JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1998, p. 526–530 Vol. 36, No. 2
0095-1137/98/$04.00+0

Evaluation of PCR Primers for Early Diagnosis of Cytomegalovirus Infection following Liver Transplantation

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Received 4 June 1997/Returned for modification 8 October 1997/Accepted 5 November 1997

The availability of microbiologic methods that detect early replication of cytomegalovirus (CMV) posttransplantation will enhance the process of initiating preemptive antiviral therapy prior to the appearance of CMV disease. Using PCR techniques we sought to determine which region of the CMV genome present in peripheral blood leukocytes (PBLs) or serum provides the highest sensitivity for the detection of CMV posttransplantation. Blood samples were prospectively collected weekly for at least 8 weeks from a cohort of 21 consecutive liver transplant recipients not receiving anti-CMV prophylaxis. Results of PCR assays were correlated with recovery of CMV in cell cultures and histopathological findings from biopsy specimens of infected organs to assess clinical symptomatic infection. Of 148 specimens, primer pairs directed to the *Hin*dIII-X fragment region of CMV detected target DNA with a 94% sensitivity, compared to an 87% sensitivity with primer pairs directed to *Eco*RI fragment D, 32% sensitivity with primer pairs directed to the immediate-early antigen 1 gene (IEA1 gene), and 20% sensitivity with primer pairs directed to the major immediate-early (MIE) gene. The performance characteristics in terms of the sensitivity of primers for amplifying CMV DNA associated with symptomatic infection ranged from 100% (*Hin*dIII-X) to 20% (MIE gene); however, specificity was inversely related (*Hin*dIII-X, 45%; MIE gene, 91%) to primers directed to these gene targets. When *Hin*dIII-X and *Eco*RI-D primer sets were used, CMV DNA from PBLs was a more sensitive target than CMV DNA from serum for the early detection of symptomatic CMV infection (17 versus 12 days). Importantly, CMV DNA was not detected in five patients with no evidence of this viral infection. In conclusion, primers directed to the *Hin*dIII-X fragment region were the most optimal for the early detection of CMV DNA in PBLs and sera from symptomatic liver transplant recipients.

Cytomegalovirus (CMV) is the most frequent cause of infection in liver transplant recipients (15). CMV has been associated with an immunosuppressive state, superinfection with other opportunistic pathogens, and allograft rejection, causing significant morbidity and mortality in this patient population (19).

With the availability of clinically effective antiviral therapy, early and sensitive laboratory diagnosis has become increasingly important. Although antiviral agents such as ganciclovir have been used to treat symptomatic CMV infection in liver transplant recipients, the role of prophylaxis in this group of patients remains unclear since not all patients are at risk and only 20 to 25% develop CMV disease. In addition, there are concerns such as the increasing cost of therapy, the emergence of resistant viral strains, and side effects associated with antiviral prophylaxis regimens.

A proposed alternative to universal prophylaxis has been preemptive therapy in which the antiviral drug is administered only to a subgroup of patients deemed to be at risk for symptomatic infection but is administered prior to its occurrence (14). Identification of the patient at risk could be achieved by detecting CMV early in the posttransplantation period. To achieve this goal, it is necessary to evaluate and compare several different laboratory techniques such as shell vial cell cultures, CMV antigenemia assays, and molecular biology-based methods, including PCR.

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The detection of CMV by DNA amplification techniques (PCR) provides the potential for rapid and early diagnosis (6, 18, 21). PCR is able to selectively amplify and detect specific CMV DNA; a variety of amplification targets have been used so far for the detection of this viral nucleic acid. However, variations in the sequences of the viral genomes found in different populations of patients including immunocompromised patients have been shown to affect the performance of PCR for detecting CMV DNA targets (2). Empiric comparisons of different regions of the CMV genome to obtain the optimal diagnostic sensitivities and specificities of PCR assays for this clinical application therefore require careful evaluation.

In this study, the efficacies of four sets of primers able to amplify different regions of the CMV genome for the early diagnosis and longitudinal detection of CMV infection for a preemptive therapy trial were evaluated with a cohort of 21 liver transplant recipients. The performance of these four primer sets, homologous to different regions of the CMV genome, was evaluated, and the results were correlated with those of cell culture diagnostic techniques.

MATERIALS AND METHODS

Clinical samples and viral culture. Whole blood and serum samples were collected before and every week after transplantation from 21 sequential orthotopic liver transplant recipients for a minimum of 8 weeks. Peripheral blood leukocytes (PBLs), collected in EDTA-coated tubes, were separated by using Histopaque 1119 (Sigma, St. Louis, Mo.). PBLs and serum samples were stored at −70°C until the samples were tested by PCR. In addition, tissue and body fluid were obtained when indicated from patients in whom CMV infection was suspected.

PBLs isolated from blood and urine specimens were inoculated into conventional tube cultures and rapid shell vial cell cultures seeded with MRC-5 cells

TABLE 1. Sequences of oligonucleotide primers used for the amplification and early detection of human CMV genome

Gene region	Primer pair	Sequence	Product length (bp)	Reference
*Hin*dIII-X fragment	P1	5′-GGATCCGCATGGCATTCACGTATGT-3′	406	3
	P2	5′-GAATTCAGTGGATAACCTGCGGCGA-3′		
*Eco*RI fragment D	P1	5′-GATCCGACCCATTGTCTAAG-3′	152	17
	P2	5′-GGCAGCTATCGTGACTGGGA-3′		
IEA1 gene	P1	5′-CAAGCGGCCTCTGATAACCAAGC-3′	438	11
	P2	5′-CTCTTCCTCTGGGGCAACTTCCTC-3′		
MIE gene	P1	5′-GGGTGCTGTCCTGCTATGTCTTA-3′	370	4
	P2	5′-CATCACTCTGCTCACTTTCTTCC-3′		

(13). Shell vials were incubated for 24 h, and conventional cultures were incubated for 14 days. Tissue specimens were homogenized, 0.2 ml of homogenate was added to each of the shell vials, and the vials were centrifuged at 2,000 rpm ($700 \times g$) at 30°C for 40 min, and then 1.0 ml of maintenance medium was added to each of the shell vials and the vials were incubated at 37°C for 24 h.

Clinical definitions. CMV infection was defined as the isolation of CMV from any body fluid or tissue of the detection of CMV in tissue specimens by characteristic histologic findings, immunohistochemistry and/or DNA hybridization, or detection of immunoglobulin M directed to CMV in serum. CMV infection was considered asymptomatic when clinical symptoms, signs, and laboratory abnormalities were absent and symptomatic when clinical symptoms or signs or documented evidence of organ invasion (as evidenced by a tissue biopsy specimen demonstrating cytomegalic inclusion bodies, positive cultures, positive DNA hybridization, or positive immunofluorescence for CMV) was present.

Extraction of nucleic acids, oligonucleotides, and PCR. The IsoQuick extraction method (ORCA Research, Inc., Bothell, Wash.) was performed according to the manufacturer's instructions for processing serum samples. Viral nucleic acid was extracted from PBLs by the lysis method described previously (4). For the lysis method, the leukocyte fraction is spun down, the resultant pellet is washed in 500 μl of phosphate-buffered saline, the pellet is resuspended in 125 μl of lysis buffer, 10 μl of proteinase K is added, and the mixture is incubated at 55°C for 1 h, then boiled for 8 to 10 min, and placed at room temperature before PCR is performed.

PCR for the detection of CMV was performed by using previously described oligonucleotide primers and probes from the *Hin*dIII-X fragment region (406 bp) (3), major immediate-early (MIE) gene (370 bp) (4), the immediate-early antigen 1 (IEA1) gene (438 bp) (11), and the *Eco*RI fragment D region (152 bp) of CMV AD169 (17) (Table 1). These primers and probes have previously been shown not to amplify other herpesviruses or cellular DNA. The probes corresponding to a region between these oligonucleotide primers were synthesized and labeled for chemiluminescence by using the enhanced chemiluminescence kit from Amersham.

Reaction mixtures consisted of 5 μl of target, 100 pmol of each of the oligonucleotide primers, 1.25 U of the enzyme *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μM (each) deoxynucleotide triphosphate (Boehringer Mannheim, Indianapolis, Ind.), 5 μl of 10× reaction buffer (500 mM KCl, 100 mM tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.01% gelatin), 10 μl of a 50% glycerol solution, 25 μg of isopsoralen per ml, and high-pressure liquid chromatography-grade distilled water to a total volume of 50 μl in a microcentrifuge tube. A no-target control reaction tube received 50 μl of reaction mixture only. The tubes were overlaid with 2 drops of mineral oil and were subjected to 35 cycles of amplification (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) by using a DNA thermal cycler (480; Perkin-Elmer Cetus, Emeryville, Calif.). After the cycling was completed, the tubes were placed in a UV transilluminator (HRI-100; HRI Associates) for 15 min at 4°C to activate the isopsoralen to control amplicon carryover (5). The amplified PCR products were electrophoresed on an agarose gel (1.5% Nusieve [FMC, Rockland, Maine] and 1.5% electrophoresis-grade agarose [Bethesda Research Laboratories, Gaithersburg, Md.]; a total of 3% agarose gel) and were visualized with UV light as a single band by staining with ethidium bromide (10 μg/ml). No other bands aside from the product were visualized. The products were then transferred to a nylon membrane (Nytran; Schleicher & Schuell) by Southern blotting and were hybridized with the corresponding labeled probe for 4 h at 42°C. We have tested the sensitivity of the Southern blotting at 4 h versus that at 24 h, and we have found no differences in the sensitivities of these results. The membranes were washed twice with 40 ml of wash buffer (6 M urea, 0.4% sodium dodecyl sulfate, 0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) at 42°C for 20 min and twice with 100 ml of 2× SSC at room temperature for 5 min. The temperatures used for both hybridization and washing are those recommended by the manufacturer of the enhanced chemiluminescence detection kit (Amersham, Arlington Heights, Ill.). The membranes were then placed in 30 ml of detection reagent, provided in the enhanced chemiluminescence detection kit, for 1 min at room temperature and were then exposed to X-ray film for 1 h. The film was then developed by a Kodak X-Omat X-ray film processor (4). For each PCR batch, four tubes containing the reaction mixture but no target DNA were processed. All tubes containing no-target DNA yielded negative results. In addition, for each PCR, four samples containing DNA extracted from MRC-5 cells infected with CMV AD169 were used as positive controls and were consistently positive.

RESULTS

The first CMV infection following transplantation was considered the end point for analysis of the results. One hundred forty-eight PBL samples and 136 serum samples from 21 consecutive liver transplant recipients were tested with each set of primers for the detection of CMV DNA. Overall, primer pairs directed to the *Hin*dIII-X fragment were more frequently associated with a positive result than the other primer pairs. The number of positive results detected with PBLs was greater than the number detected with serum for all the primer pairs (Table 2).

CMV infection was detected in 16 of 21 (76%) patients by conventional detection methods; of these 16 patients, 10 were symptomatic and 6 developed invasive CMV disease. The remaining five patients had no laboratory evidence of infection by conventional techniques or by PCR and remained free of clinical symptoms (Table 3).

CMV DNA was detected by PCR in PBLs and serum from all 16 patients diagnosed with CMV infection by conventional methods. Primer pairs directed to the *Hin*dIII-X fragment showed the highest sensitivity for the detection of CMV infection in PBLs and serum compared with the other primer pairs, while the specificity for all primer pairs was 100%. The performance characteristics in terms of the sensitivities of the primers for amplifying CMV DNA associated with symptomatic CMV infection in PBLs and serum ranged from 100% (*Hin*dIII-X) to 20 and 50% (MIE gene) in PBLs and serum, respectively; however, the specificities with both types of samples were inversely related (Table 4). The positive predictive value for symptomatic CMV infection associated with the detection of CMV DNA in PBLs and serum was 62% for the *Hin*dIII-X fragment primer, while the negative predictive value was 100%.

CMV DNA was detected in PBLs at a mean of 17 days prior to the onset of symptomatic CMV infection for the *Hin*dIII-X and *Eco*RI fragment D primer pairs; target sequences in serum were detected at a mean of 12 days for all primer pairs.

DISCUSSION

Our prospective comparison of different PCR primers for the ability to amplify different regions of the CMV genome demonstrated that (i) the CMV DNA detected by primer pairs in PCR assays in our study was more frequently associated with

TABLE 2. Comparison of four primer sets for the detection of positive PBL and serum samples by PCR

Primer set	No. (%) of positive samples	
	PBL samples (n = 148)	Serum samples (n = 136)
*Hin*dIII-X fragment	70 (47)	25 (18)
*Eco*RI fragment D	58 (39)	22 (16)
IEA1 gene	21 (14)	18 (13)
MIE gene	12 (8)	9 (6)

TABLE 3. Result of assays for early detection of CMV in liver transplant recipients

Patient no.	Serology pretransplantation: CMV infection status, Donor	Serology pretransplantation: CMV infection status, Recipient	Symptomatic infection	Result (no. of days posttransplantation to first positive result): PBL PCR, *Hin*dIII-X	PBL PCR, *Eco*RI-D	PBL PCR, IEA1 gene	PBL PCR, MIE gene	Serum PCR, *Hin*dIII-X	Serum PCR, *Eco*RI-D	Serum PCR, IEA1 gene	Serum PCR, MIE gene	Culture[a], Blood	Culture[a], Tissue	Culture[a], Urine
1	+	−	No	+ (18)	+ (25)	+ (25)	−	+ (25)	+ (25)	+ (32)	+ (25)	−	−	+ (25)
2	+	+	No	+ (22)	+ (29)	+ (36)	−	−	+ (29)	+ (36)	−	+ (29)	−	−
3	+	−	Yes	+ (7)	+ (21)	+ (21)	+ (21)	+ (14)	+ (14)	+ (21)	−	+ (21)	+H (21)	−
4	+	−	No	+ (1)	+ (15)	−	−	+ (15)	+ (29)	+ (22)	−	+ (36)	−	−
5	+	−	Yes	+ (28)	+ (21)	−	−	+ (42)	−	+ (49)	−	+ (35)	−	−
6	+	+	No	+ (11)	+ (5)	−	+ (17)	+ (38)	+ (38)	−	−	−	−	−
7	+	−	Yes	+ (17)	+ (17)	−	−	+ (31)	+ (17)	+ (31)	−	+ (45)	+H (17)	−
8	+	−	Yes	+ (29)	+ (29)	−	−	−	−	−	−	−	+H (29)	−
9	+	−	Yes	+ (23)	+ (23)	−	−	−	−	−	−	−	+H (23)	−
10	+	−	Yes	+ (34)	−	−	−	+ (41)	+ (41)	−	+ (41)	+ (48)	−	−
11	+	−	Yes	+ (39)	+ (39)	−	−	+ (46)	−	+ (46)	+ (46)	−	+G (77)	−
12	+	−	Yes	+ (29)	+ (29)	−	+ (36)	+ (36)	−	+ (36)	−	+ (57)	+H (57)	−
13	+	−	No	+ (6)	+ (6)	−	−	+ (27)	−	−	+ (27)	+ (13)	−	−
14	+	+	No	+ (6)	+ (6)	−	−	+ (6)	−	+ (6)	+ (6)	+ (6)	−	−
15	+	−	Yes	+ (9)	+ (9)	−	−	+ (23)	−	−	−	+ (23)	−	−
16	+	−	Yes	+ (22)	+ (22)	+ (29)	−	+ (22)	−	+ (29)	+ (29)	+ (29)	−	−
17	+	+	No	−	−	−	−	−	−	−	−	−	−	−
18	+	+	No	−	−	−	−	−	−	−	−	−	−	−
19	+	+	No	−	−	−	−	−	−	−	−	−	−	−
20	−	+	No	−	−	−	−	−	−	−	−	−	−	−
21	−	+	No	−	−	−	−	−	−	−	−	−	−	−

[a] All the patients with documented invasive disease by tissue culture for virus also had evidence of CMV infection by the presence of cytomegalic inclusion bodies and/or positive immunofluorescence for CMV. G, stomach; H, liver.

PBL specimens than serum specimens; (ii) differences were also seen between PBLs and serum with regard to the time to the detection of CMV DNA by PCR prior to the onset of symptomatic CMV infection, in which CMV DNA from PBLs was a more sensitive target than CMV DNA from serum for the early detection of symptomatic CMV infection (17 versus 12 days); and (iii) in addition, positive PCR results were also more frequently associated with the primer pair directed to the *Hin*dIII-X fragment than with the *Eco*RI fragment D, the MIE gene, or the IEA1 gene primer pairs.

Diagnostic PCR assays based upon the immediate-early region have frequently been used for the detection of CMV in clinical samples (4, 6, 8, 11). However, this region of the CMV genome has been shown to possess sporadic sequence variation among clinical strains, and primer mismatching has been shown to reduce the amplification efficiencies of PCR assays (2). In contrast, although sequence variation may also exist within the *Hin*dIII-X fragment region, a high degree of conservation has been shown among clinical CMV strains (3). The PCR product obtained by amplification of CMV DNA with the *Hin*dIII-X fragment primer pair was longer than the PCR products obtained by amplification with the other primer pairs with the exception of the IEA1 gene primer pair; this pair, although it amplified a long PCR product (458 bp), targets a

TABLE 4. Statistical analysis of results obtained with PCR primers for early detection of CMV infection[a]

Infection and primer set	PBL PCR, Sensitivity (%)	PBL PCR, Specificity (%)	PBL PCR, PPV (%)	PBL PCR, NPV (%)	Serum PCR, Sensitivity (%)	Serum PCR, Specificity (%)	Serum PCR, PPV (%)	Serum PCR, NPV (%)
CMV infection overall								
*Hin*dIII-X fragment	94	100	100	83	93	100	100	83
*Eco*RI fragment D	87	100	100	71	71	100	100	50
IEA1 gene	32	100	100	31	71	100	100	55
MIE gene	20	100	100	28	43	100	100	38
Symptomatic CMV infection								
*Hin*dIII-X fragment	100	45	62.5	100	100	54	62	100
*Eco*RI fragment D	90	54	64	85.7	63	54	50	66
IEA1 gene	20	72	40	50	75	63	60	78
MIE gene	20	91	67	56	50	63	50	64

[a] PPV, positive predictive value; NPV, negative predictive value.

TABLE 5. Published PCR conditions for primers used in this study[a]

Source	Fragment (size [bp])	Concn of each dNTP	Amt of *Taq* (U)	Vol	No. of PCR cycles	Cycles	pH	$MgCl_2$ concn (mM)
Perkin-Elmer Cetus		200 mM	2.5	100 μl			8.3	1.5
Spector and Wolf (17)	*Eco*RI-D (152)	200 mM	2.5	100 μl	35	94°C for 1 min, 55°C for 2 min, and 72°C for 3 min	8.3	5
Drouet et al. (3)	*Hin*dIII-X (406)	200 μM	2.5	100 ml	30	95°C for 30 s, 55°C for 1 min, and 72°C for 1 min	8.4	1.5
Espy et al. (4)	MIE gene (370)	200 μM	1.25	50 ml	60	94°C for 1 min, 60°C for 1 min, and 72°C for 1 min	8.3	1.5

[a] For all sources, PCR buffer and 10 mM Tris-HCl were used. dNTP, deoxynucleoside triphosphate.

region (immediate-early gene) that has been found to possess a low degree of conservation compared to other areas of the genome, and this may explain its low degree of sensitivity, despite its size. Optimal primer design and greater primer length may account for the increased sensitivities of some of these primer pairs compared with those of others. The effect of a mismatched base in longer primers may be minimized compared with the effect in primers with fewer numbers of nucleotides and may allow for a more sensitive PCR assay (1). That is, the specificity of the PCR amplifying a nucleic acid target is increased in a general direct relationship to the size of an amplicon. In addition, another advantage of longer PCR products is their ability to react more efficiently in some sterilization protocols such as those that use isopsoralen to prevent contamination due to the carryover of PCR products (5).

Our study involved assays with a total of 148 PBL samples and 136 serum samples from 21 patients with four different primer sets (total number of PCR tests, 1,136) directed to CMV DNA. Therefore, it is important to recognize that even though the PCR assays used in this study have been optimized by each one of the original authors' groups (Table 5), some modifications of their original techniques needed to be made in order to match the PCR conditions recommended by Perkin-Elmer Cetus and to be able to test and compare them simultaneously by using a standardized set of technical conditions to minimize intratest variability. Although we have no evidence, it is possible that the lack of specific customization of PCR protocols for each primer set may have reduced the sensitivity of the assay in some cases. However, the PCR conditions that we have used to evaluate all primer sets were based on the reaction conditions and the master mixture composition recommended by Perkin-Elmer Cetus in the package insert for the GeneAmp DNA amplification reagent kit, and these conditions closely matched those used by the different authors in their original publications (Table 5).

In order to evaluate the importance of using conditions that were described in the original publications for each primer set, we retested a subset of 34 specimens (PBL and serum specimens) from five patients by customized procedures (3, 4, 11, 17). Our results (positive or negative) exactly matched those obtained in the original analysis by using standard PCR conditions. In addition, with the customized PCR procedure, primers directed to the *Hin*dIII-X fragment (3) were the most sensitive (65%) for detecting CMV DNA compared to the sensitivities of the primers used by Spector and Wolf (17) (53%), Nyberg et al. (11) (15%), and Espy et al. (4) (6%). These data strongly indicate that the efficiencies of these primer pairs for detecting CMV DNA reflect differences in true sensitivity rather than bias reflective of using standard PCR conditions rather than customized PCR conditions.

Detection of CMV DNA in PBLs and serum by PCR had high levels of sensitivity and specificity for the detection of CMV infection and a high degree of sensitivity but a lower degree of specificity for the detection of symptomatic CMV infection. Primer pairs directed to the *Hin*dIII-X fragment showed the highest degree of sensitivity for the early detection of symptomatic and asymptomatic CMV infection in PBLs and serum. However, despite its high degree of sensitivity and ability to detect the onset of symptomatic CMV infection, the specificity and positive predictive value of PCR for the detection of symptomatic CMV infection in PBLs and serum were low. This low degree of specificity may be improved with the use of DNA quantitation, which has previously been demonstrated (10) to increase the specificity and the positive predictive value (association of high viral loads with symptomatic infection) for the diagnosis of CMV disease.

Our results are in contrast to those of previous studies of PCR of plasma and serum (12, 16, 17). However, the different characteristics of CMV infection and disease in these populations (patients with AIDS and bone marrow and renal transplant recipients) in comparison with those in liver transplant recipients or differences in PCR methodology or priming efficiency may account for some of these different results.

Overall, although PBLs and serum appear to be roughly equivalent in terms of their high degrees of sensitivity for the detection of symptomatic CMV infection in liver transplant recipients when the primers with the highest sensitivity (*Hin*dIII-X) are used, the rapidity of diagnosis or the detection of symptomatic CMV infection can be increased by demonstrating CMV DNA directly in PBLs, especially if the PCR assay is combined with quantitation. Importantly, previous studies have shown that increases in CMV load in PBLs are associated with the detection of viral DNA in plasma or serum, suggesting that during disseminated infection cell lysis may release DNA or whole virus into the plasma or serum (3, 22). It is likely that quantitative PCR of CMV DNA in PBLs will be an optimal laboratory marker for a preemptive therapy trial.

Antigenemia is another promising marker for preemptive therapy (7) and has been shown in heart recipients to have an 83% sensitivity as a marker for the future development of CMV disease; however, its major disadvantage compared to PCR is the timing of detection of CMV prior to the development of disease, which is only about 5 days, compared with 17 days for PCR (9). In one study, PCR performed with PBLs was the earliest signal of CMV replication, followed by PCR performed with plasma and then by the detection of antigenemia (20). In this study, we have also shown the convenience of using chemiluminescence-labeled CMV probes, which allow the rapid and safe detection of DNA amplicons, along with greater sensitivity and lower biosafety risks compared with the use of radioactively labeled probes (3).

In conclusion, we have shown that primers directed to the *Hin*dIII-X fragment region were the most sensitive for the early detection of CMV DNA in PBLs and serum from symp-

tomatic liver transplant recipients; however, PCR with PBLs offers the advantage of providing an earlier detection compared to the time to detection with serum, making the results of PCR with PBLs a more optimal marker for preemptive therapy.

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