Evaluation of Real-Time PCR Laboratory-Developed Tests Using Analyte-Specific Reagents for Cytomegalovirus Quantification

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Viral load testing for cytomegalovirus (CMV) has become the standard for the diagnosis of infection and monitoring of therapy at many transplant centers. However, no viral load test has been approved by the FDA. Therefore, many laboratories rely on laboratory-developed assays. This study evaluated the performance characteristics of two real-time PCR tests developed using the artus CMV analyte-specific reagents (ASRs). One version is distributed by Abbott Molecular and the other by QIAGEN. For plasma specimens, the Abbott test had a limit of detection of 2.3 log10 copies/ml and a linear range up to at least 6.0 log10 copies/ml. Comparison of plasma viral loads using the Abbott test and the Roche Amplicor Monitor test showed a mean difference of −0.012 log10 copies/ml. In addition, the Abbott test viral loads correlated with the Digene Hybrid Capture assay ratios. Viral loads obtained from plasma specimens tested by the Abbott and QIAGEN tests were in very close agreement (mean difference, 0.144 log10 copies/ml). When the QIAGEN test was evaluated with the QIAGEN, MagNA Pure, and easyMAG extraction methods, the viral loads for all three methods were within 0.370 log10 copies/ml. Thus, there is good agreement between viral loads obtained by the different tests using the same extraction method or by the same test using different extraction methods. The availability of real-time PCR ASRs provides additional reagents that can be used for CMV viral load testing.

Cytomegalovirus (CMV) remains an important pathogen for immunocompromised individuals. In many centers, viral load assays are the cornerstone for the diagnosis and monitoring of patients at risk for CMV disease. The clinical utility of CMV viral load testing is supported by a considerable body of literature (7, 9, 11, 12, 14, 15). These assays can be used to determine when to initiate preemptive therapy, to monitor the response to therapy, to determine the duration of therapy, and to assess patients at risk of developing relapsing infection (6, 9, 10, 12). For example, a study of liver transplant recipients showed that a viral load of 2,000 to 5,000 copies/ml in plasma, determined by the Amplicor Monitor CMV test (Roche Diagnostics, Indianapolis, IN), is predictive of the development of active CMV disease (10). Another study showed that when one is monitoring active disease for response to failure, to clear CMV DNA from plasma after a course of ganciclovir therapy increases the risk of relapsing CMV infection (14). Failure of the plasma viral load to decline after several weeks of therapy has been associated with the development of ganciclovir resistance (4). In addition, the half-life of CMV DNA in plasma is longer for patients who relapse than for those who do not (11). These studies all support the use of close monitoring of viral loads to identify those at high risk of relapse, thus allowing the intensification of therapy early in the treatment course.

In spite of the widespread use of CMV viral load measurements, no assays for the quantification of CMV nucleic acid have been approved by the U.S. Food and Drug Administration (FDA). Laboratories that perform CMV viral load testing either modify the Hybrid Capture CMV DNA test (Digene, Gaithersburg, MD) to allow for quantification, use the Amplicor CMV Monitor assay (Roche Diagnostics, Indianapolis, IN), or rely on laboratory-developed PCR tests. Based on the recent proficiency testing results (2006 ID A survey) of the College of American Pathologists, the majority of participating sites rely on laboratory-developed tests. Though these laboratory-developed tests can work very well for any given center, test performance may differ between different laboratories, and without an international standard, viral loads obtained using different tests are difficult to compare and interpret. This lack of agreement poses a significant problem for patients who may be monitored at more than one medical center.

Recently, several analyte-specific reagents (ASRs) for CMV DNA have become available. Currently, limited data are available regarding the performance of laboratory tests developed using these CMV ASRs. This study evaluated the performance characteristics of two tests developed in our laboratory using the artus CMV ASR (Hamburg, Germany), one version of which is distributed by Abbott Molecular (Des Plaines, IL) and another version by QIAGEN (Valencia, CA). Using clinical specimens, viral loads obtained by laboratory-developed tests using the Abbott CMV ASRs (referred to below as the “Abbott test”) or the QIAGEN CMV ASRs (referred to below as the “QIAGEN test”) were compared with those obtained by the Hybrid Capture and Amplicor Monitor tests. The goal was
to determine if there is adequate agreement in viral loads among the different tests, which could be used to establish the relevance of the currently published literature on the clinical utility of the Amplicor and Hybrid Capture assays for the these real-time PCR tests. In addition, the impact of extraction methods on viral loads was assessed using the QIAamp DNA minikit (QIAGEN, Valencia, CA), MagNA Pure (Roche Diagnostics), and easyMAG (bioMerieux, Durham, NC) methods for DNA nucleic acid extraction.

MATERIALS AND METHODS

Standard material and samples. A plasma sample with a high viral load (BBI, West Bridgewater, MA) was used to make serial dilutions in CMV-seronegative human plasma to concentrations of 1.4 to 6.0 log₁₀ copies/ml. The concentration of the BBI material was determined by the manufacturer using the Amplicor Monitor test. Specimens were aliquoted and stored at −70°C until testing.

For the cell-based standard, human foreskin fibroblasts (HFFs) were incubated with CMV strain AD169 at a multiplicity of infection of 0.03 (6). At 6 h postinfection, the HFFs were harvested by trypsinization, washed, and counted. Dilutions of the HFFs were made with uninfected baby calf cells to concentrations as high as 10⁵ HFFs in 10⁶ total cells. Aliquots of 200 μl containing 10⁴ total cells were stored at −70°C until testing. For the agreement studies, plasma samples were collected at the Emory Medical Laboratories following an institutional review board-approved protocol and stored at −70°C. In addition, whole-blood specimens submitted to the Clinical Microbiology Laboratory at Rush University Medical Center for the Hybrid Capture assay were collected under an institutional review board-approved protocol. Whole-blood specimens were stored at −70°C up to 7 days before testing.

Nucleic acid extraction. For the CMV Monitor assay, nucleic acid was extracted using the MagNA Pure total-nucleic-acid kit as previously described (8). A 200-μl volume of plasma was extracted and eluted in 100 μl, and 50 μl of the eluate was added to the master mix. For the ASRs, a variety of nucleic acid extraction methods were used as described below. Nucleic acid was extracted from 200 μl of sample using the QIAamp DNA minikit (QIAGEN, Valencia, CA) Blood and Body Fluid protocol with the following adjustments: for each specimen, 2 μl of carrier RNA (1 mg/ml) and 5 μl of internal control were added to 200 μl of buffer AL. The specimen was eluted in 50 μl. The MagNA Pure LC (Roche Diagnostics, Indianapolis, IN) was used to isolate nucleic acid from plasma using the total-nucleic-acid kit. For each specimen, 200 μl of plasma and 5 μl of the internal control were added to 300 μl of lysis buffer. The sample was eluted in 50 μl. For nucleic acid extraction using the NucliSens easyMAG, 200 μl of the sample plus 5.5 μl of the internal control were added to 2,000 μl of lysis buffer. Then 100 μl of magnetic silica was added, and the entire specimen was loaded onto the easyMAG instrument. The sample was eluted in 55 μl.

CMV DNA assays. The Hybrid Capture system (version 2.0) assay (Digene Corporation, Gaithersburg, MD) was performed according to the manufacturer’s protocol for the qualitative assay, which has been approved by the FDA. The Amplicor CMV Monitor test (Monitor; Roche Diagnostics) was performed according to the manufacturer’s recommendations except for the nucleic acid extraction, which was performed as described above. The Amplicor CMV Monitor test targets a 365-bp region of the polymerase gene. The linear range of the Abbott test was determined by testing aliquots of samples ranging in concentration from 2.0 to 6.0 log₁₀ copies/ml. Data shown in Fig. 1A are means (±SD) for samples tested in triplicate in two separate runs (n = 6). (B) Linear range of the Abbott test using cell-based standards. Data are means (±SD) for five to seven replicates tested in a single run.

Study design. A detailed analytical evaluation was performed using the Abbott test. Viral loads obtained by the Abbott test and the Amplicor CMV Monitor test or the semiquantitative Hybrid Capture assay were compared. Since the Abbott and QIAGEN tests use the same reagents, the analytical validation was not repeated using the QIAGEN test. The different extraction methods were compared using the QIAGEN test.

Statistical analysis. Data were log₁₀ transformed prior to analysis. Descriptive statistics and regression line equations were calculated with the analysis tool pack of Microsoft Excel 2000 (Microsoft Corp., Redmond, WA). Agreement between viral loads was assessed by the method of Bland and Altman (2).

RESULTS

Performance characteristics of the Abbott test. The linear range of the Abbott test was determined by testing aliquots of CMV DNA ranging in concentration from 2.0 to 6.0 log₁₀ copies/ml. Data shown in Fig. 1A are means (±standard deviations [SD]) for samples tested in triplicate in two separate runs. Multiple replicates of samples ranging in concentration from 1.4 to 3.0 log₁₀ copies/ml were tested to determine the limit of detection (Table 1). The assay was linear from 2.0 log₁₀ copies/ml to 6.0 log₁₀ copies/ml (Fig. 1). We did not have a high-titer specimen to measure the linear range beyond 6.0 log₁₀ copies/ml. When a larger number of replicates were tested to assess the limit of detection, the concentration at which CMV DNA was detected in 95% of the replicates was 2.3 log₁₀ copies/ml (200 copies/ml). The reproducibility of the assay (Table 2) varied through the
linear range of the assay; the assay has the greatest precision at higher viral loads and is least precise with viral loads at or below 2.3 log_{10} copies/ml.

The linear range, limit of detection, and reproducibility of the Abbott test were also assessed using a cell-based standard, which was designed to mimic a white blood cell sample. This sample type was tested because whole-blood samples are used for clinical testing in many laboratories. The test was linear from 1.7 log_{10} copies/ml to 5.2 log_{10} copies/ml, with a limit of detection of 1.7 log_{10} copies/ml (Fig. 1B). As seen with the plasma standard, the test was less reproducible near the limit of detection. The coefficient of variation ranged from 1.7% at 5.7 log_{10} copies/ml to 34.1% at 1.7 log_{10} copies/ml (data not shown).

Agreement between the Abbott test and the Amplicor CMV Monitor assay. A total of 101 clinical specimens that had detectable viral loads by the Amplicor CMV Monitor assay were tested by the Abbott test (Fig. 2). The one specimen that was positive by the Monitor assay and negative by the Abbott test had a viral load of 2.99 log_{10} copies/ml in the Monitor assay. For the 100 samples that were positive by both tests, the population mean (SD) was 3.51 (0.89) log_{10} copies/ml for the Monitor assay and 3.50 (0.95) log_{10} copies/ml for the Abbott test. Based on the agreement analysis, the mean difference between the two tests was -0.012 log_{10} copies/ml (95% limits of agreement, -0.869 to 0.845 log_{10} copies/ml) (Fig. 2). No bias was observed for viral loads obtained with the two tests.

Comparison of the Abbott test and the Hybrid Capture assay. Fifty specimens were tested by both the Abbott test and the Digene Hybrid Capture assay, using plasma and whole-blood specimens, respectively. Twenty-six samples were negative by both tests. Ten specimens were negative by the Hybrid Capture assay and positive by the Abbott test, with viral loads of 2.10, 3.45, and 3.83 log_{10} copies/ml by the Abbott test (Table 3). Eleven samples were positive by both assays. It was not possible to compare the viral loads for these 11 specimens, since the qualitative version of the Hybrid Capture assay was performed. However, the Hybrid Capture assay ratio did appear to correlate with the Abbott test viral load (Table 3). The lowest Abbott test viral load with a positive Hybrid Capture result was 3.73 log_{10} copies/ml. Specimens with viral loads between 4 log_{10} copies/ml and 5 log_{10} copies/ml had a mean ratio of 18 by the Hybrid Capture assay, while those specimens with viral loads greater than 5.0 log_{10} copies/ml by the Abbott test had a mean ratio of 182.

Comparison of ASRs and extraction methods. Forty-seven plasma samples were tested by the Abbott and QIAGEN tests using the QIAGEN extraction method. Viral loads were detectable in all samples by both tests. The mean viral load (SD) obtained using the Abbott test was 3.89 (1.02) log_{10} copies/ml, compared to 4.04 (1.01) log_{10} copies/ml by the QIAGEN test. The mean difference in viral loads between the two tests was

TABLE 1. Limit of sensitivity of the Abbott test using plasma samples

<table>
<thead>
<tr>
<th>log_{10} copies/ml</th>
<th>No. of positive samples/no. tested</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>16/16</td>
<td>100</td>
</tr>
<tr>
<td>2.3</td>
<td>19/20</td>
<td>95</td>
</tr>
<tr>
<td>2.0</td>
<td>18/20</td>
<td>90</td>
</tr>
<tr>
<td>1.7</td>
<td>9/13</td>
<td>69</td>
</tr>
<tr>
<td>1.4</td>
<td>7/20</td>
<td>35</td>
</tr>
</tbody>
</table>

TABLE 2. Reproducibility of the Abbott test using plasma samples

<table>
<thead>
<tr>
<th>Nominal concn (log_{10} copies/ml)</th>
<th>No. of samples tested</th>
<th>Mean viral load (log_{10} copies/ml)</th>
<th>SD (log_{10} copies/ml)</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>6</td>
<td>5.88</td>
<td>0.19</td>
<td>3.2</td>
</tr>
<tr>
<td>5.0</td>
<td>16</td>
<td>4.79</td>
<td>0.24</td>
<td>5.0</td>
</tr>
<tr>
<td>4.0</td>
<td>16</td>
<td>3.91</td>
<td>0.21</td>
<td>5.3</td>
</tr>
<tr>
<td>3.0</td>
<td>16</td>
<td>2.76</td>
<td>0.24</td>
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</tr>
<tr>
<td>2.3</td>
<td>19</td>
<td>1.93</td>
<td>0.45</td>
<td>23.3</td>
</tr>
<tr>
<td>2.0</td>
<td>18</td>
<td>1.90</td>
<td>0.46</td>
<td>24.2</td>
</tr>
</tbody>
</table>

a CV, coefficient of variation.

b Only positive samples were included.

c The Abbott test used plasma specimens, and the Hybrid Capture assay used whole-blood specimens.
Both the Abbott and the QIAGEN reagents were manufactured by *artus* and are essentially the same product, providing clinical laboratories with equivalent options for developing CMV viral load tests. When tests based on these reagents are coupled with either the QIAGEN, the MagNA Pure, or the easyMAG extraction method, the viral loads are remarkably similar. As expected, when the Abbott and QIAGEN tests were compared using the same extraction method, the viral loads were in close agreement (mean difference, 0.144 log$_{10}$ copies/ml). A larger mean difference in viral loads (0.144 to 0.370 log$_{10}$ copies/ml) was observed when the QIAGEN test was evaluated using three different extraction methods, although the greatest difference between extraction methods was only 2.3-fold. Further studies are needed to determine if this close agreement is seen when a wider range of extraction methods is used. These data support the concept that the Abbott and QIAGEN reagents provide laboratories with additional reagents that can be used to for CMV viral load testing and that may improve assay standardization.

**ACKNOWLEDGMENTS**

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**REFERENCES**


