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Optimized PCR Using Patient Blood Samples For Diagnosis and Follow-Up of Visceral Leishmaniasis, with Special Reference to AIDS Patients

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We developed a highly sensitive PCR method that enables the diagnosis and posttherapeutic follow-up of visceral leishmaniasis with patient blood. The PCR assay was thoroughly optimized by successive procedural refinements to increase its sensitivity and specificity. It was compared to in vitro cultivation as well as to direct examination of bone marrow and to serology. Two hundred thirty-seven patients presenting with clinical signs compatible with visceral leishmaniasis were included in the study. Thirty-six were diagnosed as having Mediterranean visceral leishmaniasis (MVL). Twenty-three of them, including 19 AIDS patients, were monitored during and after treatment over a period from 2 weeks to 3 years. Our PCR assay proved more sensitive than in vitro cultivation, direct examination, and serology for all patients. It is simple and can be adapted to routine hospital diagnostic procedures. For the primary diagnosis of MVL, the sensitivity of PCR versus that of cultivation was 97 versus 55% with peripheral blood and 100 versus 81% with bone marrow samples. Regarding posttherapeutic follow-up, overall, 48% of positive samples were detected by PCR only. Seven patients presented with a clinical relapse during the study; six relapses were detected at first by PCR only, sometimes a few weeks before the reappearance of signs or symptoms. We conclude that an optimized and well-mastered PCR assay with a peripheral blood sample is sufficient to provide a secure diagnosis for all immunocompromised patients and most immunocompetent patients. We also suggest systematic posttherapeutic monitoring by PCR with peripheral blood for immunocompromised patients.

The leishmaniases are parasitic diseases that are caused by various species of the protozoon *Leishmania*. These species are endemic in many countries, and infection with these species can cause a wide variety of symptoms, depending on the parasite species as well as on the host immune status. About 350 million people worldwide are at risk of contracting these diseases (4). Visceral leishmaniasis (kala-azar) is a systemic disorder which is fatal if left untreated. It is mainly prevalent in the Indian subcontinent, where it is due to *Leishmania donovani* s. st., and Eastern Africa, where *L. donovani* s. st., *L. archibaldi*, and *L. infantum* are present. In the Mediterranean Basin, visceral leishmaniasis is due to *L. infantum* and is known as "Mediterranean kala-azar." South American visceral leishmaniasis, which has a much higher incidence than the latter, is also due to the same species (originally named *L. chagasi*) (19). *Leishmania* is now considered to remain latent in the mononuclear phagocytic system after primary infection, which must therefore often be inapparent. During the past 10 years, there has been a steady increase in the prevalence of Mediterranean visceral leishmaniasis (MVL), essentially due to the appearance of this disease as a complication of human immunodeficiency virus (HIV) infection, particularly in southern Europe (2, 8, 11). The diagnosis of MVL during AIDS is difficult because patients often have unusual or nonspecific clinical signs, and the symptoms of many opportunistic infections can mimic those of leishmaniasis. The biological diagnosis of MVL requires the detection of *Leishmania* organisms in specimens of infected organs. For this, samples that must be obtained by invasive procedures, such as bone marrow (BM), lymph node, or spleen aspirates, are typically needed. Indeed, it was long believed that few or no circulating parasites were present in patients with MVL. However, several recent studies have reported peripheral blood (PB) parasitemia in patients with MVL (7, 10, 13, 16, 17, 24). On the other hand, none of the common methods routinely used for the parasitological diagnosis of visceral leishmaniasis is satisfactory: direct examination shows a poor sensitivity in most centers; in vitro cultivation has a good sensitivity but is carried out only in specialized centers, and if the results are negative or if the parasites are scanty, the results can be obtained only after several weeks; and the specific serology is unreliable for immunocompromised patients. PCR has been shown to be as good as or better than these diagnostic methods, with the advantage that it provides a more rapid result. A number of PCR assays for the diagnosis of visceral leishmaniasis due to *L. infantum* (7, 17, 20, 23) and to *L. donovani* (1, 3, 14, 20, 21, 26, 28, 29) have been developed over the past few years. Few investigators have described high PCR sensitivities for the detection of *Leishmania* in blood (1, 7), probably due to relatively low levels of parasitemia, as well as to the difficulties usually encountered in the PCR performed with blood-containing samples.

Here, we describe a PCR assay that has been optimized by successive and time-consuming procedural refinements of the reaction to detect a DNA equivalent of less than one parasite per tube in the presence of blood. This assay was assessed for as a means of diagnosing and monitoring the disease with PB from 37 MVL patients and proved highly sensitive and specific.

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It thus allows a secure diagnosis of visceral leishmaniasis while avoiding the need to obtain samples by invasive procedures, although it is also applicable to BM samples.

MATERIALS AND METHODS

Patients. A 3-year prospective study was carried out in Montpellier, France, from January 1996 to March 1999. Two hundred thirty-seven patients living in the Mediterranean area in France and presenting with clinical signs compatible with MVL were included. The patients mainly presented at the Center Hospitalier-Universitaire (CHU) of Montpellier and the CHU of Nîmes, as well as, occasionally, at the hospital of Alès. MVL was diagnosed in 36 patients, from whom 186 samples were analyzed. All MVL cases were confirmed by serology, direct examination, and/or cultivation. On the other hand, 30 patients presenting with no signs of disease whatsoever and recruited in the Obstetrics Department of the CHU of Nîmes were included as negative controls.

Sample collection. PB samples were collected in citrated or EDTA-containing tubes for in vitro cultivation (9 ml) and PCR (4.5 ml). BM samples (~0.5 to 1 ml) were collected in EDTA-containing tubes for PCR and directly in medium-containing tubes for cultivation. All samples were transported to the laboratory at ambient temperature, except when the temperature exceeded 30°C, in case of which they were put on ice. They were then stored at 4°C until processing. The samples were generally processed on the same day and, at most, within 3 days after collection.

In vitro cultivation. For blood cultures, the buffy coat collected after simple centrifugation of 9 ml of PB was seeded in three blood agar NNN (Novy-McNeal-Nicolle) culture tubes. BM aspirates were seeded in five NNN tubes immediately after aspiration and were diluted with an equal volume of 0.9% NaCl containing penicillin at 100,000 IU/ml. The cultures were incubated at 24°C and were passaged every week. A culture was declared negative after five passages.

Serology. The serological diagnosis used antigens of *L. infantum* prepared from a reference human strain (strain MHOM/FR/78/LEM75). Two techniques were used: indirect immunofluorescence (cutoff value, ≥1/40) and counter immunoelectrophoresis (cutoff value, one line).

DNA isolation. PB was prepared for PCR amplification by one of the two following methods, one with the buffy coat and the other one with whole blood. (i) Three-hundred microliters of buffy coat was collected after simple centrifugation of 4.5 ml of blood, incubated for 2 h at 58°C in 2 volumes of proteinase K lysis buffer (0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH, 10 mM Tris [pH 7.2], 320 μg of proteinase K per ml), and then boiled for 10 min. A simplified phenol-chloroform extraction was performed with 450 μl of this lysate, followed by ethanol precipitation and resuspension in 150 μl of sterile distilled water. (ii) Whole blood (4.5 ml) was incubated for 48 h at room temperature in 1 volume of 6 M guanidine hydrochloride–0.2 M EDTA (pH 8) lysis buffer (5) and was then boiled for 10 min and left at room temperature for a minimum of 2 days before being further processed or stored at 4°C, at which it is stable for more than 1 year (5; unpublished data); 200 μl of this lysate with 300 μl of sterile distilled water added was then subjected to a simplified phenol-chloroform extraction, and the DNA was precipitated with ethanol and resuspended in 150 μl of sterile distilled water. BM samples were prepared by the same methods, except that the whole BM sample was processed and the volumes used for both methods were adapted to the sample volume.

Preparation of mimic blood samples. Instead of simply adding purified parasite DNA to the final preparation, we optimized the PCR conditions using mimic samples made of live parasites in blood. Promastigotes from a 4-day-old culture of a reference strain of *L. infantum* (strain MHOM/FR/78/LEM75) were washed in phosphate-buffered saline (1×) and were precisely counted on a Thoma hemacytometer (mean of 10 countings). The contents of several EDTA-containing tubes with PB from healthy volunteer subjects were pooled, and the promastigotes were directly added either to the buffy coat or to whole blood, depending on which of the two methods for DNA isolation was used. The concentrations of parasites tested were 10,000, 1,000, 100, and 10 parasites/1 ml of blood, corresponding to 100, 10, 1, and 0.1 parasites PCR tube, respectively.

PCR amplification. The DNA target for PCR amplification was the gene coding for 18S rRNA (20 to 40-fold repeated sequence) (12). The primers used were 5′-GGTTCCTTTCCTGATTTACG-3′ (primer R221) and 5′-GGCCGGTAAAGGCCGAATAG-3′ (primer R332), which produce a 603-bp fragment upon amplification (17, 31). The reaction conditions were thoroughly optimized with mimic blood samples so as to obtain a sensitivity of less than or equal to one parasite per reaction tube. For this, the reactivities of the following were tested over the full range indicated, and all combinations were tested: $MgCl_2$ (1.5 to 5.5 mM by increments of 0.5), primers (50, 60, 75, and 100 pmol/tube), and *Taq* DNA polymerase (1.5, 2.5, 3, 4, and 5 U/tube). Moreover, annealing temperatures of 52 to 60°C were tested by increments of 1°C. The optimization assays lasted over 6 months. The optimized conditions for samples lysed by the proteinase K method (DNA isolation method (i); see above) were the following: 5 μl of 10× buffer, 0.6 mg of bovine serum albumin per ml, deoxynucleoside triphosphates at a concentration of 200 μM each, 2.5 mM $MgCl_2$, 60 pmol of each primer (primers R221 and R332), and 3 U of *Taq* DNA polymerase (Goldstar; Eurogentec), for a total reaction volume of 50 μl including 10 μl of sample DNA. For the samples lysed by the guanidine-EDTA method (DNA isolation method (ii); see above), the conditions were identical except that the $MgCl_2$ was present at 5 mM and the *Taq* polymerase was present at 4 U. The hot-start technique was used to increase specificity (Dynawax; Eurogentec). The reactions were cycled in an MJResearch thermal cycler by using the following conditions: 94°C for 4 min and 40 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s, followed by 72°C for 10 min. Each sample was tested at least in duplicate. Aside, for each sample, one semi-internal positive control tube for the detection of PCR inhibition and one DNA extraction control tube were included. The inhibition positive control consisted of the equivalent of 0.8 parasite of purified DNA from *L. infantum* promastigotes, which was added to the 10 μl of the DNA sample. The DNA extraction control procedure consisted of the amplification of a fragment of the human β-globin gene with the primers described by Saiki et al. (27) under particularly stringent conditions ($MgCl_2$, primers, temperature). Although widely used in other studies, we found that the β-globin gene amplification was not sensitive enough to detect low-grade PCR inhibitors. Finally, three negative control tubes that each received 10 μl of H_2O instead of DNA were included in each test to detect any amplicon contamination. Contaminations by amplicons were actually totally avoided by using drastic physical separation (of rooms, materials, and personnel) as well as decontamination (e.g., UV exposure of rooms, consumables, and materials and bleaching of all materials and surfaces) procedures.

PCR product analysis and hybridization. The reaction products were visualized under UV light after electrophoresis of 20 μl of the reaction solution in a 2% agarose gel. All gels were then Southern blotted and hybridized with an α-^{32}P-labeled PCR product from our reference *L. infantum* strain in order to increase sensitivity.

RESULTS

Performance of the optimized PCR assay. Our optimized PCR assay can routinely detect ≤0.05 parasite from in vitro cultures (corresponding to 1 parasite/ml) and ≤1 parasite from mimic blood samples (corresponding to 100 parasites/ml of PB). To our knowledge, the latter sensitivity (with PB) has not been assessed by other investigators. It is important that the sensitivity was not improved by Southern blot hybridization: all samples which gave no signal under UV light were also negative after hybridization. This negativity was further confirmed clinically since the diagnosis of visceral leishmaniasis was not retained for the patients concerned. The specificity of the PCR was 100%. All samples which gave a PCR signal of the expected size were confirmed as positive, technically by Southern blot hybridization and clinically by the positivity of direct examination, cultivation, and/or serology, as well as by the clinical diagnosis. No false-positive results were observed among the 30 negative control patients tested. For the 201 PCR-negative patients, the diagnosis of visceral leishmaniasis was not retained. On the other hand, for most samples the results for all PCR controls used were correct (see Materials and Methods). Complete inhibition of the PCR was observed for only three (1%) blood samples. Partial inhibition was observed for 10% (negative) of the samples and was solved by dilution of the DNA sample to 1/5 or 1/10. No PCR contamination was ever observed. Overall, all 624 negative control test tubes remained negative over the 3 years of the study.

Application to primary diagnosis and follow-up of MVL. The PCR assay was compared to in vitro cultivation for primary diagnosis and for follow-up of MVL with PB and BM samples. Overall, 36 patients were diagnosed as having MVL.

(i) Application to primary diagnosis of MVL. Thirty-one patients, including 19 adult immunocompromised (ICD) patients (15 patients with AIDS, 2 patients with liver grafts, 1 patient with a heart graft, and 1 patient with iatrogenic immunosuppression for rheumatoid polyarthritis) and 12 immunocompetent (ICT) patients (7 adults and 5 children), were diagnosed as having MVL. Fifty-two samples (21 BM samples and 31 PB samples) were collected from these patients. Among the AIDS patients, most of the patients (81%) were male, 6 of 15 (40%) were intravenous drug users, 8 of 15 (53%) contracted HIV infection from sexual relations, and 1 of 15 (6%)

TABLE 1. Comparison of PCR and in vitro cultivation with PB and BM for primary diagnosis of MVL

Results	No. of samples		
	PB[a]	BM[a]	Total
PCR positive, culture positive	17	17	34
PCR positive, culture negative	13	4	17
PCR negative, culture positive	0	0	0
PCR negative, culture negative	1	0	1
Total	31	21	52

[a] For correlation with the patient data, see Results.

was a hemophilic. The mean CD4 cell count was 65×10^6/liter (range, 3×10^6 to 322 10^6/liter). For 21 patients, both PB and BM samples were available for the parasitological diagnosis. For 10 of these 21 patients (4 patients with AIDS, 1 ICD patient, 3 ICT adult patients, and 2 ICT children), the results of both methods with both types of samples were concordant and positive. For seven AIDS patients, both methods detected *Leishmania* in the BM, but only PCR detected parasitemia. For three patients (one child, one patient with AIDS, and one ICT adult), PCR detected *Leishmania* in BM and PB, but all cultures were negative; the diagnosis was confirmed by other findings (clinical diagnosis and either high specific antibody titers or direct examination of BM). Finally, for one ICT patient, no parasitemia was detected by culture or by PCR; *Leishmania* was detected in the BM by direct examination and by PCR, but the cultures remained negative. On the other hand, for 10 patients, only PB was available for PCR and cultures. For seven of these patients (three patients with AIDS, two patients with grafts, one ICT child, one ICT adult), the results of both PCR and cultures were concordant and positive. For three patients, only PCR was positive, but the diagnosis was confirmed as described above. Overall, for primary diagnosis, the sensitivity of PCR versus that of in vitro cultivation was 97% (30 of 31) versus 55% (17 of 31) with PB and 100% (21 of 21) versus 81% (17 of 21) with BM (Table 1). The positive predictive value was 100% for both methods with both PB and BM. The negative predictive values for PCR and cultivation were 99.5 and 93.5%, respectively, with PB samples and 100 and 98%, respectively, with BM samples. By serology, 23 patients had high specific antibody titers, 3 had titers close to the cutoff threshold, and 5 were serologically negative at the time of primary diagnosis. No increase in antibody titers was observed after diagnosis.

(ii) Follow-up of MVL in ICD patients. Twenty-three ICD patients (19 patients with AIDS, 1 patient with a heart graft, 1 patient with a renal graft, 1 patient with a liver graft, and 1 patient with rheumatoid polyarthritis) could be monitored over periods from 2 weeks to 3 years. One hundred thirty-four samples (110 PB samples and 24 BM samples) were collected from these patients (Table 2). Overall, 56 samples were PCR positive, and only 29 (52%) of these were found to be positive by culture. No sample was culture positive and PCR negative. The superiority of PCR was particularly obvious with PB samples, of which only 39% (16 of 41) were positive by culture. For BM samples, of 15 PCR-positive samples, only 2 were not found to be positive by culture. One was from a patient who was gradually converting to negative during specific drug treatment, and the second one was from a patient with a relapse that was detected early. The efficacy of drug treatment was monitored with PB samples by both PCR and in vitro cultivation. A parasitemia could be detected up to about 3 months by PCR (range, 2 weeks to 7 months) and to 1 month by culture (range, 2 weeks 2 months). For one AIDS patient, PB samples remained positive by both PCR and cultivation during the 9-month follow-up period. Among the 21 patients, relapses were detected in seven patients (6 patients with AIDS and 1 patient with a renal graft) and occurred between 4 and 22 months after primary diagnosis (mean, 10 months). All patients had a CD4 cell count $<100 \times 10^6$/liter. They were all detected by the resumption of parasitemia and were confirmed clinically. For only one patient were PCR and cultures both positive. For the six other patients only PCR was positive. BM samples which were available from three of these patients were found to be positive by both PCR and cultivation. Specific serology was not helpful, because the antibody titers gave a slight increase (one patient), were stable (two patients), gave a decrease (two patients), or converted to negativity (one patient) compared with the titers at the time of primary diagnosis.

DISCUSSION

As stated above, the diagnosis of visceral leishmaniasis as a coinfection in AIDS patients may be difficult and requires both sensitive and rapid diagnostic methods such as PCR. In the study described here we have assessed the efficacy of an optimized PCR assay versus those of conventional methods for the diagnosis of MVL. The assay was thoroughly optimized with human blood samples reconstituted with whole parasites (instead of with purified parasite DNA) and by successive procedural refinements of the reaction (see Materials and Methods). The sensitivity of the optimized assay was excellent: 97% with PB and 100% with BM (versus sensitivities of 55 and 81%, respectively, for in vitro cultivation). To our knowledge, its performance is better than or equal to those of other PCR assays reported on previously. For detection of MVL due to *L. infantum*, previous studies obtained sensitivities between 64 and 96% with PB (7, 17, 20) and between 84 and 100% with BM (7, 17, 23) with cohorts which had smaller numbers of patients (13, 11, 10, and 25 patients in the studies described in references 7, 17, 20, and 23, respectively). For detection of visceral leishmaniasis due to *L. donovani*, which may have a different pathogenesis, the sensitivities obtained were between 45 and 95% with blood (1, 14, 20, 21, 29) and 100% with BM (21). The sensitivity of our PCR assay was not improved by DNA hybridization, eliminating the need for this time-consuming step, perhaps due to the thorough optimization of the technique. The specificity of the assay was also excellent (100%). It has previously been shown that *Leishmania* PCR with the 18S rRNA gene as a target is highly specific for a variety of organ-

TABLE 2. Comparison of *Leishmania* PCR and in vitro cultivation with PB and BM for posttherapeutic monitoring ICD patients

Results	No. of samples		
	PB	BM	Total
PCR positive, culture positive	16	13	29
PCR positive, culture negative	25[a]	2[b]	27
PCR negative, culture positive	0	0	0
PCR negative, culture negative	69	9	78
Total	110	24	134

[a] Seven of the 25 samples were from patients whose BM samples were positive in all cases by culture and/or PCR.

[b] One was a progressive conversion to negativity (at day 60, PB and BM were negative by culture but positive by PCR); the second one was an early detection of a relapse (clinically patent 2 months later, with positive PB and BM both by culture and by PCR).

isms (30). In our hands, no false-positive result was ever observed for specimens either in the negative control tubes or from the non-MVL or negative control patients. Finally, the preparation method used here is simple, rapid, and reliable, and in particular, it does not require the use of gradient density separation. Extraction with phenol-chloroform or with a commercial kit is essential to ensure a minimal inhibition rate (here, 1%).

The diagnosis of MVL is usually done with BM samples. Here, in all cases, the diagnosis could be established by PCR with BM (sensitivity, 100%). The cultivation of BM was also excellent (sensitivity, 81%), although lower sensitivities have been reported (22) and it requires longer delays. Direct examination of the BM has the advantage of being rapid and simple, but it has a lower reported sensitivity: from 51 to 70% (7, 22, 32). In our hands, this raised to 75% (this study) to 78% (J. Dereure, unpublished data), but this was at the expense of a minute and time-consuming microscopic examination, which is seldom practiced as part of the hospital routine.

For many physicians, the question of whether one can secure the diagnosis of MVL by a simple blood sampling technique remains (6). This study, like that by Costa et al. (7), shows that the primary diagnosis of MVL can be done with PB by PCR: for 97% of all patients and for 100% of ICD patients. The only patient whose PB was negative was an ICT patient with a typical clinical picture of kala-azar, a high specific antibody response, and a low parasite burden, as revealed by the negativity of direct examination and cultures of BM. Other techniques applied to PB samples are less sensitive: in vitro cultivation of PB was rarely reported, and its sensitivity varied from 67% (15; this study) to 88% (10). Direct examination of PB is also not commonly used. The examination of blood smears has a low sensitivity (50%) but has the advantage of being cheap, simple, and rapid (9, 16, 18); the leukocyte concentration technique is more difficult to set up, but its sensitivity was reported to be between 50% (7) and 100% (13). We conclude that a good and well-mastered PCR assay can provide a secure diagnosis of MVL with PB samples from all ICD patients and most ICT patients. This is in contrast to, for example, the findings of Martinez et al. (16) (based solely on direct examination) that HIV-negative patients have no detectable parasitemia and their suggestion that parasites were present only when CD4 cell counts were $<100 \times 10^6$/liter.

Our PCR assay was also assessed for posttherapeutic follow-up and the detection of relapses, which are frequent (60 to 90%) in AIDS patients (2). The follow-up with PB samples has the advantage of being well accepted and adapted to the hospital routine. PCR was much more sensitive than other methods for this purpose, since all relapses were detected by PCR with PB, but only one of seven was also detected by cultivation. It is also noteworthy that PCR could often detect a parasitemia a few weeks before the appearance of any clinical signs or symptoms. PCR was also highly sensitive for monitoring the efficacy of drug treatment, with a parasite clearance time threefold longer than that detected by culture. This is probably explained by the fact that the circulating drugs that are present diminish the viability of the parasite and, therefore, the efficacy of cultivation. Although it has been debated whether a PCR-positive signal may be due to the presence of intact free nucleic acid or viable cells, the high degree of correlation with the clinical diagnosis observed here suggests that a positive PCR result indicates the presence of viable *Leishmania* rather than free nucleic acid (as also suggested elsewhere [21]). From our experience, we therefore suggest the systematic monitoring by PCR with PB for ICD patients at a frequency of one test every 1 to 3 months, at least during the first year. This frequency remains to be better assessed by clinical studies.

No subclinical cases of MVL were detected in our study (except during the posttherapeutic monitoring). This is in contrast to the findings by Pineda et al. (25) that 41% of MVL cases (diagnosed by systematic BM sampling) were subclinical. This can easily be explained by the fact that in the present study the BM was sampled only in the presence of symptoms or signs compatible with MVL. In that sense, the positive predictive value with PB samples would be much higher than that with BM samples.

In total, we have set up a rapid (24-h), reliable, highly sensitive, and routine-adapted method that allows the a secure diagnosis of visceral leishmaniasis with a sample (PB) that can be obtained by noninvasive means. The assay is also applicable to other types of samples and other *Leishmania* species (unpublished data). The high sensitivity of the method raises the question of whether a low parasitemia can be considered a valid criterion for the diagnosis of visceral leishmaniasis. Again, the high correlation of the PCR result with the clinical findings observed here supports this hypothesis: a positive PCR result with PB was always correlated with a confirmed MVL, a clinically patent relapse, or the early detection of a yet asymptomatic relapse. Therefore, detection of circulating *Leishmania* by PCR appears to be a sufficient criterion for the diagnosis of visceral leishmaniasis, although studies with larger cohorts may be needed to definitively assert this point. We recommend the use of PB as the first diagnostic sample for primary diagnosis and monitoring of visceral leishmaniasis: a direct examination of a buffy coat smear may be carried out first, and if that result is negative, PCR should be performed in a laboratory with experienced technicians.

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