

VALUE OF CULTURE AND NESTED POLYMERASE CHAIN REACTION OF BLOOD IN THE PREDICTION OF RELAPSES IN PATIENTS CO-INFECTED WITH *LEISHMANIA* AND HUMAN IMMUNODEFICIENCY VIRUS

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Abstract. The use of culture and a nested polymerase chain reaction (PCR) of blood in predicting the probability of relapse was evaluated in 20 patients co-infected with *Leishmania* and human immunodeficiency virus (HIV). Fourteen of 20 patients relapsed, with 24 clinical relapses diagnosed. During clinical relapse, the parasite was detected by culture in 21 of 24 blood samples and by nested PCR in 23 of 24 blood samples. After treatment and during asymptomatic periods, the parasite was detected by culture in 18 (19.1%) of 94 blood samples and by nested PCR in 58 (61.7%) of 94 blood samples. For positive blood cultures, the Kaplan-Meier probability estimates for relapse at 6, 12, 18, and 24 months were 44%, 68%, 76%, and 76%, respectively, while for positive nested PCRs, the estimates were 20%, 33%, 45%, and 50%, respectively. For negative blood cultures, relapse probabilities for the same time points were 7%, 12%, 12%, and 12%, while for negative nested PCRs, these probabilities were 8%, 14%, 21%, and 26%. Nested PCR-positive results in asymptomatic periods indicated presence of the parasite, but not necessarily relapse. However, the presence of viable parasites during post-treatment follow-up increased the probability of relapse and showed that culture positivity could be a good relapse marker.

INTRODUCTION

Visceral leishmaniasis (VL), which is caused by *Leishmania infantum*, is endemic throughout the Mediterranean basin. It is a frequent opportunistic disease in patients infected with human immunodeficiency virus (HIV) in southern Europe, where 25–70% of adult patients with VL are co-infected with HIV, and it is estimated that 1.5–9% of patients with acquired immunodeficiency syndrome (AIDS) will develop leishmaniasis.¹

Relapse of VL is an early event, occurring in most HIV-*Leishmania* co-infected patients during the first year after treatment of VL.^{2,3} It does not depend on the clinical features of the leishmanial infection, the degree of immunosuppression, or the presence of AIDS. Despite the use of highly active anti-retroviral therapy, high VL relapse rates (26–70%) have been observed in co-infected patients.^{4,5} Clinical monitoring does not provide sufficient information to predict relapse because most patients are asymptomatic during the post-treatment period and develop symptoms only weeks before relapse.

The aim of the present study was to evaluate two diagnostic methods, culture and nested-polymerase chain reaction (PCR), in blood samples of co-infected patients to establish the value of these techniques as potential relapse markers.

MATERIALS AND METHODS

Patients. A prospective study was conducted in Barcelona, Spain from June 1998 to June 2001. Included in this study were 20 HIV-infected patients with VL at two different hospitals. All patients gave signed, informed consent for participation in this study. The study was reviewed and approved by the ethics committees of each center. Diagnosis of VL was

based in each case on an evaluation of the symptoms and signs associated with VL, including intermittent fever, hepatosplenomegaly, pancytopenia and anemia, and identification of *Leishmania* amastigotes by direct examination or/and by culture in bone marrow. The patients received one of the following treatments at standard doses: meglumine antimoniate, 20 mg of pentavalent antimony/kg/day for 28 days, or amphotericin B lipidic complex, 3 mg/kg/day for 10 days. After clinical recovery, all patients were monitored. The treatment was considered successful when patients showed clinical recovery and no parasites were detected in bone marrow one month after completion of therapy. The diagnosis of clinical relapse or therapeutic failure was confirmed by direct examination or/and culture of bone marrow.

Samples and methods. Nine milliliters of peripheral blood obtained by sterile venipuncture and collected into tubes containing EDTA for *in vitro* cultivation and nested PCR were collected at monthly intervals during post-treatment follow-up and, in patients who relapsed, upon diagnosis of relapse. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by the Ficoll-Paque™ Plus procedure (Amersham Pharmacia Biotech, Piscataway, NJ). Preparations were divided into two aliquots, one used immediately for culture and the other stored at –40°C for nested PCR.

The PBMC samples were seeded into Schneider's insect culture medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated fetal calf serum, 1% sterile human urine, and gentamicin (25 µg/mL) (Sigma). Cultures were maintained at 24–26°C, examined by inverted microscopy twice a week, and sub-cultured every 2 weeks for 6 months before being pronounced negative.

Nested PCR amplification was performed on PBMCs according to a standard technique.⁶ DNA was extracted from 0.2 mL of PBMCs adjusted to a concentration of 1×10^6 PBMCs/mL with the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instruction. External primers Ext (5'-AAT TCG ACG ATC ACG AGG TC-3') and E2b

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(5'-CGA CTC GGT TGG CAC ACT GC-3') and internal primers P-1 (5'-ACG AGG TCA GCT CCA CTC C-3') and P-2 (5'-CTG CAA CGC CTG TGT CTA CG-3') were used. The PCR mixture contained 5 µL of DNA, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 0.01% gelatin), 1.5 mM MgCl₂, 0.1 mM of each deoxynucleotide triphosphate, 0.2 µM of each primer to include Ext/E2b (Amersham Pharmacia Biotech), and 0.5 units of RedTaq polymerase (Sigma) in a final volume of 20 µL. Reactions were cycled in a PTC-200 thermocycler (MJ Research, Waltham, MA) using the following conditions: 96°C for 5 minutes; 30 cycles at 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 5 minutes. The second-round amplification contained 2 µL of the first-round amplification product as the template and primers for P-1 and P-2. Reactions were visualized by electrophoresis on a 3% agarose gel. Samples were positive when a product of 100 basepairs was detected.

Statistical analysis. The Kaplan-Meier method was used in survival analysis, and curves for the two groups (positive versus negative by culture, and positive versus negative by nested PCR) were compared by the Mantel-Cox log-rank and Breslow tests (SPSS Inc., Chicago, IL).

RESULTS

During the study period, the efficacy of specific drug treatment was monitored by blood samples over a period of one month to three years. Fourteen of the 20 patients exhibited clinical relapse, with 24 clinical relapses diagnosed. Nine patients had a single relapse, two had two, one had three, and two had four. The remaining six patients remained free of new episodes throughout the study period.

A total of 118 peripheral blood samples were collected and analyzed simultaneously by culture and nested PCR. These two methods gave concordant results in 72 cases, 37 of which were positive and 35 negative. In two instances, culture was positive and nested PCR negative; one of these cases was a relapse. In 43 instances, PBMC culture was negative and nested PCR positive; three of the cases were relapses. At time of clinical relapse, the parasite was detected in blood by culture in 21 of 24 samples and by nested PCR in 23 of 24 samples. During the post-treatment monitoring, and in absence of clinical symptoms, the parasite was detected by culture in 18 (19.1%) of 94 samples and by nested PCR in 58 (61.7%) of 94 samples, with corresponding negative predictive values of 80.8% (76 of 94) and 38.2% (36 of 94), respectively (Table 1).

TABLE 1

Results of blood cultures and nested-polymerase chain reactions (PCRs) obtained during post-treatment in 20 patients with human immunodeficiency virus-*Leishmania* co-infection during both clinical relapse and asymptomatic periods

	Number of blood samples						Total
	At time of clinical relapses			Post-treatment follow-up during asymptomatic period*			
	Culture+	Culture-	Total	Culture+	Culture-	Total	
PCR+	20	3	23	17	41	58	81
PCR-	1	0	1	1	35	36	37
Total	21	3	24	18	76	94	118

* Negative predictive value of culture = 80.8% (76 of 94); negative predictive value of nested PCR = 38.2% (36 of 94).

Kaplan-Meier estimates of the probability of relapse at 6, 12, 18, and 24 months were 8% (95% confidence interval [CI] = 0–14%), 14% (95% CI = 5–22%), 21% (95% CI = 10–33%), and 26% (95% CI = 12–39%), respectively, in those patients with a negative blood culture. However, in those patients with a positive culture, relapse probabilities were 44% (95% CI = 22–67%), 68% (95% CI = 46–89%), 76% (95% CI = 54–97%), and 76% (95% CI = 54–97%), respectively (Figure 1). There were significant differences in the probability of relapse between patients with negative and positive blood cultures ($P < 0.001$).

Kaplan-Meier estimates of the probability of relapse at 6, 12, 18, and 24 months were 7% (95% CI = 0–16%), 12% (95% CI = 0–24%), 12% (95% CI = 0–24%), and 12% (95% CI = 0–24%), respectively, in patients with negative nested PCR results, and 20% (95% CI = 10–31%), 33% (95% CI = 20–46%), 45% (95% CI = 30–60%), and 50% (95% CI = 33–67%), respectively, in those patients with positive nested PCR results (Figure 2). Differences in the probability of relapse between patients with negative nested PCR results and patients with positive nested PCR results were significant ($P < 0.001$).

DISCUSSION

The high percentage of relapses in co-infected patients following treatment^{1–3} highlights the need not only for post-treatment parasite control, but also for alternative diagnostic techniques that use non-invasive samples. Peripheral blood has the advantage over such conventional samples as bone marrow or lymph aspirate in ease of collection and being a relatively simple and non-invasive procedure. Blood culture and/or PCR in VL diagnosis has been used extensively and shows a range of sensitivities depending on the study: 55–88% for culture^{7–10} and 82–98% for PCR.^{9,11–16} Our results show that culture and the nested PCR showed high sensitivity during clinical episodes, when parasitemia is generally higher (87.5% and 95.8%, respectively).

Post-treatment monitoring of parasitemia during asymptomatic periods showed markedly different sensitivities be-

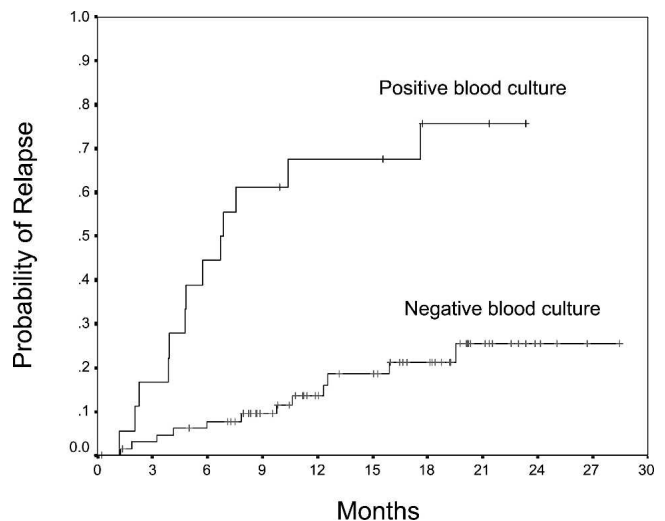


FIGURE 1. Kaplan-Meier probability estimates for relapse in patients co-infected with *Leishmania* and human immunodeficiency virus during post-treatment monitoring based on blood culture results.

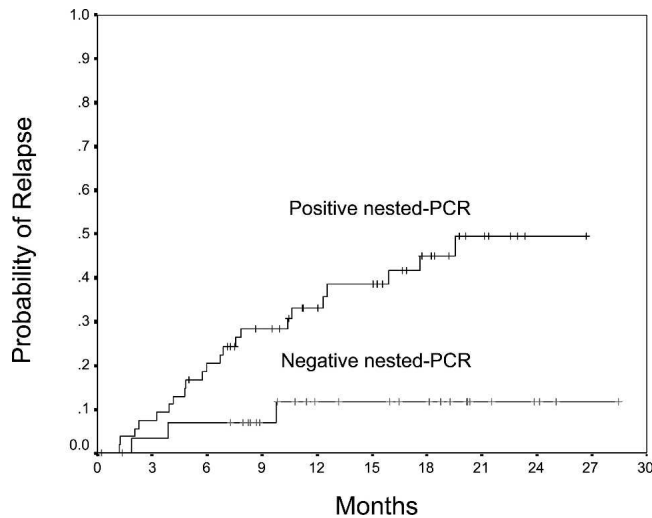


FIGURE 2. Kaplan-Meier probability estimates for relapse in patients co-infected with *Leishmania* and human immunodeficiency virus during post-treatment monitoring based on nested polymerase chain reaction (PCR) results.

tween the two diagnostic methods used in this study. Although *Leishmania* DNA was detected in 58 (61.7%) of 94 blood samples, only 17 were associated with the presence of parasites in blood culture. No association between positive results and clinical relapse during asymptomatic periods was observed. These differences were observed by Lachaud and others,⁹ as well as in a preliminary study done for the work presented here.¹⁵ Low parasitic loads, which are likely present after treatment and during asymptomatic periods, could be the principle cause of low culture sensitivity, and may explain the high sensitivity of the nested PCR in detecting infections, even when levels of circulating parasites are low. However, inoculum size affects the growth of *Leishmania*; cultures initiated with low inoculums failed to promote cell growth.¹⁷ Recent studies on blood donors in disease-endemic areas as southern France and Spain reported low blood culture sensitivity, versus PCR, when parasitemia was low.^{18,19}

Multivariate proportional hazard analyses showed that positive result in the nested PCR was not always associated with clinical disease and that at 24 months post-treatment the probability of relapse was 50%. These data differ from those of another group who reported positive PCR results predictive of clinical relapse.²⁰ Most likely, the use of a nested PCR procedure, compared with a conventional PCR, resulted in an increase in overall sensitivity, but with a decrease in the negative predictive value with respect to the culture (38.2% versus 80.8%). However, when the culture was positive, risk of relapse increased to 76%. These results suggest that positive PBMC cultures, in relation to greater parasitic loads, are associated with higher risks for a new clinical episode, thereby corroborating the data of Bossolasco and others,²¹ who reported that clinical relapse was preceded by a substantial increase in blood parasite levels, as quantified by real-time PCR. However, during follow-up, the risk of relapse in patients with a negative nested PCR result was low (12%), whereas patients with a negative blood culture had a two-fold risk of relapse (26%).

In conclusion, nested PCR is an appropriate technique for

monitoring long-term efficacy of treatment. A negative result is strongly indicative of successful control of the infection and efficacy of treatment, whereas a positive nested PCR result during an asymptomatic period is indicative of the presence of the parasite, but not necessarily of relapse. In contrast, the probability of relapse when a blood culture is positive is significantly higher than when a nested PCR result is positive. The presence of viable parasites during post-treatment follow-up increases the likelihood of relapse, demonstrating that a positive culture could be a good relapse marker.

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