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Use of Noninvasive Markers To Detect *Leishmania* Infection in Asymptomatic Human Immunodeficiency Virus-Infected Patients▽

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Visceral leishmaniasis (VL) caused by *Leishmania infantum* is a common disease in human immunodeficiency virus (HIV)-infected people in the Mediterranean basin. However, most such cases are asymptomatic, and little information about the prevalence of these infections in HIV-infected individuals is available. The aim of this study was to assess the prevalence of subclinical infection and the relationship between several *Leishmania* infection markers by noninvasive methods in asymptomatic HIV-infected patients from Southern Spain. Ninety-two HIV-infected patients, who were consecutively attended at the participant hospitals in 2004, were invited to participate in this study. These patients were asymptomatic and without any history of cutaneous or visceral leishmaniasis. *Leishmania* kinetoplast DNA (kDNA) was amplified from peripheral blood samples from 28 (30.4%) of these HIV-infected subjects. Sera from three (3.5%) patients tested positive for *Leishmania* by an enzyme-linked immunoassay. Two patients (2.4%) showed a specific 16-kDa band by Western blotting. In contrast, none of the patients showed a positive agglutination of urine. The leishmanin skin test was positive for four (4.3%) patients. None of the patients with a PCR-positive result showed a positive reaction by enzyme-linked immunoassay or by specific bands in Western blotting or had a positive leishmanin skin test. In conclusion, *L. infantum* kDNA was detected in a large proportion of asymptomatic HIV-infected patients, although a demonstrable cellular or humoral immune response to this parasite was not shown. Conversely, *Leishmania* antigen in urine was not detected in these patients.

Visceral leishmaniasis (VL) caused by *Leishmania infantum* is a common coinfection in human immunodeficiency virus (HIV)-infected people in Spain (3), although most patients remain asymptomatic (15). Tissue culture or direct examination shows *Leishmania* promastigotes or amastigotes, respectively, in a considerable proportion of asymptomatic HIV-infected subjects (15). However, invasive techniques that are not often well tolerated by asymptomatic individuals are required to obtain these samples.

Recently, a number of noninvasive methods that are easier to perform and better tolerated have been developed for the diagnosis of leishmaniasis and asymptomatic VL. Serology and leishmanin skin tests (LST) are easy to use but have low sensitivity in HIV- and *Leishmania*-coinfected patients (3, 14). More recently, PCR-based methods for detecting *Leishmania* species have been used for testing peripheral blood samples (7, 8, 13, 21). In addition, diagnostic techniques have also been developed for the identification of *Leishmania* antigen in urine (4, 6, 23, 26, 27). Some studies have been undertaken in order to determine the prevalence of *Leishmania* infection in blood donors or asymptomatic individuals (5, 11, 21). However, the prevalence of asymptomatic VL in HIV-infected subjects is not well known. Moreover, little is known about the relationships between the results obtained by both PCR or urine antigen tests and those yielded by serology and LST.

The objective of this study was to assess the prevalence of asymptomatic leishmaniasis infection in asymptomatic HIV-infected people by PCR in peripheral blood and by urine antigen detection. Results from the these procedures were compared with the results obtained by enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB) methods to detect specific antibodies in sera and in an LST.

MATERIALS AND METHODS

Population studied. Ninety-three consecutive HIV-infected patients attended at two hospitals in Sevilla and Córdoba (southern Spain) in 2004 were invited to participate in a cross-sectional study. All patients were clinically asymptomatic, had had a regular follow-up in the previous 6 months, and had no history of cutaneous or visceral leishmaniasis. All patients provided blood and urine samples, answered a structured questionnaire, and underwent a skin test. The questionnaire included demographic and clinical data and identified the areas where the patients lived and their possible contact with dogs.

Diagnostic procedures. All samples were aliquoted and frozen immediately at −70°C until tested. The investigators performing laboratory tests were blinded to the results of the other techniques.

Skin tests. A suspension containing 10^6 *L. infantum* promastigotes/ml with 0.5% phenol was used (16). Solutions containing 10% candidal antigen or 20% tetanus toxoid and 0.5% phenol were used as positive or negative controls, respectively. All antigens were administered intradermally (0.1 ml) at the same time in the volar surface of the forearm. The test results were read after 48 h, using the ballpoint technique (1, 24). A result for a specific antigen was considered to be positive when a cutaneous induration of ≥5 mm was recorded.

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TABLE 1. Main characteristics of the study population according to the PCR results

Parameter[b]	No. (%) of subjects that were:		*P* value
	PCR positive (n = 28)	PCR negative (n = 64)	
Male gender	21 (75)	48 (75)	0.59
Median (Q1–Q3) age (yr)	40.9 (35.2–44.2)	39.1 (36.1–43.9)	0.9
Intravenous drug user	16 (57.1)	32 (50)	0.58
Median (Q1–Q3) CD4 cell count	419 (285–813)	630 (435–1,063)	0.25
Patients with HIV viremia under 1,000 copies/ml	22 (78.6)	54 (84.4)	0.19
Clinical AIDS	9 (32.1)	11 (20.9)	0.25
HAART as the first therapy of HIV infection[a]	15 (57.7)	33 (52.5)	0.42
Living in an urban area at the time of the study	9 (32.1)	27 (44.2)	0.25
Contact with dogs at any time	23 (83.3)	53 (79.6)	0.48
Contact with dogs at the time of the study	7 (25)	23 (39.9)	0.21
Canine leishmaniasis	0	0	

[a] HAART, highly active antiretroviral treatment.
[b] Q1–Q3, interquartile range.

Serum antibody testing. ELISA and WB methods were used to detect anti-*Leishmania* antibodies. Anti-human immunoglobulin G conjugated with a second antibody (horseradish peroxidase enzyme, diluted 1/15,000; Sigma, St. Louis, MO) was used for ELISA, as described previously (22). The results of the reactions (in units) were quantified using a positive serum sample as a calibrator and arbitrarily set at 100 units. The result was considered positive when a value above 20 units was observed.

WB was performed on 0.1% sodium dodecyl sulfate-13% polyacrylamide gels using a Mini-gel Bio-Rad system as described previously (2). Detection was performed with an anti-human immunoglobulin G conjugated with horseradish peroxidase. The appearance of a 14- and/or a 16-kDa *L. infantum* antigen band was considered a positive result (2).

PCR procedure. To detect *L. infantum* kinetoplast DNA (kDNA), frozen whole-blood samples were tested by a PCR-ELISA by following the methodology described previously (12, 13). Briefly, 200 μl of blood was used to isolate the DNA. After extraction with phenol, the DNA was resuspended in 20 μl of double-distilled sterile water. Aliquots (1 μl, 2 μl, and 3 μl) of the DNA suspension obtained from each patient were used for PCR amplifications. For each PCR, 5 ng and 10 ng of human DNA and a sample without DNA were used as negative controls, and the DNA obtained from 1,000 *L. infantum* promastigotes was used as a positive control. Absorbance values were read at 405 nm, and a value of ≥1 was considered positive. The result was considered positive when at least two of the three DNA suspensions were positive or if an increase in the absorbance value happened when the DNA concentration was increased (as long as an absorbance value of ≥1 was shown with 3 μl of the DNA suspension). The analytical sensitivity of the method was estimated to be 0.1 parasites/ml (12).

Urine antigen detection. Urine antigen detection was carried out, according to manufacturer's instructions, by a latex agglutination test (KAtex, Kalon Biological Ltd., United Kingdom) (4). The result of latex agglutination was considered to be positive or negative.

Statistical analysis. Continuous variables are expressed as medians (interquartile range), and categorical variables are presented as the percentage of numbers of cases. Frequencies were compared by the chi-square or Fisher's exact test, if a cell had an expected count of less than five. Continuous variables were compared by Student's t test if normal distributions were proven; otherwise the Mann-Whitney U test was used. Statistical analysis was done by using an SPSS statistical package 12 for Windows.

Ethical considerations. The ethics committee of each participant's center approved the study. All patients gave their written informed consent to participate.

RESULTS

Population features. Ninety-two HIV-infected subjects agreed to be included in the study. The study population included both previous intravenous drug users and non-drug injectors. Although most patients reported they had been dog owners at some time in their lives, none of them noticed a leishmaniasis episode in their dogs. The characteristics of the subjects according to the relationship between PCR results and other parameters are summarized in Table 1.

***Leishmania* infection markers.** *L. infantum* kDNA from 28 (30.4%) asymptomatic HIV-infected subjects was amplified. The relationship between the PCR results and those yielded by the diagnostic procedures for leishmaniasis infection are displayed in Table 2. Two of the patients with a positive result by ELISA (66.6%) also revealed a specific band of 16 kDa by WB and a positive LST.

DISCUSSION

Our results illustrate that a significant proportion of asymptomatic HIV-infected individuals show markers of *Leishmania* infection. Specifically, *Leishmania* kDNA was detected even in the absence of a demonstrable cellular or humoral immune response in asymptomatic HIV-infected individuals. Conversely, *Leishmania* antigen in urine was not detected in these individuals.

A high proportion of positive PCR results were found in the asymptomatic HIV-coinfected subjects in our study. To confirm our finding, PCR was repeated three times using a different DNA suspension. In addition, the detection of *L. infantum* kDNA in peripheral blood in our study is similar to data reported for asymptomatic individuals without HIV infection and at risk for parenterally transmitted diseases in the same area of endemicity in Spain (1) and in other countries (5, 10). *Leishmania* DNA was also detected in a similar proportion of blood donors from foci of endemicity in Monaco and the Balearic Islands (10, 21). These findings are in agreement with the evidence for an increased risk of *L. infantum* infection among intra-

TABLE 2. Positive results of the *Leishmania* infection markers according to the PCR results

Parameter	% PCR positive (n = 28)	% PCR negative (n = 64)
ELISA	0	3 (3.5)
Western blot	0	2 (2.4)
Antigen in urine	0	0
Leishmanin skin test	0	4 (4.3)

venous drug users or subjects at risk for the spread of parenterally transmitted agents by the use of nonsterile tattooing, piercing, or cocaine snorting with shared devices (17).

The presence of *L. infantum* kDNA in the blood of most of our patients did not lead to either a cellular or a humoral response in our patients. Surveys conducted to detect cryptic leishmaniasis in dogs also showed a higher prevalence by PCR-positive results than by antibody detection techniques (9, 22). Le Fichoux et al. (10) suggested that in healthy *L. infantum* carriers, episodes of parasitemia might be of low intensity and, therefore, a cellular or humoral immune response may not be triggered. The finding of kDNA in blood, without evidence of an immune response, could reflect this situation. In agreement with this suggestion, the transmission of *Leishmania* to individuals who received blood from kDNA-harboring blood donors has not been reported (10). Moreover, the levels of parasites detected in asymptomatic individuals are lower than those found in patients with active VL (5). That suggests that the blood parasitemia load would not be high enough to lead to overt disease. Another possible explanation could be that the blood kDNA detected by PCR methods in asymptomatic patients could be only fossil DNA without the ability to trigger the immune system. However, the reasons for these findings are not clear, but the evidence of *L. infantum* kDNA in blood found in subjects with no history of VL raises questions concerning their clinical consequences.

Conversely to that found in the patients included in this study, in asymptomatic people without HIV infection, it was observed that the greater the antibody titer (for *L. infantum*), the higher the proportion of PCR-positive samples (1). Discordance in the results of molecular and serological techniques for the diagnosis of leishmaniasis have previously been noticed. A positive result by serological techniques does not represent an active infection and may be related to the detection of immunological memory in the absence of the parasite; also, a negative result does not discount infection, mainly in cryptic forms (21).

In HIV-infected people, serological tests have been described as limited diagnostic tools (14, 18). Detectable levels of *Leishmania* antibodies were not found in more than 40% of HIV- and VL-coinfected subjects (3). Additionally, it is estimated that specific antiparasite antibody levels in patients with HIV coinfection are 50-fold lower than in patients with normal immunity. This fact has been associated with the pronounced disregulation of the immune system that occurs in HIV infection (14). Thus, the functional damage of cell-mediated immunity due to HIV infection could result in the absence of an antibody response to *Leishmania* infection, even if a low parasitemia is present. This impairment in the immune system could lead to the elevated percentage of false-negative *Leishmania* serology results detected in HIV-infected individuals (25).

The *Leishmania* antigen detection method in urine is an alternative diagnostic tool in subjects suspected of having VL (4, 6, 19, 23, 26, 27). Recently, antigen detection in urine was found to be an appropriate technique for monitoring the efficacy of treatment among HIV-infected patients with VL (19). However, little is known about the utility of the urine antigen detection procedure for asymptomatic HIV-infected patients. *Leishmania* antigen was not detected in urine in any of the individuals in our study. This may be due to the low level of circulating parasites in asymptomatic *Leishmania* carriers. Moreover, the detection of antigen in urine becomes negative after a successful chemotherapy regimen, and no relapses are observed in the majority of these patients, as happens with cultures (20). However, detection of parasitemia by PCR gives positive results for longer, and PCR is a more sensitive technique for detecting infection in the absence of clinical features. As mentioned above, the levels of *Leishmania* parasitemia detected in healthy individuals are lower than those found in patients with active VL (5). These observations support our hypothesis that the elimination of *Leishmania* antigen in urine is related to the level of parasitemia. Furthermore, under such conditions, the urine antigen detection method may not be a valid procedure to detect asymptomatic *Leishmania* infections in HIV-coinfected patients.

In spite of the high proportion of PCR-positive results found for asymptomatic HIV-infected people in our study, most of the asymptomatic *Leishmania* carriers may not show symptoms of clinical infection. Moreover, although the PCR method is a valid procedure for epidemiologic studies, the utility of the PCR method for the diagnosis of VL may be limited in HIV-coinfected patients, because the detection of kDNA in blood is not a marker of clinical disease (20). In order to clarify these issues, prospective studies should be undertaken to survey the long-term outcomes of asymptomatic infections by *L. infantum* in HIV-infected patients.

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