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DETECTION OF *LEISHMANIA INFANTUM* KINETOPLAST DNA IN PERIPHERAL BLOOD FROM ASYMPTOMATIC INDIVIDUALS AT RISK FOR PARENTERALLY TRANSMITTED INFECTIONS: RELATIONSHIP BETWEEN POLYMERASE CHAIN REACTION RESULTS AND OTHER *LEISHMANIA* INFECTION MARKERS

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Abstract. Individuals with frequent exposures to agents spread through the parenteral route show a high prevalence of *Leishmania* seropositivity in Spain. However, the frequency of positive polymerase chain reaction (PCR) results for *Leishmania* in blood in this setting remains unknown. In this study, *L. infantum* kinetoplast DNA (kDNA) was found in blood from 23 (24%) of 95 asymptomatic individuals with a serum *Leishmania* antibody titer ≥ 1:20 and in none of 44 seronegative individuals. The greater the antibody titer, the greater the proportion of PCR-positive samples, but 16 (20%) of 81 individuals with antibody titers ≤ 1:40 tested positive by PCR. Nine (37%) PCR-positive and 22 (19%) ($P = 0.03$) PCR-negative individuals showed a positive leishmanin skin test result. This results show that a remarkable proportion of asymptomatic *Leishmania*-seropositive individuals at risk for parenterally transmitted infections carry *Leishmania* kDNA in blood. This is more common in subjects with a high serum *Leishmania* antibody level and a positive leishmanin skin test result.

INTRODUCTION

Leishmania infantum infection is frequent in the Mediterranean basin. In this area, rural endemic and urban epidemic foci have been identified.[1–3] In some urban areas, this disease appears mainly in individuals with a high grade of parenteral exposure, such as intravenous drug users.[3,4] Thus, leishmaniasis could be an anthropozoonosis in the Mediterranean countries since, besides the conventional zoonotic cycle, *L. infantum* can spread among humans through the sharing of injecting devices.[3,5,6] The emergence of an epidemic of infection with human immunodeficiency virus (HIV) among intravenous drug users in Spain led to a parallel visceral leishmaniasis outbreak in co-infected patients. This outbreak has involved hundreds people,[7] but it is currently subsiding.[8]

In southern Spain, 8% of individuals at risk for acquiring blood-borne infections show serum antibodies to *Leishmania* at a titer ≥ 1:80.[3] In Monaco, another area where the frequency of *Leishmania*-seropositive people is very high, blood of 21% of asymptomatic blood donors harboring serum antibodies to *Leishmania* also tested positive by culture or a polymerase chain reaction (PCR).[9] Similar results have been observed in asymptomatic persons from Brazil.[10,11] Recently, our group has developed a PCR technique for detection of *L. infantum* minicircle kinetoplast DNA (kDNA) that has been shown to be highly sensitive and specific,[12] even when peripheral blood samples are used.[13] Based upon these facts, we hypothesized that *L. infantum* DNA could be detected by this PCR technique in blood from a high proportion of healthy persons with frequent parenteral exposures. If this were the case, the reasons why this parasite often spreads among this population would be more easily understood. Thus, the objective of this study was to search for *L. infantum* kDNA in peripheral blood from *Leishmania*-seropositive individuals at risk for blood-borne infections by using a high-performance PCR technique. We also investigated the relationship between the results of the PCR and those of other tests for the diagnosis of *Leishmania* infection.

MATERIALS AND METHODS

Population studied. From April 1998 to July 1999, 170 individuals living in the province of Seville in southern Spain participated in an epidemiologic survey on *L. infantum* infection.[3] All participants were asymptomatic and denied a history of cutaneous or visceral leishmaniasis. All HIV-seropositive patients were excluded. The group included both intravenous drug users and non-drug injectors. However, most of the latter reported other practices that enabled the spread of parenterally transmitted agents, such as unsterile tattooing, piercing, and cocaine snorting with shared devices.[14] All participants underwent a leishmanin skin test (LST) and submitted peripheral blood for laboratory studies. Both the LST procedure and the negative and positive controls used have been described in detail elsewhere.[3] Serum antibodies to *Leishmania* were tested in all subjects by an immunofluorescent antibody test (IFAT). A whole blood sample, collected into tubes containing EDTA, was aliquoted and frozen at –70°C for PCR determinations. The IFAT showed no reaction in 75 individuals (44%). A titer of 1:20 was found in 51 participants (30%) and the remaining 44 (26%) had antibody titers to *Leishmania* ≥ 1:40 (Tables 1 and 2). These 95 subjects and 44 additional participants, chosen at random among 75 individuals without serum antibodies to *Leishmania*, were the population selected for this study.

Serum antibody testing. A suspension of *L. infantum* zymodeme 1 promastigotes from strain MCAN/ES/91/DP204 was used as antigen in the IFAT. Geometric dilutions from 1:20 to 1:640 of each sample were analyzed as previously reported.[1] Human antiglobulin at a dilution of 1:400 was used. The titration was done using a control serum (titer = 1:5,120) collected from a patient with visceral leishmaniasis. The HIV serology was tested by a commercial enzyme immunoassay (Genescreen HIV-1/2, version 2; Pasteur Diagnostics, Paris, France). As an indirect marker of parenteral exposure, the presence of antibodies to hepatitis C virus (HCV) was determined by an enzyme immunoassay (EIA-3; Ortho Diagnostic Systems, Raritan, NJ).

TABLE 1
Relevant characteristics of the study population*

Parameter	No. (%) with serum *Leishmania* antibody titer: 0 (n = 44)	No. (%) with serum *Leishmania* antibody titer: > 0 (n = 95)	OR (95% CI)
Male sex	42 (95)	87 (92)	0.52 (0.10–2.55)
IDU	20 (45)	50 (53)	1.33 (0.65–2.73)
Active IDU	3 (7)	5 (5)	0.76 (0.17–3.32)
Positive LST result	12 (27)	19 (20)	0.67 (0.29–1.53)
Positive for HCV serum antibodies	18 (41)	34 (36)	0.80 (0.39–1.67)

* OR = odds ratio; CI = confidence interval; IDU = intravenous drug users; LST = leishmanin skin test.

Polymerase chain reaction procedure. To detect *L. infantum* kDNA, frozen whole blood samples from the 139 study individuals were tested by a PCR–enzyme-linked immunosorbent assay (ELISA) following the technique described previously.[12] Briefly, 175 μL of blood was used to isolate the DNA. After extraction with phenol, the DNA was resuspended in 10 μL of double-distilled sterile water. Aliquots (0.5 μL and 1 μL) of the suspension was used for PCR amplifications. In each PCR, 5 and 10 ng of human DNA were used as negative controls. The DNA obtained from 1,000 *L. infantum* promastigotes was used as a positive control. Forward and reverse primers were used to amplify a 75-basepair fragment contained within the kDNA of *L. infantum.* For ELISA detection, an internal oligonucleotide probe (18 bases) modified at the 5′ position with biotin was used. Hybridization was carried out by agitation at 50°C for three hours. Absorbance values were read at 405 nm and values ≥ 1 were considered positive. The absorbance values obtained for human DNA were always less than 0.1. The sensitivity of this PCR procedure was 1 fg of *L. infantum* DNA. To check the quality of the results, a second DNA preparation and amplification, independent of the first, was done in 36 samples selected at random, 18 from seropositive participants and 18 from seronegative subjects. The investigators who performed the PCR procedures were blinded to the remaining data of the individuals from whom the samples came.

Statistical analysis. The proportions of samples that yielded a positive result by the PCR stratified by the titers of serum antibodies to *Leishmania* were compared by a chi-square test for linear trend using Epi-Info, version 3 (Centers for Disease Control and Prevention, Atlanta, GA). The remaining frequency comparisons were done by the chi-square test or the Fisher's exact test, if a cell had an expected count < 5. Age was compared using the Mann-Whitney U test. The statistical package SPSS 10 for Windows (SPSS, Inc., Chicago, IL) was used in these analyses.

Ethical considerations. The Ethics Committee of each participant center reviewed and approved the study. All patients provided written informed consent before participating in the study.

RESULTS

Population features. The mean ± SD age of the population studied was 32.4 ± 6.6 years. The remaining characteristics are summarized in Table 1. Seventy participants (50%) reported having injected illicit drugs at least once, but only eight (16%) used intravenous substances at the moment of inclusion in the study. A very high prevalence (30%, 21 of 70) of serum antibodies to HCV was found among non-intravenous drug user participants.

Polymerase chain reaction results. *Leishmania infantum* kDNA was detected in 23 (24%) antibody-positive individuals but was not detected in any participant in whom serum antibodies were not found. The greater the serum antibody titer to *Leishmania*, the higher the proportion of samples that tested positive by the PCR, but 16 (20%) of 81 individuals with a titer of 1:20 or 1:40 were PCR positive (Table 2). The results of the first PCR were confirmed in the 36 samples that were retested. The mean ± SD ages of PCR-positive and PCR-negative subjects were similar (32.6 ± 5 versus 32.2 ± 7.1 years; $P = 0.36$). The relationship between PCR-positive results and other data is shown in Table 3. An analysis restricted to seropositive people yielded similar results. The individuals harboring *L. infantum* kDNA in their blood were more likely to show a positive LST result that the remaining participants (odds ratio = 2.74, 95% confidence interval = 1.05–7.15).

DISCUSSION

These results show that a high proportion of asymptomatic *Leishmania*-seropositive individuals at risk for parenterally transmitted diseases harbor *L. infantum* kDNA in their peripheral blood. This proportion is greater than that reported previously in blood donors.[9–11] Moreover, we have found that the occurrence of positive PCR results is more frequent as serum antibody titer increases. However, one-fifth of low antibody titer (≤ 1:40) carriers tested positive by the PCR. The LST reactivity is also associated with the detection of *L. infantum* kDNA in blood.

The finding of a positive PCR result for *L. infantum* in blood from asymptomatic individuals might raise questions about possible false-positive results. To verify our observa-

TABLE 2
Relationship between PCR results and *Leishmania* serum antibody titers*

Antibody titer	No.	Positive PCR no. (%)†
0	44	0 (0)
1:20	51	8 (16)
1:40	30	8 (27)
1:80	10	4 (40)
1:160	2	1 (50)
≥ 1:320	2	2 (100)

* PCR = polymerase chain reaction.
† $P < 0.0001$, by chi-square test for linear trend.

TABLE 3
Characteristics of the population according to the PCR results*

Parameter	No. (%) of individuals: PCR positive (n = 23)	No. (%) of individuals: PCR negative (n = 116)	*P*
Male sex	23 (100)	106 (90)	0.21
IDU	13 (56)	57 (49)	0.52
Active IDU	4 (31)	4 (7)	0.03
Positive LST result	9 (39)	22 (19)	0.03

* PCR = polymerase chain reaction; IDU = intravenous drug users; LST = leishmanin skin test.

tions, we tested blood from 44 seronegative individuals and found negative results in all cases. In addition, a panel of 36 randomly selected samples was retested and all yielded the same result. Conversely, the observation of *Leishmania* DNA in asymptomatic individuals is not a new finding, since it had been previously reported in blood donors and in individuals living with patients with visceral leishmaniasis.[9–11]

The frequency of positive PCR results in the population studied was greater than that reported previously in blood donors.[9-11] This finding is consistent with the fact that injecting drug users and other individuals exposed to blood-borne diseases, such as those included in this study, are at high risk for infection with *L. infantum*.[3]

The detection of *Leishmania* kDNA in LST-positive individuals is relatively surprising. Recently, this finding has also been reported in asymptomatic subjects living with patients with visceral leishmaniasis in Brazil.[11] These observations prove that specific cell-mediated immunity is not able to completely eradicate *Leishmania* infection. In fact, despite having developed cutaneous hypersensitivity to *Leishmania* antigens, some patients may present with asymptomatic positive PCR results in their blood, which in many cases coincides with a positive blood culture.[9] However, these individuals do not show signs of visceral leishmaniasis, which suggests that cell-mediated immunity exerts some effect against the infection. The fact that the levels of circulating kDNA detected in asymptomatic individuals are lower than those found in patients with active visceral leishmaniasis is consistent with this hypothesis.[11]

Serology is a useful tool in the diagnosis of visceral leishmaniasis. The IFAT has been shown to be highly sensitive and specific.[15,16] However, the most appropriate cut-off level to be used has not yet been defined. In many studies, values $\geq$ 1:80 have been used.[17–20] However, HIV+ patients coinfected with visceral leishmaniasis may show values of 1:40,[20] as occurs in asymptomatic dogs, in which the parasite is detected by culture or a PCR yields positive results.[12,21] In this study, 16% and 27% individuals showing a serum antibody titers 1:20 and 1:40, respectively, had *L. infantum* kDNA in their blood, which is probably indicative of an active infection. This finding shows that these titers should be regarded as significant, both in clinical diagnosis and in seroepidemiologic studies.

The observation of a high frequency of asymptomatic patients with a positive PCR result in blood agrees with the high rate of *Leishmania* infection markers previously found, both by serologic analysis and LST, in individuals at risk for blood-borne infections in southern Spain.[3] These apparently healthy subjects are frequently engaged in high-risk behavior. In addition, the spread of *L. infantum* through the sharing of penetrating devices is possible, as experimental[6] and epidemiologic[3,4] data have suggested. Thus, such practices put uninfected people at high risk of being infected. The detection of *Leishmania* spp. DNA in a high proportion of syringes used by intravenous drug users living in Spain is consistent with our results.[5]

The frequency of symptomatic visceral leishmaniasis in HIV-uninfected adults in Spain is disproportionate to the rate of PCR-positive individuals found in this study, since overt disease is relatively uncommon in such a setting. This discordance is probably due to the fact that most patients harboring *L. infantum* kDNA will never develop active visceral leishmaniasis. If this were the case, the rate disease/infection could be much lower than the 1:18 reported previously in Brazil.[22] Because of this, prospective follow-up surveys should be undertaken to study the long-term outcome of asymptomatic carriers of *Leishmania*. Only in this way we will able to know the proportions of *Leishmania*-infected cases show self-cure, persistent cryptic infection, or overt visceral leishmaniasis.

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