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Application of PCR to Multiple Specimen Types for Diagnosis of Cytomegalovirus Infection: Comparison with Cell Culture and Shell Vial Assay

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Human cytomegalovirus (CMV) is a herpesvirus that is responsible for significant morbidity and mortality in congenitally infected infants and immunocompromised patients. Antiviral therapies are available, thus making timely diagnosis of significant importance to at-risk patients. A PCR system was devised. The newly devised system, unlike previously described systems, can be applied to a wide variety of specimen types in a clinical microbiology laboratory setting. Specimens from all sites routinely accepted for CMV culture were shown to be acceptable for CMV PCR. Sensitivity and specificity were established in comparison with those of both monolayer culture and shell vial assay (SVA). The sensitivity and specificity of PCR for detection of CMV in specimens exclusive of urine and blood were 97.5 (77 of 79 specimens) and 87.2% (41 of 47 specimens), respectively. The sensitivity and specificity of PCR for urine and blood specimens were 100 (10 of 10) and 95.7% (45 of 47) and 66.7 (4 of 6) and 78.8% (41 of 52), respectively. Discrepancies of positive PCR results with negative culture or SVA results occurred for specimens flanked chronologically by other culture- or SVA-positive specimens and were likely culture failures, increasing the specificity (100%) of PCR. Discrepancies of negative PCR results with positive culture or SVA results occurred in specimens with few cells or infectious foci by SVA or culture and may represent sampling variability associated with low virus titers.

Human cytomegalovirus (CMV) is a DNA virus which is a member of the *Herpesviridae*. Significant morbidity and mortality from CMV infection occur in two groups of patients, infants exposed to congenital infection and patients with acquired or induced immunosuppression (1, 18). Otherwise healthy individuals are typically asymptomatic or experience mild infection by early adulthood. However, patients with AIDS and those subjected to immunosuppression for purposes of organ transplantation may encounter disseminated viremia or organ syndromes such as retinitis or interstitial pneumonia (10, 32, 33). Antiviral therapies, including ganciclovir and foscarnet, are now available, making the timely diagnosis of CMV-induced disease a significant factor in the management of at-risk patient populations (8, 19, 21, 41).

Current diagnostics for CMV are awkward either because of a prolonged response time or problems with sensitivity. The "gold standard" diagnostic test is viral culture in fibroblast monolayers (30). Cell culture (CC) has a fundamental limitation in that a prolonged interval is required for development of a visible cytopathic effect (CPE). Although it may appear as early as 1 to 2 weeks, 6 weeks may be required for recovery from some specimens. A shell vial assay (SVA) has subsequently been developed with monolayer growth on coverslips subjected to examination with a monoclonal antibody to an immediate-early protein of CMV (16, 17). Results are available within 24 to 72 h; however, problems exist with sensitivity related to a number of variables including specimen type and virus concentration, the number of vials inoculated, and specimen toxicity for the monolayer (24, 28, 29). Staining with monoclonal antibodies to early and late CMV antigens is available for direct examination of infected cells in clinical specimens. Again, however, proper technique and specimen requirements may interfere with sensitivity (13, 39). Specimens tested have been limited to tissue, in situ examination, bronchoalveolar lavage (BAL) cells, and leukocytes. Nucleic acid hybridization techniques have been developed both with and without amplification. These techniques also have typically been developed for limited specimen types, for example, urine, whole-blood, or serum samples (5, 9, 27, 36). Variations on original PCR protocols have included nested primer sets and alternative detection of CMV mRNA (4, 6).

The purpose of the study described here was to apply PCR for the diagnosis of CMV infection within the clinical microbiology laboratory environment. The goals were twofold: (i) to adapt CMV PCR so that all specimen types typically received for culture could also be tested by PCR, and (ii) to determine the sensitivity and specificity of CMV PCR compared with those of monolayer CC and SVA. A simplified specimen handling technique was devised for all specimen types, including tissue biopsy specimens, various respiratory sources, urine, and blood. PCR was performed on processed specimens by using two sets of primers to the major immediate-early (MIE) antigen and late antigen (LA) gene regions of CMV. Results were then compared with those of monolayer CC and SVA. CMV was detected by PCR with significant sensitivity and specificity by using simplified specimen handling methods applied to a wide range of specimen types and processed within the clinical microbiology setting.

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MATERIALS AND METHODS

Specimens. CC and SVA were compared with PCR for the detection of CMV in 241 specimens including 34 BAL, 45 other respiratory (tracheal suction, bronchial wash, throat, nasopharyngeal), 35 tissue (lung, liver, gastrointestinal, brain), 12 miscellaneous (fluids, bone marrow, eye, stool, skin), 57 urine, and 58 blood specimens. Specimens were placed in 2 ml of sucrose-phosphate-glutamic acid-albumin (SPGA) viral transport medium (Carr Scarborough Microbiologicals, Decatur, Ga.); tissues were finely minced prior to transfer to SPGA. Heparinized blood was processed by standard methods to obtain leukocytes for virus culture (PMN-Polymorphprep; Robbins Scientific, Sunnyvale, Calif.) (26). DNA for PCR was extracted from whole blood as outlined below. A CMV isolate (VR-2, 1989) from the College of American Pathology (CAP) proficiency testing standards and human CMV strain AD-169 (ATCC VR-538) were selected as positive controls.

CC. Specimens in SPGA were thoroughly vortexed and inoculated (0.3) into one human foreskin fibroblast (HFF) tube (Bartels, Issaquah, Wash.) for CC. Separated leukocytes were resuspended in 2 ml of Eagle minimum essential medium (MEM) and inoculated (~0.8 ml) into two HFF tubes. The inoculum was allowed to adsorb for 2 h at 37°C and was then replaced with fresh MEM. Cultures were maintained at 37°C for 4 weeks and were observed for the development of a typical CMV CPE. CMV strain AD-169 and the CAP proficiency sample were also grown in HFFs as positive controls.

SVA. Each specimen (0.3 ml) was inoculated into one vial containing HFFs grown on 12-mm coverslips (Bartels) as described previously (16, 17). Shell vial monolayers were fixed with acetone at two days postinoculation, stained with monoclonal antibodies directed against an immediate-early protein (DuPont, Billerica, Mass.) (37) and an early protein (43 kDa; Dako, Carpinteria, Calif.) (46) by indirect immunofluorescence assay, and examined for nuclear staining.

Viral template control. CMV strain AD-169 was grown in HFF tube cultures until a CPE was observed in 50 to 75% of the infected monolayer. DNA was extracted by standard procedures (43) and was used in 1- to 5-μl volumes as a positive control template in amplification protocols. To assess PCR reproducibility, cell-free supernatant was obtained from HFFs inoculated with VR-2 1989 (CAP CMV), diluted with SPGA in 10-fold dilutions, and boiled for 15 min. Five microliters of each dilution was tested on four different dates, and on one date each dilution was tested twice for same-day results. An aliquot (0.3 ml) of each dilution was also inoculated for CC and SVA, one each, for comparison with PCR.

PCR. Two pairs of oligonucleotide primers for the MIE (MBIR designation, HS5MIE4) and LA (MBIR designation, HS5PPBC) gene regions of CMV and complementary oligonucleotide probes homologous to internal sequences of the amplified genes were obtained from Synthetic Genetics (San Diego, Calif.). The MIE and LA primers amplified 435- and 400-bp sequences, respectively, of CMV DNA; the amplifications were performed as described previously (9). Specimens in SPGA were placed in a boiling water bath for 15 min. DNA was extracted from heparinized whole blood by using the IsoQuick extraction kit (MicroProbe Corp., Garden Grove, Calif.) according to the manufacturer's recommendations. Briefly, 100 μl of whole blood was lysed with the lysis solution, and the manufacturer's extraction matrix was then added for isolation of template DNA. The DNA was subsequently precipitated by using isopropanol and 70% ethanol and was resuspended in 100 μl of RNase-free water. Urine specimens were prepared for PCR by filtration through Ultrafree MC Filter Units 100K (Millipore Corp., Bedford, Mass.) as described previously (22). Briefly, 200 μl of urine was filtered by centrifugation at 3,000 × *g* for 5 min. The retentate was rinsed once with sterile distilled H_2O (200 μl) by centrifugation at 3,000 × *g* for 5 min, resuspended in 200 μl of sterile distilled H_2O, and boiled for 15 min. Fifty microliters of filtered urine and 5 μl of blood DNA extract or specimen in SPGA were used as templates. PCR mixtures were prepared sequentially, as follows: sterile distilled H_2O, 10× reaction buffer as provided by the manufacturer (Perkin-Elmer, Norwalk, Conn.), deoxynucleoside triphosphate mixture (200 μM each), primers (0.5 μM), and template (5 to 50 μl of prepared specimen). *Taq* or AmpliTaq (2.5 U; Perkin-Elmer) was then added, and the reaction mixture was overlaid with 100 μl of mineral oil (Sigma, St. Louis, Mo.). The 10× reaction buffer consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.01% gelatin. Amplification was performed in a DNA thermal cycler (model 480; Perkin-Elmer) as follows. Samples were heated to 94°C for 5 min; this was followed by 40 cycles consisting of denaturation (2 min, 94°C), primer annealing (90 s, 65°C), and primer extension (90 s, 72°C), with a final extension of 10 min at 72°C. After the final cycle, reaction tubes were stored at 4°C.

Product detection. PCR product was detected by direct gel analysis and Southern transfer and hybridization. The amplified products (20 μl plus 5 μl of loading buffer) were electrophoresed through Mini-PROTEAN II Ready Gels (10% acrylamide in TBE buffer) (Bio-Rad, Richmond, Calif.) or 1% agarose gels. Gels were stained with ethidium bromide and visualized on a UV transilluminator. Each gel contained CMV (positive) and water (negative) controls. Controls and selected specimens exhibiting a range of reactivities were examined by Southern transfer and hybridization with a probe to the internal sequence to confirm product specificity. Oligonucleotide probes to MIE- and LA-amplified products were 3′ end-labeled with [^{32}P]dATP by using terminal deoxynucleotidyl transferase (GIBCO BRL, Gaithersburg, Md.). Ten microliters of PCR product was run in a 1 to 1.4% agarose gel and transferred to a GeneScreen nylon filter as described by the manufacturer (NEN DuPont, Boston, Mass.). The probe was hybridized overnight at 45°C by using the manufacturer's protocol; this was followed by three rinses with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), a wash with 2× SSC at 37°C for 15 min, and a wash with 2× SSC at 22°C for 15 min. Filters were dried and exposed to XAR film (Kodak, Rochester, N.Y.) overnight.

RESULTS

Specimens for PCR could be divided into three groups on the basis of the ease of template preparation. The first group included all specimens exclusive of urine and blood for which template preparation consisted of boiling in SPGA for 15 min. The second group included all urine specimens which were filtered to remove PCR-inhibiting substances and then boiled, and the third group included blood specimens which required nucleic acid extraction to concentrate the viral DNA as well as to remove amplification-inhibiting substances. Results from the three groups are shown in Table 1. Discrepancy analysis of specimens exclusive of urine and blood based on both primer pairs included two

TABLE 1. Comparison of cell culture and SVA with PCR for detection of CMV

PCR specimen, primer, and result	No. of specimens CC or SVA:		Sensitivity (%)	Specificity (%)	Agreement (%)
	Positive	Negative			
Specimens exclusive of urine and blood					
MIE primer			87.3 (69/79)	93.6 (44/47)	89.7 (113/126)
Positive	69	3			
Negative	10	44			
LA primer			92.4 (73/79)	87.2 (41/47)	90.5 (118/126)
Positive	73	6			
Negative	6	41			
Both primer pairs			97.5 (77/79)	87.2 (41/47)	93.7 (118/126)
Positive	77	6			
Negative	2	41			
Urine, both primer pairs			100 (10/10)	95.7 (45/47)	96.5 (55/57)
Positive	10	2			
Negative	0	45			
Blood, both primer pairs			66.7 (4/6)	78.8 (41/52)	77.6 (45/58)
Positive	4	11			
Negative	2	41			

samples which were PCR negative and CC or SVA positive. These samples generally appeared to contain lower virus concentrations, as indicated by the degree of CPE (usually less than three plaques) or number of stained cells in the SVA (one to two stained cells per coverslip) and length of time to CPE (≥21 days). The observed differences in results may therefore reflect sampling error associated with low virus titers. There were six PCR-positive and CC- and SVA-negative samples (two bile, two BAL, one tracheal suction, one throat). These specimens were all obtained from patients yielding positive cultures from other specimens (blood, urine, respiratory, tissue) collected at about the time that the PCR result was positive. Presuming that these samples are true positives and culture failures, the revised sensitivity and specificity of PCR were 97.6% (83 of 85 specimens) and 100%, respectively. CC or SVA detected 92.9% (79 of 85) of positive specimens. Sensitivity and specificity were unchanged when ethidium bromide-stained gel results were compared with Southern blot analysis results.

Agreement between PCR and CC and SVA results for urine specimens was 96.5% (55 of 57 specimens). The two PCR-positive and CC- and SVA-negative results are likely culture failures. Urine specimens collected from this patient within 1 to 3 days of the samples in question were CC or SVA positive. Discrepancy analysis of results obtained for blood specimens included two samples which were PCR negative and CC or SVA positive. In both cases, CC was negative and SVA yielded 2 and 10 CMV-infected cells per coverslip, respectively. There were 11 PCR-positive and CC- and SVA-negative results. CC- or SVA-positive blood specimens and, in some instances, BAL, tissue, and urine specimens were obtained from these patients at about the same time (range, same day to 2 weeks) that the PCR result was positive.

Specimens and CMV control (CAP, VR-2, 1989) diluted through 10^{-6} in SPGA gave reproducible amplifications over time. Results for the CMV control with both primer pairs agreed with results for CC and SVA at all dilutions, ranging from the strongest reactivity at 10^{-1} to the weakest reactivity at 10^{-6}. In addition, dilutions which had been stored frozen for 1 year gave almost identical results when retested. Twelve specimens from sites other than blood and urine and representing weak and strong positive PCR results as well as negative results were also tested for reproducibility. Specimens were tested on two or three dates. Again, reproducibility was comparable to that for the control dilutions, with those specimens producing a minimal CPE or positivity by SVA sometimes producing trace quantities of PCR product by visual inspection (comparable to PCR product in the control dilution of 10^{-6}). Specimens in SPGA could also be stored at −70°C either before or after boiling and still amplify product reproducibly.

DISCUSSION

The most common methods for the laboratory diagnosis of CMV infection include CC, SVA antigen detection, and direct detection in specimens. CC is considered the standard of comparison but may require from 4 to 6 weeks for detection of CPE and may detect only up to 90% of true-positive results (24). SVA antigen detection is used routinely as a supplement to culture to decrease the time to detection of a positive result to 1 to 2 days. The sensitivity of this method depends on a number of factors, including the specimen type, the number of vials inoculated, the type of antibodies used, the incubation time prior to staining, the type and age of cells, the concentration of virus in the specimen, potential toxicity of the specimen for the cell monolayer, and length of time cultures were maintained (2 versus 4 to 6 weeks) for comparison with SVA detection (16, 17, 24, 28, 29, 42). The sensitivity of SVA for detection of CMV in blood specimens ranges from 50 to 75%, while other sites yield reported sensitivities ranging from 75 to 100% compared with culture (16, 17, 24, 29). Direct detection of viral antigen and nucleic acid in specimens is rapid (<4 h), but it is usually limited to specific specimen types such as BAL, tissues, or leukocytes and has variable sensitivity

when compared with CC (15, 25, 27, 39, 40). We evaluated PCR as a means of rapidly detecting CMV in a much wider range of specimens than currently used for direct detection and the same range as those acceptable for culture.

The specimens evaluated were grouped by template preparation method, ranging from simplified to more complex. Specimens from all sites exclusive of urine and blood were placed in a viral transport medium (SPGA) and were boiled for 15 min for simplified template preparation. The sensitivity and specificity of PCR compared with CC and SVA were 97.6 and 100%, respectively. CC or SVA detected 92.9% of CMV-positive specimens, and in a comparison of CC with SVA, when both results were available, SVA detected 77.4% of CC-positive specimens. Our system detected more CMV-positive specimens than either CC or SVA and also yielded reproducible results at different times and dates and after extended periods of frozen storage.

Specimens in the second group included urine specimens which required additional filtration to remove PCR-interfering substances. In a preliminary assessment with urine for amplification without pretreatment and only the MIE primer set, sensitivity was 61% (28 of 46 specimens), specificity was 100% (16 of 16 specimens), and agreement with results of CC and SVA was 74.2% (46 of 62 specimens). These results were viewed as insufficient for clinical testing. The urea in urine has been shown to be inhibitory at a level of 50 mM (22). Since most adult urine specimens contain greater than 300 mM urea, unreliable amplification conditions are present. The additional filtering step resolved inhibition and, together with the use of both primer pairs, improved the sensitivity of PCR to 100% in this group.

The third group of specimens included heparinized blood which required an additional extraction step for the removal inhibitors and the purification of viral nucleic acid. A commercially available kit (IsoQuick) capable of yielding nucleic acid of a purity and yield comparable to those obtained by phenol-chloroform extraction but without the use of hazardous chemicals and requiring ~30 to 45 min to perform was used. The sensitivity and specificity of PCR compared with CC and SVA were 67 and 79%, respectively. The observed differences among PCR, CC, and SVA results are probably related to low virus concentrations in blood. Of the samples positive by CC or SVA, SVA alone detected 67% of CMV-positive specimens, while CC and SVA together detected 33% of CMV-positive specimens, indicating a significant sampling error if one test alone was performed. Similarly, two CMV-positive specimens not detected by PCR were also CC negative and yielded only 2 and 10 CMV-positive cells per SVA coverslip, respectively. Specimens (n = 11) positive by PCR alone were obtained from patients with previous or subsequent CMV-positive blood cultures. These findings are in agreement with those presented in published reports comparing PCR with CC, SVA, or antigenemia (11, 12, 14, 20, 44). PCR was positive earlier and remained positive longer than other CMV detection methods. Although detection of viral DNA in leukocytes in the absence of positive culture or antigenemia was not associated with clinical symptoms and did not necessarily correlate with the appearance of clinical disease, PCR did predict a risk of relapse. Detection of CMV DNA in serum, however, correlated with viremia and clinical disease in patients with primary CMV as well as reactivated infections (5). Future protocols with serum or plasma as the template may simplify specimen handling further and correlate more specifically with clinical disease. The utility of PCR in the detection of CMV viremia has been described for monitoring antiviral therapy, both for the early initiation of treatment as well as a predictor of the efficacy of treatment (11, 14, 44).

The issue of the presence of interfering substances remains when performing PCR on specimens obtained from various body sites and by different collection methods. Besides urea, other interfering substances that have been reported include hemoglobin (31) and heparin (3). Other substances that are suspected of causing interference but that are not yet well-documented interfering substances include mucus (23) and phosphates (34). In some cases, nesting primer sets have overcome interference (4). The presence of phosphates in the SPGA transport medium used in the present study did not seem to interfere significantly. Hemoglobin and heparin interference in blood specimens was avoided with a rapid modified DNA extraction system.

Separate reactions with two primer pairs were sensitive indicators of a positive CMV culture in the present study. The use of primer pairs to two separate genomic targets also avoided potential false-negative results because of strain variation (7). Coamplification of human β-globin as an internal control could also be added to assess the presence of adequate DNA and amplification conditions (2). Other eventual options include the development of special PCR collection kits containing appropriate lysing and clarifying agents and a multiplex system to avoid separate reaction tubes for the MIE and LA primer sets. The ideal system, however, would allow the use of current multiuse specimen collection systems for subsequent PCR analysis. We have shown that standard viral transport medium can be used for PCR analysis for CMV.

With an increase in the number of transplant recipients and human immunodeficiency virus-positive patients, timely diagnosis of CMV becomes increasingly important (32, 35). Also, the possibility of effective therapy with ganciclovir or foscarnet emphasizes the need for rapid laboratory diagnosis (8, 41). Although organ disease may not be predicted in patients with AIDS and transplant recipients by blood or urine culture positivity, the presence of infectious viral particles in other organ sites, specifically lower respiratory tract, ocular, or gastrointestinal lesion specimens, may be an indicator of CMV pathogenesis (38, 45). Rapid detection of CMV from these sites would have a significant impact on patient management through early treatment. The system described here for the rapid and minimal handling of specimens for the production of PCR template addresses these needs. Specimens exclusive of urine and blood, collected in SPGA, can be used for culture and PCR. Duplicate specimens for PCR detection of specific CMV-related organ disease are, therefore, not required. Detection of CMV in blood and urine by PCR can also be performed on the same specimens submitted for culture with relatively moderate modification of the PCR protocol. Rapid diagnosis of CMV by PCR will allow improved analysis of antiviral therapy as well as provide a new means of examining CMV pathogenesis in the immune deficient host.

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