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Detection of Cytomegalovirus in Urine from Newborns by Using Polymerase Chain Reaction DNA Amplification

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Polymerase chain reaction (PCR) amplification was used to detect cytomegalovirus (CMV) in tissue culture and in urine specimens from newborns. Synthetic oligonucleotide primer pairs were used to amplify DNA from the major immediate-early and the late antigen genes of CMV. Amplified products were detected by gel electrophoresis and by dot-blot hybridization with oligonucleotide probes. Using one or both of the primer pairs and associated probes, we found 46 different tissue culture isolates of CMV that were positive; no reaction products were detected when the same primers and probes were used to amplify other herpes family viruses or human genomic DNA. Urine samples from 44 congenitally infected infants were positive when tested with one or both primer pairs and probes. When compared with tissue culture, detection by gel electrophoresis provided a sensitivity of 93%, a specificity of 100%, and a predictive value of a positive result of 100%. Dot-blot analysis raised the sensitivity to 100%. We conclude that PCR amplification may be a valuable tool for diagnosing congenital CMV infection.

Approximately 1% of all newborns in the United States are congenitally infected with cytomegalovirus (CMV). It is the most common congenital viral infection in humans, and it is the leading infectious cause of mental retardation and nonhereditary sensorineural deafness [1]. A rapid, inexpensive, yet reliable test for congenital CMV infection is needed.

Tissue culture isolation of virus from urine in the first week of life remains the standard procedure for detecting congenital CMV infection. Alternative methods that have been evaluated to detect CMV in urine include electron microscopy to directly visualize particles of virus; ELISA to detect viral antigen; shell vial assays; and DNA hybridization analysis using both isotopic and nonisotopic, cloned CMV-DNA probes [2–6]. Serological methods for diagnosing congenital CMV infection, including CMV IgM antibody determination in cord blood, are insensitive [7]. In vitro DNA amplification via the polymerase chain reaction (PCR) is a promising, new technology that may be adapted to this clinical problem.

Among its many applications, PCR has been used in the prenatal diagnosis of genetic diseases such as hemophilia A, sickle cell anemia, β-thalassemia, phenylketonuria, and α-antitrypsin deficiency [8–12]. Published reports of the adaptation of PCR to infectious disease diagnosis have focused almost exclusively on the detection of viral agents, specifically human immunodeficiency virus in blood from infected adults and children, human T cell lymphotropic virus type I in lymphoma, and human papilloma virus (HPV) in tissue specimens [13–18]. We report the use of PCR to detect CMV in tissue culture and urine specimens. This report describes the suitability of two synthetic oligonucleotide primers and probes for detecting a variety of clinical isolates of CMV, as well as their practical application to the diagnosis of congenital CMV infection.

Materials and Methods

Tissue culture of urine specimens. As part of an ongoing study on the effects of CMV infection on mothers and their infants, newborns at The Woman's Hospital of Texas (Houston) were screened for congenital CMV infection by tissue culture of the urine. Urine specimens for isolating CMV were pretreated,

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inoculated, and maintained according to a standard tissue culture technique [19, 20]. CMV was identified by production of its characteristic CPE on human foreskin fibroblasts (HFF) and by failure to grow in the HEp-2 cell line. Since January of 1986, after being processed for tissue culture, urine specimens (0.5–3 mL) received in our laboratory were stored in glass vials at −20 C and used in the development of the PCR. Many specimens were repeatedly frozen and thawed. The strain of CMV isolated from positive urine specimens was briefly cultivated in vitro and cryopreserved at −70 C. These cryopreserved strains were not only from congenitally infected newborns but also from adults and children with acquired CMV infection. Strain AD169 of CMV used in initial experiments was obtained from the ATCC (Rockville, Md) and maintained in our laboratory.

Preparing samples for PCR. Tissue culture specimens of CMV in HFF were boiled for 10 min and then centrifuged briefly in a sealed microcentrifuge tube to remove the cellular debris. A 5-μL aliquot of the tissue culture supernatant was used in the DNA amplification reaction. Urine specimens were briefly centrifuged, and 1 μL of the supernatant was then amplified without any other preparation. Variable volumes of urine (0.5–5.0 μL) were tested during the development of the procedure.

Preparing oligonucleotide primers and probes. Two pairs of oligonucleotide primers were custom synthesized by Genetic Designs (Houston) in an Applied Biosystems DNA synthesizer (Foster City, Calif) [21, 22]. One pair, termed the LA primers, allowed amplification of a 400–base-pair sequence of CMV DNA that codes for a portion of a late antigen (LA) of CMV strain AD169 and contains a *Hin*dIII restriction endonuclease cleavage site. The other primer pair, termed the MIE primers, allowed amplification of a 435–base-pair sequence of CMV DNA that codes for a portion of the major immediate-early (MIE) antigen of CMV (Towne strain) and contains a *Ban*I cleavage site. The portion of the MIE gene that is amplified is known to be within exon 4 of this gene. Complementary oligonucleotide probes homologous to a central region of the amplified DNA sequences from both the MIE and LA of CMV were also synthesized by Genetic Designs (table 1). The synthetic oligonucleotide probes were 3′ end-labeled with ^{32}P by using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis).

DNA sequence amplification. Amplification of tissue culture samples (5 μL), urine samples from newborns (1 μL), or dilute urine samples used in inhibitor experiments was performed in a total volume of 100 μL. The reaction mixture consisted of 10 μL of a 10× concentrated reaction buffer containing 50 m*M* KCl, 10 m*M* Tris-Cl (pH 8.3), 1.5 m*M* $MgCl_2$, 0.01% gelatin, 2.4 μ*M* of each primer, 200 μ*M* of each of the four deoxynucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), water, and sample for a total volume of 100 μL. The reaction mixture was covered with 100 μL of mineral oil, and the tubes were placed in a boiling water bath for 7 min to destroy any enzymatic activity. After boiling, 0.5 mL (2.5 U) of *Thermus aquaticus* (Taq) polymerase was added. The amplification reaction was performed in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, Conn).

Each primer set was evaluated with regard to an-

Table 1. Sequence of synthetic oligonucleotide primers and complementary oligonucleotide probes and their locations in CMV genome.

Primer or probe*	Sequences (5′ → 3′)†	Length (bp)‡ of amplified product	Location of amplified product or probe§
Primer MIE-4	CCAAGCGGCCTCTGATAACCAAGCC	435	731–755
Primer MIE-5	CAGCACCATCCTCCTCTTCCTCTGG		1165–1150
Probe MIE	GAGGCTATTGTAGCCTACACTTTGG		900–925
Primer LA-1	CACCTGTCACCGCTGCTATATTTGC	400	2101–2125
Primer LA-6	CACCACGCAGCGGCCCTTGATGTTT		2500–2476
Probe LA	GTCGCCTGCACTGCCAGGTGCTTCG		2301–2325

* MIE = major immediate-early antigen region of CMV strain Towne (MBIR designation:HS5MIE4). LA = a late antigen region of CMV strain AD169 (MBIR designation:HS5PPBC).

† Patent pending on all sequences listed.

‡ bp = Base pairs.

§ Expressed as nucleotide number; nucleotides are numbered sequentially within each gene.

nealing temperature, time for primer elongation, and the number of cycles required to achieve maximum sensitivity, i.e., the highest signal-to-noise ratio when the products were evaluated by direct staining of an acrylamide gel. Annealing temperatures ranging from 55 to 65 C produced equal amplification with both primer sets. The higher annealing temperatures, however, decreased nonspecific amplification. Increasing the number of cycles from 30 to 40 and increasing the primer elongation time with a 10-s extension per cycle improved the relative sensitivity. Beyond 40 cycles, however, we found that increases in extension time, cycle number, or both produced significant undesirable nonspecific amplification. The reaction conditions that follow were found to be optimal. The samples were heated to 94 C for 120 s to denature the DNA, cooled to 65 C for 90 s to allow the primers and the DNA to reanneal, and then heated to 72 C for 60 s for primer extension. Each extension period was increased by 10 s on each subsequent cycle so that by the final cycle, the extension period was 7.5 min. A total of 40 cycles were performed in 6.5 h.

Detecting amplified product. The amplified product was detected by direct gel analysis and by dot-blot hybridization assay using oligonucleotide probes. For direct gel analysis, 5 μL of the reaction mixture was subjected to electrophoresis on 5% polyacrylamide minigels, and the DNA was visualized by UV fluorescence after staining with ethidium bromide. Molecular weight markers were included in each gel. A 400- and 435-base-pair band was seen when samples were amplified using the LA primers and the MIE primers, respectively. Specific amplification on direct gel was confirmed by restriction enzyme digestion of the amplified bands with *Hin*dIII (LA primers) or *Ban*I (MIE primers). For the restriction enzyme digestions, 8 μL of the PCR reaction mixture was mixed with 1 μL of enzyme-specific buffer preparation and 1 μL of either *Hin*dIII (12 units) or *Ban*I (12 units) and incubated for 2 h at 37 C and 50 C, respectively. Restriction enzyme digests were analyzed by electrophoresis on 5% polyacrylamide minigels, and the bands were visualized with UV fluorescence after staining with ethidium bromide.

For dot-blot analysis, 10 μL of the reaction mixture was mixed with 190 μL of 2 *M* sodium chloride and then denatured by adding 10 μL of 10 *M* sodium hydroxide for 15 min. The sample was then neutralized by adding 100 μL of 3 *M* sodium acetate (pH 5.5). The DNA was immediately applied to a nylon filter membrane (Schleicher & Schuell, Keene, NH) by vacuum filtration, and the filters were then dried by heating in a vacuum oven at 80 C for 1 h. The DNA samples were then hybridized with a homologous ^{32}P-labeled synthetic oligonucleotide probe in a hybridization solution containing 1% SDS and 6 × SSPE (0.9 *M* NaCl, 60 m*M* NaH_2PO_4 [pH 7.4], and 6 m*M* Na_2EDTA) for 18 h. Three washes were performed in 6 × SSPE and 1% SDS for 10 min, each at ambient temperature. A final wash was performed in 1 × SSPE and 1% SDS for 3 min at 68 C for the samples amplified with the LA primers and at 64 for the samples amplified with the MIE primers. Bound probe was detected by autoradiography at −70 C for 1, 4, and 18 h.

Results

Determining the sensitivity of PCR. To estimate the sensitivity of PCR relative to tissue culture, we prepared dilutions of fibroblasts infected with CMV strain AD169, with an infectivity titer of $10^{5.3}$ $TCID_{50}$/0.1 mL, and assayed these dilutions by PCR. A 5-μL aliquot of a 10^{-3} dilution of the tissue culture mixture was detected by direct gel analysis; dot-blot hybridization was positive at a 10^{-4} dilution of the same culture.

Determining specificity. The specificity of the probes and primers was evaluated using other members of the herpes family of viruses. Tissue culture samples with advanced CPE of low-passage clinical isolates of varicella-zoster virus (VZV; strain SC363) and herpes simplex virus (HSV; strain SC308) were boiled for 10 min and then centrifuged briefly in a sealed microcentrifuge tube to remove cellular debris. A 5-μL aliquot of the tissue culture supernatant was used in the PCR reaction. Two Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines, LA350 [23] and HCF6 (provided by Dr. Arthur Beaudet, Baylor College of Medicine, Houston), were used. We confirmed the presence of EBV DNA in these cell lines by using Southern blot analysis with a cloned probe (EBV FF41; *Eco*RI B fragment; provided by Dr. George Miller, Yale University, New Haven, Conn). A 0.5-μg aliquot of purified EBV DNA was added to the PCR reaction. In addition, human genomic DNA (0.5-μg aliquot) extracted from the white blood cells of a healthy CMV-seronegative donor was evaluated. When these preparations of VZV, HSV, EBV, or human genomic DNA

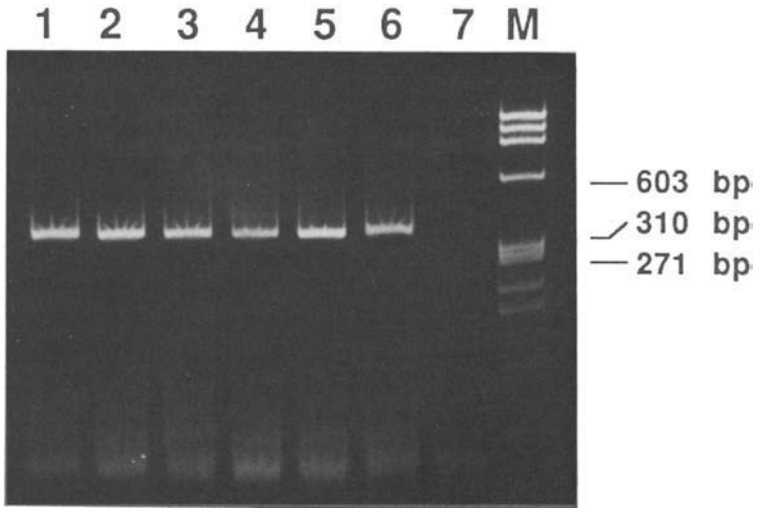

Figure 1. Dilution experiments illustrating that the inhibitory effect of urine on the PCR detection of CMV strain AD169. *Lane M*, molecular weight markers; *lane 1*, positive control: CMV strain AD169 amplified with LA primer set. *Lanes 2, 3,* and *4*, amplified bands from a 10%, 30%, and 90% dilution of urine (B) that does not inhibit the PCR reaction. *Lanes 5*, *6*, and *7*, results from the same dilutions of urine (A) that does inhibit PCR reaction. Significant inhibition was noted in the sample containing 90% urine by volume, as compared with the higher dilutions of the same urine sample.

were used as templates in the PCR reaction, no amplification was noted by direct gel analysis or by dot-blot hybridization.

Evaluation for polymerase inhibitors. Because urine is notorious for containing enzyme inhibitors, a series of experiments was performed to determine whether cultured CMV added to a variety of urines could be as readily amplified as CMV in culture medium. Initial studies using urine samples from laboratory personnel and culture-negative urine samples from newborns showed a variable degree of inhibition of amplification of cultured CMV added to these urine specimens. Of 15 urine samples containing equivalent quantities of CMV strain AD169, four were negative for amplification when examined by direct gel. Subsequently, one urine sample (A) known to cause inhibition and one urine sample (B) that did not cause inhibition were used to determine whether the inhibitor could be diluted to a point where it had little or no effect. The urine samples were diluted by adding deionized water to give solutions of 90%, 30%, and 10% urine by volume. To each dilution we added an equivalent amount (10% by volume) of cultured CMV before amplification (figure 1). A 5-μL aliquot of the dilute urine preparations was added to the PCR reaction. No difference in amplification was noted with the various dilutions of urine (B), whereas with the sample containing 90% urine (A), almost complete inhibition was observed.

Of the first 10 clinical urine specimens tested from congenitally infected newborns, three were initially negative by direct gel when 5-μL and 2-μL aliquots of the urine were used in the PCR. Use of a smaller volume of urine (1 μL) reduced, however, the inhibitory effect (figure 2, lanes 1–3 and 4–6) while retaining sufficient sensitivity for direct gel detection (figure 2, lanes 7–9).

Amplification of different CMV isolates. We evaluated the ability of the two primer sets to detect 46 different clinical isolates of CMV (29 from congenitally infected infants, 12 from children with acquired CMV infection, and 5 from women of childbearing age who experienced a primary CMV infection) collected over 2.5 y. All 46 clinical isolates were positive by direct gel with either the LA or the MIE primers (table 2). Nine (19.6%) of the 46 isolates tested had some variation when direct gel, with or without restriction enzyme analysis, and dot blot were compared for detection of the amplified product. The LA gene fragment was more frequently associated with a negative result (8 [17.4%] of 46) than was the MIE gene fragment (3 [6.5%] of 46; table 3). Using the LA primers, we found that four of the

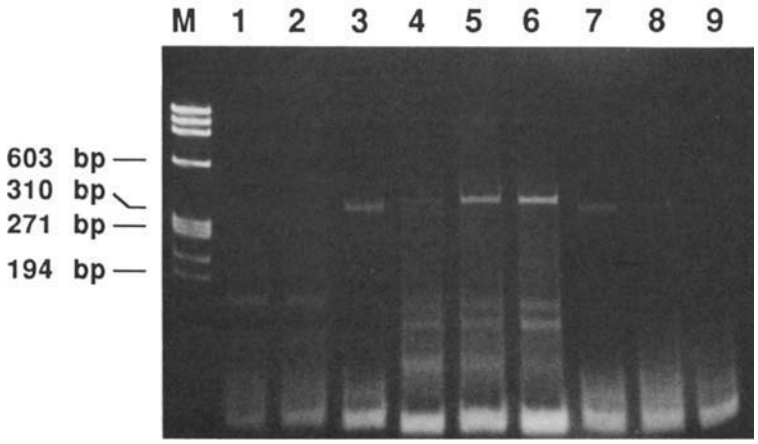

Figure 2. Effect of reduction of urine sample volume in PCR reaction mixture (from 5 μL to 1 μL) on the ability of PCR to detect CMV in the specimen. *Lane M*, molecular weight markers. Bands of 400 base-pairs (bp) and 435 bp show detection of product amplified by LA primers and MIE primers, respectively. *Lanes 1, 2,* and *3*, results of PCR reaction, using the LA primers, containing 5 μL, 2 μL, and 1 μL, respectively, of urine from congenitally infected newborns. *Lanes 4, 5,* and *6*, results of PCR reaction containing 5 μL, 2 μL, and 1 μL, respectively, of urine, from the same newborn, reacted with MIE primers. *Lanes 7, 8,* and *9*, reaction with LA primers using 5 μL, 2 μL, and 1 μL of urine, respectively, from another newborn; the smaller volume of urine does not decrease the ability of PCR to detect CMV in the specimen by direct gel analysis.

isolates (SC400, SC120, SC246, and 6351) had no amplified product detected by either direct gel or dot-blot analysis, yet these four isolates were detected with the MIE primers. In addition, three other isolates (FS1A, 42837, and 42829), when amplified with the LA primers, were detected by direct gel analysis followed by confirmatory restriction enzyme analysis but were not detected by dot blot. Using the MIE gene fragment, we found that three isolates (SC232, SC246, 6351) did not produce restriction enzyme digests on direct gel, yet two of the isolates were detected by dot blot and the third was detected by the LA primer.

Detecting CMV directly in urine specimens from newborns. The ability of the MIE and LA oligonucleotide primer pairs to detect CMV in 71 urine specimens from newborns (44 of the specimens were from congenitally infected newborns and were culture positive for CMV) was evaluated and compared with standard tissue culture (table 2). A comparison of direct gel and dot-blot analysis using both LA and MIE primer sets showed the combination of the MIE primer set (alone or with the LA primer set) and dot-blot hybridization was able to detect CMV in the urine of all 44 congenitally infected newborns. Although most specimens were detected by

Table 2. Comparison of PCR amplification to detect CMV by using MIE, LA, or both primer sets and detection by either direct gel analysis or dot-blot hybridization.

	No. of samples positive by PCR amplification*					
	LA primers only		MIE primers only		Both primers	
Detection of CMV by tissue culture	Dot blot	Gel	Dot blot	Gel	Dot blot	Gel
Clinical isolates (n = 46)	38	42	45	46	46	46
Urine specimens from newborns						
CMV isolated (n = 44)	43	41	44	41	44	41
CMV not isolated (n = 27)	0	0	0	0	0	0

* MIE = major immediate-early antigen; LA = a late antigen.

Table 3. Summary of discrepancies between PCR results when direct gel alone, direct gel with restriction enzyme digestion, and dot-blot hybridization were used to detect amplified product.

	PCR results					
	MIE primers			LA primers		
CMV isolate	Gel	Gel/RE†	Dot blot	Gel	Gel/RE†	Dot blot
SC232	+,+	−,−	−,−	+,+	+	+
FS1A	+	+	+	+,+,+	+,+	−,−
42837	+	+	+	+,+,+	+,+	−,−
42829	+	+	+	+,+	+,+	−,−
42626	+	+	+	+,+	+	−
SC400	+	+	+	−,−,−	ND	−
SC120	+	+	+	−,−,−	ND	−
SC246	+	−	+	−,−,−	ND	−
6351	+	−	+	−,−,−	ND	−

NOTE. MIE = major immediate-early antigen, LA = major late antigen, and ND = not done; restriction enzyme digests were not done when direct gel analysis was negative.

* + = Amplified product detected in each separate experiment; − = no amplified product detected in each separate experiment.

† Restriction enzyme (RE) digestion using *Ban*I and *Hin*dIII to digest product amplified by MIE and LA primers, respectively.

both primers, CMV in one urine specimen (6023C) was not detected by PCR using LA primers with either direct gel or dot blot. In no instance was amplified product detected by direct gel or dot blot in the urine of newborns whose culture for virus was negative (figure 3). Using both the LA and MIE primer sets and direct gel analysis to detect the amplified DNA product, the overall sensitivity of the amplification procedure when compared with tissue culture was 93%; specificity, 100%; predictive value of a positive test, 100%; and predictive value of a negative test, 90%. Use of dot-blot assay to detect the amplified CMV-DNA sequences increased the overall sensitivity to 100% and raised the predictive value of a negative test to 100%, while keeping the specificity and predictive value of a positive test at 100%.

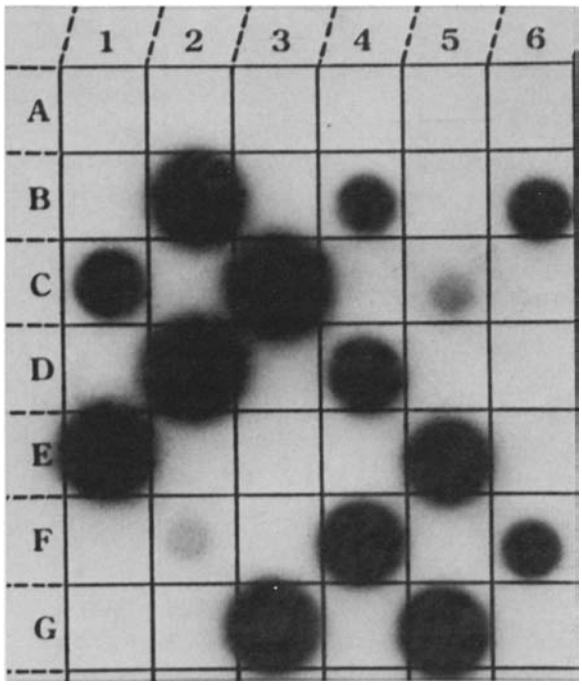

Figure 3. Detection of CMV by PCR amplification using LA primer set and detection by dot-blot hybridization with complementary ^{32}P-labeled oligonucleotide probe. Positive control of CMV strain AD169 in tissue culture is shown in *dots E1* and *G5*. *Dots E5, F2, F4, F6,* and *G3* represent five different clinical isolates of CMV detected by dot blot. *Dot G1* was also a clinical isolate not detected using the LA primer set but was detected using the MIE primer set (data not shown). *Dots A2, A4, A6, B1, B3, B5, C2, C4, C6, D1, D3, D5, E2, E4, E6, F1,* and *F3* are negative urine samples, and *dots B2, B4, B6, C1, C3, C5, D2,* and *D4* are positive urine samples.

Discussion

The choice of primers for amplification of viral DNA for clinical application requires the use of genomic sequences that are highly conserved from one generation of virus to the next. Therefore, we chose two genes from the CMV genome that are necessary for viral replication – the major immediate antigen and the late antigen, a phosphorylated matrix protein. Mutations in either of these proteins should adversely affect survival of virus. Because the mutation rate of wild-type CMV is unknown, the only way to determine the validity of our sequence selection was to test a number of clinical isolates collected over an extended period of time. This information is par-

ticularly important because the oligonucleotide sequences were derived from the sequences published for high-passage, laboratory strains of CMV.

Although all 46 isolates collected over a 2.5-y period from a variety of patients were detected by PCR, both MIE and LA primers and probes were required to detect all isolates. Nine isolates showed variable results when direct gel, with or without restriction enzyme analysis, and dot blot were compared for detection of the amplified product. Because these differences were found repeatedly in the same isolates, they most likely are not due to laboratory artifacts. Rather, they may represent a minor mutation within the targeted regions of interest in these CMV strains that was sufficient to destabilize probe hybridization or, alternatively, alter restriction enzyme analysis, but these differences were too insignificant to affect protein function and virus survival. Because the restriction enzyme sites are within 10–20 base-pairs of the sequence that is homologous with the oligonucleotide probe, one alteration may theoretically affect both restriction enzyme and dot-blot analysis, yet not abrogate the amplification and detection of a band of appropriate size, as was seen with isolate SC232. Direct sequence analysis of the amplified products of these nine CMV isolates that gave discrepant results is required to confirm our hypothesis and to define the extent of any mutation found. That mutations may cause false-negative results with PCR may be a potential limitation of the technique. Multiple primers that amplify different areas of the CMV genome, combined with different systems of detecting the amplified product similar to our approach, may be necessary to avoid false-negative results.

That CMV DNA can be detected in urine samples without prior isolation and purification of the DNA is a significant improvement over standard cloned-probe detection [5, 6]. Even though inhibitors apparently were present in some urine samples at sufficient concentration to interfere with the amplification of template DNA, simply reducing the quantity of urine in the PCR reaction eliminated the problem for all the samples tested in this series. This urine volume reduction possibly caused the apparent decreased sensitivity of direct gel compared with dot-blot analysis (table 3). Nevertheless, all urine specimens were positive by dot blot when MIE primers and probe were used for amplification and detection.

Further evidence that the PCR was sufficiently sensitive for the diagnosis of congenital CMV infection was shown when PCR detected CMV in a 10^{-4} dilution of a tissue culture suspension that had an infectivity titer of $10^{5.3}$ $TCID_{50}$/0.1 mL. The urine of congenitally infected infants has been shown to contain infectivity titers of $10^{4.3}$ to $10^{5.3}$ $TCID_{50}$/0.2 mL [24]. On the basis of these dilution studies, the PCR is theoretically capable of detecting CMV in urine samples containing infectivity titers between $10^{1.3}$ and $10^{2.3}$ $TCID_{50}$/0.1 mL.

The PCR for DNA amplification of CMV in urine offers several advantages over standard tissue culture. A very minute amount of urine (1 μL) was required for analysis. Also, the amplification reaction and detection procedures could be performed in 24–48 h, compared with a time of two to 28 days required for tissue culture isolation of CMV from congenitally infected newborns. In addition, infectious virus is not necessary for a positive result by PCR. Many of the urine specimens had been stored over two years and had undergone repeated freeze-thaw cycles. Despite these conditions, which may cause degradation of DNA, sufficient CMV-DNA template was present in the virus for PCR amplification. In fact, studies in this laboratory and others have shown that PCR can amplify CMV and human papilloma virus DNA from formalin-fixed, paraffin-embedded tissue specimens that contained degraded, cross-linked DNA template [15]. The PCR offered an advantage over standard isotopic and nonisotopic DNA hybridization assays because extensive DNA purification measures were not necessary to produce amplified DNA. In fact, the urine specimens were added directly to the PCR reaction mixture without any special sample preparation.

We chose newborns as the first clinical population for application of PCR DNA amplification for the diagnosis of CMV infection for several reasons. First, the detection of CMV in the urine of a newborn within the first week of life is diagnostic of congenital infection. Second, congenitally infected newborns usually have very large titers of virus in their urine, a fact thereby making it much more likely that CMV DNA would be detected in their specimens [23]. Third, newborns identified as congenitally infected benefit from special developmental follow-up and early diagnosis of hearing problems. Rapid diagnosis will be even more imperative once antiviral therapy for CMV infections is available. The successful use of PCR DNA amplification procedures to detect CMV in the urine of congenitally infected

newborns may lead to the use of this technique to diagnose a variety of other CMV syndromes.

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