DETECTION OF *LEISHMANIA* INFECTION BY USING NON-INVASIVE
MARKERS IN ASYMPTOMATIC HUMAN IMMUNODEFICIENCY VIRUS-
INFECTED PATIENTS

**Running title:** Non-invasive markers in *Leishmania* and HIV coinfection

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ABSTRACT

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 non-invasive 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 previous history of cutaneous or visceral leishmaniasis. *Leishmania* kDNA was amplified from peripheral blood samples coming from 28 (30.4%) of this HIV-infected subjects. Sera from 3 (3.5%) patients tested positive for *Leishmania* by an enzyme linked immunoassay. Two patients (2.4%) showed a specific 16 kDa band by western-blot. In contrast, none of the patients gave a positive agglutination in urine. The leishmanin skin test was positive in 4 (4.3%) patients. None of the patients with a PCR positive result showed a positive reaction by enzyme linked immunoassay, specific bands in western-blot or 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.
BACKGROUND

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 show *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.

In the last years, a number of non-invasive methods, that are easier 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/*Leishmania*-coinfected patients (3, 14). More recently, PCR-based methods for detecting *Leishmania* species have been used in 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 know the prevalence of *Leishmania* infection in blood donors or asymptomatic individuals (5, 11, 21). However, the prevalence in asymptomatic VL in HIV-infected subjects is not well known. Moreover, little is known about the relationship 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 leishmaniasis asymptomatic infection by PCR in peripheral blood and urine antigen detection in asymptomatic HIV-infected people. Results from the above procedures were compared
with the results obtained by enzyme linked immunoassay (ELISA) and western-blot (WB) methods to detect specific antibodies in sera and a 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 a regular follow-up in the previous six months and had no prior 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, area where the patients lived and their possible contact with dogs.

Diagnostic procedures. All samples were aliquoted and frozen at -70°C immediately 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% candida 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 tests were read after 48 hours using the ballpoint technique (1, 24). A result for a specific antigen was considered to be positive when a cutaneous induration $\geq$ 5 mm was recorded.

Serum antibody testing: ELISA and WB methods were used to detect anti-Leishmania antibodies. An anti-human immunoglobulin G conjugated with a second antibody (Horse Radish Peroxidase enzyme, dilution 1/15000; Sigma, St. Louis, MO, USA) was used for ELISA as described previously (22). The results of the reaction results (in units) were quantified using a positive serum sample as a calibrator and
arbitrarily set at 100 units. A positive result was considered when a value above 20 units was observed.

WB was performed on 0.1% SDS-13% polyacrylamide gels using a Mini-gel BioRad System as described (2). Detection was performed with an anti-human immunoglobulin G conjugated with horse radish peroxidase. Appearance of a 14- and/or 16- kDa *L. infantum* antigen band was considered positive result (2).

**PCR procedure:** To detect *L. infantum* kDNA, frozen whole blood samples were tested by a PCR-ELISA 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. In 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 1000 *L. infantum* promastigotes was used as a positive control. Absorbance values were read at 405 nm and values ≥1 was considered positive. A positive result was considered when, at least, two of the three DNA suspensions were positive or if an increase of the absorbance value happened when the DNA concentration was increased (as long as an absorbance value ≥1 was shown with a 3 µL of the DNA suspension). The analytical sensitivity of the method was estimated to be 0.1 parasites/mL (12).

**Urine antigen detection:** Antigen detection was carried, according to manufacturer’s instructions, by a latex agglutination test (KAtex, Kalon Biological Ltd, UK) (4). The result of latex agglutination was considered as positive or negative.

**Statistical analysis:** Continuous variables are expressed as median (interquartile range) and categorical variables are presented as number of cases (%). The frequency comparisons were done by chi-square or Fisher exact test, if a cell had an expected
count lesser than five. Continuous variables were compared by student t-test if normal
distributions were proven. The Mann-Whitney U test was used otherwise. Statistical
analysis was done by SPSS statistical package 12 for Windows.

**Ethical considerations.** The Ethics Committee of each participant 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 to have been dogs owners at any time in their lives, none of them noticed a leishmaniasis’ episode in their dogs. The characteristics of the subjects according to the relationship of PCR results with 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 in 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 in 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 in asymptomatic individuals without HIV infection and at risk for parenterally transmitted diseases in the same endemic area of Spain (1) and in other countries (5, 10). *Leishmania*-DNA was also detected in similar proportion of blood donors from endemic foci of Monaco and the Balearic Islands (10, 21). These findings are in agreement with the evidence of an increased risk for *L. infantum* infection among intravenous drug users or subjects at risk for the spreading of parenterally transmitted agents by using non sterile tattooing, piercing or cocaine snorting with shared devices (17).

The presence of *L. infantum* kDNA in blood in most of our patients did not lead to either a cellular or humoral response in our patients. Surveys conducted to detect cryptic leishmaniasis in dogs also showed a higher prevalence of PCR positive results than those obtained 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 therefore a cellular or humoral immune response may not be triggered. The finding of kDNA in blood, without evidence of immune response could reflect this
situation. In agreement with this suggestion, transmission of *Leishmania* in individuals who received blood from kDNA harbouring 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 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 previous 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 antibody titre (for *L. infantum*), the higher the proportion of PCR positive samples (1). Discordances in the results of molecular and serological techniques for diagnosis of leishmaniasis have previously been noticed. A positive result in serological techniques does not represent an active infection and may be related with the detection of immunological memory in the absence of the parasite; also a negative result does not discard 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 detected in more than 40% of HIV/VL-co-infected subjects with VL (3). Additionally, it is estimated that specific antiparasite antibody levels in 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*, even if a low parasitemia is present. This impairment in the immune system could lead to the elevated percentage of false negative *Leishmania* serology detected in HIV-infected individuals (25).

*Leishmania* antigen detection method in urine is an alternative diagnostic tool in subjects suspected to have 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 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 and no relapses are observed in the majority of these patients, as it happens with cultures (20). However, the PCR remains positive for longer and it 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 detected in 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 to 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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TABLE 1. Main characteristics of the study population according to the PCR results.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCR positive (n = 28)</th>
<th>PCR negative (n = 64)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender, n (%)</td>
<td>21 (75)</td>
<td>48 (75)</td>
<td>0.59</td>
</tr>
<tr>
<td>Median (Q1-Q3) ages, in years</td>
<td>40.9 (35.2-44.2)</td>
<td>39.1 (36.1-43.9)</td>
<td>0.9</td>
</tr>
<tr>
<td>Intravenous drug users, n (%)</td>
<td>16 (57.1)</td>
<td>32 (50)</td>
<td>0.58</td>
</tr>
<tr>
<td>Median (Q1-Q3) CD4 cell counts</td>
<td>419 (285-813)</td>
<td>630 (435-1063)</td>
<td>0.25</td>
</tr>
<tr>
<td>Patients with HIV viremia under 1000 copies/mL</td>
<td>22 (78.6)</td>
<td>54 (84.4)</td>
<td>0.19</td>
</tr>
<tr>
<td>Clinical AIDS, n (%)</td>
<td>9 (32.1)</td>
<td>11 (20.9)</td>
<td>0.25</td>
</tr>
<tr>
<td>HAART\textsuperscript{a} as the first therapy of HIV infection</td>
<td>15 (57.7)</td>
<td>33 (52.5)</td>
<td>0.42</td>
</tr>
<tr>
<td>Living in an urban area at the time of the study</td>
<td>9 (32.1)</td>
<td>27 (44.2)</td>
<td>0.25</td>
</tr>
<tr>
<td>Contact with dogs at any time</td>
<td>23 (83.3)</td>
<td>53 (79.6)</td>
<td>0.48</td>
</tr>
<tr>
<td>Contact with dogs at the time of the study</td>
<td>7 (25)</td>
<td>23 (39.9)</td>
<td>0.21</td>
</tr>
<tr>
<td>Canine leishmaniasis</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a}HAART: highly active antiretroviral treatment.
TABLE 2. Positive results of the *Leishmania* infection markers according to the PCR results. The results are represented as number (%) of individuals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCR positive (n = 28)</th>
<th>PCR negative (n = 64)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>0</td>
<td>3 (3.5)</td>
</tr>
<tr>
<td>Western-Blot</td>
<td>0</td>
<td>2 (2.4)</td>
</tr>
<tr>
<td>Antigen in urine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leishmanin skin test</td>
<td>0</td>
<td>4 (4.3)</td>
</tr>
</tbody>
</table>