Improved Amplification of Cytomegalovirus DNA from Urine after Purification of DNA with Glass Beads

G. J. Buffone,1,4,5,6 G. J. Demmler,1,2,3,4,5 C. M. Schimbor,1,5 and Jewel Greer2,4

Cytomegalovirus can be detected in a variety of specimens including leukocytes, urine, saliva, feces, and various tissues by polymerase chain reaction (PCR) amplification of viral DNA. Although methods for amplification are fairly standard, sample preparation is not well characterized, especially for tissue. Typically, preparation of samples for PCR amplification ranges from simple boiling to phenol/chloroform extraction and quantification before testing. Several reports have described inhibition of the PCR in some sample types. Here we show that reliable detection of cytomegalovirus DNA in urine is obtained only after some degree of DNA purification, presumably because of PCR inhibition by a yet unidentified component present in a few of the urine samples tested. Glass, in the form of fine beads, was used to adsorb DNA such that protein and other substances could be selectively eluted before the recovery of DNA for PCR amplification. Urine samples prepared by this method did not show inhibition, and results correlated well with those by tissue culture for detection of cytomegalovirus.

Additional Keyphrases: polymerase chain reaction · sample preparation

Viral shedding associated with viral infection and disease can be detected in a variety of sample types, including saliva, urine, feces, tissue, and body cavity fluids such as bronchial washings. Our laboratories have been interested in the detection of human cytomegalovirus (CMV) in many of these sample types, including fresh and fixed tissue (1, 2).7 Our initial and subsequent experiences involving the analysis and identification of CMV DNA in urine samples have shown that ~15–25% of untreated samples show partial or complete inhibition of polymerase chain reaction (PCR) amplification. Of six other reports dealing with amplification of CMV DNA from urine, four used some degree of DNA purification before amplification (3–6). Harju et al. (4) reported difficulty with CMV amplification from human urine even after partial purification, noting that one of four urine samples tested showed inhibition of CMV amplification as well as inhibition of amplification of specific human DNA sequences added to the specimens. Olive et al. (7, 8), describing amplification of CMV DNA directly from human urine collected from renal transplant patients, indicated no inhibition of CMV DNA amplification. Although PCR, rather than cell culture, is used as the benchmark in these studies, the latter technique is usually considered the referee method for detection of CMV.

Inhibition of the PCR has not been the subject of any systematic study reported thus far; what is known consists principally of anecdotal observations mentioned in various articles and trade journals. "Proteins" have been noted to be inhibitory, as are heavy metal ions and other substances such as heparin that in general would be anticipated to inhibit enzymes. Ulrich et al. (9), in a brief study of hepatitis B virus amplification from simian serum, noted that serum needed to be diluted >10-fold to prevent inhibition of the PCR. They also examined the efficacy of phenol extraction and purification of DNA with use of glass beads. Phenol extraction provided the greatest sensitivity, but differences between the two methods were noted only at a low copy number, indicating that, at least in this case, attenuation of PCR efficiency rather than total inhibition of amplification had occurred. Two other reports found amplification of human immunodeficiency virus (HIV) by PCR to be inhibited when heparin was used as an anticoagulant, and that an unidentified PCR inhibitor appeared to co-purify with DNA recovered from blood specimens (10, 11).

We have examined a series of urine samples from patients with acquired immunodeficiency syndrome (AIDS), collected during a course of antiviral therapy for CMV disease. The specimens were tested directly or purified as described before PCR amplification of the CMV DNA. About 20% of the untreated urine samples could not be amplified without purification. Experiments in which human DNA was added to these urine samples suggest that the inhibitor is a molecule that must bind to the DNA to exert its effect on PCR amplification.

Materials and Methods

Urine specimens. Voided urine specimens were obtained from adults with AIDS who were receiving antiviral therapy for CMV retinitis. The specimens were transported at 4 °C and processed within 4 h of receipt in the CMV Virology Laboratory. Any remaining specimen was aliquoted and frozen at −20 °C. Aliquots of stored samples were used in the experiments described here.

CLINICAL CHEMISTRY, Vol. 37, No. 11, 1991 1945
Quantitative CMV culture. To process urine samples for quantitative CMV culture, we mixed 3 mL of urine with 0.5 mL of an antibiotic pretreatment solution containing gentamicin and amphotericin B in Hanks Balanced Salt Solution (Gibco BRL Life Technologies, Inc., Gaithersburg, MD) to kill contaminating bacteria and fungi, then centrifuged the samples at 1000 × g for 10 min to pellet the cellular debris. The processed urine samples were then diluted 10-fold in Hanks Balanced Salt Solution and 0.2 mL of each dilution (10^0, 10^-1, 10^-2, and 10^-3) was inoculated into each of four tubes of human foreskin fibroblasts cell culture (Viromed, Minnetonka, MN). Two culture tubes were inoculated with 0.2 mL of media only, for controls. The cell cultures were placed on a roller drum, incubated at 37 °C, maintained in cell culture media M199 (Gibco BRL) containing newborn calf serum (20 mL/L), and examined for 42 consecutive days. CMV was identified by its characteristic cytopathic effect on the fibroblasts culture. The 50% end-point dilution was calculated by the Reed–Muench method and expressed as 50% tissue culture infectious dose (TCID₅₀) per 0.2 mL (12).

Sample pretreatment. DNA from urine samples was purified with use of a commercially available kit and procedure (GeneClean, Bio 101, Inc., La Jolla, CA). The procedure is essentially the same as that described by Vogelstein and Gillespie (13). Micro-centrifuge tubes containing 50 μL of urine were placed in a boiling water bath for 10 min. The samples were allowed to cool before adding 150 μL of GeneClean NaI stock solution and 8 μL of GeneClean “glassmilk” (glass bead suspension), and then mixed gently by rocking for 10 min at ambient temperature. Specimens were then centrifuged for 5 s in a microfuge at ~13,600 × g and the supernatant liquid was discarded. The glass bead pellet was eluted one washed time (abbreviated glass bead = AGB purification) or three times (glass bead = GB purification) with 1 mL of cold GeneClean NEW solution (contains NaCl, Tris, EDTA, and ethanol).

DNA bound to the glass beads was eluted by adding 10 μL of sterile H₂O₂; after a 3-min incubation at 55 °C, the supernate was removed. This process was repeated and the supernates were combined, giving a final volume of 20 μL.

Primers and PCR conditions. For preparation of specimens, preparation of reagents, and analysis of PCR products, physically separated laboratory spaces were used as well as separate equipment designated for use in each of these respective areas to minimize the possibility of contamination. For each group of specimens analyzed, several CMV-seronegative DNA specimens from laboratory volunteers, as well as template-negative samples, were used as negative controls. In addition, any seropositive specimens that were culture negative were restested with a new aliquot to confirm the results.

Primers for CMV DNA amplification were selected from the major immediate early gene and a late gene product known as pp65 (14, 15). The location of the genes, the size of the regions amplified, and the sequence for each primer are illustrated in Figure 1. The specificity of the primers and the probability of dimer formation were evaluated by using a personal-computer-based application, "Oligo" (16). The primers used in this study represent unique sequences within the target genes and form a few (or in some cases, no) internal and inter-oligonucleotide dimers. We also tested the primers with DNA from Epstein–Barr virus, Herpes Simplex virus, and Varicella Zoster virus, and were unable to amplify DNA from these human herpes viruses (data not shown). Primers for a portion of the c-K-ras gene used for control studies are described in another publication (2).

Amplification of DNA from urine samples was performed in a total volume of 50 μL. The reaction mixture consisted of, per liter, 50 mmol of KCl, 10 mmol of Tris · HCl (pH 8.3), 1.5 mmol of MgCl₂, 0.1 g of gelatin, 200 μmol each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), and 0.3 μmol of each primer, mixed with 1–5 μL of urine or 5–10 μL of a purified DNA solution. After the reaction mixture was covered with 50 μL of mineral oil, the samples were placed in a boiling water bath for 7 min to destroy any endogenous enzymatic activity. Taq polymerase (1.0 μL; 1.5 U) was added before amplification, which was performed with a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). A total of 35 cycles, each consisting of 120 s at 94 °C, 90 s at 65 °C, and 120 s at 72 °C, were used in these experiments.

Gel and dot–blot analysis of PCR products. One-tenth of the PCR reaction mixture was analyzed on 5% polyacrylamide gels stained with ethidium bromide; 20% was analyzed by a standard dot–blot technique (1). The membranes (Nytran membranes; Schleicher and Schuell, Keene, NH) were probed with a 32P-labeled oligonucleotide (CMV22 or 30) having a specific activity of 10¹⁴-10¹⁵ counts/min per gram. Hybridization was carried out overnight at 50 or 60 °C. Before autoradiography, the membranes were washed at ambient temperature for 30 min at low stringency and at the hybridization temperature for 3 min at high stringency, as previously described (1).
Results

CMV DNA in 43 urine samples from 13 patients was amplified with two sets of PCR primers. The urine samples were either purified with an abbreviated glass bead (AGB) protocol or with use of glass beads with more extensive washing (GB) as described above, or received no pretreatment ("untreated") before amplification. To assess the relative sensitivity of each method of sample preparation for either primer pair, we compared the percentage of positive samples detected (Table 1). To assess sensitivity against an objective standard, we divided the number of PCR dot–blot-positive results for each method by the number of culture-positive results and reported this as a percentage of all culture-positive results (Table 1). By all measures, the GB-purified samples gave the greatest sensitivity. Interestingly, when gel analysis was used as the detection method, both the AGB and GB were scored as similar and more sensitive than no treatment; however, this conclusion was not sustained if dot–blot detection, a more sensitive method, was applied. The data obtained by gel analysis alone showed CMV1824 primers to be more efficient primers than CMV2829, although use of the more sensitive dot–blot detection virtually eliminated any distinction. Considering dot–blot data alone, AGB-purified samples scored only slightly better than untreated urine when amplified by PCR. These conclusions were also supported by comparison of PCR and tissue culture results on the same specimens. In 10 untreated, PCR-negative, culture-positive urine specimens the viral titers ranged from + (undiluted) to +3.5 TCID$_{50}$/0.2 mL, indicating that low copy number alone was an inadequate explanation for the false-negative results observed.

False-negative results (PCR results by dot–blot with either primer pair) observed in urine receiving no pretreatment were noted in seven specimens from six of the 13 patients. Results from a series of specimens from two patients (Table 2) illustrate the disparity between individual patients in terms of PCR amplification of CMV in untreated urine samples. Comparison of dot–blot results with culture results by specimen for each method of sample preparation showed that in seven GB-pretreated samples (not the same seven samples mentioned above) PCR results were positive when culture results were negative. In all seven cases, previous specimens from that patient had been culture-positive and PCR-positive. All positive results were verified by repeat analysis performed on another aliquot of the same specimen. In contrast, for only one case were PCR results positive for untreated specimens when culture results were negative.

The urine samples that appeared to inhibit CMV DNA amplification (false-negative results) were further evaluated by adding 10 ng of human DNA to the specimens and then attempting to amplify a portion of the c-K-ras gene by using the PCR. In one experiment the DNA was added directly to the PCR buffer/reagent mixture with the urine specimen. In that case, no inhibition of c-K-ras amplification was observed. In a second experiment, DNA was added to the urine and left standing at ambient temperature for 30 min before an aliquot of the admixed sample was added to the PCR buffer/reagent mixture. When the DNA was added directly to the urine before mixing with reagent, c-K-ras amplification was completely inhibited (Figure 2, lanes 1, 4, 7). Human DNA samples dissolved in buffer (no urine) before amplification or in a urine specimen that had not shown inhibition were run as controls. c-K-ras sequences were readily amplified from urine sample F547, which had not shown inhibition, containing as little DNA as 0.5 ng/5 mL (Figure 2, lanes 3, 6, 9).

If inhibition were due to an inhibitor binding to the DNA, as indicated by these experiments, then heating the urine in the presence of a reducing agent, as described by Olive et al. (7, 8), might denature such a protein. Therefore, we heated at 93 °C for 30 min in buffer containing mercaptoethanol, 10 mmol/L, the samples that had inhibited c-K-ras amplification before testing them according to the recommendation of Olive et al. (7). We then tested these samples by using PCR amplification and primers CMV1824 and CMV2829.

Table 1. CMV Amplification by Primer and Method of Preparation

| Specimen | CMV1824 | | | CMV2829 | | |
|----------|---------|---|---|---------|---|
| UT | AGB | GB | UT | AGB | GB |
| Direct gel | 32.6 | 58.1 | 60.5 | 11.5 | 34.9 | 34.9 |
| Dot–blot | 44.2 | 55.8 | 79.1 | 46.5 | 53.5 | 79.1 |

* UT, untreated; AGB and GB, preparation methods described in Materials and Methods.

* Values for each primer pair, preparation method, and detection method are the percentage of all samples positive for CMV DNA after PCR amplification.

* Total number of CMV DNA-positive results divided by the total CMV culture-positive results obtained for 43 urine samples.

Table 2. Patient-Specific Inhibition of PCR Amplification of CMV

<table>
<thead>
<tr>
<th>Specimen</th>
<th>UTDB1824</th>
<th>UTDB2829</th>
<th>GBDB1824</th>
<th>GBDB2829</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 109</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F263</td>
<td>3.5</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>F281</td>
<td>3.5</td>
<td>–</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>F295</td>
<td>2.3</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>F331</td>
<td>2.3</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>F369</td>
<td>1.0</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>F419</td>
<td>–</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Patient 206</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F339</td>
<td>2.5</td>
<td>1</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>F355</td>
<td>1.3</td>
<td>1</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>F377</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>F404</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>F442</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>F499</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Column heads represent untreated (UT) or GB sample preparation methods and dot–blot (DB) results only. Values listed are a relative measure of the intensity of the dot–blot signal on a scale of “–” to 4, 4 being the most intense.

CLINICAL CHEMISTRY, Vol. 37, No. 11, 1991 1947
Neither primer pair produced a positive gel result for any samples tested, although two samples were slightly positive by dot–blot (data not shown).

Discussion

Symptomatic or asymptomatic shedding of CMV in body fluids such as saliva, tears, or urine can be detected by culture or, more readily, by PCR amplification of CMV DNA. However, detection does not necessarily constitute disease. Congenital infection with CMV is the only clinical condition in which detection of CMV by culture or PCR is definitely diagnostic. Testing in the newborn period is done in urine by traditional culture methods and results are usually available within a few days, as the viral titer is typically high in these patients. Some physicians advocate screening for CMV shedding in the high-risk neonatal population because early intervention can help with developmental problems usually seen in these children. PCR amplification of viral DNA could offer a high-volume, rapid, and low-cost means of testing for CMV in these patients. Other immunocompromised hosts such as AIDS, cancer, and transplant patients are also susceptible to CMV infection and disease and would benefit from rapid and sensitive methods for viral detection. In light of the clinical need for improved viral diagnostic methods, PCR amplification of viral DNA could provide an excellent means to provide faster and less expensive test results. Practical implementation requires definition of issues such as sample preparation, sensitivity relative to traditional methods, and clinical significance of PCR- derived results.

In our earlier report describing the application of PCR to the detection of CMV DNA in urine from newborns with congenital CMV infection, we noted that some neonatal urine samples apparently contained inhibitors of CMV DNA amplification by PCR, which was most readily overcome by a simple dilution of the sample in some cases (1). We have subsequently observed similar inhibition of CMV DNA amplification in urine samples from children and adults, in comparison with results obtained by tissue culture. On the basis of these observations and the need to move to clinical trials to determine the efficacy of PCR detection of viral DNA, well-delineated and reliable methods for sample preparation are required. Because protein, among other factors, has been implicated as a potential inhibitor of the PCR reaction (9), we evaluated the use of acid-washed glass beads as a simple and inexpensive adsorbent for DNA purification (17). At least one earlier publication, in addition to that by Ulrich et al. (9), reported success in purifying viral DNA from blood by using glass beads or powder before PCR amplification and detection of HIV DNA (18). Here we used a commercial preparation of glass beads, or “glassmilk,” although glass powder is readily available and can be prepared in bulk for individual laboratory use (17).

The use of glass beads or powder to purify DNA from the urine samples worked well, permitting detection of culture-positive samples regardless of the titer. Several specimens from infected patients remained CMV DNA-positive longer when tested by PCR than by tissue culture. This finding is not unexpected, particularly because the patients were receiving antiviral drugs that inhibit viral DNA polymerase and thus viral replication but do not destroy or eliminate viral DNA (19–21). Unfortunately, a further effort to save time by reducing the number of high salt washes (AGB procedure) did not provide the degree of purity needed to ensure reliable PCR amplification of the CMV DNA.

As in earlier studies, we observed a small but troublesome number of samples to be PCR-amplification-negative and culture-positive. In addition, the false-negative results were not confined to low-titer samples, but included titer as high as +3.5 TCID90/0.2 mL. Samples with titer in this range are normally detected on gels after PCR amplification and do not require dot–blot detection with labeled probes. The association of false-negative results with specific patients was also in keeping with our previous observations (data not shown).

Given that the mixing of urine, human DNA, and PCR reaction buffer at the time of testing did not inhibit c-K-ras amplification, whereas preincubation of DNA and urine was required before inhibition was observed, we concluded that a substance(s) in the urine must interact with the DNA to inhibit PCR amplification. The substance was successfully removed by purification on glass, indicating a reversible association with the DNA, but only after extensive washing designed to remove protein but not DNA bound to the glass.

Given the reported success of Olive et al. (7, 8) in amplifying CMV DNA directly from urine, we attempted to reproduce their results, expecting that the use of mercaptoethanol and ammonium sulfate in the buffer used to pretreat the urine samples during a
prolonged heating step (93 °C for 30 min) would irreversibly denature any proteins that could be responsible for PCR inhibition. However, inhibition of PCR amplification of CMV DNA was still observed in the seven urine samples in question, despite the use of the pretreatment reagents and conditions described by Olive et al. Other reports (as already stated) describing amplification of CMV DNA from human urine have used some form of sample pretreatment or purification and provide no additional insight into the problem described here.

Although the inhibitor(s) remains unidentified, the data presented here confirm that the urine of some patients contains an inhibitor(s) of PCR amplification of DNA in high enough concentration to produce false-negative results if PCR is used for diagnostic testing for viral DNA; moreover, the inhibitor(s) appears to interact reversibly with the DNA to exert its effect on the PCR. As noted, the urinary inhibitor is not limited to AIDS patients; we have observed the same phenomenon in newborns without evidence of HIV infection. Purification of DNA from urine by the method originally described by Vogelstein and Gillespie (13) effectively eliminated the problem in the samples tested here and provided excellent correlation with tissue culture results when dot-blot detection was used.

Funded in part by the Texas Advanced Technology Program, Project No. 00494029.

References