Enhanced Analytical Sensitivity of a Quantitative PCR for CMV Using a Modified Nucleic-Acid Extraction Procedure

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Accurate and rapid diagnosis of CMV disease in immunocompromised individuals remains a challenge. Quantitative polymerase chain reaction (QPCR) methods for detection of CMV in peripheral blood mononuclear cells (PBMC) have improved the positive and negative predictive value of PCR for diagnosis of CMV disease. However, detection of CMV in plasma has demonstrated a lower negative predictive value for plasma as compared with PBMC. To enhance the sensitivity of the QPCR assay for plasma specimens, plasma samples were centrifuged before nucleic-acid extraction and the extracted DNA resolubilized in reduced volume. Optimization of the nucleic-acid extraction focused on decreasing or eliminating the presence of inhibitors in the pelleted plasma. Quantitation was achieved by co-amplifying an internal quantitative standard (IS) with the same primer sequences as CMV. PCR products were detected by hybridization in a 96-well microtiter plate coated with a CMV or IS specific probe. The precision of the QPCR assay for samples prepared from untreated and from pelleted plasma was then assessed. The coefficient of variation for both types of samples was almost identical and the magnitude of the coefficient of variations was reduced by a factor of ten if the data were log transformed. Linearity of the QPCR assay extended over a 3.3-log range for both types of samples but the range of linearity for pelleted plasma was 20 to 40,000 viral copies/ml (vc/ml) in contrast to 300 to 400,000 vc/ml for plasma. Thus, centrifugation of plasma before nucleic-acid extraction and resuspension of extracted CMV DNA in reduced volume enhanced the analytical sensitivity approximately tenfold over the dynamic range of the assay. J. Clin. Lab. Anal. 14:32–37, 2000. © 2000 Wiley-Liss, Inc.

Key words: CMV; quantitative PCR; CMV viral load; plasma

INTRODUCTION

CMV infection as reflected by the presence of CMV viremia occurs in 20 to 60% of immunocompromised patients but only a fraction of those will ultimately develop CMV disease (1,2). Though progress in the prophylaxis of CMV disease has been made, CMV remains a major cause of morbidity and mortality in immunocompromised patients (1,2). Distinguishing between CMV infection and CMV disease is still a challenge. After primary infection, the virus may remain latent and become an opportunistic pathogen in hosts with impaired cellular immunity (3–8).

Viremia as detected by conventional culture (CC) is still the most reliable predictor of CMV disease, but this method is very labor intensive and can take up to 6 weeks for results (9,10). The introduction of a rapid-centrifugation shell-vial culture method reduced the time for virus detection down to 24–72 hr, although specimens with a low viral load can be a problem (11,12). The CMV antigenemia assay provides a more rapid result by directly detecting CMV antigens in white blood cells (13). Even though this assay is more sensitive than CC or shell vial (11,14), the antigenemia assay is labor intensive and its sensitivity varies greatly among different laboratories (15,16).

A number of nucleic-acid amplification methods have been developed for detection of CMV for diagnosis of CMV disease (17–21), and these methods have been applied to a wide

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variety of specimens with varying results (17–21). Most of these methods show greater analytical sensitivity than CC, shell-vial culture, and the antigenemia assay. Perhaps because of the higher analytical sensitivity, PCR shows a lower positive predictive value in seropositive patients (17,19). To overcome this problem, a number of studies have evaluated changes in the viral load as measured by QPCR to diagnose CMV disease (22–27). All of these reports demonstrated a high positive predictive value for diagnosis of CMV disease for PBMC and plasma. However, when PBMC were compared to plasma as the source of specimen, plasma samples were negative for CMV in patients with disease more often than for PBMC samples, resulting in a lower negative predictive value of the assay with plasma specimens. However, PBMC are more difficult and laborious to work with than plasma. We undertook to overcome this problem by improving the analytical sensitivity of QPCR using plasma as the specimen source.

In this report we describe a modification of a commercially available DNA-extraction procedure and QPCR assay that enhances the analytical sensitivity of the assay using plasma as the specimen source. Enhancement of sensitivity was achieved by introducing a simple and rapid centrifugation step before nucleic-acid extraction from plasma specimens and concentration of the nucleic acid at the resolubilization step. In addition, we compared the analytical performance of the enhanced QPCR method to that of the method recommended by the manufacturer.

MATERIALS AND METHODS

Sample Preparation

Peripheral blood from healthy volunteers was drawn using standard venipuncture technique with EDTA anticoagulated tubes. Plasma was separated within 2 hr of collection by centrifuging at 2,000 g for 10 min at room temperature. After processing, plasma was pipetted into 200-μl or 500-μl aliquots and stored at –80°C until tested.

DNA Extraction From Plasma (Standard Extraction Procedure)

DNA was extracted from 200 μl of plasma using the QiAmp Tissue Extraction kit (Qiagen, Boston, MA) according to the manufacturer’s recommendations. The extracted material was eluted from the columns using 200 μl of Dnase- and RNase-free H₂O (Gibco-BRL, Gaithersburg, MD) pre-warmed to 75°C. Extracted material was immediately used for PCR analysis.

DNA Extraction From Pelleted Plasma (Modified Extraction Procedure)

Five hundred microliters of plasma were aliquoted into a microcentrifuge tube and centrifuged at 15,000 g in a microcentrifuge (Eppendorf, Westbury, NY) at room temperature for 15 min. The supernatant was discarded and the pellet was resuspended in 200 μl of sterile Phosphate Buffered Saline (PBS, 0.138 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, and 0.01 M KH₂PO₄, pH:7.4; SIGMA, St. Louis, MO). DNA was extracted from the pellet using a modification of the QiAmp Tissue DNA Extraction kit. The steps were taken as described by the manufacturer except that after the sample was applied to the column and washed twice with 400 μl of AL buffer (provided by manufacturer, Qiagen, Boston, MA), two additional washes with AW buffer (provided by manufacturer) occurred. In addition, DNA was eluted from the columns using 50 μl of Dnase- and RNase-free H₂O (Gibco-BRL, Gaithersburg, MD) pre-warmed to 75°C.

CMV Quantitative PCR (QPCR)

Quantification of CMV was performed using a modification of the CMV Viral Quant test (Biosource International, Camarillo, CA) as previously described (28). Denatured amplicon and fivefold serial dilutions were detected by hybridization with either CMV or IS probes followed by the addition of horseradish peroxidase and substrate. The colorimetric reaction was read at 450 nm. An optical density reading of ≥ 0.300 was considered positive. Calculation of the CMV titer was performed by multiplying the OD value of the CMV by the respective highest dilution that gave an OD between 0.3 and 1.5, and then dividing by the product of the OD value of the IS and respective highest dilution that gave an OD between 0.3 and 1.5. This value was multiplied by 100 for the number of IS molecules and by the sample dilution factor.

CMV DNA and Purified CMV

Purified CMV DNA and CMV were obtained from a commercial source (ABI Columbia, MD). The number of viral particles present in the purified CMV had been counted by electron microscopy by the commercial source. The purified CMV preparation had a nominal titer of 9.0 × 10⁹ CMV viral particles per ml. In order to confirm the amount of CMV viral particles present in the reference material, we split the sample in half and extracted the DNA using a standard Phenol/Chloroform method including glycogen as a carrier during the precipitation step (29). After DNA extraction we calculated a titer of 4.5 × 10⁹ per ml for the purified CMV sample, which confirmed the amount of virus present in the reference sample.

Statistical Evaluation

Calculations were performed using the Stata v 5.0 statistical software package (Stata, Computing Resources Center, Santa Monica, CA).
The values represent the mean value of equivalent viral copies into the QPCR.

The values represent the calculated equivalent viral copies into the QPCR.

<table>
<thead>
<tr>
<th>Purified viral DNA</th>
<th>1,000*</th>
<th>500</th>
<th>100</th>
<th>50</th>
<th>10</th>
<th>5</th>
<th>1</th>
<th>0.5</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>1237 ± 377</td>
<td>476 ± 127</td>
<td>113 ± 36</td>
<td>48 ± 19</td>
<td>15 ± 5</td>
<td>6 ± 3</td>
<td>2 ± 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HSV-1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>EBS</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The values represent the calculated equivalent viral copies into the QPCR.

The values represent the mean value of equivalent viral copies into the QPCR ± the standard deviation of samples tested in triplicate and in three different experiments.
CMV, with purified virus and frozen at −80°C until tested. Samples were analyzed in duplicate and in three different experiments. Extraction and analysis of 200 μl of spiked plasma produced signals equivalent to the theoretical number of added viral particles (see Virus and Extraction, Table 2). Similarly, extraction of the pellet resulting from centrifuging the 500 μl of the spiked plasma resulted in signals that did not differ significantly from the analysis of the entire 500-μl sample (see Virus/pelleted plasma, Table 2). The pelleted plasma samples did include the two additional washes similar to the studies with purified DNA. Thus, using the centrifugation step and a tenfold concentration of the original plasma sample in combination with the two extra washes, we were able to pellet down the virus and at the same time remove the inhibitors present in the final extracted material.

Comparison of Performance Characteristics for QPCR Using Plasma and Pelleted Plasma for CMV

The precision of the QPCR method was therefore determined in similar fashion to that described for a quantitative HCV test (30). First, high and low control samples were prepared by spiking fresh plasma with two different amounts of purified CMV. The samples were aliquoted into single-use 500-μl or 200-μl aliquots and stored at −80°C until tested. All samples were continuously mixed and maintained on ice during mixing and aliquoting. Again, nucleic acid from the 200-μl aliquots were extracted according to manufacturer’s recommendation and the 500 μl aliquots were centrifuged before nucleic-acid extraction and processed as described above. Samples from each control were analyzed in triplicate and the experiments were repeated five times for pelleted and uncentrifuged aliquots. The titers and log titers for both the high and low controls of both plasma and pelleted plasma showed normal distributions (data not shown). Having determined that the error of the mean for both linear and log-transformed CMV concentrations as determined by the QPCR method were normally distributed, the precision of the assay was evaluated using parametric calculations. Table 3 shows the results from the precision studies for the two extraction procedures. Both extraction procedures showed very similar coefficients of variation. Use of log-transformed data reduced the coefficient of variation approximately tenfold as previously observed with QPCR for HCV (30).

Sensitivity and Linear Dynamic Range for Plasma and Pelleted Plasma for CMV QPCR

In order to determine the sensitivity and linear dynamic range of both extraction procedures we prepared a dilution of purified CMV spiked into plasma. Alternate twofold and fivefold dilution was prepared using previously tested CMV negative plasma. Each dilution was aliquoted into 200 and 500 μl and the samples were frozen at −80°C until tested. Each dilution was analyzed in duplicate for each extraction procedure and the experiment was repeated three times. Figure 1 shows the plot of the measured log-transformed concentrations against the log-transformed input value. As clearly seen in Figure 1, the QPCR was linear over a 500-fold range regardless of the extraction procedure performed. Moreover, there was complete overlap for both extraction procedure curves between 500vc/ml plasma and 10,000vc/ml plasma. The correlation coefficients for both plasma and pelleted plasma were close to 1 for both transformed and untransformed data. Even though both extraction procedures showed linearity over a 500-fold range, the extraction procedure using pelleted plasma was linear between 20 and 40,000 vc/ml plasma while the standard extraction-procedure linearity was achieved between 300 and 400,000 vc/ml plasma. As anticipated, pelleting the plasma and concentrating the extracted CMV DNA allowed the detection of virus at lower concentrations and down-shifted the dynamic range of the assay approximately tenfold.

DISCUSSION

Diagnosis of CMV disease in immunocompromised individuals remains a challenge due to the difficulty in differentiating CMV viremia from CMV disease (1–4). A number of molecular quantitative methods have been described (17–21,31–34). It has been suggested that CMV viral load corre-
lates with CMV disease and could be used as a marker for diagnosis, risk assessment, and response to therapy for CMV disease (17–21,31–34). A number of these reports have shown a high positive predictive value for diagnosis and risk assessment of CMV disease when using PBMC and plasma in immunocompromised individuals. Although plasma specimens are easier to manipulate and their integrity is easier to maintain in the clinical laboratory than are PBMC, the clinical sensitivity of the assays was lower for plasma than PBMC, thus lowering the negative predictive value of the assay. Interestingly, plasma was found to have a lower negative predictive value regardless of the patient population studied (i.e., HIV-infected, bone marrow, and solid organ transplant patients) (17–21,31–34).

In the present study we have described the enhancement of the analytical sensitivity of a rapid QPCR assay for quantitation of CMV in plasma. By introducing a simple centrifugation step before nucleic-acid extraction and resolubilization of extracted CMV DNA into a reduced volume, we were able to enhance the analytical sensitivity of the QPCR on plasma approximately tenfold. This was accomplished with no adverse effect on the assay’s precision or the magnitude of the linear dynamic range (Table 3 and Fig. 1). The QPCR assay displayed a linear dynamic range of 3.3 logs regardless of the extraction procedure. Using a standard microcentrifuge we were able to pellet down essentially 100% of CMV virions spiked into plasma (see Table 2). The centrifugation step allowed us to increase the volume of plasma evaluated from 200 μl to 500 μl. Resuspending the final DNA extracted material in a volume that was one-tenth the original sample also enhanced sensitivity. Initially, 500 μl of plasma was centrifuged and the extracted CMV DNA was eluted in 50 μl. However, we found that the concentration process introduced inhibitors of the PCR reaction into the extracted DNA. This problem was overcome by simply increasing the number of washes applied to the column used to bind the deproteinized DNA during the extraction procedure. The introduction of these two wash steps had little impact in the overall time spent by the technologist extracting the nucleic-acid specimens (data not shown).

The increased sensitivity due to concentration of the virus in the plasma sample was also reflected when we compared the sensitivity of our QPCR with others (31–34). A number of QPCR assays that used plasma as the specimen source have stated sensitivities between 200 to 2,500 vc/ml plasma (31–34). In contrast, the sensitivity of our QPCR was 20 vc/ml plasma. Thus, the sensitivity of our QPCR assay was approximately 10–100 times greater than most of other plasma QPCR assays. One point of interest is the difference in the amount of original plasma material tested in other QPCR assays compared to ours. The majority of the extraction procedures in those assays reconstituted the extracted DNA back to the original volume of plasma used for the extraction. Because these assays used 5–10 μl of extracted sample in their PCR reactions, this translated into testing an equivalent of 5 to 10 μl of original plasma for virus. In contrast, we reconstituted the extracted DNA in a volume that was one-tenth the volume of the original plasma sample and used 10 μl of extracted sample in the PCR. This translated into testing an equivalent of 100 μl of original plasma sample. Thus, the difference in the amount of the equivalent original plasma tested presumably accounts for much of the difference in the enhanced sensitiv-

Fig. 1. Linearity of the QPCR method for plasma and pelleted plasma was assessed by testing a serial dilution of purified HCMV spiked into plasma. Alternate twofold and fivefold serial dilutions were analyzed in duplicate and repeated three times. The log-transformed values for the measured concentrations were plotted against the log-transformed values of the input values for CMV.
ity of our QPCR compared to others. It is possible that the increased sensitivity of the modified QPCR assay might improve the clinical sensitivity of QPCR from plasma samples.

In conclusion, the introduction of a simple centrifugation step using a conventional microcentrifuge, coupled with concentration of the extracted CMV DNA at the resolubilization step, demonstrated an improvement in the analytical sensitivity of the QPCR using plasma as the specimen source. We increased the analytical sensitivity of the assay approximately tenfold without affecting the precision and dynamic range of the overall assay.

REFERENCES