Characterization of cutaneous isolates of *Leishmania* in Colombia by isoenzyme typing and kDNA restriction analysis


1 Department of Parasitology. University of Granada, Severo Ochoa s/n, E-18071 Granada (Spain).
2 Centro Dermatológico Federico Lleras Acosta, E.S.E., Bogotá, D. C., Colombia.
3 Department of Statistics, University of Granada, Severo Ochoa s/n, E-18071 Granada (Spain).

**ABSTRACT**

In Colombia, leishmaniasis is an endemic disease for which efforts are currently being made to establish the geographic distribution and identification of the species circulating in the country. Sixteen stocks of *Leishmania* isolated from patients of the National Dermatology Institute of Bogotá (Colombia) have been characterized by means of isoenzymatic characterization and kDNA analysis by restriction enzymes. These isolates were compared with 8 reference stocks: *L. braziliensis, L. guyanensis, L. panamensis, L. mexicana, L. amazonensis, L. colombiensis, L. peruviana*, and *L. chagasi*. The statistical study of the results gave 3 clusters. The dominant species was *L. panamensis*, with 44% and distributed throughout the country, followed by *L. amazonensis* with 25%, distributed preferentially along the Pacific coast and to the south of the country. *L. braziliensis*, with 19%, was distributed mainly in the centre and east of the country, while 12% remained undetermined. None of the isolates were identified as belonging to *L. mexicana*.

**Key words:** Leishmaniasis, Colombia, isoenzymatic characterization, kDNA analysis by restriction enzymes, PCR.

**RESUMEN**

En Colombia, la leishmaniasis es una enfermedad endémica para la cual actualmente se están dedicado muchos esfuerzos para establecer la distribución geográfica y la identificación de las especies circulantes en el país. Dieciséis aislados de *Leishmania* aisladas de pacientes del Instituto Nacional de Dermatología de Bogotá (Colombia) han sido caracterizadas mediante estudio de isoenzimas y análisis por enzimas de restricción. Estos aislados fueron comparados con 8 cepas de referencia: *L. braziliensis, L. guyanensis,*
**INTRODUCTION**

The diverse clinical forms of leishmaniasis constitute a serious public health problem worldwide. According to the WHO, 350 million people are at risk of contracting the disease, with nearly 12 million people already infected, and some 2 million new cases yearly showing different clinical forms of leishmaniasis, classified in category I as an emerging disease without control (WHO, 2004).

In Latin America, primarily in Andean countries and those sharing the Amazon basin, the disease is present in three main clinical forms: cutaneous, mucocutaneous, and visceral (Herwaldt, 1999). In Colombia, leishmaniasis is considered an endemic disease throughout the country, except for some zones and the capital, Bogotá D.C. (Ovalle et al., 2006). In 2005, the number of leishmaniasis cases rose by 3,794 (21.9%), i.e. 18,097 reported cases. Of these, 17,983 cases (99.4%), were cutaneous, 60 cases (0.3%) mucocutaneous, and 54 (0.3%) visceral leishmaniasis (Zambrano, 2006).

In the Americas, two taxonomic groups of *Leishmania* exist, the subgenera *Leishmania* and *Viannia*, which are also known as the *Brazilensis* Complex. This complex includes the species *L. braziliensis*, *L. peruviana*, *L. panamensis*, *L. lainsoni* and *L. guyanensis*. The subgenus *Leishmania* may be further divided into species complexes: the *Mexicana* Complex (*L. mexicana, L. amazonensis, L. garnhami, L. aristidisi*, and *L. pifanoi*), and the *Donovani* Complex (*L. chagasi*) (Lainson & Shaw, 1987). For more than 20 years efforts have been made to determine the geographic distribution of the *Leishmania* species in Colombia. To date, numerous species of *Leishmania* have been identified: *L. braziliensis*, *L. guyanensis*, *L. panamensis*, *L. mexicana, L. amazonensis, L. chagasi*, and other *Leishmania* spp. remaining uncharacterized (Corredor et al., 1990; Saravia et al., 1998; Ovalle et al., 2006).

The marked phenotypic diversity of *Leishmania* has given rise to a complex taxonomy of more than 20 species described, most in Latin America (Lainson & Shaw, 1987). Given the epidemiological complexity in some regions in Colombian, it is necessary to determine the species in circulation (Lucas et al., 1998). Due to, the strongly heterogeneous distribution of parasites, where the transmission cycles of the different species can overlap and several species can be found at the same focal point of the disease, broad human migration, as well as tourism, can spread *Leishmania* beyond its traditional ecological distribution (Victoir et al., 2003). That is, the ability to distinguish between *Leishmania* species is crucial when prescribing treatment as well as when determining possible control measures in epidemiological studies. Frequently, *Leishmania* species are identified based on their geographical distribution and on clinical manifestations of the resulting disease. However, geographical origin is an inadequate criterion in non-endemic areas, as well as endemic regions where multiple species of *Leishmania* may coexist. Identification of the infectious species based on clinical symptoms can be problematic, because several species cause both cutaneous and mucocutaneous disease while others cause visceral and cutaneous disease (Schönian et al., 2003).

Diverse characterization methods have been applied to the study of this genus, such as electrophoresis and isoenzymes (Chance & Walton, 1982; Shamsuzzaman et al., 2000; Belhadj et al., 2003; Rodriguez-González et al., 2006 and 2007), DNA analysis of the kDNA (Gramiccia et al., 1992; Rodriguez-González et al., 2006 and 2007), the tech-
nique of randomly amplified polymorphic DNA (RAPD) (Tybairenc et al, 1993; Giuziani et al, 2002).

The National Dermatology Institute of Bogotá D. C. (Colombia) attends patients from the entire country with different clinical forms of leishmaniasis, from which the etiological agent is isolated. The present study attempts provide new data on the distribution as well as the characterization of 16 new Leishmania isolated obtained from patients of the National Institute of Dermatology (Colombia) during the period 1995-2005. The characterization was made in comparison with reference stocks: L. braziliensis, L. guyanensis, L. panamensis, L. mexicana, L. amazonensis, L. colombiensis, L. peruviana, and L. chagasi, by isoenzyme analysis and restriction kDNA analysis using different restriction endonucleases.

MATERIALS AND METHODS

Parasite isolation and in vitro culture: The 16 isolated were isolated from patients treated by dermatologists in the special leishmaniasis clinic of the National Dermatology Institute of Bogotá D.C. (Colombia). All the patients had a confirmed diagnosis of cutaneous leishmaniasis, and their clinical histories were reviewed to gather information related to the clinical form: probable site of infection, patient’s age, sex, and origin (Table 1).

The sample was taken by aspiration of the edge of the cutaneous lesions characterized by multiple ulcerated plaques with violet edges of different sizes and at various locations of the patient’s anatomy. The sample was placed in Senekjie medium (Senekjie, 1943), incubated at 27°C, and monitored for 5 weeks. The parasites isolated were cultured in Schneider Drosophila medium (Sigma) supplemented to 10% with inactivated foetal bovine serum (LabClinics). Once in the logarithmic growth phase, these isolates were sent from the National Dermatology Institute of Bogotá D.C. to our laboratory (Granada, Spain) and were cultured in vitro in MTL medium supplemented with 10% inactivated foetal bovine serum kept in an air atmosphere at 27°C (Fernández-Ramos et al, 1999). As reference Leishmania stocks, we used L. peruvi-

Table 1. Details of 16 Leishmania strain isolated from patients of the National Institute of Dermatology (Bogotá D.C., Colombia) during the period 1995-2005

<table>
<thead>
<tr>
<th>Code</th>
<th>Clinical manifestation</th>
<th>Sex†</th>
<th>Years</th>
<th>Place of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL001</td>
<td>Cutaneous</td>
<td>F</td>
<td>31</td>
<td>Guaviare</td>
</tr>
<tr>
<td>LL003</td>
<td>Cutaneous</td>
<td>M</td>
<td>25</td>
<td>Cauca</td>
</tr>
<tr>
<td>LL004</td>
<td>Cutaneous</td>
<td>M</td>
<td>20</td>
<td>Casanare</td>
</tr>
<tr>
<td>LL005</td>
<td>Cutaneous</td>
<td>M</td>
<td>34</td>
<td>Valle del Cauca</td>
</tr>
<tr>
<td>LL008</td>
<td>Cutaneous</td>
<td>M</td>
<td>20</td>
<td>Santander</td>
</tr>
<tr>
<td>LL009</td>
<td>Cutaneous</td>
<td>F</td>
<td>36</td>
<td>Cundinamarca</td>
</tr>
<tr>
<td>LL012</td>
<td>Cutaneous</td>
<td>M</td>
<td>39</td>
<td>Cundinamarca</td>
</tr>
<tr>
<td>LL035</td>
<td>Cutaneous</td>
<td>F</td>
<td>48</td>
<td>Cundinamarca</td>
</tr>
<tr>
<td>LL092</td>
<td>Cutaneous</td>
<td>M</td>
<td>24</td>
<td>Casanare</td>
</tr>
<tr>
<td>LL104</td>
<td>Cutaneous</td>
<td>M</td>
<td>50</td>
<td>Vichada</td>
</tr>
<tr>
<td>LL106</td>
<td>Cutaneous</td>
<td>M</td>
<td>38</td>
<td>Guaviare</td>
</tr>
<tr>
<td>LL109</td>
<td>Cutaneous</td>
<td>F</td>
<td>13</td>
<td>Cundinamarca</td>
</tr>
<tr>
<td>LL110</td>
<td>Cutaneous</td>
<td>M</td>
<td>20</td>
<td>Vichada</td>
</tr>
<tr>
<td>LL113</td>
<td>Cutaneous</td>
<td>M</td>
<td>33</td>
<td>Santander</td>
</tr>
<tr>
<td>LL119</td>
<td>Cutaneous</td>
<td>M</td>
<td>31</td>
<td>Santander</td>
</tr>
<tr>
<td>LL125</td>
<td>Cutaneous</td>
<td>M</td>
<td>55</td>
<td>Boyaca</td>
</tr>
</tbody>
</table>

†F = female, M = male.
ана (MHOM/PE/1984/LC26) from department of La Libertad (Peru), L. guyanensis (MHO/BR/1975/M4147) from the Brazilian Amazon, L. colombiensis (IHAR/CO/1985/CL500) from Colombia, L. braziliensis (MHOM/BR/1975/M2903) from Pará, Brazil, L. panamensis (MHOM/PB/1971/LS94) isolated in Canal Zone (Panama), L. mexicana (MHOM/BZ/1982/BEL21) from Belize, L. amazonensis (MHOM/BR/1973/PH8) from Brazil, and L. chagasi (MHOM/BR/1974/PP75 (M2682)). The reference stocks and new isolates were maintained in our laboratory by cryopreservation and a maximum of 4 subcultures in NNN medium modified with a liquid phase of minimal essential medium (MEM) plus 10% inactivated foetal bovine serum kept in an air atmosphere at 27ºC.

Isoenzyme characterization: Crude homogenates were obtained from 300 mL of culture medium containing 2 x 10^7 cells.mL⁻¹. Cells were harvested by centrifugation at 600 x g 10 min, washed twice in a phosphate-buffered saline (pH 7.4), and resuspended in a hypotonic enzyme stabilizer solution containing 2mM dithiothreitol, 2mME-amino-caproic acid, and 2mM EDTA (Fernández-Ramos et al, 1999). The samples were frozen at -80ºC for 15 min and thawed at 25ºC. After several freezing-thawing cycles, cell lysates were centrifuged at 8000 x g for 20 min at 4ºC, and the supernatants were stored in liquid nitrogen until used. The protein concentration was determined using the Bradford method and stored at a final concentration of 1mg.mL⁻¹ of protein.

The enzymes were separated by isoelectric focusing in a PhastSystem apparatus, using Phastgel IEF 3-9 (Pharmacia, Freiburg, Germany).

The following enzymes were tested: malic enzyme (EM), glucose 6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), glucose phosphate isomerase (GPI), and superoxide dismutase (SOD). The staining procedures are described in Fernández-Ramos et al (1999).

kDNA isolation: Promastigotes were collected by centrifugation of 300 mL of culture medium, when their concentrations had reached about 2 x 10^7 cells.mL⁻¹, after about 5 days. They were washed twice in 50mL of 0.15M NaCl, 0.015M sodium citrate, and once with SE buffer (0.15M NaCl, 0.1M EDTA, pH 8.0). Kinetoplast DNA was obtained according to the procedure described by Gonçalves (Gonçalves et al, 1984).

Restriction-enzyme digestion and electrophoresis analysis: The kDNA extracts (3 mg.mL⁻¹) were completely digested with restriction endonucleases (Hae III, BamH I, HinF I, Hind III, EcoR I, and Msp I) according to the manufacturer’s prescribed buffer conditions (Boehringer-Ingelheim, Barcelona, Spain). The digestion products were electrophoresed in 1.3% agarose slab gels as described elsewhere (Riou & Yot, 1977) and the fragment sizes were estimated by comparing their mobilities with those of a 100-bp DNA ladder (Gibco-BRL, Gaithersburg, USA). The gels were stained with ethidium bromide (10 mg.mL⁻¹ for 10 min) and photographed under UV light with an Olympus Camedia digital camera, C-4000 Zoom.

Statistical study: The statistical methods were based on individual hierarchical cluster analysis, selecting the Euclidean distance to the square as the basis for measuring the associations between individuals. The Euclidean distance was calculated by the following grouping procedures of simple linkage (Rk 0.7355), average linkage among groups (Rk 0.7518), average linkage (Rk 0.7726), centroid method (Rk 0.7586), median method (Rk 0.7204), and the Ward method (Rk 0.7570). The cophenetic coefficient (Rk) measures the degree of distortion between relationships, means in terms of original distances between individuals and those existing at the end of the analysis. The one with the highest cophenetic correlation was chosen as the optimal method. In the selection procedures, the average linkage between groups was considered using the coefficient Rk of Rand, which is an index of the similarity between classifications. This analysis was made with the STATGRAPHICS program, version 5.0.

Ethical considerations: All the patients attended in the clinic of the National Dermatology Institute of Bogotá were informed on the inclusion of the isolates in an institutional biological bank for subsequent research. The protocol of the present study was submitted to the ethical committee that catalogued it as risk-free research, according to the Helsinki Declaration.

RESULTS

A total of 16 new stocks of Leishmania species from patients in the National Dermatology Institute
of Bogotá D.C. (Colombia) were isolated, and cultured. These 16 stocks, together with 8 reference stocks (*L. peruviana*, *L. guyanensis*, *L. colombiensis*, *L. braziliensis*, *L. panamensis*, *L. mexicana*, *L. amazonensis*, and *L. chagasi*) were analysed by isoenzymatic profiles using 6 enzyme systems (EM, G6PDH, IDH, MDH, GPI, and SOD). Some of the enzymatic systems revealed significant differences between the reference stocks in their number of bands and their isoelectric points (Figure 1). Five enzymatic systems (EM, MDH, IDH, GPI, and SOD) enabled us to differentiate the species *L. peruviana* from the rest of the reference stocks (Fig. 1 A, C, D, E, and F, lane 1). Meanwhile, EM, IDH and MDH differentiated *L. panamensis* from the rest (Figure 1 A, C, and D, lane 5), and *L. braziliensis* could be differentiated by EM and MDH (Figure 1 A and D, lane 4). *L. colombiensis* was distinguished by the enzymes G6PDH and MDH (Figure 1 B and D, lane 3, respectively). However, *L. amazonensis* was not identified by any of the six systems used, while *L. guyanensis* was distinguished by MDH (Figure 1 D, lane 2) and *L. chagasi* by EM, IDH, and SOD (Figure 1 A, C, and F, lane 8). When the 16 new *Leishmania* stocks were submitted to isoenzymatic analysis with the six systems, we were able to establish phenotypic relationships between these isolates and the reference stocks. The isolates LL001, LL004, LL106, LL113, LL119, and LL125 presented isoenzyme profiles resembling *L. panamensis* with less than 5 of the enzymatic systems (Figure 1 lanes 9, 11, 19, 22, 23, and 24). Similarity

![Figure 1. Isoenzyme profiles of *Leishmania* isolates. Organisms: (1) *L. peruviana* (2); *L. guyanensis*; (3) *L. colombiensis*; (4) *L. braziliensis*; (5) *L. panamensis*; (6) *L. mexicana*; (7) *L. amazonensis*; (8) *L. chagasi*; (9) isolate LL001; (10) isolate LL003; (11) isolate LL004; (12) isolate LL005; (13) isolate LL008; (14) isolate LL009; (15) isolate LL012; (16) isolate LL035; (17) isolate LL092; (18) isolate LL104; (19) isolate LL106; (20) isolate LL109; (21) isolate LL110; (22) isolate LL113; (23) isolate LL119 and (24) isolate LL125. Enzymes: (A) malic enzyme (ME); (B) glucose 6-phosphate dehydrogenase (G6PDH); (C) isocitrate dehydrogenase (IDH); (D) malate dehydrogenase (MDH); (E) glucose phosphate isomerase (GPI); (F) superoxide dismutase (SOD).]
occurred with the isolates LL009, LL035, LL092, LL104 and LL110), but in this case the profiles were similar to the profile of *L. braziliensis* (Figure 1, lanes 14, 16, 17, 18, and 21). The isolates LL003 and LL012 had a profile resembling that of *L. amazonensis* for 4 or more systems (Figure 1 lanes 10 and 15). Finally, the isolates LL005, LL008, and LL109 presented a profile which was not clearly similar to that of any of the reference stocks (Figure 1, lanes 12, 13 and 20).

All the DNA endonucleases assayed were capable of digesting the various kDNA in different-sized fragments (Figure 2). The heterogeneous restriction profiles were remarkable in all the DNAs studied. *L. mexicana* presented a restriction pattern that completely differed from the other reference stocks used in this study with 4 of the 6 endonucleases used (Figure 2 A; C; D and F, lane 6). The restriction pattern of *L. peruviana* with the endonucleases Hae III and EcoR I were differentiated from

---

**Figure 2.** Restriction endonuclease analysis of kDNA of *Leishmania* isolates. (A) kDNAs+ BamHI; (B) kDNAs+ HinfI; (C) kDNAs+HaeIII; (D) kDNAs+EcoRI; and (E) kDNAs +MspI. (F) kDNAs +HindIII. Lane: (1) *L. peruviana* (2); *L. guyanensis*; (3) *L. colombiensis*; (4) *L. braziliensis*; (5) *L. panamensis*; (6) *L. mexicana*; (7) *L. amazonensis*; (8) *L. chagasi*; (9) isolate LL001; (10) isolate LL003; (11) isolate LL004; (12) isolate LL005; (13) isolate LL008; (14) isolate LL009; (15) isolate LL012; (16) isolate LL035; (17) isolate LL092; (18) isolate LL104; (19) isolate LL106; (20) isolate LL109; (21) isolate LL110; (22) isolate LL113; (23) isolate LL119 and (24) isolate LL125. Size markers are 1 Kb DNA ladder fragments (Lane 25).
the rest of the reference stocks (Figure 2 C and D, lanes 1). *L. colombiensis* was identified with the endonucleases Hinf I and EcoR I (Figure 2 B and D, lane 3), and *L. chagasi* with EcoR I and Hind III (Figure 2 D and F, lane 8). The stocks belonging to *L. braziliensis* and *L. guyanensis* were identified with the endonuclease Hind III (Figure 2 F, lanes 2 and 4), and finally the stocks of *L. amazonensis* and *L. panamensis* were unidentifiable with any of the 6 endonucleases. The 16 new isolates presented two restriction patterns when their kDNA was submitted to the endonuclease BamH I: the isolates LL001, LL003, LL004, LL005, LL008, LL012 LL106, LL109; LL110, LL113, LL119, and LL125 had profiles similar to those of the stocks *L. panamensis* and *L. amazonensis* (Figure 2 A; lanes: 9-13, 15, and 19-24), while the rest of the isolates presented a profile similar to that of *L. braziliensis* and *L. guyanensis* (Figure 2 A). When the kDNA of the new isolates was digested by the endonuclease Hinf I, it gave 5 different restriction patterns: the isolate LL004 presented 2 fragments similar to that of *L. colombiensis*, the isolates LL008, LL035, LL119, and LL125 a single fragment. There was no correspondence to the reference stocks (Figure 2B, lanes 13, 16, 23, and 24), as happened with the isolates LL012 and LL110, which presented 6 and 4 kDNA fragments, respectively, without correspondence to the reference stocks (Figure 2 B, lanes 15 and 21). The rest of the isolates had a profile similar to those of *L. peruviana*, *L. braziliensis*, *L. panamensis*, *L. amazonensis*, and *L. guyanensis* (Figure 2 B). With the enzyme Hae III, 2 profiles related some of the isolates to some of the reference stocks, e.g. isolates LL003, LL004, LL009, LL106, LL119, and LL125 to stocks *L. braziliensis*, *L. panamensis* and *L. chagasi* (Figure 2 C lanes 10-11, 14, 18-20, 23, and 24; lanes 4-5 and 8, respectively). The other profile, which included the group of the isolates LL005, LL008, LL012, LL035, LL092, LL104, and LL113 (Figure 2 C, lanes 12, 13, 15-17, and 22), resembled the profiles of the stocks *L. colombiensis* and *L. amazonensis* (Figure 2 C, lanes 3 and 7); the isolates LL001 and LL110 presented a profile that was similar to none of the reference stocks (Figure 2 C, lanes 9 and 21). With the enzyme EcoR I, the isolate LL008 presented a profile that did not resemble any of the reference stocks (Figure 2 D, lane 13), as occurred with the enzyme MspI, which gave a different restriction pattern to the reference stocks for the isolates LL005 and LL109 (Figure 2 E, lane 12 and 20). When the kDNA of the isolates LL009 and LL012 was subjected to the action of the enzyme Hind III, the profiles differed from those of the reference stocks (Figure 2 F, lanes 14 and 15).

**DISCUSSION**

It is known that one technique alone makes the exact identification of an isolate difficult, and therefore we analysed the results by the two techniques used (Figure 3), grouping the isolates into three large clusters. **Cluster 1** in turn contained two subclusters: on the one hand, *L. chagasi*, which, according to Lainson & Saw (1987) would form the Donovani Complex (subcluster 1-1), while **Subcluster 1-2** was comprised of *L. guyanensis*, *L. peruviana*, and *L. braziliensis*, together with the isolates LL104, LL092, and LL035. Phylo-
netically, these are very close to L. braziliensis, and thus can be considered L. braziliensis. From this analysis, we deduce that L. peruviana and L. braziliensis are related, supporting the observation of previous authors who related the two species by isoenzyme studies (Sierra et al., 2006). Cluster 2 is formed by another two subclusters: Subcluster 2-1 would include L. panamensis and the isolates LL113, LL001, LL106, LL125, LL004, LL119, and LL009; and Subcluster 2-2 formed by L. colombiensis. Subcluster 1-2 and Cluster 2 would form the traditional Braziliensis Complex. Cluster 3 would be composed of Subcluster 3-1, which includes L. amazonensis and the isolates LL003, LL005, LL109, and LL110, which we consider to be L. amazonensis; Subcluster 3-2, composed of the isolates LL008 and LL012; and finally Subcluster 3-3, formed by L. mexicana. This Cluster 3 would form the Mexicana Complex. Isolates LL008 and LL012 are loosely related to L. amazonensis, although they cannot be considered as this species and perhaps would be a variation within the species or some other species of this same complex not included in this study and therefore we do not have the patterns for comparisons.

Isolates LL003, LL004, LL005, LL008, and LL009, were classified at the level of a complex by monoclonal antibodies, isoenzymatic analysis, and PCR (Ovalle et al., 2006). The results provided in the present study partially agree with those of the previous work in that the isolates LL003, LL005, and LL008 belong to the Mexicana complex and we can identify them as L. amazonensis; and we identify isolates LL004 and LL009 as L. panamensis.

The dominant species in Colombia was L. panamensis (44%), followed by L. amazonensis (25%), and L. braziliensis (19%), with a portion yet to be determined (12%). These data are consistent with those reported by other authors (Corredor et al., 1990; Cupolillo et al., 1998; Ovalle et al., 2006). The small number of stocks analysed does not lead us to draw conclusions concerning the geographic distribution of this species (Figure 4). Nevertheless, we can suggest that L. panamensis was distributed uniformly throughout the country, L. braziliensis was located in the centre and east, while L. amazonensis was found along the Pacific coast and in the south of the country. Until now, L. amazonensis has been isolated at a very low percentage (Grimaldi et al., 1989) and with a geographic distribution coincidental with our results. Despite that different authors have reported the presence of L. mexicana throughout Colombia (Ovalle et al., 2006), we have not identified any new isolates as belonging to L. mexicana. This does not mean that the species L. mexicana does not exist in Colombia, but rather indicate us that the monoclonal antibodies or the isoenzymatic typing and even the PCR are of limited value by themselves in differentiating L. amazonensis from L. mexicana. However, the combined use of phenotypic and genotypic characteristics can differentiate the two species. This leads us to conclude that it is necessary to use several techniques to identify the species level, such as isoenzymatic analysis together with a kDNA study by means of endonucleases that in most cases identify the species level.

REFERENCES

1. BELHADJ S, PRATLONG F, HAMMAMI M, KALLEL K, DEDET JP, CHAKER E. 2003. Human cutaneous leishmaniasis due to Leishmania infantum in the...


Acknowledgments: We wish to thank Encarnación Guer- rero for technical help in culture media preparation. This work was supported by grants from the Project CGL-2006- 27889-E and CGL-2008-03687-E of the Ministerio de Cien- cia y Tecnología (Spain).