCAAGCC	435
MIE-5	CAGCACCATCCTCCTCTTCCTCTGG	
LA-1	CACCTGTCACCGCTGCTATATTGC	400
LA-6	CACCACGCAGCGGCCCTTGATGTTT	
CP-15	GTACACGCACGCTGGTTACC	256
CM-3	GTAGAAAGCCTCGACATCGC	

^aExpressed in base pairs.

DNA Amplification—Multiplex PCR

The amplification was performed with a Perkin-Elmer GenAmp PCR system 9600. Forty amplification cycles of 20 sec at 94°C, 20 sec at 60°C, and 20 sec at 72°C were carried out in a 50 µl final volume containing 5 µl of 10× reaction buffer (Minotech, Kreta, Greece), 0.3 mM of each deoxy-nucleotide triphosphate (dNTP), 0.3 µM of each of the 8 primers, 2 units of Taq Polymerase (Minotech, Kreta, Greece) and 2 mM MgCl₂. The control experiments used 5 µl of appropriate DNA sample (positive control CMV strain AD 169 or negative control uninfected MRC-5 cells) which were added to the reaction mixture. After the last cycle, the samples were incubated for 15 min at 78°C to complete the extension of the primers. To optimize the buffer concentration of multiplex PCR, we performed a series of titrations of the enzyme and primer concentrations and then titrated the Mg²⁺ and dNTP levels. To avoid parasite bands that can occur during nested PCR which also needs several primer pairs (34), the annealing temperature was raised to 60°C where all primer pairs continue to amplify equally with the same sensitivity (35). Ten µl of each amplified product were analysed by agarose gel electrophoresis on 3.5% agarose (Gibco BRL, ultra pure agarose, electrophoresis grade) containing 1 µg/ml of ethidium bromide in TBE buffer and visualized on an UV transilluminator FOTO/PHORESIS I, FOTODYNE (Hartland, WI).

All procedures were carried out under conditions that minimized the risk of contamination with exogenous viral DNA during the PCR. Separate rooms and sets of pipettes with plugged pipette tips were allocated for each step of the PCR, reaction mixture preparation, template addition, and amplified product analysis. DNA from uninfected MRC-5 cells gave negative results in each assay run, confirming the efficiency of these preventive measures.

Specificity and Sensitivity

The specificity of the PCR reaction was tested with 100 ng, 10 ng, and 1 ng of genomic MRC-5 cell DNA infected with CMV in presence of 10 µg of uninfected MRC-5 cell DNA to verify the absence of parasite bands. The eventual crossreactivity of the HCMV specific primers was tested on the following DNAs: Herpes Simplex virus type 1 (HSV 1), strain F; Herpes Simplex virus type 2 (HSV2), strain G; Varicella-Zoster virus (VZV), strain Ellen and Epstein-Barr virus (EBV), strain P-3 (American Type Culture Collection, Rockville, MD). One hundred ng of each viral DNA were subjected to amplification to test the specificity of the primer pair CP-15/CM-3; that of the other three primer pairs has been previously reported (19,21). To evaluate the sensitivity of the PCR, a decreasing scale (100 ng, 10 ng, 1 ng, and 0.1 ng) of genomic MRC-5 cell DNA infected with CMV was amplified. The specificity and sensitivity assays were performed for each primer pair and for the combined four primer pairs in the multiplex PCR.

RESULTS

Optimization of Multiplex PCR

The two most critical events in the PCR are the dNTP/MgCl₂ concentrations and the annealing temperature. Different concentrations of dNTP and MgCl₂ were assayed in order to optimise the buffer system for the single and the multiplex PCR. The MgCl₂ and dNTP concentrations are dependent on each other, as well as on the concentration of primers. The primer concentrations were first titrated (0.10–1 µM). The selected concentration for all eight primers was 0.30 µM, as all the primers in the reaction anneal with equal efficiencies.

MgCl₂ and dNTP were titrated simultaneously as the MgCl₂ concentration used for the amplification is dependent on free dNTPs. Optimal results were obtained with 2 mM MgCl₂ and this level was used to determine the optimal dNTP concentration which was 1.2 mM dNTPs (Fig. 1, lanes 1 and 2), while lower levels resulted in a decrease in the efficiency of amplification (Fig. 1, lanes 4, 5, 7, and 8). Reduction of the dNTP concentrations can lead to a considerable increase in the formation of primer artifacts as a consequence of the excess of free Mg²⁺ resulting from the lowered dNTP concentration. Lower levels of dNTPs also led to inconsistent amplification results and frequently the reactions produced only a subset of the targeted amplicons (Fig. 1, lanes 4 and 7). The T_m is comprised between 55°C and 60°C for all the eight primers which are reported in the Table 1. The optimal temperature for annealing was tested between 55 and 60°C and the best sensitivity and specificity were reached for 60°C. The polymerase extension time was extended from 20 sec to 1 min when the four primer pairs were added simultaneously, as the 589 bp-long-P-1/P-2 fragments amplify less well than others (Fig. 1, lane 10). At each stage in the optimization of the single pair of primers, the two-, three- and four-primer pair PCRs, serial 10-fold dilutions of CMV, and MRC-5 genomic DNA were used to define the sensitivity and specificity achieved.

Specificity and Sensitivity

The aim was to achieve the lowest possible detection limit for all four-primer pairs in the multiplex DNA amplification. The specificity of the PCR reaction was tested with 100 ng, 10 ng, and 1 ng of genomic MRC-5 cell DNA infected with CMV in presence of 10 µg of uninfected MRC-5 cell DNA. When specific primer pairs were added to a single reaction tube in the presence of 10 µg of negative controls, bands of the predicted size were visualised on agarose gel by ethidium bromide staining (Fig. 2a, lanes 2, 3 and 4). In such conditions, negative control DNAs were always negative (Fig. 2a, lane 1). The specificity of the primer pair CP-15/CM-3 is illustrated in Figure 2b where the amplification occurred only with the CMV strain (Fig. 2b, lane 9) but not with the HSV 1, HSV 2, VZV, and EBV DNAs (Fig. 2b, lanes 5 to 8, respectively). The lack of crossreactivity allowed the use of the

Fig. 1. Optimization of dNTPs concentration. Amplification was produced with 1.2mM (lanes 1–3), 0.8mM (lanes 4–6), and 0.4mM dNTPs (lanes 7–9). The concentration of $MgCl_2$ was constant at 2mM. All four primer pairs were used simultaneously at the concentration of 0.30 μ M. Amplification conditions were as follows: 40 cycles of 20 sec at 94°C, 20 sec at 60°C, and 20 sec at 72°C. The amplified products were subjected to a 3.5% agarose gel electrophoresis containing 1 μ g/ml of BET. M, Molecular weight markers corresponding to a HaeIII digest of Φ X174 RF DNA (GIBCO BRL, Life Technologies); Lanes 1, 4, and 7, Amplification from 10ng of genomic MRC-5 cell DNA infected with HCMV; Lanes 2, 5, and 8, Amplification from 1ng of genomic MRC-5 cell DNA infected with HCMV; Lanes 3, 6, and 9, Amplification from 10 μ g of uninfected genomic MRC-5 cell DNA; Lane 10, Amplification conditions as in lane 1, except that extension time was raised to 1 min. The predicted size of each amplicon is shown on the right.

quadriplex PCR as a method for a direct detection and specification of the studied infected cell DNA.

Various amounts of genomic MRC-5 cell DNA infected with CMV were amplified. The sensitivity of the reaction was equivalent with each of the four primer pairs. On electrophoresed 3.5% agarose gel stained with 1 μ g/ml ethidium bromide, CMV was consistently detected from 10ng of genomic MRC-5 cell DNA for each primer pair (Fig. 3, lanes 1, 3, 5, and 7). Faint bands of the predicted size were also observed from 1ng of genomic MRC-5 cell DNA (Fig. 3, lanes 2, 4, 6, and 8). When all four sets of primers were combined in a single reaction tube, the sensitivity of the assay was equivalent to 10ng of genomic MRC-5 cell DNA (Fig. 3, lane 9), whereas amplification from 1ng genomic MRC-5 cell DNA produced only a subset of the amplicons in which the 589-bp band disappeared (Fig. 3, lane 10).

Fig. 2. Specificity studies. **a.** Specificity of the quadriplex PCR to detect HCMV. Lane M, Molecular weight markers corresponding to a HaeIII digest of Φ X174 RF DNA (GIBCO BRL, Life Technologies); Lane 1, Negative control performed on 10 μ g of uninfected MRC-5 cell DNA; Lanes 2–4, Amplification performed on 100ng, 10ng, and 1ng of genomic MRC-5 cell DNA infected with CMV in presence of 10 μ g of uninfected, respectively. The predicted size of each amplicon is shown on the right. **b.** Specificity of primer pair CP-15/CM-3 to detect HCMV. Lane M, Molecular weight markers corresponding to a HaeIII digest of Φ X174 RF DNA (GIBCO BRL, Life Technologies); Lanes 5–9, Amplification performed on 100ng of the following viral DNAs: HSV-1, HSV-2, VZV, EBV, and HCMV, respectively. The predicted size of each amplicon is shown on the right.

Concerning clinical specimens, HCMV can only be present in a few DNA copies and in such conditions, at least one of the four primer pairs should allow a detection. In case of negative results, the DNAs should be independently assayed for potential PCR inhibition by using primers designed to detect the β -globin gene or the HLA dq- α locus.

DISCUSSION

The results reported in the present paper illustrated both the feasibility and difficulties of developing a quadriplex PCR for HCMV. In a multiplex PCR protocol, there are several points that have to be considered which may affect primer-template affinities and competition between different primers of the same sense. A sensitive assay was established by careful choice of primers and adjustment of $MgCl_2$, dNTPs, and primer concentrations. The rather high number of 40 cycles was chosen to drive PCR into the linear phase of amplification when the amount of primers or enzyme became limiting. The same was for the choice of a hybridization temperature of 60°C which avoided parasite bands while maintaining a normal amplification rate. The melting temperature

Fig. 3. Sensitivity of quadriplex PCR applied to 100ng and 10ng of the genomic MRC-5 cell DNA infected with CMV. Lane M, Molecular weight markers corresponding to a HaeIII digest of Φ X174 RF DNA (GIBCO BRL, Life Technologies); Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, Amplifications with primer pairs P₁/P₂, MIE 4/5, LA 1/6, and CP-15/CM-3 respectively; Lanes 9 and 10, Amplification with the four sets of primers; Lanes 1, 3, 5, 7, and 9, Amplifications performed on 10ng of MRC-5 cell DNA infected with CMV; Lanes 2, 4, 6, 8, and 10, Amplifications performed on 1ng of MRC-5 cell DNA infected with CMV. The predicted size of each amplicon is shown on the right.

of primer-template complexes has a major influence on the efficiency of amplification. It is dependent on the length of the primer, the relative amount of G/C vs. A/T nucleotides and the number of mismatches to the target sequence. The reduction in primer-template affinity, for example, to 40% in each primer, should theoretically result in only 20% product amplification efficiency (33). The relative position of the primers to one another is important. If, for instance, there are two primers of the same sense and the same affinity to the template competing with one antisense primer, the one sense primer located more closely to the antisense primer has an advantage if the reaction is not limited by too low primer concentration (33). If both sense primers bind to one template molecule at the same time, the strand elongation initiated by the outer primer will stop at the position of the inner primer, as this primer already blocks the template molecule. The importance of adjusting the primer sequence and concentration reflects the competition between simultaneous DNA amplifications occurring in the same tube.

The multiplex PCR raised the problems of the interference

between the different primers, which could lead to nonspecific amplimers, and of the coordinate amplification of the bands. In our method using four primer sets, several template concentrations should be compensated, since smaller amounts of template would be amplified exponentially during more cycles, before the plateau phase of the PCR was reached. Such a compensation step should reduce the possible influence of differences in the primary template concentrations on the final results. In each PCR amplification, two units of Taq DNA polymerase were used. This initially represented an excess of enzyme molecules versus the number of starting template molecules that allowed full extension of PCR products. The increase in the cycle number and the product concentration could lead to less than one molecule of Taq DNA polymerase per primer-template complex being present. In these conditions, the efficiency of extension decreased, yielding less than full-length product after every cycle, especially for longer targets (i.e., P-1/P-2 amplicon of 589 bp). The advantage of increasing the Taq polymerase concentration was that it provided adequate enzymatic activity when one DNA amplification was less efficient than another in a simultaneous reaction. And, the annealing temperature chosen was as high as possible, taking care not to reduce the sensitivity of the assay (Fig. 3, lanes 9 and 10). The four primer pairs directed against different regions of the genome can compete for a limited copy number of the target, lowering the sensitivity of the assay (Figure 3, lane 10) but, as mentioned above, at least one PCR product should be detected. The dynamics of multiplex PCR are, in part, empirical yet this method allows a direct typing of the detected DNAs.

It is accepted that quantification is the way to follow an infection that appears after a treatment, as is done with HIV in the viral load assays. This approach allows the detection of resistance development among the clinical isolates for the therapeutic agents being administered. It has been claimed that active HCMV infection could be distinguished from the latent one by PCR with no need for quantification (36–38). However, other studies have developed quantitative PCRs for HCMV with a view to differentiating the two kinds of infection (39–43). Another way to distinguish latent from active infection is to detect viral RNA via RT-PCR which is more labor intensive but which can be masked partially by the RT activity of the Taq-polymerase (44). The mere presence of the organisms in the body in a latent manner could lead to the detection of its DNA, but it should not be transcribing itself into RNA, and then protein. The latter phenomena occur only in actively replicating virus where its capacity to lead to a frank infection is manifested by the presence of the full gamut of molecules found in mature virion, which would have to include viral RNA as the necessary intermediary between the viral DNA and its protein translate. The sequence variability of clinically isolated HCMV poses a serious problem resulting in false negative results (30). The use of four primer sets to different genomic regions achieved maximal sensitivity and

specificity, while the semiquantitative modification of our quadruplex PCR should provide discrimination between active and latent infection.

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