Relapses versus Reinfections in Patients Coinfected with Leishmania infantum and Human Immunodeficiency Virus Type 1

Miguel A. Morales,1 Israel Cruz,1 Jose M. Rubio,2 Carmen Chicharro,1 Carmen Cañavate,1 Fernando Laguna,3 and Jorge Alvar1

In the Mediterranean basin, Leishmania infantum is a major opportunistic parasite in people with acquired immunodeficiency syndrome (AIDS), and up to 9% of the patients with AIDS suffer from newly acquired or reactivated visceral leishmaniasis. Distinguishing between reinfections and relapses in these patients is important because some apparent treatment failures occur in patients with new rather than reactivated infections. Isoenzyme characterization is limited for use in determining relapsed versus newly acquired leishmaniasis in human immunodeficiency virus (HIV)–infected patients because of the variability of L. infantum and the predominance of the MON-1 zymodeme in people coinfected with HIV. A seminested polymerase chain reaction (PCR) was used to amplify L. infantum minicircle kinetoplast DNA, and, after digestion, the restriction fragment–length polymorphism (RFLP) profiles showed that 3 (7.5%) of 40 patients coinfected with L. infantum and HIV had a new infection, whereas isoenzyme characterization indicated that all 40 patients had infection relapses. These results suggest the utility of this PCR-RFLP analysis in detecting leishmaniasis reinfection in HIV-positive patients.

In the Mediterranean basin, Leishmania infantum is the infectious agent of both visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) and has been shown to be an important opportunistic parasite in patients with AIDS. VL is the clinical form most frequently associated with human immunodeficiency virus type 1 (HIV-1) and AIDS in southwestern Europe, and up to 9% of people with AIDS suffer from newly acquired or reactivated VL. Another cause for concern is the increased number of cases of coinfection in eastern Africa and the Indian subcontinent, which is due to the simultaneous spread and geographic overlap of both diseases and periodic epidemics of VL [1].

The VL and CL relapse/reinfection dichotomy is a major concern in patients with AIDS, who are at risk for opportunistic infections. In areas where the diseases are endemic, the rate of reinfections might be higher than estimated. This could lead physicians to believe that treatment for relapsed infection failed when, in fact, the patient has a newly acquired infection. Therefore, the inability to distinguish relapses from reinfections might be an important impediment to the evaluation of leishmaniasis treatment protocols.

Enzymatic characterization is the most common method used to study the variability of Leishmania species, and this method has shown the extreme variability of L. infantum in HIV-positive patients. However, half the Leishmania isolates from HIV-infected persons belong to a single zymodeme (MON-1), and isoenzyme characterization cannot differentiate relapsed infections from newly acquired infections when sequential isolates from the same individual exhibit the same zymodeme. Several researchers have developed molecular methods, mainly based on restriction fragment–length polymorphism (RFLP) analysis, to resolve this problem [2, 3]. The use of polymerase chain reaction (PCR) and RFLP analysis of the amplified product to detect reinfections was successful in a preliminary study with 10 L. infantum stocks from 5 HIV-positive patients [4]. The kinetoplast contains ~10000 minicircles of DNA, and the conserved region of these minicircles is an ideal target for PCR assays.

Almost 80% of patients with VL respond initially to classic chemotherapy (pentavalent antimony); however, the disease shows a clear tendency to relapse in HIV-positive patients (27% and 60% of the patients relapse within 6 and 12 months, respectively, after treatment), with the time between the first and second relapse being shorter than the time between the first episode and first re-

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All patients gave signed, informed consent for this study, which had ethics committee approval from all hospitals involved.

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of diagnosis (before treatment). The results of this trial are the subject of a different publication (authors’ unpublished data).

Materials and Methods

Patients. Forty HIV-1–infected patients from Spain who were suspected, on the basis of clinical data, of having leishmaniasis were studied. HIV positivity was established by ELISA and confirmed by Western blot analysis. All patients belonged to an open, randomized, multicenter trial comparing the efficacy of amphotericin B lipid complex against antimonials. The results of this trial are the subject of a different publication (authors’ unpublished data). Thirty-one patients were confirmed as having leishmaniasis, as determined by bone marrow culture and/or microscopy; the other 9 cases were confirmed by PCR. Characteristics for 3 of the study patients who were determined to have new infections are summarized in table 1.

Collection of biologic samples. Bone marrow aspirate (200 μL) and EDTA-treated blood (10 mL) samples were obtained on the day of diagnosis and 1 month after treatment ended. When possible, samples were also obtained during follow-up (1–20 months after treatment ended). When possible, isolates of L. infantum were obtained from bone marrow. The techniques used for culturing and extracting total DNA have been described elsewhere [4, 7].

kDNA snPCR-RFLP analysis. DNA extraction from blood samples and bone marrow aspirates has been described elsewhere [8]. In brief, for the first amplification, 5 μL of DNA solution was added to 20 μL of PCR mix containing 15 pmol specific Leishmania primers D3J, 5′-CGA TTT TTG AAC GGG ATT TCT GCA C-3′; and KLK2, 5′-CTC CGG GGC GGG AAA CTG G-3′; 0.2 mM dNTP (Amersham Pharmacia Biotec); 2 mM MgCl2; 5 mM KCl; 75 mM Tris HCl (pH 9.0); 2.0 mM (NH4)2SO4; 0.001% bovine serum albumin; and 0.7 U of Tth (Thermus thermophilus) DNA polymerase (Biotechnologies B&M Laboratories). A GenAmp System 2400 thermocycler (Perkin-Elmer) was used with the following cycling conditions: 80°C for 2 min, 94.5°C for 5 min, 35 cycles at 94°C for 30 s and at 68°C for 30 s, with an extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min. Amplification products were visualized on a 1.6% agarose gel, with a 100-bp DNA ladder (Boehringer Mannheim) as a molecular weight marker, and stained with ethidium bromide (10 mg/mL). Samples with an 800-bp PCR product were considered to be positive for Leishmania DNA.

In the snPCR amplification, the reaction consisted of the reamplification of the previous PCR product, to improve the sensitivity and specificity of the reaction: 5 μL of a 1:200 dilution of the first PCR product was added to 20 μL of PCR mix under the same conditions as the first amplification, with 7.5 pmol D3J primer and 7.5 pmol of new Leishmania-specific primer AJS31, 5′-GGGTTGGTGTAA-AATAGGGCCGG-3′ [9], 0.7 U of Tth, and a new annealing temperature in the cycling parameters (70°C). Amplification reactions were visualized on a 1.6% agarose gel, and positive samples yielded a PCR product of 780 bp.

Table 1. Data for human immunodeficiency virus type 1–positive patients in Spain who were determined to have newly acquired Leishmania infections, by use of seminested polymerase chain reaction–restriction fragment–length polymorphism analysis of kinetoplast minicircles of DNA (kDNA snPCR-RFLP).

<table>
<thead>
<tr>
<th>Patient code, date biologic samples obtained*</th>
<th>Result of kDNA snPCR-RFLP analysis</th>
<th>Therapeutic regimenb</th>
<th>Zymodeme type</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRJ 26 Oct 1998, A 1 Dec 1998, B 19 May 1999, C</td>
<td>Shift of the RFLP pattern, indicating reinfection</td>
<td>Amphotericin B lipid complex (3 mg/kg) intravenously once daily for 5 days</td>
<td>MON-34</td>
</tr>
<tr>
<td>JAE 16 Apr 1998, A 24 Jun 1998, B 13 Oct 1999, C</td>
<td>Shift of the RFLP pattern, indicating reinfection</td>
<td>Amphotericin B lipid complex (3 mg/kg) intravenously once daily for 10 days</td>
<td>MON-1</td>
</tr>
<tr>
<td>APB 7 Oct 1997, A 28 Oct 1997, B</td>
<td>Shift of the RFLP pattern, indicating reinfection</td>
<td>Amphotericin B lipid complex (3 mg/kg) intravenously once daily for 5 days</td>
<td>MON-24</td>
</tr>
</tbody>
</table>

* A–C indicate, respectively, samples obtained on the day of diagnosis (before treatment), 1 month after treatment ended, and during follow-up (up to 20 months after treatment ended).

b Treatment failed in all 3 patients.
In all assays, negative controls without DNA and negative controls with healthy human DNA were used. A positive control (DNA from 10 promastigotes) was also used.

Restriction analysis. Digestions were carried out as described elsewhere [4]. To assure that partial digestions were not occurring, samples were electrophoresed at a wide range of digestion times.

Cloning and DNA sequencing. Fifty randomly selected PCR products were subcloned in a vector system (pGEM-T; Promega). Ten clones from each product were sequenced on a DNA sequencer (ABI Prism, model 377; Applied Biosystems).

Parasite cloning. The cloning method we used has been described elsewhere [10]. In brief, a single parasite was isolated from a limiting dilution into a 96-well plate and transferred onto a blood-agar plate after examination by microscope. It was then cultured under the same conditions as the parent strain.

Results

The first PCR, using DNA from *L. infantum*, resulted in a single product of 800 bp, whereas the second PCR product was a fragment of 780 bp. Subcloning these 2 products in a commercial vector system and sequencing revealed that a major minicircle class was always amplified in each isolate, indicating that restriction patterns did not randomly appear.

DNA samples from nonpathogenic flagellates (*Leptomonas*, *Phytomonas*, *Herpetomonas*, *Criithidia*, *Endotrypanum*, and *Blastocrithidia* species) were also used to test the specificity of this PCR, and no amplification was observed.

Two or more pre- and posttreatment sequential samples were studied for 40 patients who were coinfected with *Leishmania* and HIV and treated for VL. To distinguish between relapses and reinfections, we tested 256 clinical samples and 56 *Leishmania* stocks (derived from the bone marrow of patients) that were cultured in NNN medium. PCR-RFLP analysis of the samples revealed that 3 (7.5%) of the 40 patients had a newly acquired infection, whereas all the patients were considered to have relapses by isoenzyme characterization.

Figure 1B shows sequential isolates from 1 of the HIV-*Leishmania*–coinfected patients. PCR was done on 6 bone marrow

![Figure 1](https://academic.oup.com/jid/article-abstract/185/10/1533/837148)
samples, and the product was digested using Hpa II. The restriction patterns are identical, revealing a typical relapse, which was also confirmed by isoenzyme analysis (zymodeme MON-1 was characterized in all stocks). The last isolate sample for the patient was obtained 2 years after the first. Another patient with relapse is represented in figure 1A, giving evidence of the maintenance of the profiles, regardless of the enzyme (Hpa II or Rsal) used for RFLP analysis.

Figures 1C, 1D, and 1E present examples of reinfection in 3 HIV-Leishmania–coinfected patients. Isoenzyme analysis had determined the infections to be relapses (MON-1, MON-34, and MON-24 were typed in the 3 stocks, respectively, before and after treatment), but kDNA snPCR-RFLP analysis clearly shows different patterns, as determined by use of different restriction endonucleases. Sequence data from isolates taken before and after treatment from patient GRJ (GenBank access numbers: AJ275326 and AJ275331) confirms the shift of RFLP profile.

To confirm that the different restriction patterns observed in reinfections were not due to a mosaic structure population, we cloned (in blood-agar plates) pre- and posttreatment strains isolated from a coinfected and reinfected patient. Eight clones from each strain were identical (in regard to PCR-RFLP profiles) to each other and to the parental strain, suggesting that the new pattern, which was thought to be a reinfection, was not formerly present in the population of that strain.

When possible, we isolated and cultured the parasite from clinical samples (56 Leishmania stocks isolated from bone marrow samples of patients). In all these cases, PCR-RFLP profiles were identical whether we worked directly with biologic samples or with their respective cultured Leishmania stock, indicating that cultures do not select any of the clones or affect the latter’s restriction patterns.

Discussion

In the Mediterranean basin, L. infantum is extremely variable in patients coinfected with HIV. Enzyme analysis has been widely used to characterize these infections and it remains a useful technique for epidemiologic purposes or as a taxonomic tool; however, isoenzyme characterization has certain limitations when individual stocks from the same HIV-coinfected patient need to be tracked through time. In addition, >50% of L. infantum stocks isolated from HIV-positive subjects are MON-1 [11], and the heterogeneity of stocks identified as MON-1 has been described after analysis with more discriminating methods [12]. Thus, in most cases, enzyme analysis does not reveal whether leishmaniasis infections are due to relapses or newly acquired infections.

As expected, the snPCR detected extremely small amounts of L. infantum in biologic samples from these patients, and we found that the PCR was highly specific for Leishmania species and that nonpathogenic flagellates, which are usually present in samples from immunocompromised patients, were not amplified. The primers are expected to amplify all L. infantum minicircles present in the sample, and, as discussed elsewhere [4, 13], only one major minicircle class is found in the PCR product. In addition, the kDNA snPCR-RFLP analysis that we used has shown excellent reproducibility and reliability. We must take into account that the use of 6 different restriction enzymes (1 per digestion) ensures the similarity or lack of similarity between the profiles analyzed. Unlike results with isoenzyme characterization, kDNA snPCR-RFLP results can be obtained and interpreted within a few hours once the total DNA has been extracted.

It is remarkable that, in leishmaniasis relapses occurring long after the original infection, the same restriction pattern has persisted even though it has been proposed that minicircle sequences evolve rapidly [14]. However, recent evidence [15] has shown that the restriction sites utilized in PCR-RFLP analysis of the major minicircle classes are conserved over a considerable time (at least 2–3 years, the longest time patients in this study have been monitored).

snPCR-RFLP analysis revealed that the restriction profiles for the 56 Leishmania stocks, which were cultured in NNN medium, were identical to those from their respective biologic samples, providing confirmation that culturing does not select any specific clone and providing support for the use of these faster molecular techniques to complement data from isoenzyme analysis. Moreover, in certain situations (e.g., when contamination is present), it is not possible to culture the parasite.

In this study, 3 (7.5%) of the 40 patients had a newly acquired, rather than a relapsed, Leishmania infection. The shift in RFLP patterns might be due to the detection of a minority Leishmania population remaining after chemotherapy. However, to our knowledge, mixed infections due to L. infantum have not been reported, thus providing support for the data presented here.

As determined by isoenzyme characterization of zymodemes, all patients in this study had a relapse of leishmaniasis, and although Leishmania–HIV–coinfected patients have the tendency to relapse after treatment [8], the fact that most of these HIV-positive patients are intravenous drug users increases their risk of reinfection. Among these subjects, the habit of sharing syringes provides a mechanical substitute (the syringe) for the natural vector (the sandfly) and confirms the increased risk of acquiring new infections for such individuals [16].

In conclusion, use of kDNA snPCR-RFLP analysis could provide more information for physicians to determine whether leishmaniasis infections that fail to respond to treatment are newly acquired infections rather than isoenzyme analysis–determined relapses.

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