Utility of Urine and Leukocyte Cultures and Plasma DNA Polymerase Chain Reaction for Identification of AIDS Patients at Risk for Developing Human Cytomegalovirus Disease

Mika Shinkai, Samuel A. Bozzette, William Powderly, Peter Frame, and Stephen A. Spector

Urine and blood leukocyte cultures and qualitative plasma polymerase chain reaction (PCR) and quantitative competitive (QC) PCR were evaluated for their ability to identify AIDS patients at risk for human cytomegalovirus (HCMV) disease. AIDS patients were followed with urine and blood specimens every 3 months. During a mean follow-up of 12 months, 26 (28%) developed HCMV disease. The sensitivity, specificity, positive predictive value, and negative predictive value for urine culture were 85%, 29%, 31%, and 83%; for leukocyte culture were 38%, 74%, 69%, and 81%; for qualitative plasma PCR were 89%, 75%, 58%, and 94%; for QC-PCR (>1000 copies/µL) were 35%, 100%, 100%, and 80%; and for QC-PCR (>100 copies/µL) were 73%, 90%, 73%, and 90%, respectively. Of 41 patients identified by qualitative PCR to have HCMV DNA in plasma, the 24 who developed HCMV disease had 1510 ± 448 (mean ± SE) peak copies of HCMV DNA/µL by QC-PCR, versus 161 ± 52 for the 17 patients who did not develop disease (P = .0007). Thus, plasma PCR is superior to culture for identification of AIDS patients at risk for HCMV disease, and quantitation of plasma DNA further identifies high-risk persons.

Human cytomegalovirus (HCMV) is a common pathogen of severe illness in immunocompromised persons, including those with AIDS, who may develop HCMV retinitis, colitis, encephalitis, or other HCMV-related disease [1-6]. Early diagnosis and, preferably, identification of persons at highest risk for developing subsequent disease are important for initiating early treatment or instituting targeted prophylaxis for those most likely to benefit. Isolation of HCMV in conventional or shell vial cultures has been of limited usefulness in identifying persons at highest risk of developing disease [7, 8]. HCMV diseases have been diagnosed traditionally by identification of the typical viral inclusions in biopsy specimens or cytology specimens enhanced by direct detection of viral antigens or nucleic acid, by viral culture using human fibroblasts with or without immunostaining, or by a rise in HCMV-specific antibody titers [9, 10]. However, diagnosis by these methods is often either inaccurate or too delayed to be clinically useful.

In an attempt to improve both the sensitivity and timeliness of diagnosis, numerous groups have applied the detection of the HCMV matrix protein, pp65, or viral DNA by polymerase chain reaction (PCR) in blood leukocytes for improved identification of HCMV disease in immunocompromised patients [11-19]. More recently, PCR has been applied to the detection of HCMV DNA in plasma of persons with AIDS and following organ transplantation [18-23]. Although the detection of pp65 in leukocytes or HCMV DNA in cells or plasma have been found to be useful in identifying persons with HCMV-related disease, it is thought that quantitation of virus load would be of additional use in prospectively identifying persons at highest risk for developing disease [18]. Moreover, the natural history of HCMV replication before development of disease can be better understood by monitoring virus load before the clinical recognition of disease.

In the current study, PCR for HCMV DNA in plasma, urine cultures, and blood leukocyte (buffy coat) cultures were performed longitudinally on human immunodeficiency virus (HIV)-infected persons. Additionally, a quantitative competitive PCR (QC-PCR) procedure was developed and applied for the quantitation of HCMV DNA in positive plasma samples obtained longitudinally from persons with AIDS.

Materials and Methods

Study participants and clinical specimens. Specimens were obtained from a subset of HIV-infected persons who had participated in the AIDS Clinical Trials Group (ACTG) protocol 181 at the University of Cincinnati, Washington University (St. Louis), and University of California, San Diego. This protocol was designed to be a natural history study of HIV-related opportunistic infections and consisted of a subset of persons who participated in ACTG protocol 081/981 [24, 25]. Participants did not receive anti-HCMV therapy during the course of the study.
A total of 437 plasma specimens, 425 evaluable urine cultures, and 405 evaluable leukocyte cultures were obtained from 94 HCMV-seropositive HIV-infected persons. Plasma specimens were obtained every 3 months and stored at -20°C until assayed. Cultures were performed in real time at each site.

Study participants had a median baseline CD4 lymphocyte count of 54/mm^3. All were HCMV-seropositive but had no known past or current HCMV disease.

**HCMV cultures.** HCMV was isolated from peripheral blood leukocytes and urine specimens by the viral diagnostic laboratories at each of the three sites using standard methods for virus isolation or shell vial procedures [9]. Leukocytes for HCMV cultures were generally obtained from the same blood samples as the fractions.

**Documentation of HCMV disease.** Patients were considered to have HCMV disease if they had retinitis consistent with HCMV confirmed by an experienced ophthalmologist or if they had gastrointestinal disease, including gastroenteritis, esophagitis, or colitis, confirmed by endoscopy with biopsy. Routine ophthalmologic examinations were not required but were obtained either as a result of visual symptoms or during routine eye examination obtained as part of standard care at individual sites. CMV pneumonia was diagnosed by the detection of CMV on histopathology of lung with no other pathogen identified in a patient with a history of respiratory deterioration.

**Plasma viremia PCR (plasma PCR).** Qualitative plasma PCR for HCMV was done as described [20, 21].

**QC-PCR and standard curves.** A DNA fragment of the same length and sequence as the target genomic DNA (except for a 20-nucleotide insertion placed in the middle of the amplicon, which allows it to be easily distinguishable from the amplicon after gel electrophoresis) was constructed as the competitor for the quantitative PCR procedure [26, 27]. A constant quantity (1000 copies) of competitor was used in QC-PCR as the internal control. A standard curve was constructed from the data obtained by quantitative analysis of the ratio between PCR products of competitor and wild type DNA in a set of samples containing a known amount of fragment D DNA ranging from 6 x 10^6 copies to 6 copies. Standard curves were generated for each assay.

QC-PCR of plasma specimens was done identically as for the standard plasma PCR procedure [20, 21]. Quantities used for QC-PCR were 1 or 0.1 μL of plasma that was positive by plasma PCR and 1000 copies of competitor. Specimens were run in duplicate with the mean of the two values used for the sample copy number.

Amplified product was quantitated by determining the radioactivity of each band (the 152-bp wild type and the 172-bp competitor) by direct counting from the exposed gel in a scintillation counter (Beckman LS 6000SC; Beckman Instruments, Fullerton, CA). A standard calibration curve was constructed from the data obtained by quantitative analysis of the ratio between PCR products of competitor and wild type DNA in a set of standard samples. The standard curve was calculated using a least squares analysis and was used to quantitate the amount of wild type template (152 bp) in unknown samples by using the ratio of the 152-bp to the 172-bp PCR products [28]. A comparison between the QC-PCR procedure used for these studies and one under development at Roche Molecular Systems (Branchburg, NJ) found a correlation (r) of .997 for the two assays [29].

**Statistical analysis.** The ability of plasma PCR, urine culture, or blood culture to identify persons at risk for development of HCMV disease was assessed using Fisher’s exact test. Medians of HCMV DNA were compared by use of the Wilcoxon rank sum test, whereas differences between categories were assessed by use of Fisher’s exact test. All tests were two-sided. Sensitivity, specificity, positive predictive value, and negative predictive value were determined using standard formulas.

The overall performance characteristics of the tests were compared through the use of logistic regression and the construction of receiver operating characteristic (ROC) curves [30]. ROC curves plot the sensitivity against 1 minus specificity. A diagonal line drawn from 0.0 to 1.1 would represent a test of no value. Curves that pass higher and to the left are better, with the area under the curve representing the overall value of the test. The curve for a perfect test would pass through 0,1 at the upper left and enclose 1.0 of the possible area. The point at which each curve is closest to the 0,1 point represents the optimal overall cutoff for the test. Data for each patient were reduced to one point before running the regressions by dividing study participants into those who were ever positive or never positive for cultures and qualitative PCR. For quantitative PCR data, the highest observed values for each patient were used.

**Results**

**Development of HCMV disease.** Of the 94 study participants, 26 (28%) met criteria for development of HCMV disease. HCMV diseases identified included retinitis in 19, colitis in 4 (1 patient also had retinitis), esophagitis in 1, disseminated disease in 1, and pneumonia in 1. The median follow-up period during which study participants had specimens obtained was 12 months (mean, 12). All participants who were alive at the conclusion of the period of specimen collection were followed for an additional 6 months for development of HCMV-related disease.

**Relationship of urine and blood culture results to HCMV disease.** Urine cultures were positive during the median 12-month study period in 70 (76%) of 94 study participants. Of the 26 persons who developed HCMV disease, 22 (85%) had at least 1 positive urine culture before diagnosis (table 1). However, 4 persons (15%) who developed organ disease associated with HCMV remained urine culture-negative. The detection of HCMV in urine was not significantly associated with the development of HCMV-related disease (relative risk [RR], 2.3; 95% confidence interval [CI], 0.7-7.5; P = .2), mostly due to the large number of study participants who were urine culture-positive and never developed HCMV disease.

Blood leukocyte cultures were positive on at least one occasion in 16 (17%) study participants (table 1). Of the 26 persons who developed disease, only 11 (42%) had positive blood leukocyte cultures before or at the time of development of CMV disease. However, 11 (69%) of the 16 persons with positive leukocyte cultures developed HCMV disease, and blood leukocyte cultures were significantly associated with the development of disease (RR, 9.2; 95% CI, 2.8-30.6; P = .002).

**Qualitative plasma PCR of plasma specimens and development of HCMV disease.** Of the 94 HIV-infected persons eval-
Table 1. Urine or blood cultures and qualitative plasma DNA PCR—correlation with HCMV disease.

<table>
<thead>
<tr>
<th>Test, result</th>
<th>HCMV disease (n = 26)</th>
<th>No disease (n = 68)</th>
<th>Relative risk</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11 (42)</td>
<td>5 (7)</td>
<td>9.2</td>
<td>2.8–30.6</td>
<td>.0002</td>
</tr>
<tr>
<td>Negative</td>
<td>15 (58)</td>
<td>63 (93)</td>
<td>2.3</td>
<td>0.7–7.5</td>
<td>.2</td>
</tr>
<tr>
<td>Urine culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>22 (85)</td>
<td>48 (71)</td>
<td>2.3</td>
<td>0.7–7.5</td>
<td>.2</td>
</tr>
<tr>
<td>Negative</td>
<td>4 (15)</td>
<td>20 (29)</td>
<td>23</td>
<td>6.1–86.3</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Plasma PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23 (89)</td>
<td>17 (25)</td>
<td>24</td>
<td>6.1–86.3</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>3* (11)</td>
<td>20 (29)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%), except as indicated.
* 1 patient was PCR-positive only after diagnosis of HCMV retinitis.

QC-PCR of clinical specimens and development of HCMV disease. In the group of 41 study participants who were plasma PCR-positive at any time during the study period, the 24 patients with HCMV disease had a median of 473 (mean ± SE, 1510 ± 448) HCMV DNA copies/μL in their plasma, compared with a median of 35 (161 ± 52) HCMV DNA copies/μL for the 17 plasma PCR-positive patients who never developed HCMV disease (P = .0007) (table 2). Of the 9 patients who had a peak of >1000 copies/μL of HCMV DNA in plasma, all developed HCMV retinitis. In contrast, of the 15 study participants who had a peak of <100 copies/μL, 5 (33%) developed HCMV disease (RR, 36.3; 95% CI, 1.8–748; P = .002; table 2). Study participants with a peak of 100–1000 copies had a 59% risk of developing HCMV disease. Thus, persons with the highest mean peak copies of HCMV DNA in their plasma were at the highest risk of developing HCMV-related disease; however, some persons with an apparent low HCMV DNA load in their plasma still developed disease.

Sensitivity, specificity, and predictive value of urine culture, blood leukocyte culture, qualitative PCR, and quantitative PCR for HCMV disease. The qualitative detection of HCMV DNA in plasma was consistently superior or equivalent to the culture procedures in identifying those persons at highest risk for development of HCMV disease (figure 1). The sensitivity of plasma PCR, urine culture, and blood leukocyte culture for detection of HCMV disease was 89%, 85%, and 38%, respectively. The specificity for the three assays was 75%, 29%, and 74% for plasma PCR, urine culture, and blood leukocyte culture, respectively. Thus, the combined sensitivity and specificity favored the qualitative plasma PCR assay. Positive and negative predictive values were also highest for the qualitative plasma PCR assay, except for the blood leukocyte culture having the highest positive predictive accuracy.

When plasma specimens had >1000 copies/μL, the specificity and positive predictive accuracy for development of CMV disease was 75% and 33%, respectively. In contrast, when plasma specimens had <100 copies/μL, the specificity and positive predictive accuracy for development of CMV disease was 85% and 29%, respectively.

Table 2. Quantitative competitive HCMV plasma PCR results and development of HCMV disease in subjects previously identified to be positive by qualitative plasma DNA PCR.

<table>
<thead>
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<th>Peak copies/μL</th>
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<td>&gt;10009</td>
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<td>100–1000</td>
<td>10 (59)</td>
<td>7 (41)</td>
</tr>
<tr>
<td>&lt;10007</td>
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<td>10 (67)</td>
</tr>
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<td>Mean ± SE HCMV copies/μL1</td>
<td>1510 ± 448</td>
<td>161 ± 52</td>
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<td>Median HCMV copies/μL2</td>
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NOTE. Data are no. (%) with disease or no disease at copy range, except as noted.
* P = .006 disease vs. no disease (3-way χ²).
† P = .002 >1000 copies/μL vs. <100 copies/μL (Fisher’s exact test).
‡ P = .0007 (Wilcoxon rank).

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Virologic Monitoring for HCMV Disease

The qualitative detection of HCMV DNA in plasma by PCR has been previously demonstrated to be a rapid and useful method to diagnose HCMV disease in HIV-infected persons and transplant recipients [19–23, 31, 32]. In the current study, the qualitative plasma PCR procedure used to detect HCMV DNA in plasma was compared with urine and blood cultures when applied to specimens obtained from HIV-infected persons every 3 months. The sensitivity, specificity, and predictive value of the plasma PCR procedure was superior or equivalent to both culture procedures. Urine cultures were positive before diagnosis of HCMV disease in 85% of study participants; however, the specificity of urine culture was only 29%. In contrast, blood cultures had a low sensitivity, being positive in only
38% of persons developing disease, but a high specificity of 74%.

In an attempt to improve the ability of the plasma PCR procedure to identify those persons at greatest risk for HCMV disease, we developed a competitive PCR procedure using an internal competitor as a standard for quantitation of the PCR product. Our findings indicate that HIV-infected persons with the highest peak HCMV DNA load in their plasma are at greatest risk for development of subsequent HCMV disease. Using a cutoff of a peak HCMV DNA copy level of 1000 copies/μL, 100% of persons with greater than this level of circulating viral DNA developed HCMV disease. For persons with >100 copies/μL, 76% developed HCMV disease, whereas 33% of those with peak HCMV DNA levels of <100 copies developed disease. A multiple regression analysis demonstrated that the quantitative PCR was clearly superior to blood or urine culture but only marginally significantly superior to the qualitative PCR. Thus, persons with the highest quantities of HCMV DNA in their plasma are at greatest risk for developing HCMV retinitis and other HCMV-related diseases. However, when specimens are obtained every 3 months, some persons who have persistently low levels of HCMV DNA still may develop HCMV-related disease.

The longitudinal monitoring of persons with advanced HIV infection revealed a number of important features of HCMV infection and disease in this population. As previously described both for persons with AIDS and for those following transplantation, there is a group of persons who despite having acute viremia with HCMV do not develop clinically recognized disease [33]. It should be noted, however, that for this study the diagnosis of HCMV disease required either ophthalmologically confirmed retinitis or histopathologically confirmed HCMV organ disease. Fever or other clinical manifestations that may have been due to HCMV viremia were not considered end points for this study. Thus, the association of HCMV DNA in plasma with HCMV disease may have been underestimated.

Of additional interest, persons with HCMV DNA in their plasma developed visceral disease a median of 6 months following the first detection of viral DNA in their plasma. This finding contrasts greatly with our previous observations in bone.

Figure 2. Receiver operating characteristic curve for urine culture (Cx), blood (leukocyte culture), qualitative (Qual) plasma DNA polymerase chain reaction (PCR), and quantitative (Quant) DNA PCR.

Figure 3. HCMV DNA copies in plasma of 2 study participants who developed HCMV retinitis after having high levels of HCMV DNA in their plasma. One patient was PCR-negative for HCMV DNA; other had 357 copies/μL at time of diagnosis of HCMV retinitis. Arrows indicate time HCMV retinitis was diagnosed.
marrow transplant recipients, in which the median time between having HCMV DNA detected in plasma and the development of HCMV-related disease was 3 weeks [21]. Therefore, the pathogenesis of HCMV disease in persons with AIDS is considerably different from that of transplant recipients; this suggests that the presence of HCMV DNA in the plasma of persons with AIDS may be an early indicator of viral seeding of an end organ months before disease becomes manifest clinically. In support of this hypothesis is the finding in this study that some AIDS patients had extremely high levels of HCMV DNA in their plasma months before they were diagnosed with HCMV retinitis and, at the time of diagnosis, had little or no HCMV DNA present in their plasma. Moreover, peak levels of HCMV in plasma were identified a median of 4 months before the clinical diagnosis of HCMV disease. We suggest that in many cases the retina is infected with HCMV some months before diagnosis and that the virus replicates slowly but persistently within the eye until a plaque of viral replication manifested as HCMV retinitis becomes detectable on ophthalmologic examination.

The findings of the current study compare favorably with those previously published that have examined culture, antigenemia, or PCR for identification of persons at risk for development of HCMV disease [16, 18, 19, 22]. Studies examining either blood leukocyte or urine culture have found them either not useful or of limited clinical assistance in identifying persons at risk for HCMV disease [7, 8]. Although numerous studies have found the antigenemia assay to be useful for identification of high-risk persons, standardization of the procedure among multiple institutions has not as yet been accomplished [29]. In other studies, quantitative PCR of leukocyte HCMV DNA has been found to be useful for predicting persons at risk for developing HCMV disease. The plasma PCR procedure has the advantages over both of these assays in the ease of sample preparation and the ability for multiple sites without highly trained technicians to collect specimens, which can be stored and processed in a single laboratory. Thus, the data presented here indicate that detection of HCMV DNA in plasma compares favorably with previously published procedures and provides useful data for identifying persons at highest risk for developing HCMV disease. Similarly, quantitation of HCMV DNA in cerebrospinal fluid has been useful for identification of AIDS patients with HCMV-related polyradiculopathy and encephalitis and has been used to monitor antiviral therapy [34].

In summary, our findings indicate that the qualitative detection of HCMV DNA in plasma by the PCR procedure is more useful than either urine or blood culture in identifying over time those persons with advanced HIV disease who will develop HCMV disease. Quantitation of HCMV DNA in plasma further identifies persons who are at highest risk for development of disease. However, when monitored every 3 months, some persons appear to maintain low levels of HCMV DNA in their plasma despite development of disease. Furthermore, our findings indicate that persons with advanced HIV infection may have active HCMV infection for months before clinically recognized HCMV disease. These data suggest that the plasma PCR procedure should be useful in helping to identify persons at risk of developing HCMV disease and in helping to guide decisions regarding HCMV prophylaxis and treatment.

References