Distinguishing Cytomegalovirus (CMV) Infection and Disease with CMV Nucleic Acid Assays

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Human cytomegalovirus (CMV) continues to be a significant cause of morbidity and mortality among transplant recipients. Molecular assays have been developed for the detection and quantification of CMV nucleic acid. In evaluating the clinical utility of these assays, correlations with clinical outcome are essential. The Amplicor CMV Monitor and NucliSens CMV pp67 tests were compared to the CMV antigenemia assay for 45 transplant recipients and 1 patient with Wegener’s granulomatosis. Twenty-three patients remained antigenemia negative throughout the monitoring period, none of whom developed CMV disease. In this patient group, both the Amplicor and NucliSens assays showed very high specificity; only 1 of the 324 specimens assayed by NucliSens and none of the 303 specimens assayed by Amplicor were positive. Twenty-three patients were antigenemia positive during the monitoring period, 12 of whom developed 13 episodes of symptomatic CMV disease. In this patient group, the NucliSens assay was positive at or before the development of symptoms in 12 of the 13 episodes of CMV disease. All eight patients with symptomatic CMV disease who were tested by the Amplicor assay were positive at or before the development of disease. For the 11 asymptomatic patients, the NucliSens assay was positive less frequently than the antigenemia or Amplicor assays. The NucliSens assay was more likely to be positive at higher antigenemia or viral load levels. Both the NucliSens and Amplicor assays appear to have clinical utility in monitoring patients for CMV disease.

Cytomegalovirus (CMV) is a major cause of disease in organ and cell transplant recipients (12, 25) and patients who are immunocompromised due to other clinical conditions, such as human immunodeficiency virus type 1 infection and chronic steroid use. Furthermore, CMV is associated with an increased risk of bacterial and fungal infections (11, 13, 31, 32) and graft rejection (8, 24), increased health care costs (9, 18), and decreased survival (7, 10) in transplant recipients. While treatment of disease with specific anti-CMV drugs reduces the severity and mortality of CMV disease in transplant recipients (17, 29), prophylaxis and preemptive therapy are more effective in preventing CMV disease (5, 6, 15, 21). The success of preemptive therapy in particular is dependent upon the availability of sensitive, specific, and timely diagnostic tests for CMV infection. At our institutions, the CMV pp65 antigenemia assay has been heavily relied upon for this purpose with considerable success (19, 20). However, the antigenemia assay is highly labor-intensive, not automated, susceptible to inaccuracy when not performed on very fresh specimens, and subjective in its reading.

Recently, several molecular assays for detection of CMV in blood have become commercially available. Several recent publications support the clinical utility of the Amplicor Monitor CMV test (Roche Diagnostics, Indianapolis, Ind.), a quantitative CMV DNA PCR assay, for preemptive therapy monitoring, diagnosing active disease, and monitoring response to therapy (4, 16, 27, 28). The hybrid capture assay, which also measures CMV DNA in leukocytes, has also been shown to be useful in predicting the risk of CMV disease when used in a quantitative format (1). The NucliSens assay (Organon Teknika, Boxtel, The Netherlands) is a qualitative assay that detects a late mRNA (pp67), rather than CMV DNA, to better distinguish between active and latent disease. Several recent studies have evaluated the clinical utility of this assay for solid-organ transplant recipients (2, 3, 14, 23, 33).

The key to evaluating the clinical utility of molecular assays for the detection of CMV disease is clinical correlation with the laboratory results. Studies that compare two assays without including information about which patients go on to develop CMV disease are difficult to interpret. For example, if one of the molecular tests has poor clinical specificity and detects CMV DNA in the absence of disease, then the other test may appear insensitive. Only by comparing assays to clinical outcome can their true clinical performance be determined. We have therefore compared the correlations with clinical outcome of Amplicor Monitor, NucliSens, and CMV antigenemia assays for 46 immunocompromised patients.

**MATERIALS AND METHODS**

**Patient and clinical specimens.** Forty-six patients were studied, 45 of whom were transplant recipients while 1 patient had Wegener’s granulomatosis and was receiving corticosteroids. The distribution of transplants was 12 renal, 11 liver, 3 heart, 12 lung, 1 renal and bone marrow, and 6 bone marrow. Blood and plasma samples were collected sequentially from the patients during the monitoring...
period. Specimens were tested at either the Massachusetts General Hospital (MGH) Clinical Microbiology Laboratory or the University of Pittsburgh Medical Center (UPMC) Clinical Virology Laboratory. Antigenemia assays were performed in real time, and the results were used for patient management decisions. Plasma samples from blood anticoagulated in EDTA or acid citrate dextrose were frozen at −70°C and subsequently batch tested using the Amplicor Monitor assay. For the NucliSens assay, 100 μl of whole blood anticoagulated in either EDTA or heparin was placed in NucliSens lysis buffer, rocked at room temperature for 20 to 30 min, and then frozen at −70°C. Samples for the NucliSens assay were batch tested retrospectively. Chart review was done to obtain information regarding clinical symptoms and treatment.

Each study was approved by the corresponding institutional review board. At both the MGH and the UPMC, the antigenemia assay is the standard test used for the detection of CMV infection from blood specimens.

**Definition of CMV disease.** CMV infection was defined as a positive CMV antigenemia assay. Active CMV disease was defined as a positive CMV antigenemia assay and any of the following: the presence of appropriate symptoms (fever, malaise, and/or diarrhea) or signs (leukopenia or transaminitis), the presence of retinitis on ophthalmologic examination, or a tissue biopsy specimen that was CMV positive by either culture or immunohistochemical staining.

**CMV antigenemia assay.** The CMV antigenemia assays were done by standard procedures in either the MGH or UPMC clinical virology laboratory, as previously described (20, 30). The results were reported as the number of cells staining positive per 200,000 leukocytes.

**Molecular assays.** The Amplicor Monitor assay is a quantitative CMV DNA PCR assay. The target is a 362-bp region of the polymerase gene. The linear range of the assay is 400 to 100,000 copies/ml of plasma. The NucliSens assay is a qualitative nucleic acid sequenced-based amplification (NASBA) assay. The assay amplifies pp67 mRNA, a late mRNA produced during viral replication. Results are reported as positive or negative. The Amplicor Monitor and NucliSens pp67 assays were performed according to the manufacturers’ recommendations.

**RESULTS**

Of the 46 patients studied, 23 were antigenemia negative throughout the monitoring period, and none of these patients developed active CMV disease. A total of 324 specimens were
collected from these 23 patients. All of the specimens were tested using the NucliSens assay, and only one was positive for pp67 mRNA. Three hundred and three specimens (18 patients) were tested with the Amplicor assay, and none were positive for CMV DNA.

The remaining 23 patients were antigenemia positive, and the results of antigenemia, NucliSens, and PCR testing of representative patients are shown in Fig. 1 and 2, along with information regarding antiviral therapy. Antigenemia and NucliSens testing were performed on all 23 patients, and Amplicor testing was performed on 16 of these patients (8 symptomatic and 8 asymptomatic). Eleven patients (examples are shown in Fig. 2) had no clinical signs or symptoms of CMV disease, in spite of positive test results, and were defined as having asymptomatic CMV infection. Twelve patients (examples are shown in Fig. 1) had CMV disease. Of the 11 patients who were CMV
antigenemia positive without symptoms, 3 were treated with acyclovir only (Fig. 2d and e) and did not develop active disease. Two of these patients had one positive NucliSens assay, and the third patient did not have any positive NucliSens results. Each of these patients had multiple specimens that were positive in the antigenemia assay. For the remaining eight patients with positive antigenemia and no symptoms, the NucliSens assay was positive less frequently than the antigenemia assay. Whether this represents overtreatment based on the antigenemia results is difficult to assess due to the use of preemptive therapy.

There were 12 patients who developed 13 episodes of active CMV disease for which NucliSens and antigenemia results are available (Fig. 1). For 12 of the 13 episodes of active disease, the NucliSens assay was positive at or before the development of symptoms. In the one episode of symptomatic disease (CMV colitis) in which the NucliSens assay was not positive, there were only two positive cells in the antigenemia assay (data not shown). The patient was on ganciclovir at the time colitis was diagnosed, which may explain the negative NucliSens assay. The treatment regimen for the patient was not changed, and the symptoms resolved. Higher levels of antigenemia positivity were associated with an increased likelihood of NucliSens positivity (Table 1). Correlation data are presented for all results and for time points when patients were not receiving therapy. Most of the data (112 of 160; 70%) were collected while the patients were off therapy. Of the 48 data points where the patients were off therapy, when the NucliSens assay was negative and the antigenemia was positive, the patients were always asymptomatic. There was a very good association between the Amplicor and antigenemia assays in all patients evaluated (Fig. 1 and 2). All eight patients with symptomatic CMV disease who were tested by the Amplicor Monitor assay were positive at or before the development of CMV disease. The declines in the values of the two assays after initiation of therapy were not always similar, presumably due to the fact that the antigenemia assay measures antigen in peripheral blood mononuclear cells (PBMC) while the Amplicor assay measures viral DNA in plasma. Of the three assays, the NucliSens tended to become negative after treatment more rapidly than the antigenemia or Amplicor assays. A comparison of antigenemia and Amplicor results is shown in Fig. 3. All time points in which both assays were positive are included in the graph, regardless of whether the patient was receiving therapy. Time points were included for both symptomatic and asymptomatic patients. The correlation coefficient is 0.77. When the Amplicor and NucliSens assays are compared, the higher the viral load by Amplicor, the more likely the NucliSens assay was to be positive (Table 2). This is consistent with what was seen when comparing the NucliSens and antigenemia assays. There were 26 data points collected when patients were off therapy; 12 were Amplicor and NucliSens positive. All 14 specimens that were NucliSens negative and Amplicor positive were from asymptomatic patients, and the viral load was <5,000 copies/ml. There are too few time points to make any definitive statements, but the trend is similar to that seen when comparing the NucliSens assay with the antigenemia assay. A total of 710 specimens were tested using the NucliSens assay, including specimens from antigenemia-positive and -negative patients. Of these, 5.5% were invalid in the assay due to failed amplification of the internal calibrator.

During the study, the lyophilization process for the enzymes was changed to improve stabilization and performance. After this change, the rate of invalid results decreased to <0.5%. The rate of amplification inhibition in the Amplicor Monitor assay was 1.9% (12 of 635 specimens).

### DISCUSSION

Laboratory parameters are essential for guiding decisions concerning anti-CMV preemptive therapy in immunocompromised patients (26). The present standard of care for organ transplant recipients includes close monitoring for clinically important CMV infections by quantitation of pp65 antigenemia or DNA viral load or by detection of CMV mRNA. Though the clinical utility of CMV antigenemia and DNA assays has been documented, they can be positive in patients without disease (20–22). Recent studies with the NucliSens assay for CMV pp67 mRNA in blood have confirmed the high clinical specificity but have questioned the sensitivity of the assay (2, 3, 14). Our study is unique in that it compares the CMV antigenemia assay, the Amplicor Monitor assay for CMV DNA, and the NucliSens assay in a group of patients with known clinical outcomes.

Our data show that the Amplicor Monitor and NucliSens assays have good clinical specificity, as both assays are rarely positive in asymptomatic patients with negative antigenemia results. The NucliSens assay detects pp67 mRNA, which is expressed at high levels during active CMV replication (22), and this likely contributes to the greater specificity of the assay. The high specificity of the Amplicor Monitor assay may be due to the use of plasma specimens and the fact that the assay was deliberately designed with a clinically appropriate level of sensitivity (400 copies/ml).

We found a very good association between the CMV antigenemia and Amplicor Monitor assays for the detection of CMV infection and disease. These results are in agreement with those observed in other studies supporting the clinical utility of the Amplicor Monitor assay (4, 16). The Amplicor and antigenemia results mirrored each other, and both assays were effective in diagnosing CMV infection, monitoring response to therapy, and deciding when to initiate preemptive therapy. The values observed for the Amplicor assay were 1 to 2 log_{10} units higher than that observed for the antigenemia assay. The fact that the values from the two assays do not match is not surprising, considering that the Amplicor assay is measuring plasma DNA while the antigenemia assay is measuring CMV pp65 antigen-positive PBMC. There is a wide

### TABLE 1. Comparison of antigenemia and NucliSens assays

<table>
<thead>
<tr>
<th>No. antigenemia positive²</th>
<th>No. NucliSens positive/total no. of specimens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All specimens</td>
</tr>
<tr>
<td>≥50</td>
<td>36/36 (100)</td>
</tr>
<tr>
<td>49–11</td>
<td>30/40 (75%)</td>
</tr>
<tr>
<td>≤10</td>
<td>27/84 (32%)</td>
</tr>
</tbody>
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² Number of antigenemia-positive cells per 200,000 leukocytes.
³ CMV-specific therapy; for all NucliSens-negative, antigenemia-positive specimens, patients were asymptomatic.
range of CMV DNA levels for any given pp65 antigen level. This may be due to the fact that most data points were collected when the patients were receiving therapy, and the rates of decline of CMV DNA in plasma and pp65 antigen in PBMC after initiating therapy were not always similar. These results are useful for clinicians when changing their monitoring assay from antigenemia to the Amplicor Monitor assay.

Regarding clinical utility, the NucliSens assay was positive in 12 of 13 episodes of active CMV disease at or before symptoms developed. In the one episode of disease that was not detected by the NucliSens assay, there were only two positive cells by antigenemia and the patient was receiving ganciclovir therapy. Though our results show good clinical sensitivity, previous studies (2, 3, 14) have raised concerns about the sensitivity of the NucliSens assay compared to those of the antigenemia assays and in-house-developed PCR assays. However, a recent study showed that the sensitivity of the NucliSens assay for detection of CMV infection was 97% and the specificity was 94% (23) when CMV infection was defined retrospectively based on the results of serology. The definitions of disease and comparative assays were different for these studies, which may explain the differences in sensitivity of the NucliSens assay. Moreover, it is clear from our results that the antigenemia and Amplicor assays are positive more frequently than the NucliSens assay. However, an evaluation of the sensitivity of an assay for the detection of CMV requires correlation with the clinical outcome. We identified three patients without symptoms who did not receive anti-CMV therapy and who did not develop symptomatic disease. Two of the 28 specimens from these patients tested by NucliSens were positive, while multiple specimens were positive by antigenemia (19 of 41 specimens from three patients) or Amplicor Monitor (4 of 9 specimens from one patient). For these patients, the NucliSens assay was able to distinguish infection from disease more effectively than the antigenemia or PCR assays. It is difficult to assess the significance of a NucliSens-negative, antigenemia-positive result while the patient is receiving therapy. Based on the data, the rates of decline of the CMV pp65 antigen (antigenemia) and pp67 mRNA (NucliSens) were not always the same. For some patients, the mRNA declines more quickly than CMV antigenemia. This may explain the large number of antigenemia-positive NucliSens-negative specimens. Though the number of specimens collected while patients were off therapy was relatively low, all of the patients with an antigenemia-positive (or Amplicor Monitor-positive), NucliSens-negative specimen were asymptomatic when the specimen was collected. This suggests that the NucliSens assay may be useful in monitoring response to therapy. Indeed, a recent report showed that patients who were NucliSens positive when therapy was discontinued experienced a relapse in CMV infection and received further therapy (23).

In patients without symptoms, monitoring sequential specimens with the NucliSens assay may decrease the use of preemptive therapy. Whether episodes of disease would be missed cannot be determined by this study, because preemptive therapy was based on the antigenemia assay. Prospective clinical trials with solid-organ and bone marrow transplant patients are ongoing to address this question. Studies of both groups are essential, since different analytical sensitivities may be required for different types of transplants.

Based on the findings of this study, if the NucliSens assay were to be used to monitor patients for CMV disease, it would be appropriate to initiate therapy whenever the assay is positive. On the other hand, the Amplicor Monitor assay is frequently positive at low levels in patients without symptoms. In liver transplant recipients, a cutoff of 2,000 to 5,000 copies/ml in the Amplicor assay has been shown to have good correlation with predicting clinical disease (16). Similarly, in our study, for the eight patients with active disease who were tested with the Amplicor Monitor assay, seven had a viral load of >5,000 copies/ml at the time of symptoms and six had a viral load of greater than 20,000 copies/ml at the time of symptoms. Therefore, our findings would support a 2,000- to 5,000-copy/ml cutoff in the Amplicor assay for the initiation of therapy. Furthermore, this apparently higher level of sensitivity may provide earlier warning for the emergence of resistant virus.

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REFERENCES


