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Polymerase Chain Reaction Assay for Detection of Human Cytomegalovirus

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Direct detection of human cytomegalovirus (HCMV) from clinical specimens was examined by using the polymerase chain reaction (PCR) for amplifying HCMV DNA. The efficiency of the amplification reaction was examined by using three different buffers and concentrations of deoxynucleotide triphosphates. The PCR assay was most efficient with a reaction mixture containing 17 mM ammonium sulfate, 67 mM Tris hydrochloride (pH 8.5), 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 170 μg of bovine serum albumin per ml, and each deoxynucleotide triphosphate at a final concentration of 1.5 mM. After 35 cycles of amplification, 0.15 fg of a plasmid containing the cloned target gene (corresponding to approximately six gene copies) was detected. The PCR assay correctly identified all of 24 clinical isolates of HCMV. Virus in urine specimens could be disrupted by heating at 93°C for 30 min. The viral DNA was amplified directly from 5 μl of preheated urine, with no further treatment before amplification. We tested the PCR assay on urine specimens from patients who had undergone renal transplantation that had been screened for the presence of HCMV by enzyme-linked immunosorbent assay, hybridization assay, and direct virus isolation. Specimens that were positive by one or more of these assays were screened by PCR. HCMV was consistently detected by PCR in all specimens that were positive by at least one other test. No cross-reactivity to other herpesviruses or MRC-5 cellular DNA was observed.

Human cytomegalovirus (HCMV) usually causes mild or asymptomatic infections in healthy individuals (8, 18). However, severe infections occur among congenitally infected infants (29, 32, 39) and immunocompromised patients, including those with acquired immunodeficiency syndrome (9, 13, 33, 37). Diagnosis of HCMV infection is clinically difficult and usually depends on the isolation of virus by tissue culture methods. Since antiviral therapy is available for severe HCMV infections, rapid methods to identify infected patients early are needed so that therapy can be instituted and monitored (1, 5). Efforts have been made to replace time-consuming tissue culture methods for detection of HCMV with more rapid yet reliable methods. However, none of the alternative methods, which include detection of viral antigens by enzyme-linked immunosorbent assay (ELISA) (11, 23, 30, 40) and shell vial assay coupled to immunofluorescence assay (14, 15) and detection of viral nucleic acid by DNA hybridization (4, 26, 38), have proven effective for detection of HCMV directly from clinical specimens.

Saiki et al. (34) have described a system for amplifying the concentration of specific nucleic acid sequences as much as 10^6-fold. The polymerase chain reaction (PCR) has been used successfully to detect human immunodeficiency virus type 1 (16, 19, 21, 28), human T-cell lymphotrophic virus type I (10), hepatitis B virus (20), human papillomavirus (35), and toxigenic *Escherichia coli* (24, 25). Here we describe the development of a PCR assay for direct detection of HCMV from urine specimens. The PCR assay was specific for HCMV and, on the basis of calculations with cloned plasmid DNA, could detect as few as six viral particles. When applied directly to clinical specimens, the PCR assay detected HCMV more reliably than did viral isolation in tissue culture, ELISA, or a DNA hybridization assay using a biotinylated probe. The assay was rapid, with final results being achieved in 5 h. The PCR assay should greatly facilitate the diagnosis and treatment of HCMV-mediated disease.

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MATERIALS AND METHODS

Clinical specimens and characterization. Pre- and post-transplant urine specimens were collected from renal transplant patients. Urine samples were clarified by centrifugation at 700 × *g* for 10 min. For virus isolation, urine specimens were passed through a 0.45-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.) before inoculation onto human MRC-5 cells. The infected cultures were maintained in Eagle minimal essential medium supplemented with 1% fetal bovine serum for 6 weeks and observed for cytopathic effects due to HCMV. Infected cell lysates from 24 patients were collected and stored at −70°C before PCR assay.

Isolation of plasmid DNA. The 24.6-kilobase plasmid pAT153-HCMV-*Hin*dIII-E contained the HCMV AD169 *Hin*dIII E DNA fragment (27), which contains the HCMV immediate-early gene. Plasmid pAT153-HCMV-*Hin*dIII-O contained the HCMV AD169 *Hin*dIII O fragment and was used as a probe for detection of HCMV by hybridization assay (26).

Bacteria containing the plasmid were grown as described by Maniatis et al. (22). The bacteria were harvested by centrifugation, and plasmid DNA was extracted by the alkaline method (2). Plasmid DNA was purified by centrifugation to equilibrium on cesium chloride-ethidium bromide gradients (22).

Hybridization probe assay. The pAT153-HCMV-*Hin*dIII-O fragment was labeled with biotin-19-dUTP by the oligolabel-

ing method (12). The hybridization assay for detection of HCMV has been previously described (26).

ELISA for HCMV. The ELISA procedure and reagents used were as previously described (11, 26, 40). The virus was detected by using a monoclonal antibody directed against the HCMV DNA polymerase, which is synthesized early during HCMV infection (40).

Oligonucleotide primers. The primers used in the PCR assay were based on a conserved region of the sequence of the HCMV major immediate-early gene (3). The primers were purchased from Collaborative Research, Inc. (Waltham, Mass.). The HCMV sequences were amplified by using primer P_1 (5'-dGCAGAGCTCGTTTAGTGAACC-3' [nucleotides −21 to −2]) and either P_2 (5'-dCCGTTCCCG GCCGCGGAGGC-3' [nucleotides 66 to 85]) or P_3 (5'-dGGC ACGGGGAATCCGCGTTCC-3' [nucleotides 82 to 102]).

PCR. For amplification, 10 μl of pAT153-HCMV-*Hin*dIII-E plasmid DNA (100 ng), 5 μl of virus stock solution containing 50 50% tissue culture infective doses ($TCID_{50}$) of HCMV, or 5 μl of urine was used in the reaction mixture. This sample was combined with 10 μl of 60 mM nucleotide stock solution (15 mM each deoxynucleotide triphosphate [dNTP]), 20 mM nucleotide stock solution (5 mM each dNTP), or 8 mM nucleotide stock solution (2 mM each dNTP). Three buffers were tested. Buffer A consisted of 500 mM KCl, 100 mM Tris hydrochloride (pH 8.5), 25 mM $MgCl_2$, and 0.1% gelatin. Buffer B consisted of 500 mM KCl, 100 mM Tris hydrochloride (pH 8.5), 70 mM $MgCl_2$, and 0.1% gelatin. Buffer C contained 170 mM ammonium sulfate, 670 mM Tris hydrochloride (pH 8.5), 70 mM $MgCl_2$, 100 mM 2-mercaptoethanol, and 1.7 mg of bovine serum albumin per ml. Each reaction mixture contained 10 μl of buffer A, B, or C. In addition, each reaction mixture contained 3 μl of each primer (30 ng) and 2.5 U of *Thermus aquaticus* DNA polymerase in a total volume of 100 μl. Before amplification, samples containing whole virus or urine were combined with 10 μl of buffer C and 65 μl of H_2O, covered with 100 μl of mineral oil, and heated at 93°C for 30 min. The samples were cooled and used directly for the PCR. Samples containing plasmid DNA were not preheated but were amplified directly. Amplification cycles consisted of heating at 93°C for 2 min followed by cooling at 50°C for 2 min. The heating and cooling cycles were carried out 30 to 35 times, using two water baths. An additional unit of enzyme was added after the first 10 cycles were completed. After the last extension, 10 μl was removed from each reaction and subjected to electrophoresis on a 3% Nusieve–1% HGT agarose gel (FMC Corp., Philadelphia, Pa.). The products of the PCR were visualized by staining the gels with ethidium bromide (10 μg/ml).

RESULTS

PCR conditions. The sizes of the PCR products resulting from amplification with each primer set are shown in Fig. 1. The PCR primers cover a region either 106 (P_1-P_2) or 133 (P_1-P_3) base pairs in length. The reaction was carried out in buffer B containing 1.5 mM each dNTP. Several reaction buffers have been reported in the literature for use in the PCR assay (16, 21, 34). We tested the *T. aquaticus* DNA polymerase from one manufacturer with three different buffers and dNTP concentrations. The PCR assay was very inefficient with buffer A containing 2.5 mM Mg^{2+}, regardless of the concentration of the dNTPs (Fig. 2, lanes 7 through 9). Raising the Mg^{2+} concentration to 7 mM and using a final concentration of 1.5 mM each dNTP resulted in a weak

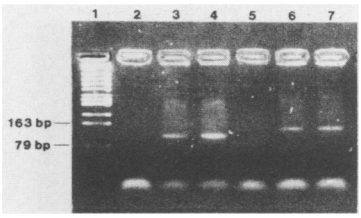

FIG. 1. Products of the PCR assay after amplification of cloned target DNA. The plasmid pAT153-HCMV-*Hin*dIII-E (10 ng), containing the immediate-early gene of HCMV AD169, was subjected to 30 cycles of amplification in buffer B with a final concentration of 1.5 mM each dNTP. Primer pair P_1-P_2 or P_1-P_3 was used. Lanes: 1, marker DNA fragments (sizes [in base pairs] are shown on the left); 2, unamplified reaction mixture of P_1-P_2; 3 and 4, PCR products of P_1-P_2; 5, unamplified reaction mixture of P_1-P_3; 6 and 7, PCR products of P_1-P_3.

signal after 30 cycles of amplification (Fig. 1, lanes 3, 4, 6, and 7; Fig. 2, lane 3). Significantly better amplification, however, was achieved with buffer C (Fig. 2, lanes 4 through 6). The reaction with buffer C was most efficient when the dNTP concentration was 1.5 mM each, but even a final concentration of 500 μM each dNTP resulted in a strong signal.

Sensitivity and specificity of the PCR assay. Because of the high ratio of noninfectious to infectious particles in any given preparation of HCMV (6, 31), it was difficult to calculate the number of genome equivalents that could be detected by the PCR assay. Therefore, we determined the sensitivity of the PCR assay on a plasmid of known molecular length. The plasmid pAT153-HCMV-*Hin*dIII-E, which contains the major immediate-early gene of HCMV, was serially diluted and subjected to 35 cycles of amplification. The amplification was done by using buffer C and a final concentration of 1.5 mM each dNTP. Under these conditions, as little as 0.15 fg of plasmid DNA was amplified and detected (Fig. 3). On the basis of a molecular length of 24.6 kilobases for pAT153-HCMV-*Hin*dIII-E, this result corresponds to detection of six genome equivalents of target DNA.

The specificity of the assay was examined by amplifying DNA from a number of other herpesviruses as well as from MRC-5 cellular DNA. The PCR assay did not cross-react with herpes simplex virus type 1 or 2, varicella-zoster virus, Epstein-Barr virus, or uninfected MRC-5 cellular DNA (Fig. 4). However, the PCR assay was positive when tested on HCMV-infected MRC-5 cells (Fig. 4). Twenty-four clinical isolates of HCMV as well as the Towne strain were tested by

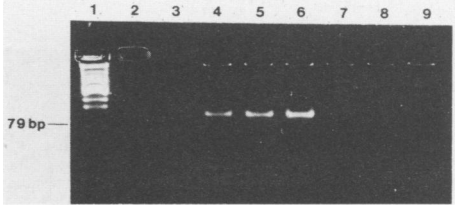

FIG. 2. Effects of buffer and dNTP concentration on PCR efficiency. Plasmid DNA (10 ng) was amplified for 30 cycles, using primer pair P_1-P_3. Lanes: 1, marker DNA fragments (size [in base pairs] is shown on the left); 2, buffer B and 500 μM each dNTP; 3, buffer B and 1.5 mM each dNTP; 4, buffer C and 200 μM each dNTP; 5, buffer C and 500 μM each dNTP; 6, buffer C and 1.5 mM each dNTP; 7, buffer A and 200 μM each dNTP; 8, buffer A and 500 μM each dNTP; 9, buffer A and 1.5 mM each dNTP.

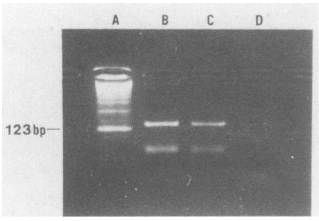

FIG. 3. Detection of serially diluted target plasmid DNA. Serial dilutions of pAT153-HCMV-*Hin*dIII-E DNA were amplified for 35 cycles, using primer pair P_1-P_3. Lanes: A, marker DNA fragments (size [in base pairs] is shown on the left); B, 1.5 fg of target DNA; C, 0.15 fg of target DNA; D, 0.015 fg of target DNA.

PCR. All 25 strains of virus were detected by using either the P_1-P_2 or P_1-P_3 primer pair.

PCR detection of HCMV in urine specimens. Urine specimens from six renal transplant recipients which had been previously characterized as positive for the presence of HCMV by either ELISA, hybridization assay with a biotin-labeled probe, or direct isolation (26) were tested for the presence of HCMV by the PCR assay. Pretransplant specimens from each patient were also tested. Pretransplant urine specimens from all patients were negative for the presence of HCMV by all four techniques (Table 1). In addition, 12 specimens from volunteers who had no history of HCMV infection and had not undergone renal transplantation were also negative for the presence of HCMV (data not shown). The PCR products indicating the presence of virus in some of the HCMV-positive urine specimens are shown in Fig. 5 and summarized in Table 1. The PCR primer pairs P_1-P_2 and P_1-P_3 gave equivalent results (data not shown). The PCR assay detected the presence of HCMV in all specimens that were positive by at least one other assay technique.

DISCUSSION

The PCR primers used in this study were chosen to correspond to a conserved region of the major immediate-early region of the HCMV genome. Because of the short length of the region amplified, we found it unnecessary to use three different reaction temperatures for denaturation, annealing, and polymerization. Instead, amplification was performed at two temperatures, 50 and 93°C. In addition, virus present in urine could be disrupted and amplified directly, without further purification. These factors significantly shortened the time to complete the assay.

The *T. aquaticus* DNA polymerase appeared to be significantly affected by the concentrations of Mg^{2+}, dNTPs, and the salt used. The conditions described here work well for amplification of viral as well as bacterial and cellular genes (unpublished data). It should be noted, however, that our observations concerning enzyme activity may be specific to the particular enzyme source used.

TABLE 1. Detection of HCMV in urine specimens from renal transplant recipients

Specimen	Patient	Detection by:			
		ELISA	Probe	Isolation	PCR
1[a]	A	−	−	−	−
2	A	+	−	+	+
3	A	−	+	+	+
4	A	+	+	−	+
5	A	−	+	−	+
6[a]	B	−	−	−	−
7	B	+	−	−	+
8	B	+	+	−	+
9	B	+	+	+	+
10[a]	C	−	−	−	−
11	C	−	+	−	+
12	C	+	+	+	+
13[a]	D	−	−	−	−
14	D	+	+	−	+
15	D	−	+	+	+
16	D	+	+	−	+
17	D	−	−	+	+
18[a]	E	−	−	−	−
19	E	−	+	+	+
20[a]	F	−	−	−	−
21	F	+	−	−	+
22	F	+	−	+	+
23	F	+	+	+	+
24	F	+	+	−	+

[a] Pretransplant urine specimen.

The ELISA used in this study has a reported lower limit of detection of $10^{1.7}$ $TCID_{50}$ (11), whereas the lower limit of the hybridization assay is 10^3 $TCID_{50}$ of virus (26). One $TCID_{50}$ of HCMV represents approximately 10^4 virus particles (6, 31). Similar to the findings of Shibata et al. (36), the PCR assay described here had a lower detection limit of six gene copies, which indicates that it is by far the most sensitive assay for HCMV detection. The assay was specific and did not cross-react with other herpesviruses or uninfected human cellular DNA yet detected 24 clinical isolates of HCMV previously recovered from renal transplant patients. Demmler et al. (7) and Shibata et al. (36) have recently described PCR assays for detection of HCMV in the urine of infected neonates and the blood of patients with acquired immunodeficiency syndrome, respectively. Both reported that a single primer pair was not sufficient to detect all clinically isolated HCMV. Although the sensitivity of our assay was

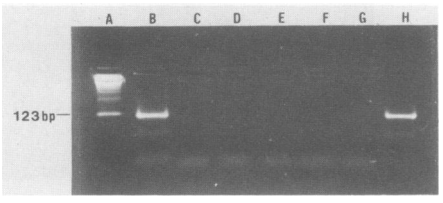

FIG. 4. Specificity of the PCR assay. Nucleic acids from HCMV and other herpesviruses were subjected to 30 cycles of amplification, using primer pair P_1-P_2. Lanes: A, marker DNA fragments (size [in base pairs] is shown on the left); B, pAT153-HCMV-*Hin*dIII-E DNA; C, herpes simplex virus type 1; D, herpes simplex virus type 2; E, varicella-zoster virus; F, Epstein-Barr virus; G, uninfected MRC-5 cells; H, HCMV-infected MRC-5 cells.

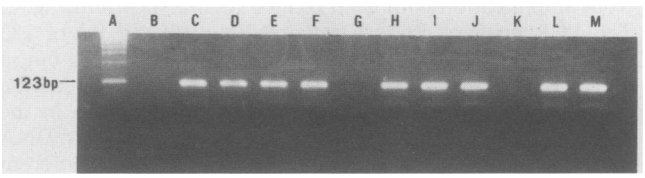

FIG. 5. PCR detection of HCMV in urine obtained from renal transplant recipients. Urine specimens were amplified for 35 cycles, using buffer C, 500 μM each dNTP, and primer pair P_1-P_2. Lanes: A, marker DNA fragments (size [in base pairs] is shown on the left); B through M, clinical specimens 1 through 12 (Table 1).

100%, the sequence variability of clinically isolated HCMV (17) could pose a problem resulting in false-negative results. Therefore, in some cases it may be necessary to use a second primer set to a different genomic region, similar to those previously described (7, 36), to achieve maximal sensitivity.

The utility of the PCR assay can be seen from its use for detection of HCMV in the urine of renal transplant patients. Pretransplant and control specimens showed no background and were uniformly negative by PCR. The PCR assay consistently detected HCMV in specimens that were positive by virus isolation, hybridization assay, or ELISA. In specimens positive by virus isolation and PCR, the amount of virus present was probably below the detection limits of either the ELISA or hybridization assay, whereas the specimens positive only by PCR and either ELISA or hybridization may have contained defective virus. The PCR assay may therefore prove useful for monitoring HCMV infection as well as the effectiveness of antiviral treatment in both immunocompromised individuals and infected neonates.

The PCR assay is a rapid, sensitive test for the presence of HCMV in clinical urine specimens or tissue culture fluids and can be completed in approximately 5 h. The reaction can be automated by using a thermal cycling instrument to carry out the temperature shifts, which would allow mass screening of specimens. The PCR assay may be useful for detecting other viral pathogens that are currently difficult to detect, such as enteroviruses or respiratory viruses. Conceivably, any virus for which characteristic nucleic acid sequences are known can be detected by the PCR assay.

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