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PCR Enzyme-Linked Immunosorbent Assay for Diagnosis of Leishmaniasis in Human Immunodeficiency Virus-Infected Patients

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A PCR enzyme-linked immunosorbent assay (ELISA) involving the use of bone marrow aspirates (BMA) and blood samples (BS) for the diagnosis of visceral leishmaniasis (VL) in human immunodeficiency virus-infected patients was developed with primers selected from the sequence of the small-subunit rRNA gene and compared with direct examination and in vitro cultivation. The PCR was optimized for routine diagnosis: processing of samples with lysis of erythrocytes without isolation of leukocytes, enzymatic prevention of contamination, internal control of the reaction, and ELISA testing in a microtitration plate hybridization. Of 79 samples (33 BMA and 46 BS) from 77 patients without VL, all the results were negative. Fifty-three samples (9 BMA and 44 BS) were obtained from 13 patients with VL: 6 samples drawn during anti-*Leishmania* treatment were negative whatever the technique used, and 47 samples (9 BMA and 38 BS) were positive with at least one technique. The sensitivities were 51% (24 of 47), 81% (38 of 47), and 98% (46 of 47) for direct examination, culture, and PCR, respectively. Thus, PCR ELISA is reliable for diagnosing VL in human immunodeficiency virus-infected patients, and blood sampling should be sufficient for the follow-up.

Visceral leishmaniasis (VL), a protozoan infection transmitted by sandflies, is an opportunistic disease in AIDS patients (1). In France, numerous human immunodeficiency virus-infected patients from the Mediterranean basin, where leishmaniasis is endemic, are seen in clinics (10). Currently, the diagnosis of VL is achieved in our laboratory by in vitro cultivation, which allows characterization of isolates by phenotypic methods (9). Although the sensitivity of cultivation is usually satisfactory, negative results cannot be obtained rapidly. This hampers therapeutic decisions, as VL is usually recurrent in AIDS patients (1).

To circumvent the slowness of cultures, a lot of PCR assays have been developed, but no consensus exists on the target DNA and the primers (6, 8, 12). We chose primers in a very well conserved region in the small-subunit rRNA gene (4, 11) to ensure amplification of every *Leishmania* sp. and developed a competitive PCR technique designed to avoid false-negative results due to amplification inhibitors, as we had for *Toxoplasma gondii* (2). We also developed a detection method for PCR products by capture in microtitrate wells and colorimetric visualization, named "PCR enzyme-linked immunosorbent assay" (PCR ELISA). This method avoids electrophoresis in a gel and checks the specificity of the PCR products. Moreover, the reliability of PCR on blood was tested, as blood is more easily and less painfully obtained than bone marrow aspirates (BMA).

Blood samples (BS) and/or BMA were obtained for suspected VL from 91 human immunodeficiency virus-infected patients at the Henri Mondor hospital (Créteil, France) from August 1993 to December 1994. Each sample was coded, and amplification was performed blind and subsequently compared with other data. Peripheral blood and BMA were collected in EDTA Vacutainer tubes (Becton Dickinson). Two 100-μl aliquots of each sample were processed as previously reported (2).

The target for amplification was the small subunit rRNA-coding region of *Leishmania donovani* (GenBank accession number, X07773) (4). The following primers were used: 5′-TTAGACCGCACCAAGACGAACT-3′ (sense; nucleotides 1158 to 1178) and 5′-GGGTGTCATCGTTTGCAGTGTG-3′ (antisense; nucleotides 1264 to 1284). Their specificity was controlled with DNAs of different collection strains and other parasites (Table 1). The lower primer was 5′ fluorescein labeled to allow detection of PCR products using microtitration plates (see below).

A positive internal control using M13mp18 phage DNA was designed as previously done for *T. gondii* (2). First, composite primers were synthesized which contain the M13mp18 sequence flanked by the *L. donovani* sequence used in the PCR amplification: 5′-TTAGACCGCACCAAGACGAACTCGGT TTGCGTATTGGG-3′ and 5′-GGGTGTCATCGTTTGCA GTGTGGTGGACCGCTT-3′. The PCR with the composite primers generated a M13mp18 fragment with the *L. donovani* sequence incorporated at the ends. This product was diluted until the lowest concentration which constantly gave a positive amplification (around 10 molecules per μl) was reached. Moreover, hot start was achieved with a monoclonal antibody to *Thermus aquaticus* DNA polymerase (TaqStart antibody; ClonTech, Palo Alto, Calif.).

The samples were amplified, in duplicate, in a 50-μl reaction mixture containing 2 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2.5% glycerol; 0.2 mM each dATP, dGTP, and dCTP; 0.4 mM dUTP; 0.4 μM each *L. donovani* primer; 1 μl of the internal control; 0.5 U of uracyl-D-glycosylase (Gibco BRL, Cergy Pontoise, France); 35 nM anti-*Taq* antibody; and 1.25 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Saint Quentin-en-Yvelines, France). The samples were initially incubated for

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TABLE 1. Reference microorganisms used to test the specificity of the PCR

Strain
Leishmania aethiopica MHOM/ET/72/L100
Leishmania braziliensis MHOM/BR/00/LTB300
Leishmania donovani MHOM/IN/80/DD8
Leishmania guyanensis MHOM/BR/75/M4147
Leishmania infantum MHOM/TN/80/IPTI
Leishmania major MHOM/SU/73/5-ASKH
Leishmania mexicana MHOM/BZ/82/BEL21
Leishmania tropica MHOM/SU/74/K27
Trypanosoma brucei brucei TREU667
Trypanosoma brucei brucei GVR35/Cl2
Trypanosoma brucei brucei fast strain[a]
Toxoplasma gondii RH
Plasmodium falciparum FCR3
Candida albicans ATCC 10231

[a] Pasteur Institute.

2 min at 50°C to promote the action of uracyl-D-glycosylase. This incubation was followed by a 5-min denaturation at 95°C prior to temperature cycling (40 cycles at 94 and 57°C for 30 s each and 72°C for 1 min) in a 48-well thermal cycler (Perkin-Elmer Cetus). After 40 cycles, primer extension was continued for 10 min at 72°C, and then an equal volume of chloroform was added to inactivate uracyl-D-glycosylase. Each amplification run contained several negative controls (heat detergent extraction buffer) and positive controls (internal positive control in heat detergent extraction buffer).

Amplification products were detected by PCR ELISA using fluorescein to label the PCR products. First, two 20-nucleotide probes within the amplified sequences but not overlapping the primers were selected: the *Leishmania* probe (5′-CAAAGT GTGGAGATCGAAGA-3′) (nucleotides 1223 to 1242) and the internal probe specific for the M13mp18 sequence. Both oligonucleotides were biotin labeled to allow binding to a microplate well by biotin streptavidin affinity. The detection protocol was adapted from the DIG detection ELISA kit (Boehringer, Mannheim, Germany). The main difference was the substitution of fluorescein for digoxigenin, but all the reagents came from this kit.

All of the following steps were performed directly in wells of a streptavidin-coated microtitration plate (Boehringer). A 15-μl aliquot of the PCR product was mixed with 20 μl of denaturation solution in a well. After a 10-min incubation at room temperature, the well was filled to 250 μl with hybridization buffer containing 20 nM either probe. After an incubation of 2 h at 55°C, the solution was discarded and the wells were washed with washing buffer. Then, 200 μl of peroxidase-conjugated, anti-fluorescein Fab fragment (Boehringer), diluted in conjugate buffer at 150 mU/ml (final concentration), was added to each well. The plate was subsequently washed five times, and each well received 200 μl of the tetramethylbenzidine peroxidase substrate. After 5 min, the reaction was stopped by adding 100 μl of 1 M sulfuric acid per well. The absorbance was read at 450 nm. The absorbance of a reagent blank, in which the test sample was replaced with distilled water, was subtracted from each test sample. A test was considered positive if the absorbancy was at least five times that of the blank control. A biological sample was considered positive if the signal with the *Leishmania* probe was positive. A clinical sample was considered negative if only the signal with the internal control probe was positive. A clinical sample was inconclusive if no signal was observed with both probes.

TABLE 2. Comparative results of the diagnostic methods

Direct examination	In vitro cultivation	PCR	*n*[a]
Positive	Positive	Positive	21
Positive	Negative	Positive	3
Negative	Positive	Positive	16
Negative	Negative	Positive	6
Negative	Positive[b]	Negative	1
Negative	Negative	Negative	6

[a] Of 53 samples (9 BMA and 44 BS) from 13 patients with VL.
[b] Positive with the second in vitro passage.

Direct examination was performed after leukocytoconcentration of 1 ml of blood or BMA as described elsewhere (3). Briefly, after lysis in 0.4% saponin and centrifugation, the pellet was resuspended in 0.9% saline and cytocentrifugated. The spot was stained with May-Grünwald-Giemsa and examined under oil immersion (magnification, ×1,000). In vitro culture was performed on Novy-MacNeal-Nicolle medium empirically modified in our laboratory by overlaying with 1 ml of Schneider's medium, 1 ml of fetal calf serum, and antibiotics (penicillin, vancomycin, and amikacin). Two tubes were seeded with 1 ml of each sample and kept at 27°C. Microscopic examination was done after 1 week, and samples were subcultured twice if a negative result was obtained.

DNAs from different *Leishmania* strains (Table 1) gave identical results as expected from the analysis of the sequences in the GenBank database. Despite one mismatch in the lower primer, the three *Trypanosoma brucei* DNAs (Table 1) were equally amplified. However, the *Leishmania* probe was chosen to encompass the identified mismatches between the sequences of *Leishmania* spp. and *Trypanosoma brucei*. Thus, the hybridization temperature at 55°C in a microplate distinguished between these parasites, as the *Leishmania* probe did not hybridize to the *Trypanosoma brucei* DNA. Other blood microorganisms usually observed in our laboratory, but not in this study, such as *Plasmodium falciparum*, *T. gondii*, and *Candida albicans*, gave negative results upon amplification.

One hundred twenty-six samples from 91 human immunodeficiency virus-positive patients were tested. One BMA from a leishmaniasis-free patient had residual amplification inhibitors and was excluded from the analysis. Seventy-seven patients (55 males and 22 females; mean age, 38 years [range, 26 to 64 years]) did not develop leishmaniasis over the next 12 months. Thirteen males (mean age, 35 years [range, 29 to 50]) had at least one positive culture over that time.

From 77 patients without VL, 79 samples (33 BMA and 46 BS) were negative with each of the diagnostic techniques used. From 13 patients with VL, 53 samples (9 BMA and 44 BS) were obtained, and the results are listed in Table 2. Only one BS was culture positive and PCR negative, but the culture was positive only after the second passage. For samples positive by at least one technique (47 samples), the sensitivities were 51% (24 of 47), 81% (38 of 47), and 98% (46 of 47) for direct examination, culture, and PCR, respectively. BMA and BS were simultaneously withdrawn six times (three from the *Leishmania*-negative group and three from the *Leishmania*-positive group), and the results were identical regardless of the technique used.

The PCR technique we developed for the diagnosis of VL showed an excellent congruence with direct examination and in vitro cultivation. First, among 77 patients without VL, no false-positive results were observed. Second, among 13 patients with VL, when direct examination and/or cultivation were positive,

the PCR was positive too, except for one sample (Table 2). This finding is probably due to the very small number of parasites and the unequal apportionment of the original sample. Indeed, only 200-μl samples were processed for the PCR, whereas culture tested 2 ml of blood. Conversely, PCR was positive six times when the other techniques were negative (Table 2). These results were obtained from five patients undergoing anti-*Leishmania* treatment. PCR is, then, expected to be more sensitive, as microorganisms can fail to thrive after initiation of treatment. However, PCR results did not always remain positive once the diagnosis of VL was made. PCR became negative with six samples (Table 2) for 4 patients undergoing treatment even if a relapse occurred several months later.

In our study, BS was as efficient as BMA for the diagnosis of leishmaniasis in AIDS patients. Of 13 patients with VL, all but one (for whom only BMA was available) had at least one positive BS. Moreover, when we were able to test BS and BMA in parallel (six pairs), no difference was seen. Our results are similar to those obtained by others with blood from human immunodeficiency virus-negative patients suffering from VL (7). Therefore, BMA could be avoided for the comfort of the patients. Our findings equally emphasize the usefulness of direct visualization of *Leishmania* sp. amastigotes in peripheral blood smears, as around 50% of the samples from patients with VL are positive, as previously stated (5).

The PCR ELISA can make the diagnosis of VL with BS in AIDS patients. This will be useful for easy monitoring of the efficacies of different treatments. Cultivation should be needed only when the PCR is positive and the strain is to be obtained for further characterizations.

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