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Quantitative PCR Determination of Human Cytomegalovirus in Blood Cells

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We evaluated a rapid and sensitive method to determine human cytomegalovirus (CMV) DNA levels in blood cells using a quantitative polymerase chain reaction (PCR) technique. This method is based on real-time detection of PCR using a dual fluorescence-labeled probe and a sequence detector. Ten copies of CMV DNA were detected, when 1 μg of DNA from blood samples was used with this method, and a good correlation was obtained between increased concentrations of copy numbers calculated and measured copy numbers of CMV DNA (r = 0.999). Forty normal subjects exhibited no copies of CMV DNA. On the other hand, a 6-month-old girl tested positive for increased levels 4 weeks after liver transplant. This method is simple, accurate, and sensitive for the quantitative detection of CMV DNA in vivo, indicating possible applications for the diagnosis and monitoring of CMV infection. J. Clin. Lab. Anal. 15:122–126, 2001.

Key words: polymerase chain reaction (PCR); quantitative PCR; cytomegalovirus (CMV); liver transplantation

INTRODUCTION

Polymerase chain reaction (PCR) techniques are widely applied for detecting viral DNA in samples from patients with pathological conditions. Conventional PCR, however, is not quantitative, and PCR detection of some kinds of viral DNA, such as cytomegalovirus (CMV)(1,2) and Epstein–Barr virus (EBV) does not always reflect the infection in vivo because these viruses are persistently present even in normal subjects who may lack clinical manifestations. In spite of these limitations, the detection of CMV DNA in blood, or in an affected organ, is important in order to understand the clinical states associated with CMV infection, which may cause severe pneumonia or erythrophagocytic syndrome. In this regard, the development of new ways to quantify PCR products is quite important. Competitive PCR is one such representative method for this purpose (3,4), but it is time consuming and too cumbersome for clinical use.

Recently, a new method called real-time quantitative PCR has been developed (5). It is based on the use of the 5′nuclease activity of *Taq* DNA polymerase to cleave a nonextensible hybridization probe during the extension phase of PCR amplification (5,6) and measures PCR products with a dual-labeled fluorogenic probe (7). The sequence detector allows for the continuous measurement of fluorescent spectra of the wells of thermal cycler during PCR amplification with the PE Applied Biosystems model 7700 Sequence Detector System (TaqMan). We have developed a quantitative PCR assay useful for the clinical evaluation of CMV infection.

MATERIALS AND METHODS

Samples

Blood samples were obtained from 40 healthy individuals and a patient having undergone transplantation of the liver. DNA was extracted from blood samples by phenol-chloroform and ethanol precipitation. Human DNA from the Raji cell line of Birkitt lymphoma (American Type Culture Collection, ATCC), DNA from MRC-5 cells infected with CMV strain Towne (ATCC), and genomic DNA from the human leukemic cell line K562 (ATCC) were also prepared and used as positive or negative controls.

PCR Amplification

Nested PCR was performed to amplify CMV DNA. The primers used for CMV PCR corresponded to the nucleotide

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sequence of a major immediated early region (HCMV-IE, accession no. M21295) of the CMV strain Towne genomic DNA (8). The sequences of the primers in the first PCR were 5′-TTGCTCACATCATGCAGCTCCT-3′ as a forward and 5′-ACGACGTTCCTGCAGACTAT-3′ as a reverse. The sequences of the primers in the second PCR were 5′- AGGCATTCTGCAAACATCCT-3′ as a forward and 5′- AAGAGAAAGATGGACCCTGA -3′ as a reverse, respectively.

First and second PCR primers were mixed in a final volume of 50 µl, which included 5 µl of DNA (0.2 µg/ml), 0.25 µl of *Taq* DNA polymerase (5 µ/ml, TOYOBO, Osaka, Japan), 1 µl of each primer (0.2 mg/ml), 1.25 µl of dNTP, 5 µl of reaction buffer and 32.5 µl of distilled water. Each of the mixtures was subjected to 30 cycles of amplification under 94°C for 2 minutes, 55°C for 30 sec, and 72°C for 90 sec using a DNA thermal cycler. The amplified PCR products were electrophoresed on 1.6% agarose gels and visualized with UV light as a single band of 462 bps after staining with ethidium bromide.

Plasmid Construction of CMV DNA

In order to calculate the amount of genomic DNA equivalent to that from PCR amplifications, PCR products of 462 bps amplified from the Towne strain were inserted into multicloning sites of 3,003 bps pGEM-T (Promega, W1) plasmid DNA, and used as a cloning vector. Plasmid DNA from cultured bacteria was extracted and purified as described by Maniatis et al (9). The original number of CMV copies was determined by the optical density (OD) values against the molecular weights of different dilutions of this plasmid DNA. Namely, the reference value for the copy numbers of CMV DNA was established at 1×10^9 molecule/3.7ng/µl.

Quantitative PCR

The amplification reaction was performed with a total volume of 50 µl, containing 1 µl of DNA sample (1 µg/µl), 5 µl of reaction buffer, 4 µl of dNTP (200 µMdATP, 200 µMdCTP, 200 µMdGTP and 400 µMdUTP), 8 µl of 25 mM $MgCl_2$, 0.5 µl of AmpErase uracil N-glycosylase (1u/ml, Perkin Elmer Applied, Norwalk, CA), 0.25 µl of Ampli Taq Gold (5u/µl, Perkin Elmer Applied), 1 µl of each primer, and 28.75 µl of distilled water. The base sequences of the primers used were as follows: upstream, 5′-TCCAGAGTTGGCCGAAGAAT-3′; downstream, 5′-TACAGGACCGTCTTGAGCCA-3′. Although these primers were not the same primers based on HCMV-IE using a conventional method, these were established within the region amplified with a nested PCR.

An oligonucleotide probe with a 5′ fluorescent FAM (blue) label and a 3′ fluorescent TAMRA (green) label was employed. The reactions also contained 100 nM of one among the following detection probes: CMV probe: 5′-(FAM)- TGACCGAGGATTGCAACGAGAACC -(TAMRA)- p-3′, where *p* indicates phosphorylation. The thermal cycling conditions were 2 minutes at 50°C and 10 minutes at 95°C. Thermal cycling was performed with 40 cycles at 95°C for 15 sec and at 60°C for 1 minute. All reactions were performed using a 7700 Sequence Detector (Perkin Elmer Applied), with a GeneAmp PCR System 9600. A calibration curve was run in parallel and in duplicate with each analysis.

Copies	CT
10^0	35.6
10^1	32.6
10^2	29.0
10^3	25.7
10^4	22.1
10^5	18.6
10^6	15.4

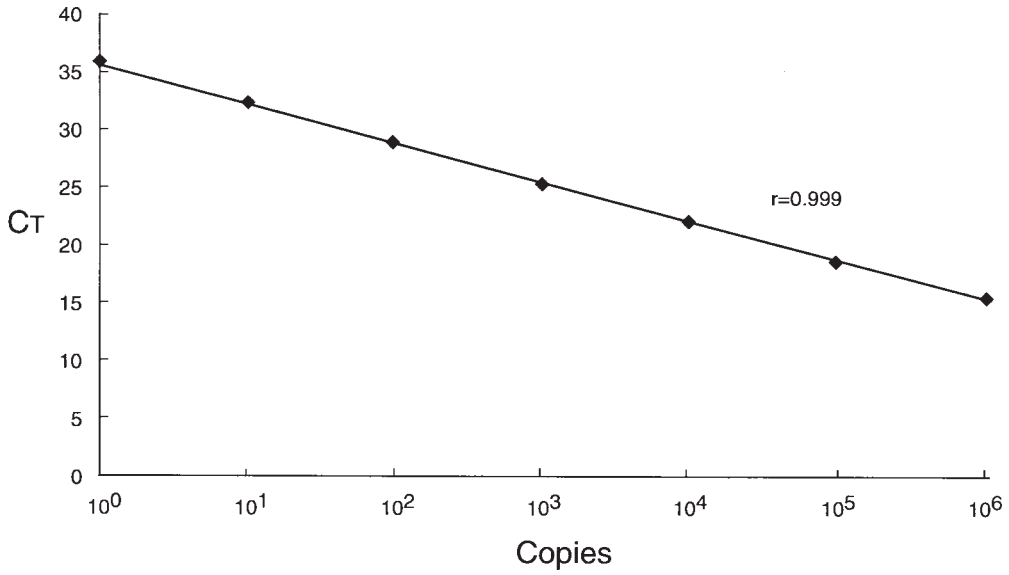


Fig. 1. Standard curve log of starting copy numbers versus threshold cycle (C_T). The relationship between DNA concentrations and C_T shows linearity (r = 0.999). In this curve, data for each copy number are plotted as mean values of duplicate PCR amplifications. C_T values are defined as an amplification plot, which C_T crosses the threshold, plotted values versus copy numbers (the sample dilution).

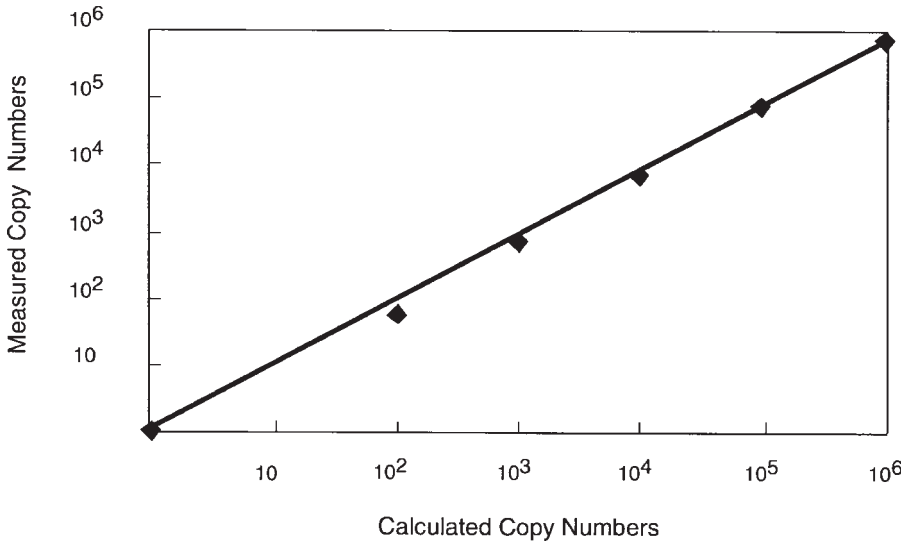


Fig. 2. Relationship between calculated copy numbers and measured copy numbers. Numbers indicate amplification plots of target DNA (r =0.999).

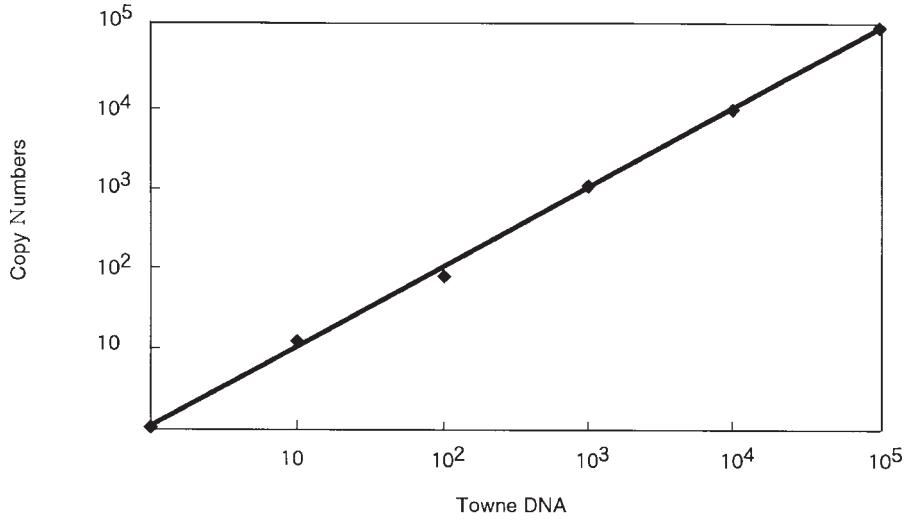
Serology

CMV serology was performed by an enzyme-immunoassay (EIA) for IgG and IgM (Enzygnost Anti-CMV/lgM and IgG, Dade Behring Inc., Deerfield, IL). Quantification of anti-IgG or -IgM was determined using a limiting dilution. The cut-off value in serum samples was defined with titers of < 41.

RESULTS

Amplification Plots for Sensitivity

In preliminary studies we examined and established appropriate conditions for the quantitative PCR assay of the β-actin gene. Sensitivities of the qualitative and quantitative PCR assays were determined by using a template from plasmid DNA inserted into the CMV strain Towne fragment. Under the above



Towne DNA	10	100	1000	10000	100000
Copy Numbers	12	81	1099	10265	109955

Fig. 3. Quantitation study with diluted CMV DNA and genomic DNA. Each point represents the numbers of CMV DNA copies from CMV strain Towne virus, which were amplified as described in Materials and Methods (r = 0.999).

conditions, as little as 1 fg of plasmid DNA was amplified and visualized in gels. In Figure 1 the standard curve, where a known amount of DNA was analyzed by PCR, shows a good correlation (r = 0.999) between the copy numbers of target CMV DNA amplified and the threshold cycle (C_T). Figure 2 illustrates a linear correlation between calculated copy numbers and measured copy numbers of CMV DNA (r = 0.999). Ten copies of CMV DNA were the minimum number for detection under the conditions used, and detection with fewer than 10 copies was not reproducible. In order to determine whether the amplification may be affected by the presence of chromosomal DNA, CMV DNA was mixed with or without genomic DNA from a CMV-negative donor at an equal concentration. The results showed no differences between the mixture and CMV DNA alone (Fig. 3).

Next, the specificity of the assay was examined by amplifying DNA from other types of herpes viruses, as well as MRC-5 cellular DNA. The primers for CMV PCR, however, failed to amplify the DNA from herpes simplex virus type 1 or 2, varicella-zoster virus, Epstein–Barr virus, or uninfected MRC cellular DNA (data not shown).

Between-Run Precision

When serial dilutions of target CMV DNA with 1 to 10^5 copies were tested 20 times, the coefficients of variation (CV) were 17.2 to 20.9%, except for the CV of 162% with one copy as shown in Table 1. The CV for copy numbers of β actin DNA were 7.8 and 13.5% for 4,000 and 1,000 copies, respectively.

To determine the precision of the assay, 20 replicates of three different copies in plasmid DNA including PCR products was amplified on three separate days. The mean values, standard deviation (SD), and the CV for CMV DNA and β actin DNA were calculated for each day to define the intra-assay precision. CV values for each concentration of CMV DNA were found to be 22.2 to 29.5% of SD with 10 copies, and 9.6 to 13.5% with 10^3 copies (Table 2). The precision of amplification for three days of β actin DNA ranged from 4.5 to 16.0% for a low number copies, 11.0 to 22.1% for an intermediate number of copies, and 0.8 to 15.4% in a high number of copies.

TABLE 1. Within-run reproducibility of quantitative PCR

CMV DNA				
No. of copies DNA	1	10	1,000	100,000
No. of tested samples	20	20	20	20
Mean	1.0	8.0	1003	84,738.0
SD	1.62	1.67	200.7	14,570.5
CV (%)	162.0	20.9	20.0	17.2

β actin DNA	Low number of copies	Intermediate number of copies	High number of copies
No. of copies DNA	(200)	(1,000)	(4,000)
No. of tested samples	20	20	20
Mean	183.0	946.0	3,862.0
SD	18.73	127.7	302.63
CV (%)	10.2	13.5	7.8

TABLE 2. Day-to-day reproducibility (CV%)

CMV DNA	Low number of copies	Intermediate number of copies	High number of copies
No. of copies DNA	(10)	(100)	(1,000)
No. of tested samples	20	20	20
First day	13.5	12.1	6.3
Second day	29.5	9.6	6.7
Third day	27.0	13.5	7.9

β actin DNA	Low number of copies	Intermediate number of copies	High number of copies
No. of copies DNA	(10)	(100)	(1,000)
No. of tested samples	20	20	20
First day	4.5	22.1	1.7
Second day	16.0	11.0	0.8
Third day	4.6	18.0	15.4

PCR Detection of CMV in Healthy Donors

Blood specimens from 40 healthy donors were tested for the presence of CMV DNA by both qualitative and quantitative PCR assays. Genomic DNAs in blood samples of all donors were negative by the qualitative PCR assay, while plasmid DNA was positive. In addition, the copy numbers of CMV DNA in blood cells from all donors were zero as determined by the quantitative assay (data not shown).

Time-Course Study With a Liver Transplant Recipient

A six-month-old girl who received a liver transplant was monitored for CMV infection during the transplantation. She was negative for CMV DNA on the date of her surgery, but was CMV-positive 2 weeks after the transplantation, when she developed fever and pneumonitis. At that time, quantitative PCR showed increased numbers of copies of CMV DNA in the blood (Fig. 4). She was administered high doses of gammaglobulin and ganciclovir. The copy numbers of CMV DNA then decreased ultimately to zero.

DISCUSSION

We examined a quantitative method to determine the copy numbers of CMV DNA using fluorescent hybridization probes and a sequence detector. This method made it possible to quantitate absolute numbers of CMV DNA in samples, in contrast to

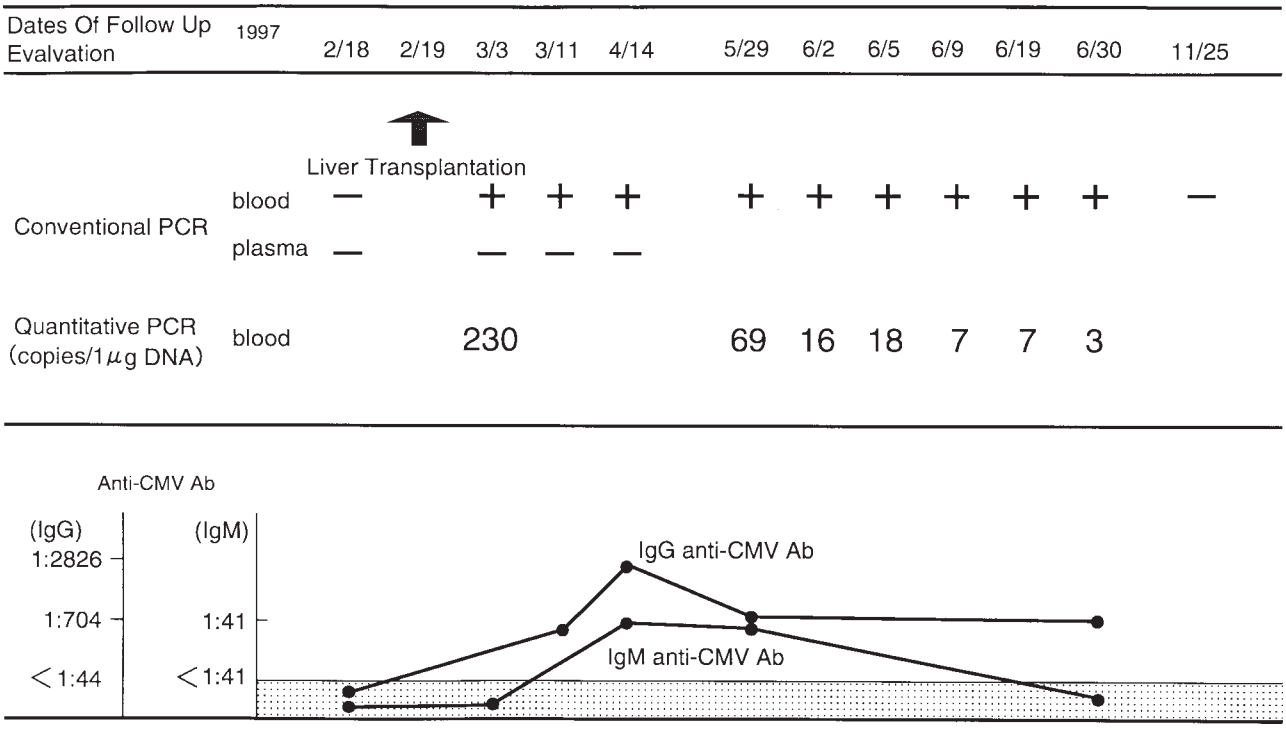


Fig. 4. Follow-up study of 6-month-old girl with a liver transplant. Anti-CMV antibodies in serum were determined by EIA and indicated in titers.

previously reported competitive PCR or semiquantitative methods (10–12). This method is based on determining comparative C_T values by following real-time PCR kinetics, which eliminates the necessity of a competitor to be co-amplified with the target. This was confirmed by preparation of the standard curve and determination of the known copy numbers by comparison with the standard curve.

Our data also show that this method is reproducible for detecting quantities greater than ten copies of CMV DNA, indicating that the sensitivity may be adequate for clinical use. We did not detect significant numbers of copies of CMV DNA in blood samples from the 40 normal subjects, but CMV DNA was positive for one patient in an immunosuppressive state after transplantation of the liver. In particular, the copy numbers of CMV DNA in this case were increased as detected by increased titers of antibodies. Thus this method may be useful for the early detection of CMV infection.

In conclusion, our results indicate that real-time quantitation of PCR is accurate, reproducible, and sensitive. This method will be valuable for monitoring not only CMV infection, but also a variety of clinical conditions such as minimum residual disease in leukemias.

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