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Automated Extraction and Quantification of Human Cytomegalovirus DNA in Whole Blood by Real-Time PCR Assay

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The measurement of human cytomegalovirus (HCMV) DNA in blood is becoming the standard method for monitoring HCMV infection in immune-suppressed and unsuppressed patients. As various blood compartments can be used, we have compared the HCMV DNA measured in whole blood (WB), peripheral blood leukocytes (PBL), and plasma by real-time PCR. We tested 286 samples: HCMV DNA was extracted automatically from WB and PBL with the MagNA Pure instrument (Roche Molecular Biochemicals) and manually from plasma samples. The HCMV DNA from WB, PBL, and plasma was measured by real-time Light Cycler PCR. Primers and probe were located in the UL 83 region. HCMV DNA was detected more frequently in WB (88.5%) than in the PBL (65.7%) (P < 0.0001) or the plasma (55.2%) (P < 0.0001). There was a good correlation between the positive results in WB and in PBL (r = 0.68; P < 0.0001), and 3.15 log10 genome copies in 200,000 PBL, equivalent to 3.4 log10 genome copies in 200 µl of WB. WB was shown to be suitable for automated extraction and the quantitation of HCMV DNA by real-time Light Cycler PCR by analysis of serial samples from representative patients of various populations. This system may be very useful for monitoring of immune-suppressed and unsuppressed patients.

Human cytomegalovirus (HCMV) is the most important opportunistic pathogen in immune-suppressed patients, especially those who have received solid organ or bone marrow transplants. While antiviral treatment reduces the mortality and morbidity rates, the development of sensitive, specific techniques for detecting HCMV infection has now made possible preemptive treatment.

A wide variety of techniques have been developed, including both nonmolecular and molecular tests. Because of its great sensitivity, qualitative PCR was the first method used (8, 18), but this method cannot differentiate between infection and disease in transplants. In contrast, quantitative PCR methods have shown that immune-suppressed patients with a high virus load are at great risk of developing HCMV disease (3, 9, 16, 19–21). PCR tests developed in-house and the first commercially available tests are time consuming and labor intensive because they require a post-PCR detection step. The development of automated real-time PCR instruments that can quantify DNA has led to a significant change in the routine diagnosis of CMV infection (6, 10, 17).

The virus loads in several blood compartments, such as peripheral blood leukocytes (PBL) and plasma, have been compared to pp65 antigenemia (4, 13). Virus was most frequently detected in the PBL, and the virus loads were significantly higher than in the plasma (1, 2, 12). More recently, real-time quantitative PCR using the Tagman PCR or Light Cycler PCR methods have been used to measure virus DNA in various blood compartments of immune-suppressed patients, and the results were compared with pp65 antigenemia (6, 7, 10) or other PCR techniques (14). One study found that whole blood (WB) was the most suitable for quantifying HCMV DNA (15).

Our study was therefore carried out to compare the performances of HCMV DNA measured after automated extraction from WB and PBL with the MagNA Pure instrument and real-time Light Cycler PCR. The results were compared with those obtained by manually extracting HCMV DNA from plasma. We analyzed samples from various populations to assess the usefulness of WB for automated extraction prior to quantifying HCMV DNA by real-time Light Cycler PCR in the follow-up of immune-suppressed and unsuppressed patients.

MATERIALS AND METHODS

Patients and clinical specimens. The specificity studies were done on 24 blood samples from five HCMV-uninfected (seronegative) patients. We tested 286 blood samples from 95 HCMV-infected patients, namely, 157 samples from 48 solid organ transplant recipients, 77 samples from 10 allogeneic bone marrow transplant (BMT) recipients, 26 samples from 18 human immunodeficiency virus-infected patients, 6 samples from 5 unsuppressed patients with a primary HCMV infection, and 20 samples from 14 patients suffering from other disorders.

The group of solid organ transplant patients included 34 men and 14 women, and their mean age was 48 years. There were 29 renal transplant patients, 12 liver transplant recipients, 6 cardiac transplant patients, and 1 heart and renal transplant recipient. The BMT recipients were 9 men and 1 woman, and their mean age was 46 years. The human immunodeficiency virus-infected patients were 15 men and 3 women, and their mean age was 44 years. The unsuppressed patients suffering from a primary infection were 1 man and 4 women, and their mean age was 52 years. The other patients were 10 men and 4 women, whose mean age was 61 years.

Blood was collected into potassium EDTA tubes. An aliquot of WB was kept at 4°C until use, and another aliquot was used to separate leukocytes and plasma by sedimentation through Histopaque-1119 and Histopaque-1077 solutions (Sig-
ma Chemical Co., St Louis, Mo.). The leukocytes were then counted and divided into aliquots of 700,000 cells per ml.

**HCMV DNA detection and quantification.** DNA was extracted from 200 μl of WB and 200 μl of PBL with the MagNA Pure instrument (Roche Molecular Biochemicals). The MagNA Pure LC DNA Isolation Kit I was used according to the manufacturer’s instructions. DNA was extracted manually from 200-μl samples of plasma with a QIAamp DNA blood minikit (Qiagen).

HCMV DNA was detected and quantified with the Light Cycler system (5). The primers and probe, defined in the UL83 region, were as follows: pp549s (sense), 5'-GTC AGC GTT CGT GTT TCC CA-3'; and pp812as (antisense), 5'-GGG ACA CAA CAC CGT AAA GC-3'. The fluorogenic probe was 5'-(6-Fam)CCC GCA ACC CGC AAC CCT TCA TG (phosphate)-3'. Real-time PCR was carried out with the Fast Start DNA master hybridization probes (Roche Molecular Biochemicals). Extracted DNA (10 μl) was added to the PCR mixture containing 5 mM MgCl₂, 0.5 μM concentrations of each primer and probe, and 0.025 U of uracil DNA glycosylase/μl. The mix was incubated for 10 min at room temperature prior to PCR. The conditions were as follows: initial denaturation of one cycle of 10 min at 95°C, followed by 45 cycles of 10 s at 95°C and 45 s at 65°C. The reaction, data acquisition, and analysis were all done by using the Light Cycler instrument.

The pBluescript II KS plasmid (kindly provided by F. Freymuth) containing the target sequence was used as a reference standard. The plasmid was diluted to give a standard curve of 1 to 5 log₁₀ genome copies per reaction volume. The Light Cycler software generated a best-fit line from this log-linear region of each curve that defined the crossing line. The point of intersection between the emitted fluorescence and the crossing line defined the crossing point. The concentration of target DNA was calculated by plotting the crossing point of each sample on the standard curves by using the LightCycler software. The HCMV DNA in WB and PBL were expressed as log₁₀ genome copy numbers per 200 μl of WB and as log₁₀ genome copy numbers per 200,000 PBL.

Contamination of the PCR was checked by including a negative sample and a sample with distilled water in each run. The detection limit of the method was 10 genome copies per 10 μl of reaction mix. This corresponds to 100 (2 log₁₀) genome copies in 200 μl of WB or 200 μl of a suspension of 700,000 leukocytes per ml of PBL.

**SEROLOGICAL MARKERS.** Immunoglobulin G (IgG) and IgM were detected with the ETI-CYTOK-G Plus and ETI-CYTO-M reverse (DIA Sorin, Antony, France) assays according to the manufacturer’s instructions.

**Statistical analysis.** The proportion of positive and negative results were compared by using the chi-square test. Correlations between quantitative values were checked using the Spearman test. P values of less than 0.05 were considered significant.

**RESULTS**

**Specificity.** The specificity of the real-time Light Cycler PCR method was determined by testing three compartments (WB, PBL, and plasma) extracted from 24 blood samples from nine HCMV-seronegative patients. All the results were negative, indicating 100% specificity.

**TABLE 1. Detection of HCMV DNA in WB and in PBL**

<table>
<thead>
<tr>
<th>PBL result</th>
<th>WB sample result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. positive</td>
<td>182</td>
<td>188</td>
</tr>
<tr>
<td>No. negative</td>
<td>71</td>
<td>98</td>
</tr>
<tr>
<td>Total</td>
<td>253</td>
<td>286</td>
</tr>
</tbody>
</table>

**TABLE 2. Detection of HCMV DNA in WB and in plasma**

<table>
<thead>
<tr>
<th>Plasma result</th>
<th>WB sample result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. positive</td>
<td>154</td>
<td>158</td>
</tr>
<tr>
<td>No. negative</td>
<td>99</td>
<td>128</td>
</tr>
<tr>
<td>Total</td>
<td>253</td>
<td>286</td>
</tr>
</tbody>
</table>

**FIG 1.** Correlation between quantitative PCR results in WB and in PBL. Results of quantitative PCR show close correlation between HCMV DNA log₁₀ copy numbers in PBL and in WB.
FIG 2. Monitoring the HCMV load in WB and the PBL of five solid organ transplant recipients (SOT-1 to SOT-5) by using real-time Light Cycler PCR. The results of serial samples collected from five patients are shown, as are the periods of treatment with intravenous ganciclovir (IV GCV) and Zelitrex (Val ACV). Diamonds show HCMV DNA copy number in WB, and squares show HCMV DNA copy number in PBL. Qualitative results in plasma are also shown.
Detection of HCMV DNA in WB and PBL. Of the 286 samples tested, 182 were positive in both compartments, while 27 were negative in both. The WB of 71 samples were positive and their PBL was negative, whereas the PBL from 6 samples were positive while WB was negative. HCMV DNA was detected significantly more frequently in WB (88.5%) than in PBL (65.7%) (P < 0.0001) (Table 1).

Detection of HCMV DNA in plasma. There were 154 samples that were positive in WB and the plasma, whereas 29 were negative in both compartments. Ninety-nine samples were positive in WB and negative in plasma. The plasma of four samples was positive and their WB was negative.

HCMV DNA was detected significantly more frequently in WB (88.5%) than in plasma (55.2%) (P < 0.0001).

One hundred and thirty-two samples were positive in PBL and plasma, and 72 were negative in both. Fifty-six samples were positive in PBL and negative in plasma, while 26 samples were negative in PBL and positive in plasma. HCMV DNA was significantly more frequently detected in PBL than in plasma (P < 0.0001) (Table 2).

Thus, the WB is the most sensitive substrate for the qualitative detection of HCMV DNA.

Correlation between HCMV DNA in PBL and HCMV DNA in WB. All the samples that gave positive PCR results in WB and in PBL were analyzed (n = 165). There was a good correlation between the HCMV DNA log_{10} genome copy number in WB and the HCMV DNA log_{10} genome copy number in PBL (r = 0.68; P < 0.0001) (Fig. 1).

A clinical threshold value of 50 positive polymorphonuclear cells in 200,000 leukocytes was equivalent to 3.15 log_{10} genome copies in 200,000 cells (i.e., 2 log_{10} genome copy number per capillary) (11). The linear regression line for the qualitative PCR results in WB and in PBL indicated that 3.15 log_{10} genome copies was equivalent to 3.4 log_{10} genome copies in 200 µl of WB.

Kinetics of HCMV DNA markers on serial samples. Five unsuppressed patients with primary HCMV infections and
seven immune-suppressed patients who had been given anti-HCMV therapy were studied. These treated patients included five solid organ transplant recipients and two BMT recipients, from whom 5 to 27 serial samples had been taken.

**Analysis of the unsuppressed patients.** The clinical manifestations were those typical of a primary HCMV infection. They all had IgM antibodies, and two showed IgG seroconversion. Qualitative PCR done on WB and PBL was always positive, while it was negative for three plasma samples.

**Analysis of solid organ transplant recipients.** Two patients remained asymptomatic. The three others showed clinical manifestations at a mean time of 47 days posttransplant (range, 41 to 53 days posttransplant). Virological markers were always positive at that time. Patients were given intravenous anti-HCMV therapy (250 mg of intravenous ganciclovir twice a day, adapted to renal function), starting when HCMV DNA was detected. The rates at which the virus loads in WB and in PBL changed were identical and followed antiviral treatment (Fig. 2).

**Analysis of BMT recipients.** Virological markers were positive 51 days posttransplant for patient 1 and 31 days posttransplant for patient 2, at which time they were asymptomatic. They were given intravenous anti-HCMV therapy (250 mg of intravenous ganciclovir twice a day or 3 g of Foscavir twice a day), starting when HCMV DNA was detected. The rates at which the virus loads in WB and in PBL changed were identical and followed antiviral treatment (Fig. 3).

**DISCUSSION**

The use of immune-suppressive chemotherapy has enabled the number of transplants performed to increase, and these patients all require follow-up. This led to the need for an easy-to-use method for detecting HCMV infection, as a very large number of samples are tested daily. The PBL must be separated from plasma, washed and counted, and then aliquoted and frozen. Clearly, if WB could also be tested, this would require much less processing. An automated method for

[FIG 3. Monitoring of HCMV load in WB and the PBL of two BMT recipients, BMT-1 (A) and -2 (B), by using real-time Light Cycler PCR. The results of serial samples are shown, as are the periods of intravenous ganciclovir (IV GCV) and Foscavir (IV PFA) treatment. Diamonds show HCMV DNA copy number in WB, and squares show HCMV DNA copy number in PBL. Qualitative results in plasma are also shown.]
extraction of the nucleic acid used in molecular tests is also needed. Several instruments are now commercially available.

We have compared the results obtained by extracting HCMV DNA from WB and PBL with the MagNA Pure instrument and PCR by using the real-time Light Cycler. The PCR results obtained for plasma extracted with a QIAamp blood DNA minikit were also analyzed.

This is the first study showing that the automated extraction and quantification of DNA from WB provides acceptable results, although similar results were obtained after manual extraction (7, 10, 15). HCMV DNA was detected more frequently in the WB specimens than in the PBL or plasma samples, confirming the suitability of this substrate for assays. This is particularly important for BMT recipients, as these patients are often aplastic or leukopenic, which might impair the detection of HCMV DNA in WB. However, we found that this compartment was always adequate for detecting HCMV DNA in these patients.

There were some discrepancies between the data obtained from the WB and PBL. For example, six samples were WB negative and PBL positive. The concentrations of HCMV DNA were low and below the limit of quantification. These differences also occurred during the follow-up of four patients who were previously or subsequently positive in both compartments.

There was a good correlation between the virus loads in WB and the PBL. We have previously shown that a clinical threshold of 50 positive polymorphic leukocytes per 200,000 cells was equivalent to 3.15 log_{10} genome copies in 200,000 cells (i.e., 2 log_{10} genome copies per capillary). The linear regression formula of the curve established between the qualitative PCR results in WB and in PBL indicates that 3.4 log_{10} genome copies from 200 μl of extracted WB is a relevant clinical threshold. Longitudinal studies are now needed to validate this.

The results for the seven immune-suppressed patients showed a dramatic decrease in the virus loads in WB and in the PBL during the antiviral treatment, indicating a good response to treatment.

In summary, we have shown that a high-throughput automated system that has three easy steps has considerable clinical potential. The three key points are as follows: the use of WB instead of separated, counted PBL; automated extraction; and fast real-time PCR using the Light Cycler. A total of 32 samples can be tested in each run, and the results are available less than 3 h after arrival of the sample in the laboratory. The good correlation between the virus loads in WB and the PBL indicates that WB can be used to monitor HCMV-infected patients.

REFERENCES


