Genetic variability within the species *Leishmania infantum* by RAPD. A lack of correlation with zymodeme structure

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**Abstract**

The infraspecific variability of the species *Leishmania infantum* is studied by using genetic markers generated by random amplified polymorphic DNA (RAPD). We have applied this technique, using 18 primers of arbitrary sequence, to 33 strains of the parasite belonging to 18 zymodemes isolated in different clinical forms and hosts. Other strains belonging to the species *L. donovani*, *L. major*, *L. tropica* and *L. mexicana* were used as a reference. The RAPD technique produced very different genetic profiles between *L. infantum* and *L. major*, *L. tropica* and *L. mexicana* with all primers used, whereas 11 of the 18 primers distinguished *L. infantum* strains from the species *L. donovani*. All primers except 1 (TAF 300), generated polymorphism in the *L. infantum* strains. The dendrograms constructed with the isoenzyme data and with RAPD are congruent in relation to the separation of the different species but show little agreement within the *L. infantum* species, reflecting the genetic heterogeneity of the strains belonging to one zymodeme. A geographical structuralisation is observed with two diverging groups that evolve independently whereas there is no relation between the genotype of the parasite and the host or between the former and the clinical form of the disease. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords**: *Leishmania infantum*; RAPD; Infraspecific genetic variability; Zymodeme

1. Introduction

*Leishmania infantum* is the aetiological agent of Leishmaniasis in Spain. In this country, in man, it causes both the cutaneous (CL) and the visceral forms (VL), including a high proportion of cases of *Leishmania*-human immunodeficiency virus (HIV) coinfection. In dogs, which are the main reservoir for the parasite, it produces canine leishmaniasis. The main vectors are Phlebotomus perniciosus and, to a lesser extent, *P. ariasi*, both of the subgenus Larroussius.

The gold standard technique for the characterisation of *Leishmania* species is isoenzyme electrophoresis that involves examination of a panel of 15 enzymatic loci [1]. This technique is also useful for infraspecific characterisation and in *L. infantum* has revealed the existence of a wide variety of zymodemes with a heterogeneous geographical distribution throughout the Mediterranean Basin [2–6]. Several attempts have been made to find a relationship between these zymodemes and the different clinical forms of the disease using concepts such as dermotropic, viscerotropic and apathogenous [7].

In southern Spain, enzymatic polymorphism studies of strains of the parasite isolated in man, dog and sand fly, have revealed the existence of 20 [4,5,8,9] of the 39 zymodemes that currently constitute the *L. infantum* complex [1,2].

MON-1 is the most common zymodeme and is widely distributed throughout the Mediterranean basin. It is the zymodeme most frequently isolated in dogs also in southern Spain. Other enzymatic variants have

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*Abbreviations*: CL, human cutaneous leishmaniasis; HIV, human immunodeficiency virus; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphisms; RAPD, random amplified polymorphic DNA; VL, human visceral leishmaniasis.

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only occasionally been identified in the canine reservoir [2,9,10] and the identity of the reservoirs of a great many zymodemes remains unresolved.

On the other hand, some authors have identified a high degree of additional genetic variability between strains belonging to zymodeme MON-1 [11] and others have suggested a strong host selection [12]. Noyes et al. 1997 [13] found only small differences between strains of L. chagasi MON-1 from Honduras by random amplified polymorphic DNA (RAPD), differential display, pulsed-field gel electrophoresis and schizodemes. This suggests the need to use other genetic markers, in addition to isoenzymes, for the infraspecific study of L. infantum.

Recently, polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) and RAPD have been shown to be valid techniques to demonstrate the genetic variability within and between different Leishmania species, including the L. donovani complex [12,14,15]. The RAPD technique has the added advantage of not requiring previous knowledge of the DNA sequence to design the primers and of only using small amounts of DNA. The latter, is, a priori, an important advantage over isoenzyme analysis that requires an amount of DNA. The latter, is, a priori, an important advantage of not requiring previous knowledge of the DNA sequence to design the primers and of only using small amounts of DNA. The latter, is, a priori, an important advantage of not requiring previous knowledge of the DNA sequence to design the primers and of only using small amounts of DNA. The latter, is, a priori, an important advantage of not requiring previous knowledge of the DNA sequence to design the primers and of only using small amounts of DNA.

In this work, we analyse the infraspecific variability of the species L. infantum in the geographically limited region of southern Spain by using genetic markers generated with the RAPD technique. The strains used in the study are highly polymorphic from an isoenzymatic perspective (18 zymodemes) and have been isolated from different clinical forms and hosts, permitting these factors to be studied as well.

2. Materials and methods

2.1. Leishmania strains

Genomic DNA was extracted by proteinase K digestion and ethanol precipitation [16] from 33 Spanish strains of L. infantum. All the strains have been previously characterised by isoenzymes (15 enzymatic loci) [4,5,8,9] and belong to 18 different zymodemes (Table 1). In each isolate only one zymodeme was identified. Other reference strains used belong to the species, L. donovani (1 strain), L. major (1), L. tropica (1) and L. mexicana (1).

2.2. Random amplified polymorphic DNA

Sixteen arbitrary primers and two L. infantum specific primers (PLID2-9 and PLID2-83) (Table 2) were used upon the 37 Leishmania strains. Each 25 ul of RAPD reaction contained: 2 mM MgCl₂, 0.2 mM dNTPs, 25 pmol primer, 1.25 U Taq polymerase (Bioculter) in the buffer recommended by the manufacturer ([(NH₄)₂]SO₄) and the DNA equivalent to 1000 parasites. Amplifications were carried out using, one cycle of 94 °C for 2 min; 40 cycles of, 94 °C for 30 s, 36 °C (60 °C for primers M13 and 037) for 1 min and 72 °C for 2 min; one cycle of 72 °C for 2 min. RAPD products were separated on 1.5% agarose gels in TBE buffer, with ethidium bromide, at 90 V for 5 h.

RAPD profiles were scored as presence or absence. Dendrograms were built from a distance matrix (Jac-
card index, 1 – (a/a + b + c)) using unweighted pair group method with arithmetic averages (UPGMA) clustering algorithm. Computer analyses used program STAT-ITCF. Phylogenetic relationships among Leishmania strains were analysed using mixed method parsimony (MIX) program in the phylogenetic inference package (PHYLIP) and bootstrap analysis.

2.3. Reproducibility and sensitivity of RAPD

The reproducibility of the RAPD fingerprints obtained under the amplification conditions specified above was confirmed. To do this, DNA from the randomly selected strains DP210, DP170 and DP154 was repeatedly amplified (three to four times) with each one of the 18 primers and the profiles of the bands obtained were compared.

Moreover, in an attempt to establish the minimum amount of cultured promastigotes that could be amplified with completely reproducible results, we started with a known number of parasites counted with a Thoma camera and carried out DNA extraction as described previously. This DNA was resuspended in sterile water and appropriate dilutions were made to obtain the DNA equivalent to 1, 10, 100 and 1000 cultured promastigotes, that we had repeatedly amplified (three to four times) with each of the primers recorded in Table 2.

3. Results

3.1. Reproducibility and sensitivity of RAPD

The results were completely reproducible under the amplification conditions used, and both within each dilution and between dilutions. This demonstrates that the technique is sensitive to quantities of DNA equivalent to 1–1000 promastigotes.

3.2. RAPD fingerprints

The RAPD technique produces very different genetic profiles between the L. infantum species and L. tropica, L. major and L. mexicana, with all tested primers. The primers ILO 509, ILO526, ILO 872, ILO 876, ILO 878, TA150, TA610, M13, 037, PLiD2-9 and PLiD2-83 separated the L. infantum strains from the L. donovani species. Most of the differences between the genetic profiles of theses last two species are provided by primers ILO 878, PLiD2-9 and PLiD2-83. In total, RAPD has generated 485 genetic markers of which only 1.6% are shared by the five species studied.

Within the L. infantum species, variation is observed with all the primers except with TAF 300. The most polymorphic genetic profiles between strains of the L. infantum species were generated by the following primers, ILO 872 with 11 of the 14 fragments amplified being polymorphic, TA-160 with 10 of the 11 amplified, ILO 875 with nine of the 13 fragments being polymorphic, TA-150 with 10 of the 11 amplified, ILO876 and ILO878 were highly conserved with only one polymorphic DNA fragment of the 25 amplified with ILO876 and one of the 26 amplified with ILO878 (Figs. 1 and 2). With primer TAF-300 a single 880 bp fragment was amplified for L. infantum and L. donovani species.

3.3. Estimation of genetic diversity

The results obtained with the isoenzyme (Fig. 3) and RAPD data (Fig. 4) were consistent with the separation of species. In both UPGMA dendrograms, strains of the L. infantum species were grouped together with genetic distances shorter than 0.310 and 0.229, respectively. The cluster as a whole was independent from the other species studied. The genetic distance between the species L. infantum and L. donovani was estimated as 0.467 in the dendrogram generated with the isoenzymatic data and 0.412 in that constructed from the RAPD results.

Within the L. infantum species, the dendrograms obtained with both techniques showed very little overlap. The RAPD dendrogram separated the L. infantum strains into two main clusters, with 26 and 7 strains, respectively (Jaccard’s distance = 0.229). The two clusters are linked to geographical origin, the first included strains isolated from Huelva and Malaga provinces in the central-western region of Spain. The second corresponded to 26 strains isolated from eastern Spain (Almeria and Granada provinces).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Sequence data of the primers used</th>
</tr>
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<tbody>
<tr>
<td>Primer code</td>
<td>Sequence (5’–3’)</td>
</tr>
<tr>
<td>ILO 509</td>
<td>CGGCAGCTGT</td>
</tr>
<tr>
<td>ILO 524</td>
<td>CGCGCCGC</td>
</tr>
<tr>
<td>ILO 526</td>
<td>GCGTCCGA</td>
</tr>
<tr>
<td>TA 150</td>
<td>ATCCGATGGATGTTGAG</td>
</tr>
<tr>
<td>TA 610</td>
<td>TCACCGATTACATAAACA</td>
</tr>
<tr>
<td>ILO 876</td>
<td>GGGAGCCTTC</td>
</tr>
<tr>
<td>ILO 872</td>
<td>CCCGCCATCT</td>
</tr>
<tr>
<td>ILO 525</td>
<td>CGAGCCTCG</td>
</tr>
<tr>
<td>ILO 878</td>
<td>GTGCCGGAG</td>
</tr>
<tr>
<td>ILO 509</td>
<td>TGTCAGCTGA</td>
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<tr>
<td>ILO 868</td>
<td>CAGCCTCGGC</td>
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<tr>
<td>ILO 875</td>
<td>GTCCGTGAGC</td>
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<tr>
<td>TAR 752</td>
<td>TGAACGACCTATGTCC</td>
</tr>
<tr>
<td>TAF 300</td>
<td>CACCTCAAACATACCC</td>
</tr>
<tr>
<td>M 13</td>
<td>TGACCGGCAGCAAATG</td>
</tr>
<tr>
<td>037</td>
<td>TGGATCCGGGAGTTTCCGTCAC</td>
</tr>
<tr>
<td>PLID2–9</td>
<td>CAAAAGTCCCCACCAATCCC</td>
</tr>
<tr>
<td>PLID2–83</td>
<td>AAACCTGTGCTTGGAGGCTTAG</td>
</tr>
</tbody>
</table>
With the RAPD technique, none of the strains were identical, i.e. no pair of strains had a Jaccard distance equal to zero. The smallest genetic difference calculated was between DP 289 and DP 418, that had a \(J = 0.038\). Both strains were isolated from humans in the Granada province, the first from a cutaneous form and DP 418 from a case of visceral leishmaniasis with HIV-coinfection. These belonged to the different zymodemes MON-80 and MON-34, respectively.

The UPGMA dendrogram derived from the isoenzyme data separated the \(L.\) infantum strains into two different clusters \((J = 0.310)\) these were not related to either the host, clinical manifestation of the disease or the geographical origin. Neither was any structuralisation into dermotropic, viscerotropic or apathogenous zymodemes apparent. As expected, the genetic distance among the group of strains belonging to the same zymodeme was zero.

### 3.4. Evolutionary trees

Phylogenetic trees were constructed using the Wagner parsimony method with \(L.\) mexicana as the out-group. The consensus tree generated with the isoenzyme data was not supported by bootstrap analysis (data not shown). The evolutionary relationships obtained with the RAPD data are shown in Fig. 5. The trees clustered the \(L.\) infantum strains placing \(L.\) donovani outside the group. This clustering was supported by 100% of bootstrap replicas. Within the \(L.\) infantum species, the separation of the two geographical groups corresponding to the eastern strains (from Granada and Almeria) and the central-western strains (from Huelva and Malaga) was also supported by 100% of bootstrap replicas.

### 4. Discussion

The possibility of applying RAPD to amounts of DNA equivalent to only one promastigote, with completely reproducible results, is a clear advantage over using isoenzyme electrophoresis as a characterisation method that requires hundreds of millions of parasites.
A total of 51.25% (103 of 201) of the fragments amplified in the \textit{L. infantum} strains studied were polymorphic corroborating the great intraspecific heterogeneity of this species in southern Spain, previously demonstrated by isoenzyme data. Indeed, the 20 zymodemes found in our study area represent the greatest isoenzymatic polymorphism described in an area in which \textit{L. infantum} is endemic. Six of the zymodemes included in the study were represented by several strains of the parasite whereas the rest were represented by only one. None of the amplified fragments could be used as a genetic marker of any of these zymodemes and the strains belonging to each of these were more or less scattered around the dendrogram (Fig. 4). This reflected a lack of genetic uniformity within the zymodeme and confirmed the results of Bañuls et al., 1999 [11], with strains belonging to MON-1. Nevertheless, the strains identified by isoenzymes as zymodeme MON-24 appeared to present a higher degree of genetic homogeneity with a mean distance between them of 0.117. Of these, DP423 was the strain most genetically differentiated from the others (mean distance from the group of 0.121), with the peculiarity that this strain was isolated from a human case of VL in an apparently immunocompetent individual. MON-24 is one of the zymodemes classically considered as dermotropic. This has a very wide geographical distribution (detected in seven countries) and has not been isolated in dogs although it has been found in a sand fly vector (\textit{P. perniciosus} and \textit{P. ariasi}) [4,5].

The strains DP418 and DP435, both identified as MON-34, were isolated from the same HIV-positive patient, with a year between the two events. The isolation in different clinical episodes in one individual of different strains assigned to the same zymodeme has on several occasions been interpreted as an indicator of relapse. Isolation of two different zymodemes, however, has been interpreted as reinfection [17,18]. The differences revealed by RAPD show that, in this case, this could be a reinfection although we cannot rule out that the small differences detected (Jaccard’ distance = 0.073) could reflect the existence of different clones of a single strain (the strains were not cloned). In this respect, Bastien et al., 1990 [19] detected in \textit{L. infantum} a
degree of interclonal polymorphism that affected one or two chromosomes.

The almost total absence of overlap between genetic polymorphisms of the species *L. infantum* and its zymodeme structure has also been demonstrated by Guerbouj et al., 2001 [12] and Mauricio et al., 2001 [14] in a study of genes gp63 by PCR-RFLP. In any case, this is the first time that such a large number of zymodemes of this species have been included in a study of these characteristics.

The *L. infantum* strains analysed by RAPD are structuralised into two genetic clusters each of a different geographical origin (eastern and central-western; Figs. 4 and 5). These two clusters diverge and evolve independently. The geographical structuralisation of the *L. infantum* strains has been reported by Guerbouj et al., 2001 [12] for strains isolated in different countries. In a study in which we applied the RAPD technique to the genetic polymorphism in sympatric species of the genus *Phlebotomus* [20] we found differences between specimens from the eastern (Huelva) and the western populations (Granada and Almeria) of the species *P. ariasi*.

One of the vectors of *L. infantum*. This could reflect a degree of co-evolution between the parasite and the vector. The Jaccard distance between the two genetic groups was 0.211. On the other hand, the leishmaniasis focus in the Huelva province from where most of the central-western strains included in our study proceeded presents special epidemiological characteristics [21].

In conclusion, our results suggest, (1) the validity of RAPD for the identification and characterisation of *Leishmania* species in general, and especially *L. infantum*; (2) the lack of overlap of the RAPD results with the zymodeme structure of *L. infantum*, reflecting the genetic heterogeneity of the strains belonging to the same zymodeme; (3) the existence in these strains of a geographical structuralisation with two phylogenetically distinct groups that can be associated with certain epidemiological differences in their foci of origin; (4) the lack of any relation with the host, that could indicate no selection by the host, at least of these strains; (5) the absence of any association between parasite genotype and clinical form suggesting, perhaps, an importance of the immune status of the host.

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Fig. 4. UPGMA dendogram generated from the results obtained with the RAPD technique. The binary data matrix was constructed with 485 characters (DNA fragments). The matrix of genetic distances was constructed using Jaccard’s distances (*J*). CL, human cutaneous leishmaniasis; VL, human visceral leishmaniasis; VL-HIV, coinfection *Leishmania*-HIV, dog (v), strain isolated from visceral lesions of a dog, dog (c), strain isolated from cutaneous lesions of a dog, Pp, *Phlebotomus perniciosus*. 
Fig. 5. Evolutionary tree (consensus tree) constructed with 485 genetic markers generated with the RAPD technique using the Wagner parsimony method. The number in each bifurcation indicates the number of times the two groups are separated in the 100 trees constructed with bootstrap analysis. We used the species *L. mexicana* as an outgroup.

References


