Detection of *Toxoplasma Gondii* and Human Cytomegalovirus DNA in Blood from Transplant Recipients Using Multiplex Nested Polymerase Chain Reaction

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ABSTRACT

Evidences from many studies suggested a polymerase chain reaction (PCR) as a valuable method for diagnosing infectious disease in the transplant recipients. We used this method for detection of *Toxoplasma, gondii* and human cytomegalovirus in blood specimens from patients after bone marrow or kidney transplantation. DNA of both infectious agents were detected using two separate sets of nested primers in the PCR. The conditions for a multiplex nested PCR providing simultaneous identification of both pathogens in one tube were optimized. This assay provides an application in clinical research for diagnosis of infections in post-transplant recipients. *Iran. Biomed. J 2: 89-93, 1998*

INTRODUCTION

Toxoplasmosis and reactivated human cytomegalovirus (HCMV) infection are important causes of complications in transplant recipients [1-4]. Clinical manifestations of these diseases give no direct evidences for their diagnosis. However, the effect of patient treatment may be improved if disease be diagnosed at the very early stages. Immunosuppressive treatment of the patients makes difficulties for serological investigations [5, 6]. The necessary level of a specificity and sensitivity for pathogen testing can be achieved in a short time using the methods based on direct detection of its DNA in a clinical sample, e.g., PCR or hybridization with labeled DNA probe [7-10].

Such issue has greatly increased the importance of application of PCR-based methods to studies on clinical specimens from patients suspected of having post-transplant complications. Here, we present data for a PCR assay specific for detection of HCMV and *T. gondii* in clinical specimens.

MATERIALS AND METHODS

Samples tested. Specimens were received from patients after bone marrow or kidney transplantation.

Details of age, sex, clinical presentation and diagnostic findings were recorded in routine way. Isolation of HCMV from clinical specimens was done by treatment and inoculation of the samples in human embryonic lung cell line in accordance with the conventional techniques [11]. Characteristic cytopathic effect (CPE) on human fibroblast was an evidence for the presence of HCMV in a specimen. Laboratory strain of HCMV (AD-169, ATCC, Bethesda, MD) was used as a standard control. *Toxoplasma gondii* parasites were cultivated in BHK21 cell line [12].

DNA extraction. Blood specimens from patients were processed for isolation of leukocytes by density gradient centrifugation in Ficoll-Hypaque. Then, DNA samples from 10^5 purified leukocytes were prepared by phenol-chloroform extraction protocol and were dissolved in 200 .tl of TE buffer (10 mM Tris-HC1, 1 mM EDTA, pH 8.0). Samples also were incubated with 200 .tl of 0.05% Sarcosyl at $37^°$C for 15 mM and centrifuged at 10.000 rpm for 5 mM. 2 .tl of supernatant was used directly for PCR.

Amplification primers. Four Oligonucleotide primers relevant to HCMV MIE (Major Immediate Early) exon 3-exon 4 were examined previously [13]. The outer HCMV primers Cl, 5'-

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GCA ACG AGA ACC CCG AGA AAG-3' and C4, 5'-AAG CCA TAA TCT CAT CAG GG-3' produced a 698-bp amplimer. The inner primer set consisted of CO, 5'-GCG CAT AGA ATC AAG GAG CAC ATG-3' and C2, 5'-CAAGCCATCCACATC TCCCGC-3' flanking a 222-bp amplimer. B1 gene repeat in *T. gondii* genome was a target for other nested primers. The T *gondii* specific outer primers T3, 5'-AAA AGA GGA AGA GAC GCT GCC GCT G-3' (position 572-596) and T4, 5'-CAT TTT CTG AGC ATC CCT TCC G-3' (position 1160-1137), amplifying a 589-bp fragment, were designed using standard recommendations [14]. The inner set consisted of primers T1 and Tol, equivalent to oligo 1 (5'GGA ACT GCA TCC GTT CAT GAG-3') and oligo 3 (5'-GG CGA CCA ATC TGC GAA TAC ACC-3') proposed by Burg et al. [8]. Final PCR product was a 160 by DNA fragment. The designed oligonucleotides were synthesized and purified according to manufacturer recommendations (Applied Biosystems, Inc.).

Amplification conditions. The two round PCR assay was done as follow. Reaction mixture (100 pi) contained 67 mM Tris-HC1 pH 8.8, 16 mM $(NH_4)_2SO_4$, 3.0 mM MgC1$_2$, 0.001% gelatin, 0.2 mM of each of dNTPs, 0.3-0.5 tM of each pair of outer primers (C1 and C4 for HCMV or T3 and T4 for T *gondii),* and 0.03 units/pi of Taq polymerase (BioTaq; Biomaster, Russia). The reaction mixture was covered with 100 IA of mineral oil and boiled for 3 min and thermal cycling was performed in DNA thermal cycler (Pharmacia; or Biokom, Russia). The protocol for temperature cycling included 30 cycles of incubation at 94°C for 1 min, 53°C for 1.0 min and 72°C for 1.0 min followed by final extension at 72°C for 3 mM. Then, 1-2 tl of the first reaction mixture, containing outer primers, was diluted ten times and used for the second round of amplification (with primers CO and C2 or T1 and Tol.) under the same thermal and time protocol.

Gel electrophoresis. PCR products were electrophoresed on 1.5 agarose gel in 0.5 x TBE (45 mM Tris-Borate, 45 mM boric acid, 1 mM EDTA, pH 8.0) containing 0.5 pg/m1 ethidium bromide. The specific nature of the final HCMV PCR product was repeatedly approved by Taq I restriction endonuclease digestion of 222-bp amplimer to 132 by and 90 by fragments. We

followed all necessary recommendations to prevent false positive results during DNA sample preparation [15]. The presence of inhibiting substances inducing false negative results was repeatedly checked by adding standard template, DNA of *T. gondii* or HCMV, into all samples that showed negative results in a preliminary detection.

RESULTS

The presence of a pathogen in blood seems to be one of the important evidences for diagnosing infection. We used two-round PCR with two sets of nested species-specific primers for rapid detection of T *gondii* or HCMV in a blood specimen of patients after bone marrow or kidney transplantation. At first, all clinical specimens had been used in standard PCR with *T gondii* specific primers described previously [8]. However, none of them was positive by direct examination of PCR products in the agarose gel. Preliminary amplification of the samples with specially designed outer primers T3 and T4 was chosen as a necessary step for reproducible detection of *T. gondii* in leukocytes (Figure 1).
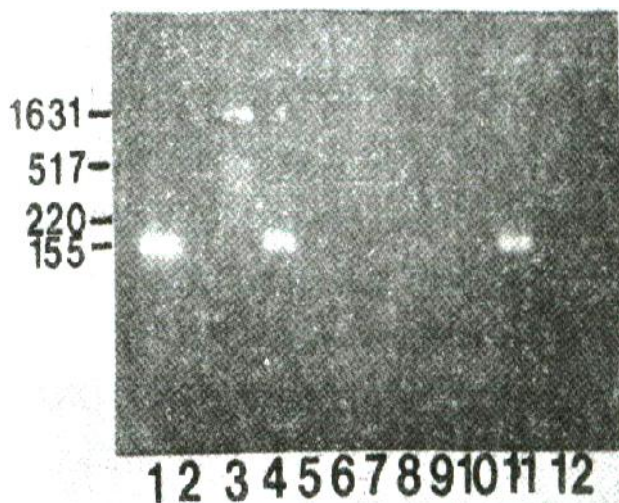

Fig. 1. Products of PCR with primers specific for *T. gondii* and leukocytes specimens from patients after kidney or bone marrow transplantation. (1) positive control (10^2 fold dilution of *T. gondii* culture sample in TE buffer); (2) negative control; (3) DNA molecular size markers (restriction endonuclease Hinfi digest of pBR 322); for each next line (4-12) 1111 of DNA sample purified from leukocytes by standard phenol-chloroform procedure was subjected to nested PCR with T3-T4 outer pair and T 1 -Tol. inner pair of primers and electrophoresis in a 1.5% agarose gel.

After this improvement, Toxoplasma was clear: cut detected in five specimens using nested PCR. In both examinations, we used two different protocols for leukocyte treatment, standard procedure for extraction of DNA with phenol-chloroform and the treatment of purified leukocytes with 0.05% Sarcosyl (see Methods). Addition of Sarcosyl to leukocytes at concentrations not inhibiting Taq polymerase activity greatly simplified the protocol giving the results practically identical to the data obtained by standard phenol-chloroform extraction procedure (Figure 1). To check the presence of inhibitors of Taq polymerase in the negative samples, we added 1 µl of diluted positive soecimens or standard template of *T. gondii* and HCMV. This addition has changed the negative samples to positive one indicating the lack of any pre-existed Taq polymerase inhibitors. In parallel, we used part of the same DNA samples for nested PCR with HCMV specific primers (Table 1). Out of 41 clinical specimens, six were HCMV positive by specific HCMV Ag assay (EIA), seven by nested PCR and also five positive specimens for T *gondii*. The PCR testing with recommended primers gave superior sensitivity in comparison with Ag assay.

Finally, we combined two sets of nested primers for simultaneous detection of both pathogens in one PCR tube. Two-fold increase of the concentration of Toxoplasma specific primers produced HCMV or *T. gondii* specific amplimer with comparable intensity of DNA bands (Figure 2). Amplification in one of the specimens produced both specific bands similar to PCR products received in specially mixed positive control containing DNA of the both organisms.

DISCUSSION

PCR assay with one set of primers for detection of HCMV or *T. gondii* in clinical specimens have been developed in several laboratories. However, some
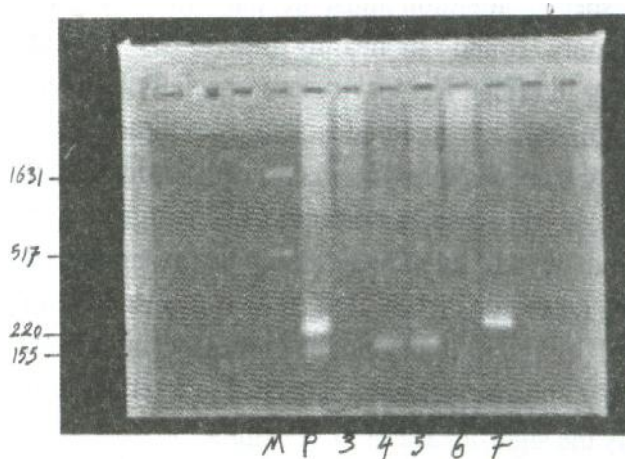

Fig. 2. Products of multiplex nested PCR of leukocytes specimens from patients after kidney or bone marrow transplantation with primers specific for T. gondii and HCMV. (M) DNA molecular size markers (restriction endonuclease HinfI digest of pBR 322); (P) positive control (mixture of T. gondii and HCMV culture samples in TE buffer); for each next line (3-7) 1 µl of DNA sample purified from leukocytes by standard phenolchloroform procedure was subjected to nested PCR with T3-T4 pair and C1-C4 pair of primers in the first round of amplification and with T1-To1 pair and C2-C0 pair of primers for the second round of multiplex PCR; (8) negative control.

of them were not of appropriate sensitivity in diagnosing the cases of suspected disease [10,16, 17]. Previous studies have shown that a few HCMV particles or *Toxoplasma* parasites could be detected by PCR with nested primers [9, 13, 15, 17]. Recently, a definite variability of DNA sequence in a favorite target for HCMV primers, MIE region, has been found [9]. In order to guarantee the detection of different viral strains with variable DNA sequences, we decreased the annealing temperature of thermocycling for the outer pair of primers. The further amplification with inner primers improved the specificity and sensitivity of the reaction. So, the nested PCR is preferable because of less stringent temperature conditions for primers annealing do not influence the high specificity and sensitivity provided by four primers combination instead of two primers in standard PCR.

Recently, the lack of correlation between pathogen detection and titers of IgG and IgM in sera of immunocompromised patients was demonstrated [3, 4]. In practice, the diagnosis of secondary reactivation of HCMV or *T. gondii* cannot be made with good confidence [15]. It calls for direct detection of antigens or pathogen DNA for diagnosis of opportunistic infections in patients with AIDS or after treatment with

Table 1. PCR results for clinical samples from patients after transplantation.

Patient's specimens	Nested PCR		Ag assay HCMV
	T. gondii	HCMV	
Positive	5	7	6
Negative	36	34	35

immunosuppressive drugs. PCR detection of HCMV and *T. gondii* is becoming one of the important tests to diagnose these patients.

A special attention must be paid to pathogens commonly found in healthy individuals without causing ill effect. Herpesviruses, especially HCMV, and Toxoplasma are good examples of these pathogens, which can present in clinically insignificant doses. In this case, importance of PCR signals will depend on the source of specimen, the amount of target detected. Even if a 'positive' result is obtained by PCR, it may not be clinically significant. Much clinical experience is needed to interpret correctly the significance of PCR results.

The rapid PCR methods for HCMV and T. *gondii,* which we present here, are reliable for simultaneous identification of both pathogens in one tube. Early diagnosis is of a great importance for transplant recipients. High sensitivity of PCR guarantees the detection of the pathogens at the very beginning of their reactivation, overtaking an influence of probable inhibitors from blood on Taq polymerase activity. Finally, the assay we have discussed here is recommended for direct detection of HCMV and *T gondii* in blood. Multiplex two-round amplification of target DNA with subsequent approval of DNA product by treatment with an appropriate restriction endonucleases may be recommended for routine diagnostic use at an inexpensive price.

ACKNOWLEDGMENTS

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