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# Highly Sensitive Single-Step PCR Protocol for Diagnosis and Monitoring of Human Cytomegalovirus Infection in Renal Transplant Recipients

OTÁVIA L. CABALLERO,[1,2,3] CARLA L. P. MENEZES,[4] MARIA CLARA S. L. COSTA,[5] SILVANA C. FERNANDES,[4] TAÍS M. ANACLETO,[6] RICARDO MANOEL DE OLIVEIRA,[4] ESTEVAM A. VIOTTI,[7] EDUARDO R. F. TÁVORA,[7] SANDRA S. VILAÇA,[7] EMIL SABBAGA,[8] FLÁVIO J. DE PAULA,[8] PAULA F. TÁVORA,[9] LUISA L. VILLA,[1] AND ANDREW J. G. SIMPSON[6]*

*Laboratory of Virology[1] and Laboratory of Cancer Genetics,[6] Ludwig Institute for Cancer Research, Departamento de Bioquímica, Instituto de Química, USP,[3] Departamento de Patologia Clínica e Hemoterapia, Hospital A. C. Camargo,[4] Hemagen S/A,[5] and Unidade de Transplante Renal, Hospital das Clínicas, Universidade de São Paulo,[8] São Paulo, and Departamento de Ciências Biológicas, Faculdade de Medicina do Triângulo Mineiro, Uberaba,[2] Hospital Felício Rocho, Belo Horizonte,[7] and Laboratório São Marcos, Belo Horizonte,[9] Minas Gerais, Brazil*

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**A multiplex, single-step PCR protocol for the detection of human cytomegalovirus (HCMV) DNA is described. The protocol amplifies regions of the viral LA and IE genes and employs elevated temperatures for both reagent mixing and primer annealing together with product detection by silver staining on polyacrylamide gels. This assay detects one to five HCMV genomes in clinical samples containing up to 100 ng of human DNA, a level of sensitivity equivalent to that of more complex assays involving either nested PCR or postamplification hybridization. As well as being of importance in clinical situations where high-sensitivity qualitative diagnosis is required, this assay is also applicable to the monitoring of HCMV infection in renal transplant recipients. Due to its multiplex format the assay provides quantitative information, in that samples from which a single target is amplified contain on average sevenfold fewer viral genomes per $10^6$ leukocytes than those from which both targets are amplified. When weekly blood leukocyte DNA preparations from renal transplant patients were assayed, findings of three consecutive tests in which both HCMV targets were amplified were highly indicative of patients who had developed very high loads of HCMV (100% sensitivity, 88% specificity). We thus show that the same simple PCR assay which permits highly sensitive HCMV diagnosis can also be used for the efficient identification of transplant recipients at risk of clinically significant infection.**

The diagnostic application of PCR presents challenges in response to which sophisticated modifications of the basic technique are continually being developed. For example, to ensure very-high-sensitivity qualitative diagnosis (one to five target genes in a clinical sample), nested PCR or a postamplification hybridization protocol has become routine (1, 9). Where viral load is an issue, quantification is normally undertaken with external competitive standards and/or limiting dilution and multiple replicates (7, 22). In general, such quantitative assays do not exhibit the levels of sensitivity of nested PCR or hybridization and are thus not appropriate where initial qualitative diagnosis is required. The plethora of alternative PCR strategies and the diverse requirements of the assay have led to a situation in which the clinical application of PCR has become both complex and costly.

We have investigated whether a single, simple, carefully applied PCR assay might be able to substitute both for the use of nested PCR and postamplification hybridization for high-sensitivity qualitative diagnosis and for complex quantification protocols for viral load assessment of the same infection. The model with which we chose to work is human cytomegalovirus (HCMV). High-sensitivity detection of HCMV is required for screening of blood donors, evaluation of amniotic fluid in anti-HCMV immunoglobulin M (IgM)-positive pregnant women, and analysis of spinal fluid samples. On the other hand, quantitative viral load assessment is required for monitoring of immunosuppressed individuals in order to distinguish low-level, innocuous infections from those which may be of clinical relevance (5, 6, 11, 17).

We show that a single PCR assay is readily capable of detecting fewer than five HCMV genomes in clinical samples without recourse to nested or hybridization protocols. Furthermore, the same assay used to monitor HCMV infection in immunosuppressed renal transplant recipients distinguishes individuals with and without significantly elevated viral loads (>50,000 viral genomes per $10^6$ cells, as measured by limiting DNA dilution) with 100% sensitivity and 88% specificity.

## MATERIALS AND METHODS

**Subjects and specimens.** A total of 40 samples from 40 patients who were HCMV-seropositive for IgM and 412 samples from 34 renal transplant recipients were included in this study. Five-milliliter EDTA-blood samples were obtained from the first group of patients when anti-HCMV IgM was positive and from the second group of patients weekly after renal transplantation for up to 12 weeks.

**DNA preparation.** DNA was isolated from the peripheral blood leukocytes (PBL) contained in 1 ml of EDTA-blood. Erythrocytes were lysed by three rounds of mixing with 1 ml of 10 mM Tris-HCl (pH 7.6)–1 mM EDTA (TE) in a 2.2-ml microcentrifuge tube and centrifugation for 1 min 30 s at 14,000 × *g*. The final PBL-containing pellet was resuspended in 100 μl of TE, and the DNA

* Corresponding author. Mailing address: Laboratory of Cancer Genetics, Ludwig Institute for Cancer Research, Rua Professor Antonio Prudente, 109, 4th floor, 01509-010 São Paulo, Brazil. Phone: 55-11-270-4922. Fax: 55-11-270-7001. E-mail: asimpson@node1.com.br.

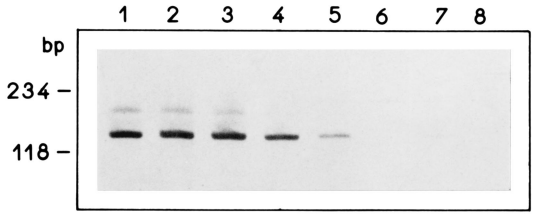



FIG. 1. Sensitivity of the PCR protocol. A silver-stained 6% polyacrylamide gel with amplification products of serial 10-fold dilutions of mixtures of human DNA and a plasmid containing the fragment amplified by the LA primer pair in different copy numbers is shown. Lanes 2 to 6 show 3,000, 300, 30, 3, and 0.3 HCMV gene copies, respectively, in 50 ng of human DNA. Lanes 7 and 8, negative controls, contain no DNA and HCMV-negative human DNA, respectively. Lane 1, positive control (plasmid containing the LA DNA fragment used to prepare the mixtures). The numbers on the left indicate the positions of molecular size markers (ϕX *Hae*III digested).

was prepared with a RapidPrep genomic DNA isolation kit, as described by the manufacturer (Pharmacia Biotech).

**PCR.** The quality of the DNA preparation of all samples was checked by amplification of a 110-bp fragment of the β-globin gene with primers PC03 (5′ ACACAACTGTGTTCACTAGC 3′) and PC04 (5′ CAACTTCATCCACGTT TCACC 3′) (13). For HCMV DNA detection, two pairs of oligonucleotide primers, specific for the fourth exon of the HCMV immediate early gene (IE) and HCMV late antigen gp64 (LA) were used to generate fragments of 393 and 136 bp, respectively. The sequences of the IE primers (3) and the LA primers (16) are as follows: IE, 5′ GCTGCGGCATAGAATCAAGGAGCA 3′ and 5′ GGTTGGTGGTCTTAGGGAAGGCTGAG 3′; LA, 5′ CGCAACCTGGTGC CCATGG 3′ and 5′ CGTTTGGGTTGCGCAGCGGG 3′.

All PCR mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM (each) deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 12.5 pmol of each primer, and 2 U of *Taq* DNA polymerase (Cenbiot, Porto Alegre, Brazil) in a final volume of 50 μl. The thermal cycling conditions included a wax-mediated hot start (4), which precluded the initiation of the reaction until the mixture had reached 80°C. For amplification of HCMV sequences, an initial denaturation step at 94°C for 3 min was employed, followed by 40 cycles in which each cycle consisted of 30 s at 94°C and 2 min at 72°C, followed by one final extension at 72°C for 8 min.

For amplification of the β-globin sequence, the program was the same except that an annealing step at 55°C for 1 min was included in each cycle following the 94°C step.

All experiments had two negative controls, one without DNA and the other with a human PBL DNA preparation known to be negative for HCMV DNA.

**Cloning and sequencing of the 136-bp HCMV DNA fragment amplified by the LA primers.** The HCMV DNA fragment amplified with the LA primer pair was cloned into pUC18 exactly as described elsewhere (14), and the insert was sequenced with a Cy 5 Autocycle sequencing kit (Pharmacia) and an ALF Express semiautomatic fluorescent sequencer.

**Semiquantification of HCMV DNA in clinical samples by limiting dilution.** The total DNA concentration in each sample was estimated by comparison with the Low DNA Mass ladder (Gibco-BRL, Gaithersburg, Md.) following electrophoresis on 1.5% agarose gels stained with ethidium bromide. This value was used to calculate the number of cell equivalents in the reactions, since even at the very highest viral loads the HCMV DNA makes an imperceptible contribution to the total quantity of DNA present. Twofold dilutions of the samples were prepared in water, and each dilution was submitted to DNA amplification with the LA primers, as described above. The endpoint was taken as the highest consecutive dilution that resulted in amplification, which we considered to contain two viral genomes. Endpoint titers were then converted to HCMV DNA copies per $10^6$ leukocytes (7, 19).

**PCR product detection by silver-stained polyacrylamide gel electrophoresis.** Four microliters of the PCR products was run on 6% polyacrylamide gels with a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, Calif.). The staining procedure consisted of initial gel fixation for 3 min in 10% ethanol–0.5% acetic acid followed by staining in fixing solution containing 0.2% silver nitrate for 5 min. After a wash in Milli-Q water (Millipore, Bedford, Mass.) for 2 min, the gels were developed for approximately 5 min in 3.0% NaOH and 0.1% formaldehyde (15). All solutions were prepared with Milli-Q water. Following staining, the gels were fixed for 5 min and dried on a glass plate between two sheets of porous hydrophilic cellophane.

## RESULTS

**High-sensitivity HCMV DNA detection by high-temperature PCR cycling.** We selected, from the literature, a primer pair specific for a portion of the HCMV LA gene (16). The length and GC content of these primers are sufficiently high to permit the use of a two-temperature PCR amplification protocol of 94°C for denaturation and 72°C for annealing and extension. In a preliminary experiment, the LA primer pair was shown to be capable of specifically amplifying the HCMV sequence, by using this high temperature cycling profile together with the hot-start procedure, from template DNA prepared from the leukocytes of individuals with positive anti-HCMV IgM. From 40 such DNA preparations, a single amplification product of 136 bp was obtained in 15 cases (data not shown). The other samples were entirely negative, showing no bands on silver-stained gels. All DNA preparations were tested separately with primers specific for the human β-globin gene and were all positive. The 136-bp product, derived from one of the patients, was cloned and sequenced. The sequence exhibited 100% homology with the expected fragment of the HCMV LA gene (GenBank accession no. K02531). These data were thus consistent with a very low background and highly specific amplification for the targeted region of the HCMV LA gene.

To test the sensitivity of the reaction, the plasmid containing the HCMV LA gene sequence was added to normal human leukocyte DNA to produce reaction mixtures containing from 5,000 to 0.1 viral gene copies in 50 ng of normal human DNA. In replicate experiments, with independent mixes, the lowest LA gene copy number detected was always between one and five. The results of a representative experiment are shown in Fig. 1, where mixes containing 3,000 to 0.3 gene copies in the presence of 50 ng of human DNA were amplified and the presence of 3 copies was detectable.

The effects of variable levels of human DNA in the reaction mix were tested in an experiment in which 50 gene copies were mixed with increasing amounts of human DNA and amplified (Fig. 2). Amplification products of identical intensity were produced independent of the amount of human DNA in the reaction mixture.

**Multiplex amplification of distinct HCMV gene sequences by using high-temperature PCR cycling.** A multiplex reaction was established by the inclusion of a second pair of primers, which amplify a 393-bp portion of the IE gene and allow annealing at 72°C (3). The multiplex reaction was applied to 412 clinical samples of leukocyte-derived DNA containing variable amounts of HCMV DNA as estimated by limiting dilution. The results were of the same quality as those produced by the LA primer pair alone in that only the specific products were amplified from clinical specimens, with complete absence of any detectable background amplification product (Fig. 3). On this basis we presume that the two primer

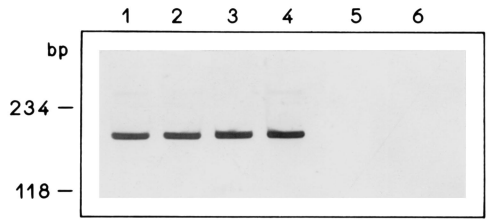



FIG. 2. Lack of competition from host DNA for the amplification of HCMV DNA. A silver-stained 6% polyacrylamide gel with amplification products derived from 50 copies of the plasmid containing the HCMV LA gene sequence in the presence of increasing amounts of HCMV-negative human leukocyte DNA is shown. Lanes 1 to 4 correspond to the amplification of the 50 HCMV copies without human DNA and with 1, 10, and 100 ng of human DNA, respectively. Lanes 5 and 6, negative controls (human DNA used to prepare the mixtures and no DNA added, respectively). The numbers on the left indicate the positions of molecular size markers (ϕX *Hae*III digested).

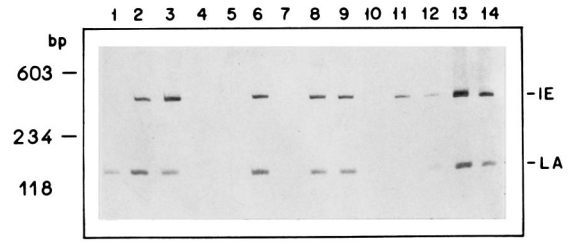



FIG. 3. Multiplex PCR. A silver-stained polyacrylamide gel with the PCR products of the HCMV LA and IE genes derived from the PBL of 13 renal transplant patients. Lanes 1 to 3, 6, 8, 9, and 11 to 13 are from HCMV DNA-positive patients; lanes 4, 5, 7, and 10 are from HCMV DNA-negative patients. Lane 14, positive control (HCMV DNA-positive patient). Note that in lanes 1 and 11 only one of the two possible amplification products is visible. The numbers on the left indicate the positions of molecular size markers (ϕX *Hae*III digested).

pairs have similar levels of sensitivity and specificity. This presumption is further supported by the observation that of the 412 samples tested, 211 were positive for the LA fragment and 169 were positive for the IE fragment.

Table 1 shows the distribution of results positive for one or both products. Based on the argument that those samples positive for single products would have been false negatives if the primer pair which did not amplify were used alone, the use of the multiplex format increased the number of positive samples by an average of 12% over a single primer pair.

As well as increasing the sensitivity, the multiplex assay also provides quantitative information. The number of copies of viral DNA in the samples positive for both targets was significantly higher than in those positive for only one of the targets (Table 1), and the proportion of results positive for both gene targets varied in relation to the concentration of HCMV DNA. With <1,000 HCMV genomes per $10^6$ leukocytes, 65% of the results were positive for both gene products. With 1,000 to 10,000, 10,000 to 50,000, and >50,000 HCMV genomes per $10^6$ leukocytes, 72, 94, and 100% of the results were positive for both gene products, respectively.

**Monitoring of HCMV infection in renal transplant recipients by multiplex PCR.** The results obtained with the multiplex assay were compared with the estimates of viral genome copy numbers in serial leukocyte DNA samples taken from 34 patients during the first 12 weeks post-renal transplantation. During this period eight patients developed very high levels of HCMV DNA (>50,000 genomes per $10^6$ leukocytes), and three patients displayed clinical symptoms attributable to HCMV infection (fever, leukopenia, pneumonia, or gastrointestinal ulcers).

All patients were positive at least once for the presence of HCMV DNA during the time frame studied, indicating that qualitative PCR alone is of little value in identifying high-risk patients. For 23 patients the results were intermittent, with interspersed negative samples and positive results in which either one or both targets were amplified and with no instance in which three consecutive samples were positive for both gene targets (for examples, see Fig. 4). In all of these cases the estimates for viral DNA levels never rose above 50,000 HCMV genomes per $10^6$ leukocytes (Table 2). None of the patients developed any clinical symptoms of HCMV.

For 11 patients there were at least three consecutive examples which were positive for both gene targets (Fig. 5). This group contained all eight cases in which the estimated viral load rose above 50,000 viral genomes per $10^6$ leukocytes, including the three patients who developed clinical symptoms of HCMV infection (Table 2). For all eight patients for whom the maximum number of genomes rose above 50,000 per $10^6$ leukocytes, the third consecutive double-positive result (indicated by arrows in Fig. 5) either coincided with or preceded the peak level of DNA. The use of three consecutive positive results of either type alone did not yield the same high values of sensitivity and specificity for high-level infections (Table 3).

## DISCUSSION

We have demonstrated that attention to the conditions of a diagnostic PCR to avoid unwanted background amplification, together with the use of silver staining to detect the reaction products, results in a simple, single-step assay with a sensitivity of less than five HCMV genomes in a reaction mix containing 50 ng of human DNA. In order to achieve this level of sensitivity in PCR-based diagnosis, two approaches are generally adopted: nested PCR and postamplification hybridization (1–3, 8, 21). Consultation of standard texts on PCR diagnosis of infectious disease (9) reveals that almost all protocols use one or the other of these adaptations, and some use both. These adaptations are undoubtedly effective, but they also considerably complicate the clinical application of PCR. For example, in nested PCR the necessity of the second round of PCR delays results, the manipulation of the amplified material greatly increases the possibility of contamination, and the use of two separate primer pairs requires that two sets of positive and negative controls routinely be used. Although postamplification hybridization does not cause increased contamination, it does considerably delay results and is relatively costly and complex.

We took the view that nested PCR and postamplification hybridization may be necessary to achieve high specificity and sensitivity only when the stringency of the reaction is not correctly adjusted. In this situation, unwanted background products are amplified due to nonexact interaction of the primers with the template DNA. Multiple products clearly necessitate either nested or hybridization protocols to identify unambiguously the specific product of interest. Furthermore, PCR is a competitive process, and nonspecific products decrease the efficiency of the amplification of the target and hence the sensitivity of the reaction (10). We have routinely been using silver-stained gels for PCR-based diagnostic assays due to both their superior sensitivity and their easy storage, which avoids the necessity of photography to record all results (15). We have noted that many diagnostic protocols, when analyzed with silver staining, result in extensive background amplification due to inappropriate primer-template interactions.

Since we were concerned with maximizing sensitivity, we employed a multiplex format. Our rationale was that at single-copy concentrations, and with very sensitive assays, Poisson distribution becomes a factor in whether a particular sample is

TABLE 1. Amplification of the IE and LA target sequences by multiplex PCR from leukocyte DNA samples from renal transplant patients in relation to the number of viral genomes per $10^6$ cells

| Test result | No. of samples ($n$ = 412) | Geometric mean (SD) of the no. of HCMV genomes/$10^6$ leukocytes[a] |
|---|---|---|
| Negative | 196 | |
| Positive for either IE or LA | 52 | $1.84 \times 10^3$ (3.75) |
| Positive for both IE and LA | 164 | $4.96 \times 10^3$ (6.68) |

[a] Means are significantly different ($P < 0.001$) according to an unpaired $t$ test.

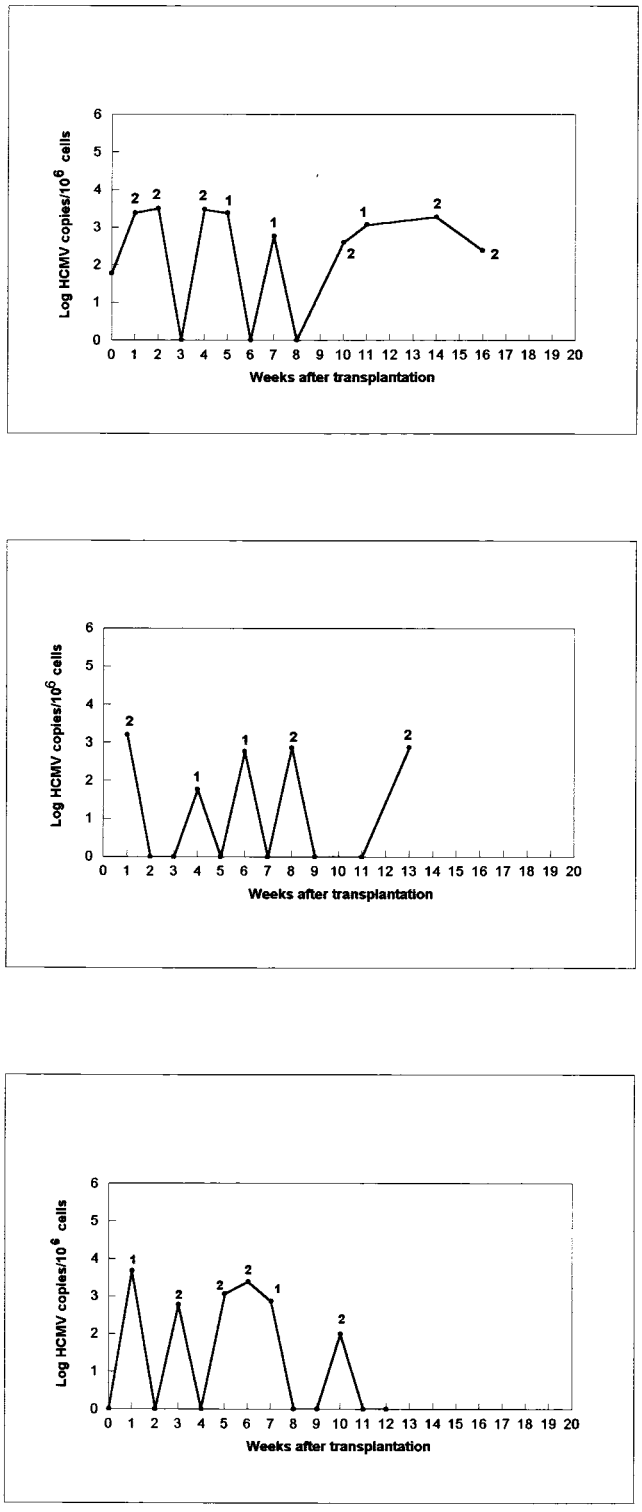



FIG. 4. Examples of the results of multiplex PCR compared with HCMV genome copy number estimates in renal transplant patients for whom there were never three consecutive assays positive for both gene products. 1, assay positive for either the LA or IE gene; 2, assay positive for both LA and IE. Each graph represents an individual renal transplant patient.

TABLE 2. Correlation between the maximum number of HCMV genomes during the 12 weeks following renal transplant and the results of multiplex PCR positive for both products[a]

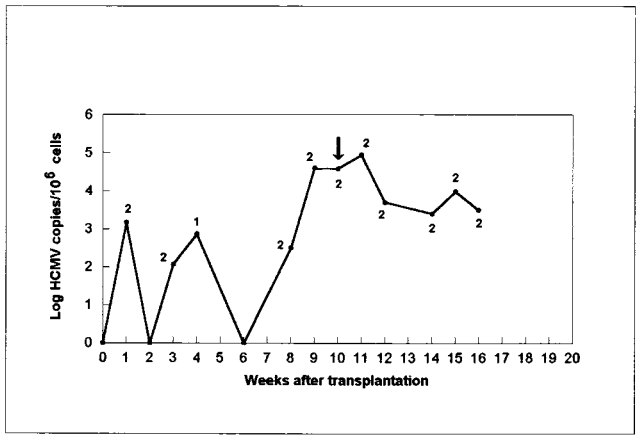
| No. of consecutive PCR assays positive for LA and IE | HCMV copies/$10^6$ leukocytes | | |
|---|---|---|---|
| | >50,000 | <50,000 | Total |
| 3 or more | 8 | 3 | 11 |
| Less than 3 | 0 | 23 | 23 |
| Total | 8 | 26 | 34 |

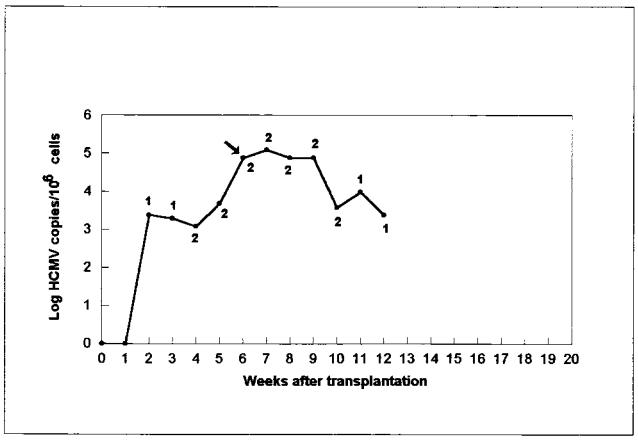
[a] Sensitivity, 100%; specificity, 88%; negative predictive value, 100%; positive predictive value, 73%.

scored positive or negative (19, 20). Thus, replicate assays are often employed to increase the probability of the sample containing the genome target. We hypothesized that the HCMV DNA (approximately 150 kb) is likely to be fragmented following DNA preparation and that the amplification of separate genes (in this instance separated by approximately 50 kb in the HCMV genome [12, 18]) would be an alternative approach to undertaking replicate assays for the same gene fragment. This approach proved to be successful, as judged by the number of results positive for one of the two genes as compared with the number positive for both in a survey of leukocyte DNA samples from renal transplant recipients. As expected, the average amount of HCMV DNA in those samples from which a single gene was amplified was significantly less than in those from which both genes were amplified. Likewise, the proportion of samples from which both genes were amplified increased with the estimated relative concentration of HCMV DNA. This assay, combining two very sensitive amplifications, is of clear clincal value in situations in which establishing the presence or absence of HCMV DNA is of critical concern.

The use of two primer pairs also endows the assay with an element of quantitative discrimination, since samples with small amounts of viral DNA are more likely to lead to the amplification of only one of the two gene targets. It should be noted that in assessing the samples for HCMV with the multiplex assay, the total amount of DNA in the assay was not normalized. Thus, variation in the concentration of DNA in the preparation is a factor in the outcome of the reaction. Nevertheless, the preparations were sufficiently homogeneous for the adopted approach to be effective. This crude approach to assessing viral load may be of limited value in assaying single samples, but when serial samples were used we found it to provide clinically valuable data. The quantity of HCMV DNA in immunosuppressed individuals who do not develop active infection is at about the level of sensitivity of the assay, so serial samples are intermittently positive and positive samples intermittently contain one or two gene targets. On the other hand, individuals who develop high-level infections which are highly likely to produce clinical symptoms have sufficient viral DNA for the serial samples to be always positive for both gene targets. This difference serves to differentiate the two patterns of infection if a lower limit of three consecutive double-positive results is taken as the cutoff. If the necessity of using three samples collected over a period of 15 days led to a delay in the diagnosis of high-level infection, the assay would not be of clinical value. However, our observations show that the third consecutive double positive either coincides with or precedes the peak level of DNA irrespective of its absolute value.

Although the data presented here pertains to HCMV infection, the principles are applicable to any DNA-based PCR



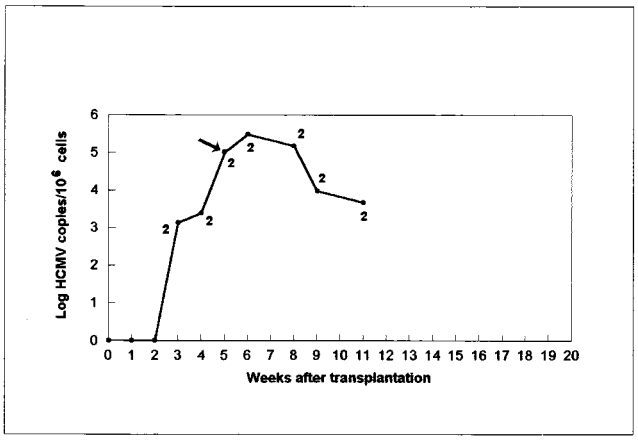




FIG. 5. Examples of the results of multiplex PCR compared with HCMV genome copy number estimates in renal transplant patients in which there were at least three consecutive assays positive for both gene products. 1, assay positive for either the LA or IE gene; 2, assay positive for both LA and IE. Each graph represents an individual renal transplant patient. The arrows indicate the third consecutive assay positive for both products.

TABLE 3. Correlation between the maximum number of HCMV genomes during the 12 weeks following renal transplant and the results of multiplex PCR positive for one or both products[a]

| No. of consecutive PCR assays positive for LA and/or IE | HCMV copies/$10^6$ cells | | |
|---|---|---|---|
| | >50,000 | <50,000 | Total |
| 3 or more | 8 | 11 | 19 |
| Less than 3 | 0 | 15 | 15 |
| Total | 8 | 26 | 34 |

[a] Sensitivity, 100%; specificity, 58%; negative predictive value, 100%; positive predictive value, 42%.

diagnostic assay; the combination of hot start, an elevated annealing temperature, and silver staining should permit the development of assays of sensitivity similar to that described here for other infectious organisms. Likewise, the concept of using multiplex PCR both for increased sensitivity and for initial assessment of viral load in monitoring situations should be applicable in other situations. We believe that the development of such simple but sensitive assays could have a significant effect on the clinical application of PCR.

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