Quantitative Real-Time PCR with Automated Sample Preparation for Diagnosis and Monitoring of Cytomegalovirus Infection in Bone Marrow Transplant Patients

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Background: In bone marrow and stem cell transplant patients, the widespread use of preemptive cytomegalovirus (CMV) antiviral therapy necessitates faster, more precise, and more sensitive quantitative laboratory methods for serial viral load monitoring.

Methods: We quantified CMV viral load assay by real-time PCR after plasma DNA was prepared by an automated robotic workstation. Fluorescent hybridization probes were directed at the glycoprotein B (gB) gene (or EcoRI D region) of CMV. The β-globin gene was amplified in parallel to control for the efficiency of the extraction and PCR steps. Samples were collected from patients for whom final diagnoses were divided based on other tests and clinical history.

Results: The assay was linear (R = 0.999) from 125 to 5 × 10⁶ copies/mL with a PCR efficiency of 1.975 (gB) or 2.02 (EcoRI D). The viral loads determined by PCRs directed at these two different viral targets were no different (n = 53; R = 0.928). The interassay CV was 3.5%, and the intraassay CV was 1–4%. Compared with a commercially available quantitative competitive PCR assay (Roche Monitor; R = 0.59), the mean results were 3.1 times higher (mean ratio; P = 0.002). The diagnostic sensitivity and specificity were 96% and 100%, respectively (n = 147), compared with 74% and 98% for a qualitative competitive PCR assay (Roche Ampliprimer). On a subset of samples, sensitivity of viral culture was 50% (n = 44). Of 1115 clinical referral samples from 252 patients, 10% of the samples and 18% of the patients had low-level CMV viremia (median, 500 copies/mL). In this predominantly (85%) bone marrow transplant testing cohort, serial CMV viral load results were the predominant clinical trigger for the initiation, monitoring, and cessation of preemptive antiviral therapy.

Conclusions: The combination of automated DNA preparation and semiautomated real-time fluorescent PCR detection allows for a sensitive, precise, and accurate high-throughput assay of CMV viral load that can be used as the laboratory trigger for preemptive antiviral therapy.

Cytomegalovirus (CMV)¹ disease is a major and often deadly complication of allogeneic bone marrow transplantation, with an incidence of 20–35% (1). Because the pharmacologic treatment of symptomatic CMV disease is difficult, toxic, and expensive, most transplant centers use these antiviral agents either prophylactically in all patients at risk of viral activation or preemptively only in those patients with early presymptomatic laboratory evidence of infection. The laboratory methods that have been used to guide preemptive CMV antiviral therapy include viral culture (traditional or “shell vial”), direct immunostaining methods (typically the pp65 antigenemia assay), and molecular methods (typically PCR-based). The culture-based methods for CMV are laborious, have long turnaround times, and are widely regarded as being too analytically insensitive (1–4). The pp65 antigenemia assay, although more analytically sensitive than culture, requires a minimum neutrophil count (often problematic after bone marrow transplantation) and rapid sample processing (to microscope slides), and can give false negatives even with active disease (5).

The direct detection and quantification of CMV DNA

¹ Nonstandard abbreviations: CMV, cytomegalovirus; gB, glycoprotein B; FRET, fluorescence resonance energy transfer; and Cp, crossing point.
by PCR methods provides the opportunity to substantially improve the detection of very low-level viremia (enabling earlier preemptive therapy) as well as to directly monitor the efficacy of antiviral therapy. In bone marrow transplant patients, preemptive antiviral therapy guided by a PCR-based CMV assay leads to earlier detection of viremia; decreased overall usage of toxic, expensive antiviral agents; and most importantly, a lower incidence of active CMV disease (2, 6, 7). The advantages of quantitative PCR-based CMV detection have led to the creation of a variety of different quantitative methods, the most popular being those based on either real-time fluorescent PCR (8–11) or competitive PCR (12, 13). Although the real-time fluorescent PCR assays for CMV typically have a wider dynamic range, lower analytical sensitivity, better analytical accuracy, higher reproducibility, and lower costs than the commercially available quantitative competitive PCR assays (11, 12, 14), their practical use in clinical diagnostic laboratories will require major reductions in assay complexity (and thus cost). The labor savings afforded by automating the detection phase of real-time PCR have not, however, been applied to the tedious and imprecise DNA sample preparation steps of these assays. Manual nonautomated DNA sample preparation is thus not only extremely laborious (and thus costly) (14, 15), but are also the major source of total assay imprecision in real-time PCR methods (16). Toward the goal of a higher throughput, less costly, and precise CMV assay for use in the routine clinical diagnostic laboratory, we developed a real-time fluorescent PCR method with an automated robotic DNA extraction step. In this study we show that this new assay is sufficiently sensitive, specific, and precise for routine monitoring of bone marrow transplant patients before, during, and after preemptive antiviral therapy.

Materials and Methods

Specimens
The specimens tested for CMV by any of several different methods were a subset of residual samples referred to the Oregon Health & Science DNA Diagnostic Laboratory for qualitative or quantitative analysis of CMV DNA. The majority (85%) of these clinically referred samples were from patients who had undergone allogeneic bone marrow or stem cell transplantation. The electronic medical records of patients with discordant clinical laboratory results were reviewed in an attempt to resolve the discrepancy. This study was ruled exempt from full Institutional Review Board review by the Oregon Health Sciences University Institutional Review Board.

Nucleic Acid Extractions
For real-time quantitative CMV PCR, 250 ng of human genomic DNA (from a CMV seronegative donor) was initially added to the lysis buffer used to extract each 200-μL plasma sample to control for subsequent extraction and amplification efficiencies. DNA was then extracted with the MagNa Pure LC instrument (Roche Applied Science) and the MagNa Pure LC Total Nucleic Acid Isolation Kit according to the manufacturer’s instructions. This instrument uses robotics, precision pipetors, and magnetic glass particles to purify DNA from various sample types. Briefly, the samples were dissolved by incubation in lysis/binding buffer and proteinase K. Magnetic glass particles were added to each sample to bind the nucleic acid, and unbound substances were then removed by washing steps. A magnetized robotic arm was used to “hold” the magnetic glass particles. Finally, purified total nucleic acid was eluted from the particles with 100 μL of elution buffer. The instrument can process up to 32 samples in 1.5 h with minimal hands-on effort.

Quantitative Real-Time PCR for CMV
We amplified 10 μL of each autoextracted DNA sample by PCR in a LightCycler instrument (Roche Applied Science) using CMV glycoprotein B (gB) gene primers and probes (final reaction volume, 20 μL). The PCR product was detected in real time by fluorescence resonance energy transfer (FRET) using fluor-labeled CMV gB hybridization probes labeled with fluorescein or LC-Red 640. The principles of FRET have been described previously (17). The primer and probe sequences, PCR conditions, and instrument settings for fluorescent data acquisition and analysis have been described previously (11), the only modification being a reduced PCR annealing temperature (to 56 °C). After 45 PCR cycles to generate, if present, a 254-bp gB amplicon, the specificity of the acquired fluorescent signal was checked by melting curve analysis to determine the melting temperature of the fluorescent product in comparison with the expected CMV gB product (with a specific melting temperature of 59.2 °C).

An alternative CMV real-time amplification target was the EcoRI D region of the virus, which yielded a 152-bp amplicon. The primer and FRET probe sequences, PCR conditions, and instrument settings have been described previously (3), except that the PCR conditions were modified to be the same as those for the gB amplifications. For both CMV amplification targets (gB and EcoRI D), the amplification curve baseline adjustment was carried out in the arithmetic mode. The second derivative maximum mode of the LightCycler software was then used to calculate the amplification curve crossing point (Cp), defined as the PCR cycle at which the amplification curve first increases to above background. The lower limit of detection of the real-time PCR was defined by the lowest dilution of template DNA that yielded a PCR negativity rate <37% in replicate experiments, as derived from the Poisson distribution (18).

The amplification of human β-globin gene sequences was used as an internal control for DNA extraction and PCR amplification efficiencies and to assess the presence of possible inhibitors in the clinical samples. A 110-bp fragment of the human β-globin gene was amplified with
the reagents and protocols from the LightCycler Control Kit DNA and the LightCycler DNA master hybridization probes reagent sets (both from Roche Applied Science). Briefly, 5 μL of DNA from the same DNA preparation as the CMV amplifications was amplified in a separate 10-μL reaction containing 4 mM MgCl₂, 0.5 μM each β-globin primers, 0.2 μM fluorescein-labeled FRET probe, and 0.4 μM LC-Red 640-labeled FRET probe. For the β-globin amplifications, the DNA samples were denatured at 95 °C for 30 s, followed by 45 cycles denaturation at 95 °C for 0 s, annealing at 55 °C for 10 s, and extension at 72 °C for 5 s. FRET-based fluorescence data were obtained during the annealing period with the “single” mode and the channel setting F2/F1 within the LightCycler software package.

Qualitative PCR-Southern Blot for CMV
For this in-house-developed method, DNA was extracted from 200 μL of a cell suspension drawn from the buffy coat of a slowly centrifuged (800g for 15 min) anticoagulated blood tube. The DNA extractions were performed according to the manufacturer’s instructions with Generation capture columns (Genta Systems). From the final DNA elution volume of 200 μL, 10μL was used for a 50-μL PCR reaction containing 0.2 mM each deoxynucleotide triphosphate (Roche Molecular Biochemicals), 1 ng/μL each oligonucleotide primer, 2.5 mM MgCl₂, and 50 μL/μL Taq polymerase (Roche Molecular Biochemicals). The amplification primers were from the CMV gB gene (gB 1319, 5'-TGA ACT GGA ACG TTT GGC-3'; gB 1604, 5'-GAA ACG CGC GCG CAA TCG G-3'). The PCR was carried out with an initial 1-min denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s. After the final cycle, the reaction was extended 3 min at 72 °C, and the PCR product was separated by 2% agarose gel electrophoresis. After electrophoresis, the DNA was transferred to a Biodyne B membrane and hybridized with a 32P-labeled plasmid DNA probe containing the whole 2.7-kb CMV gB gene subcloned into the pCR2.1 vector (Invitrogen). After high-stringency washing of the Southern blot, the 310-bp CMV PCR product was visualized by autoradiography.

Qualitative Amplicor CMV Test
We used the reagents and protocols from the commercially available qualitative Amplicor CMV test (Roche Diagnostics) to amplify a 365-bp fragment of the CMV DNA polymerase gene, using the biotinylated primers that came in the Amplicor reagent set and colorimetric PCR-ELISA detection. Results are expressed, as per the manufacturer’s recommendations, as positive, negative, or invalid (possible inhibitor) for CMV DNA. The lower limit of detection of this assay is 1000 copies/mL.

Quantitative Competitive PCR
We used a commercially available quantitative competitive PCR assay (Cobas Amplicor CMV Monitor test; Roche Diagnostics) to measure CMV viral load over the manufacturer’s reportable range of 400–100 000 copies/mL. This assay uses the same CMV DNA polymerase gene biotinylated PCR primers and the same internal control DNA (as a calibrator for competitive quantification) as the qualitative Amplicor CMV test. The CMV and calibration amplicons are detected, as in the qualitative Amplicor test, by separate solid-phase-bound hybridization probes.

Calibrator for Quantification
A CMV gB plasmid containing the whole 2.7-kb CMV gB gene subcloned into the pCR2.1 vector (Invitrogen) was used as the calibrator for quantification in the fluorescent real-time PCR assay. A triplicate serial dilution series of this plasmid DNA (quantified by spectrophotometry) was used to create the external calibration curve [Cp vs log(copy number)] that was used to quantify unknown clinical samples. The same external calibration curve was used for each subsequent real-time PCR batch run until routine quality-control tolerance limits (for low and high positive controls) were exceeded; a new external calibration curve was then generated. A CMV “viral calibrator” was harvested from the supernatant of CMV-infected diploid human fibroblast cultures (strain AD169). DNA from this viral stock was purified with a Generation capture column (Genta Systems), and the viral load was quantified by fluorescent real-time PCR using the plasmid external calibration curve described above.

Statistics
Because the CMV viral load data (in copies/mL) did not follow a gaussian distribution and were mathematical derivations of gaussian-distributed log scale data (Cp), descriptive and comparative statistics for real-time PCR data used either raw Cp data or log-transformed viral load values. Statistical analyses were performed with the software packages Excel (Microsoft) or Statview (SAS Institute).

Results

Two different regions of the CMV genome were chosen as quantitative amplification targets based on their high amplification efficiencies, low background signals, and absence of homology to human sequences. These diagnostic targets were the CMV gB gene (11) and the CMV EcorI D region (3). The real-time PCR assays for these two targets were optimized to use a viral DNA template that was prepared from human plasma by an automated DNA purification workstation. Serial dilution experiments (with a gB plasmid) were performed to assess the linearity and analytical sensitivity at low copy numbers of the gB-targeted assay (Fig. 1). The quantitative real-time PCR assay for the gB gene was linear over a 7.6-log dynamic range, from 10⁸ molecules/reaction (5 × 10⁶ copies/mL) to the lower detection limit of 2.5 molecules/reaction (125 copies/mL). This detection limit was determined by six
A graphical representation of three combined serial dilution experiments. The replicates), 10^2 (three replicates), 10 (three replicates), 5 (four replicates), and 2.5 input molecules generated a specific amplification product. (B) input plasmid copy numbers were 10^8 (two replicates), 10^7 (three replicates), 10^6 (two replicates), 10^5 (three replicates), 10^4 (three replicates), 10^3 (three replicates), 10^2 (three replicates), 10 (three replicates), 5 (four replicates), and 1.25 (one replicate) molecules per PCR reaction. Results of the regression analysis were as follows: slope, -3.38; y-intercept, 39.5 cycles; R = 0.999; PCR efficiency = 10^(-1/slope) = 1.975; n = 28.

Accurate quantification of the CMV viral load of patient-derived plasma specimens was accomplished by comparing the real-time PCR determined Cp to the composite external calibration curve shown in Fig. 1B. This calibration curve showed excellent linearity (R = 0.999) and could be used to determine the efficiency for the gB PCR reaction (1.975). This experimentally determined PCR efficiency is quite close to the ideal theoretical value of 2.0 (representing a doubling of PCR product with each amplification cycle). The external calibration curve equation subsequently used for determining the CMV viral load of patient plasma samples was:

\[
\text{Viral load (copies/mL of plasma)} = 50 \times 1.975^{(39.5 - \text{Cp})}
\]

where 1.975 is the PCR efficiency, 39.5 is the y-intercept of the calibration curve, and 50 is the conversion factor relating CMV molecules/PCR reaction to CMV molecules/mL of plasma.

Analogous serial dilution experiments showed that the quantitative real-time PCR assay for CMV using the alternative EcoRI D region primers and probes also yielded a quantitatively linear assay down to at least the same lower detection limit as the gB assay (2.5 molecules/PCR reaction, or 125 molecules/mL; Fig. 2). The equation for the calibration curve for CMV viral loads with use of the alternative EcoRI D primers and probes was:

\[
\text{Viral load (copies/mL of plasma)} = 50 \times 2.02^{(38.5 - \text{Cp})}
\]

The efficiency of the EcoRI D PCR reaction (2.02) was then also quite close to the ideal value of 2.0.

To validate the new quantitative PCR assay, samples sent to our laboratory for clinical CMV testing were comparatively evaluated by our standard CMV detection method (PCR with Southern blot) as well as by both the gB- and EcoRI D-based real-time PCR assays. Of 63 different plasma samples initially found to be CMV positive with the gB primer–probe set, 53 were subse-
quently confirmed as positive by the alternative EcoRI D primer–probe set (with a repeat DNA preparation). To resolve these 10 potentially discrepant results, we tested the 10 samples in alternative, direct diagnostic tests that did not use real-time PCR. Three of the 10 discordant samples were CMV positive by these alternative diagnostic tests: 2 by PCR with Southern blot (with a different set of gB primers) and 1 by quantitative competitive PCR (of the CMV DNA polymerase gene). Although the seven other discordant samples were negative in the PCR-Southern blot assay, the very low CMV viral loads in these seven samples (mean, 180 copies/mL) suggest that the gB-based real-time PCR assay may be more analytically sensitive than other comparable direct diagnostic tests for the detection of weakly positive samples. Alternatively, although there was no evidence for PCR contamination in any of these assays, we cannot definitively rule out the possibility of isolated false-positive gB PCR reactions.

Of the 53 samples in which both the gB and EcoRI D real-time PCRs yielded positive results, the viral loads determined with each of these distinct targets correlated well with one another ($R = 0.928; P < 0.0001$; regression line slope, 0.927) despite the large spread of viral load values (extending over more than 4 logs; mean viral load, 5400 copies/mL; Fig. 3). Although there was no significant difference in the viral load determined by the gB vs the EcoRI D method ($P = 0.48$), the interassay CV for the difference between the viral loads determined by these two different methods (including the variability from a repeat DNA preparation) was 7.2%, consistent with only a small degree of variation in the quantification of the CMV viral load with one real-time PCR primer–probe set vs the other.

To further assess analytical precision of the real-time PCR assay, we subjected 32 of the 53 dually CMV-positive plasma samples (with both real-time target genes) to duplicate viral load determination with the gB primer–probe set, including a repeat DNA preparation (Fig. 4). This replicate testing confirmed the low interassay variability; the interassay CV was 3.5% across a large range (>3 logs) of viral load values [mean 3800 copies/mL = mean (SD) of 3.58 (0.125) log$_{10}$ copies/mL]. As might be expected, the interassay precision for replicate PCRs with the same gB primers ($CV = 3.5\%$) was slightly better than the interassay precision obtained with two different primer pairs from distinct regions of the CMV genome (gB vs EcoRI D; CV = 7.2%).

The results of an intraassay precision study (for both of the real-time primer–probe sets) performed with 6 replicates of each of 4 different virus dilutions in which not only the PCRs, but also the DNA preparation, were repeated for each of the 24 samples used for each PCR primer pair are shown in Table 1. The intraassay CV was 0.6–1.1% for “high” CMV viral loads (>10 000 copies/mL), 0.95–2.2% for an “intermediate” viral load of 10 000 copies/mL, and 3.3–4.2% for a “low” viral load of 1000 copies/mL (Table 1). The intraassay precision of the real-time PCR was also assessed with use of a stock of control CMV virus purchased from Boston Biomedica (target viral load, 1900 copies/mL by the Roche Monitor assay) that was extracted and quantified by real-time PCR 21 different times. The mean viral load by real-time PCR was 3600 copies/mL with an intraassay CV of 2.0%.

To determine how the viral loads generated with real-time PCR compared with those generated by a widely used commercially available quantitative competitive PCR (Roche Monitor), we assayed 32 different plasma samples representing a broad range of viral load values with both assays (Fig. 5). Of the 23 samples with...
CMV viral loads within the reportable dynamic range of both assays, there was a reasonable correlation between the two quantitative methods (\(R = 0.59; P = 0.002\)), but the real-time method gave, on average, slightly higher viral loads (3.1-fold) than the quantitative competitive method (mean log difference, 0.49; \(P = 0.002\)). The intermethod variability (CV = 16%) for the viral loads determined with these two different quantitative methodologies was significantly higher than the interassay variability for replicate viral loads measured with only the real-time PCR method with either different amplification targets (gB vs EcoRI D; CV = 7.2%) or the same amplification target (gB; CV = 3.5%).

There were 9 samples (of 32) in which either (or both) the real-time or the qualitative competitive PCR assay generated a negative result or a result below the linear dynamic range of the assay. Five of these nine samples yielded a concordant “negative” result in both assays, and one sample was qualitatively concordant by being “weak positive” (<125 copies/mL) by real-time PCR and 7400 copies/mL by competitive PCR. Of the three remaining discordant samples, two had low viral loads by real-time PCR (500–600 copies/mL) but were each negative by competitive PCR, and one had a low viral load by competitive PCR (550 copies/mL) but was negative by real-time PCR. Assuming (conservatively) that the competitive PCR result was the “correct” result, the sensitivity of the comparative real-time assay is 96%. The specificity could not be accurately determined with this sample set because the majority of the samples for this comparative study were preselected for being PCR positive.

To more thoroughly determine the analytical performance characteristics of the real-time PCR assay, we dually assessed 147 consecutive samples referred to our clinical diagnostic laboratory for CMV testing by real-time PCR (with gB primers) and a comparative commercially available qualitative PCR-ELISA assay (Roche Amplicor CMV test) with a lower detection limit of ~1000 CMV copies/mL. As shown in Table 2, there were 9 samples with discrepant results between the two comparative assays and 138 with concordant results. To resolve the discrepant results, we subjected these nine samples to additional CMV assays, including PCR-Southern blot (gB primers) and real-time PCR with the alternative EcoRI D primer–probe set. All six of the discrepant samples positive by gB real-time PCR were confirmed as positive by the EcoRI D assay. The one sample that was undetectable in the EcoRI D assay (positive in the PCR-Southern blot and gB real-time PCR assays) was from a patient who was unequivocally CMV positive in consecutive samples collected 5 days before and 7 days after the discrepant sample. All six of these samples were then judged as "CMV positive". Of the three discrepant samples that were initially undetectable by real-time PCR, two were negative by PCR-Southern blot (one was negative in the EcoRI D assay, and one was not tested with the EcoRI D assay), both of which were then judged as "CMV negative". The one remaining real-time negative discrepant sample was positive by PCR-Southern blot and was from a patient who was CMV positive in two consecutive samples collected 1 and 2 weeks after the discrepant sample was collected. This final sample was judged to be "CMV positive" and thus represents a false-negative real-time PCR result, the only sample (of 147) with a real-time PCR result discordant from the final, confirmed result. When we included these final, confirmed results,
the sensitivity of the gB real-time PCR assay was 96%, and the specificity was 100%. In comparison, on the basis of these same data, the sensitivity of the Amplicor PCR-ELISA was 74%, and its specificity was 98%. We also compared real-time gB CMV PCR to routine CMV viral culture and found that, in 44 samples coordinately assayed by both methods, the sensitivity of the viral culture assay was 50% compared with 100% for the real-time PCR (data not shown).

Each DNA sample amplified with CMV primers was also amplified (in a different tube) with primers and probes for a control housekeeping gene (human β-globin). The ensuing β-globin Cp value (or its absence) was then used as a quantitative quality-control check to ensure the efficiency of the extraction and PCR amplification steps and the absence of sample-derived “inhibitors” that could create false-negative results. The CV for the β-globin Cp values was 4.0% (mean, 25.8 cycles; n > 1100). For quality-control purposes, samples with a failed β-globin amplification or those with a β-globin Cp above 27.9 cycles (2 SD above the mean) were considered “out of range” and were subjected to repeat analysis (including a repeat DNA preparation step). If the second β-globin Cp was also out of range or negative, the sample was classified as containing a “possible inhibitor”, and a new sample was requested. Of 1115 clinical samples processed, 23 samples (2.0%) had an initial out of range β-globin PCR. This 2.0% retesting rate was not significantly different (P > 0.8) from the 2.3% retesting rate that would be mathematically predicted by the use of a 2 SD cutoff for repeat testing. Of these 23 retested samples, only 3 (0.27%) had consistently increased β-globin Cp values on repeat testing and were reported as containing a possible inhibitor. One of these samples was a plasma that had been mistakenly collected into a heparin (green top) tube; heparin is a known PCR inhibitor. The remaining seven out of range samples were analyzed within seven different batch runs, implying that “random” DNA preparation (or PCR) failures represented at least 30% of the 2.0% of total samples that required repeat testing.

Our laboratory has recently begun using the real-time CMV PCR method as the primary diagnostic assay on all clinical samples referred for CMV DNA evaluations. After

Table 2. CMV real-time PCR vs PCR-ELISA (Roche Amplicor).

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<thead>
<tr>
<th>PCR-ELISA</th>
<th>Positive</th>
<th>Negative</th>
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<tbody>
<tr>
<td>Positive</td>
<td>16</td>
<td>3(^a)</td>
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<tr>
<td>Negative</td>
<td>6(^b)</td>
<td>122</td>
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\(^a\) Two of three classified as “CMV negative” based on results obtained in other assays and on the clinical history, and one classified as “CMV positive” based on results obtained in other assays and on the clinical history (see text for details).

\(^b\) All six classified as “CMV positive” based on results obtained in other assays and on the clinical history (see text for details).
9.5 months of this clinical utilization period, the gB CMV real-time assay had been performed on 1115 clinical samples from 252 different patients (mean, 4.4 samples/patient), and 85% of these samples were from patients being monitored for CMV infection after allogeneic bone marrow (or stem cell) transplantation. The CMV positivity rate in this predominantly bone marrow transplant population was 10% of tested samples (112 PCR-positive samples) with a low median CMV viral load (500 copies/mL; viral load range, 125 to 25 × 10^6 copies/mL). Forty-five different patients donated the 112 PCR-positive samples (18% of patients tested), with each of these patients donating a mean of 10 CMV PCR samples (including many CMV-negative samples).

The CMV viral load kinetics of several representative bone marrow transplant patients are shown in Fig. 6. The potential clinical usefulness of this assay for monitoring antiviral treatment efficacy was demonstrated by the sequentially falling viral loads after initiation of preemptive antiviral therapy, typically after one or two positive PCR results. Not uncommonly, after the initial course of antiviral therapy was discontinued, CMV viremia recurred (Fig. 6B), perhaps because of reinfection, inadequate initial therapy, or emergence of drug resistance.

**Discussion**

Because the quantitative detection of CMV DNA is quickly becoming the standard of clinical care for high-risk transplant (and other immunocompromised) patients, reliable, sensitive, and rapid laboratory methods are increasingly in demand. In response to this need, we developed a semiautomated method for quantification of CMV DNA, verified the analytical performance characteristics of the assay, and showed its potential clinical usefulness in the posttransplantation monitoring of hematopoietic stem cell transplant patients. This assay combines the advantages of an automated robotic DNA purification system with semiautomated fluorescent real-time PCR-based quantification of CMV DNA. To our knowledge, this particular combination of automated molecular diagnostic systems has not been described previously for use as a CMV diagnostic test.

The advantages of real-time PCR over other molecular or nonmolecular diagnostic virology methods have been described previously (1) and include a wider dynamic range, a lower detection limit, increased precision, increased accuracy, reduced hands-on technical time (and costs), and shorter turnaround time. Our CMV viral load assay has a 7.6-log dynamic range, a lower detection limit of 125 copies/mL, interassay CV of 3.5% (with a SD of 0.125 log10 copies/mL), and good correlation with other laboratory tests for CMV infection. A major obstacle facing most clinical molecular virology laboratories is the tedious, time-consuming, manual process of DNA sample preparation. The sample preparation step (and not the PCR step) has also been shown to be the major source of total assay variability in real-time PCR methods (16); it thus is an ideal target for creating more precise (and thus more clinically meaningful) quantitative molecular virology tests. To address this limitation, we incorporated an automated robotic DNA extraction step in our real-time CMV viral load assay to create an assay with reduced hands-on labor time (and costs) as well as enhanced analytical precision. In addition, although we did not directly address the issue of PCR product contamination, the reduced manual processing afforded by our assay could lower the number of false-positive results attributable to human errors in specimen handling. Similar use of robotic automated sample preparation in other clinical molecular virology assays has recently been shown for the detection of herpes viruses (15, 19), hepatitis C virus (14), HIV (20, 21), and quantitative competitive PCR for CMV (14).

The lower limit of detection of our real-time quantitative CMV PCR assay is 125 copies/mL (2.5 molecules/PCR reaction). The absolute detection limit could be even lower given that, in everyday clinical use, we often find samples that are unequivocally CMV positive after melt-
ing curve analysis but give no detectable amplification Cp above background values and, thus, no definable viral load. These samples are reported as “CMV weak positive” to reflect that their viral loads are below the confirmed detection limit of 125 copies/mL. This detection limit represents a slight improvement over other published real-time PCR assays for CMV, which have reported lower detection limits of ~10 copies/PCR reaction (500 copies/mL in our assay) (8–10, 22). In comparison, a widely used commercially available quantitative competitive PCR platform for CMV DNA (Roche Monitor) has a lower detection limit of 400 copies/mL.

Some of these other comparable CMV assays use whole blood or blood cells as starting material rather than the plasma sample that we chose to use. The analytical detection limit for CMV DNA does not, however, significantly differ for plasma vs cell-containing samples (13). Although the biological CMV DNA viral load is probably higher in cell-containing blood fractions (whole blood or leukocytes) than in plasma (13, 23), we chose plasma as our biological specimen for two reasons. First and foremost, because the intended clinical use of the CMV viral load assay in our institution was the serial monitoring of transplant patients, we chose a sample type (plasma) that had been shown to be clinically (not just analytically) useful in detecting CMV disease (not just viremia) after transplantation (7, 24). The other reason that we chose a plasma-based assay was purely practical and was based on the much simpler preanalytical processing, stability, transport, and preparation procedures for plasma vs cell-containing samples.

For any viral load assay used to serially monitor patient responses to therapy, it is of clinically important to determine the degree of viral load change (between successive measures) that likely distinguishes a “true” biological effect (attributable to treatment) from an “artefactual” change (attributable to assay imprecision). For HIV viral load assays, for example, changes of less than approximately threefold between successive laboratory measurements are not considered biologically significant (25, 26). Expert AIDS clinical trial group laboratories have thus defined an intraassay SD of <0.15 log_{10} copies/mL as a practical precision goal for HIV viral load assays (26). To be able to similarly interpret serial CMV viral loads, the analytical imprecision of the assay must also be clearly defined and, ideally, minimized. The real-time quantitative CMV PCR assay that we have developed has an interassay CV of 3.5% (with a SD of 0.125 log_{10} copies/mL) and an intraassay CV of 0.6–4.2% (with an SD of 0.03–0.14 log_{10} copies/mL). Of note is the fact that all of these replicates included a repeat DNA preparation because the primary determinant of overall assay variability in real-time PCR assays is not the PCR step, but rather the DNA preparation step (16). For example, replicates of real-time PCRs for CMV performed on the same DNA preparation gave intraassay CVs of 0.8–3.1% and interassay CVs of 0.3–2.6% (10). In comparison, when repeat (nonautomated) DNA preparations were included in the precision determinations, real-time PCR assays for CMV had much higher interassay CVs in the range of 3–8% (27) and 12–21% (22) compared with a CV of 3.5% for our assay. The precision of multistep competitive PCR methods is poorer than single-step real-time PCR methods. For example, a widely used commercially available CMV competitive PCR assay (Roche Monitor) has an assay CV of 12–24% (14). Compared with any of these other assays, the real-time CMV PCR assay that we have developed has lower imprecision and thus is suitable for its intended clinical use of serial monitoring of patients after transplantation. From a practical perspective, the interassay CV of our assay (3.5%) suggests that successive CMV viral load changes less than approximately four- to fivefold may not be biologically significant and may represent assay imprecision.

The performance characteristics of our CMV real-time PCR assay were assessed by comparisons with two other PCR-based assays for CMV disease and with clinical chart review. On the bases of these comparison, we determined that the CMV real-time PCR assay had a sensitivity of 96% and a specificity of 100%. This is comparable to the sensitivity of 95.8% and specificity of 99% reported by Schaade et al. (11) for a similar real-time PCR assay for CMV. These performance characteristics for PCR-based methods have led a group of international experts to recommend that either a PCR-based assay or the pp65 antigenemia assay (but not viral culture) be used to guide preemptive CMV therapy after allogeneic bone marrow transplantation (1).

We have shown that CMV viral loads, as measured by real-time PCR, correlate with those measured by a commercially available competitive PCR method (Roche Monitor) but that the real-time values are, on average, approximately threefold (0.5 log) higher. A similar method-dependent lack of agreement in viral loads has been described previously by two other groups of investigators (11, 12); one group reported a 0.2–0.5 log (two- to threefold) lower viral load for the Monitor assay (12), a value similar to our 0.5 log mean difference. A true determination of the quantitative “accuracy” of any CMV viral load assay cannot, however, be determined until a properly validated and internationally recognized CMV quantitative reference standard is developed.

We validated our CMV real-time PCR assay with use of two different viral targets: the gB gene and the EcoRI D region. Because the gB method has slighter better precision and clinical sensitivity, we routinely use the gB primer set to initially test all incoming clinical samples. The alternative EcoRI D reagents are then used primarily as a “back-up” to resolve indeterminate cases (with very low viral loads) and, perhaps more importantly, to accurately quantify CMV viral loads in patients with a “common” polymorphism (1.3% prevalence) in the gB probe-binding region that has been reported to adversely affect accurate viral load quantification (28). Because this poly-
morphism causes a single base-pair mismatch to one of the FRET probes used for fluorescence detection, this gB variant is easily identified by routine melting curve analysis (after gB real-time PCR) as a shift in the melting temperature from 59.2 °C (in wild-type templates) to 53.1 °C. Furthermore, because this single base-pair mismatch decreases the affinity of FRET probe binding during real-time PCR—and thus possibly adversely affects template quantification—the EcoRI D real-time PCR is always used to accurately report the viral load whenever this shifted melting curve is observed in our laboratory. In our testing population, which is predominantly bone marrow transplant patients, we have found this polymorphism in 2.2% of tested samples (2.3% of the patients).

By routinely amplifying a control human gene (β-globin) with each CMV sample, we are able to consistently ensure the quantitative efficiency of the DNA extraction and PCR amplification steps and monitor each sample for the possible presence of inhibitors. As part of this quality-control program, we have established a one-tailed 97.7% confidence interval (2 SD) defining an “acceptable” β-globin Cp, and we classify CMV samples with results outside of this range (after repeat testing) as containing a possible inhibitor. In our testing population (primarily bone marrow transplant), we have found that only 0.27% of >1100 tested samples contained such an inhibitor. This very low inhibitor rate confirms the consistent performance of the MagNa Pure automated DNA preparation system in a real-world clinical testing environment. Furthermore, this low inhibitor rate for our CMV viral load assay appears to be somewhat lower than the inhibitor or “dropout” rate that has been reported with HCV viral load testing, either without (1.1%) (29) or with (1.5%) (14) automated DNA preparation. In comparison, the statistically expected fraction of CMV samples (2.0%) required repeat β-globin PCRs after being initially above the 2 SD Cp cutoff. Although many of these initially out-of-range samples appeared to be the result of nonrandom batch-wide technical failures, at least 30% of these retested samples had initial failures that were seemingly random, perhaps the result of an otherwise undiscovered problem with the MagNa Pure DNA preparation. Despite the small number of these random failures (~0.6% of tested samples), we continued to routinely perform duplicate parallel β-globin amplifications on every sample. Although this duplicate testing policy adds considerably to the total reagent costs of the assay, we estimate that without it we would fail to detect extraction- or PCR-related problems or inhibitors in ~0.9% of all samples. For example, even with the routine incorporation of one or two “extraction controls” in every batch run (to detect batch-wide extraction failures), we would miss the 0.3% of samples that appear to contain inhibitors and the 0.6% of samples with random extraction or amplification failures. Assuming a 10% CMV positivity rate, the failure to institute parallel internal control gene testing on every sample might then lead to a falsely negative (or quantitatively underestimated) CMV result for ~0.09% of tested samples.

Another advantage of the described CMV viral load assay is the minimal hands-on labor time (and cost) required for its performance. We estimate, for example, that a full batch of 32 samples can be processed with the MagNa Pure DNA preparation method in ~1.5–2 h with only 15–30 min of hands-on time. In comparison, for a similar batch size (~28 samples plus controls), most nonautomated precipitation- or spin-column-based DNA purification methods would require ~2–3 h of labor per batch, with hands-on time representing at least 60–120 min (14, 15). Reagent costs per sample, although somewhat variable with different vendors, are in the same range for most manual methods vs the automated MagNa Pure method (at ~$1.70/sample preparation).

In summary, we have developed and analytically and clinically validated a CMV viral load assay that uses an automated DNA purification system and a semiautomated real-time PCR amplification and detection system. Despite the now routine use of this plasma-based assay in our bone marrow transplant population, as both the laboratory trigger for preemptive antiviral therapy and for subsequent serial monitoring of the efficacy of this therapy, we do not have any definite “outcomes” data assessing the effects of this new method on the prevalence or severity of clinical CMV disease. These outcomes data would, of course, be a more definitive indicator of the clinical usefulness of this new assay. In addition, because we have only limited experience with this assay in other immunocompromised patient populations (most notably solid organ transplantation patients), its potential clinical usefulness in patients other than those with bone marrow transplants, cannot be confirmed. For example, a recent prospective clinical outcomes study in liver transplant patients (30) found that the optimum method to prevent CMV disease in this population was the preemptive use of ganciclovir after PCR-based detection of CMV DNA in blood leukocytes (not plasma). An alternative observation—that leukocyte-based PCR methods can detect CMV DNA in >60% of bone marrow transplant patients (6, 13)—suggests, however, that assays using cell-containing samples may be too analytically sensitive and thus lead to expensive and toxic antiviral drug usage in many patients who might never get disease. In comparison, our plasma-based assay detects CMV in only 18% of tested patients. Another possible limitation of our study was that we did not use conventional pp65 antigenemia testing as a comparative assay method for CMV DNA. From a practical perspective, we chose to avoid antigenemia testing in this study because of the high prevalence of leukopenic bone marrow transplant patients being tested (the antigenemia assay needs 200 000 neutrophils/mL) and the time-consuming subjective visual readouts that would have been necessary. Despite these limitations, we believe that our real-time CMV viral load assay is a
sensitive, specific, and cost-effective method for diagnosing and monitoring CMV infection, particularly in a bone marrow transplant population being managed with preemptive antiviral therapy.

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