# Identification of New World *Leishmania* species from Peru by biochemical techniques and multiplex PCR assay

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## Abstract

We have characterized diverse strains or species of *Leishmania* isolated in humans that are currently circulating throughout Peru, by means of isoenzymatic characterization, kDNA analysis by restriction enzymes, and multiplex PCR assay. The cluster analysis gave five groups. Cluster 1 includes *L.* (*L.*) *donovani* together with the isolates LP4 and LP7, forming the *donovani* complex. Thus, this complex corresponds to the New World visceral form, *L.* (*L.*) *chagasi*. Cluster 2 is formed by the isolates LP1–LP3, LP6, LP10, LP9, and LP11, phylogenetically intermediate between Cluster 1 and Cluster 3, or they can be treated as hybrids. Cluster 3 is divided into two subgroups: one formed by *L.* (*V.*) *peruviana*, together with the isolates LP14 and LP5, and the second one formed by *L.* (*V.*) *brazilensis* and the isolate LP8. These two subgroups form part of the *brazilensis* complex. The three strains of *L.* (*L.*) *infantum* [*L.* (*L.*) *infantum* I and II and la LSI] make up Cluster 4. In Cluster 5, we include the three Mexican strains (LM1–LM3) forming one subgroup while we would place *L.* (*L.*) *amazonensis* in another subgroup. These two subgroups would comprise the complex *mexicana*.

## Introduction

Parasitic protozoa of the genus *Leishmania* cause a broad spectrum of disease in humans throughout tropical and subtropical regions worldwide and are considered a major public health problem (Harris *et al*., 1998). Their clinical spectrum goes from cutaneous ulcers that heal spontaneously to serious visceral infections that can compromise the life of the patient, and between these two poles lies a wide range of clinical possibilities (Gallego, 2004). Leishmaniasis represents a serious obstacle for the socio-economic development of 88 countries, this being recognized by the World Health Organization as a worldwide health problem (World Health Organization, 1990). The 72 developing countries affected by this disease include Peru, where leishmaniasis constitutes the third cause of morbidity from transmitted diseases, after malaria and tuberculosis (Ministerio de Salud del Perú, Dirección General de Salud de las Personas, Dirección del Programa de Control de Enfermedades Transmisibles, 1995). It is a parasitosis in continuous expansion: 4645 cases were reported in 1999, and 5998 in 2002 (Cáceres & Montoya, 2002). In Peru, leishmaniasis is endemic in the Andes valleys as well as on the Amazonic plain. They present two main forms of tegument leishmaniasis, defined primarily by geographical and clinical characteristics: Andean leishmaniasis or Uta, and jungle leishmaniasis or Espundia (Lucas *et al*., 1998).

In the Americas, two taxonomic groups of *Leishmania* exist, the subgenera *Leishmania leishmania* and *Leishmania viannia*; the subgenus *viannia*, which is also known as the *brazilensis* complex, includes the species *Leishmania brazilensis*, *Leishmania peruviana, Leishmania panamensis*, and *Leishmania guyanensis*, while the subgenus *Leishmania* may be further divided into species complexes: the *mexicana* complex (*Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania garnhami*, *Leishmania aristidesi,* and *Leishmania pifanoi*), and the *donovani* complex (*L. chagasi*, Laison & Saw, 1987).

In Peru, five *Leishmania* species have been identified: *L. (Leishmania) amazonensis, L. (Viannia) guyanensis,* and *L. (V.) braziliensis* are etiological agents of cutaneous and

mucocutaneous leishmaniasis (Espundia) in the Amazon region; *L. (V.) peruviana* causes Andean cutaneous leishmaniasis (Uta) (Rodriguez, 2000); and, in provinces situated to the east of the Andes, *Leishmania (V.) lainsoni* is the pathogen (Lucas *et al.*, 1994).

*Leishmania* species are morphologically very similar and species identification is possible using standard biochemical methods (lectin agglutination, isoenzyme analysis, analysis of kinetoplast DNA (kDNA) restriction fragment using different restriction endonuclease, etc.) (Andrade & Saraiva, 1999; Shamsuzzaman *et al.*, 2000; Belhadj *et al.*, 2003; Sampali *et al.*, 2003; Rodríguez-González *et al.*, 2006). 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 nonendemic areas, as well as endemic regions where multiple species of *Leishmania* may coexist. Identification of the infecting 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).

In a recent publication, we characterized eight *Leishmania* promastigotes isolated from different geographical areas: Peru, Mexico, and Spain (Rodríguez-González *et al.*, 2006). The stocks were characterized by carbohydrate cell-surface residues using agglutinations with lectins, by isoenzyme analysis, and by analysis of kDNA restriction fragment length polymorphism using four different restriction endonucleases. These isolates were compared with four reference stocks and typified as *Leishmania* (*L.*) *donovani*, two stocks of *Leishmania* (*L.*) *infantum*, and one species of *L.* (*V.*) *peruviana*. The Spanish isolate was identified as belonging to *L. (L.) infantum*, the stocks from Mexico as the Mexican complex, and the four isolates from Peru as *L. (V.) peruviana* (Rodríguez-González *et al.*, 2006). On this occasion, we characterized 10 new stocks, all isolated in humans from different regions of Peru, by the National Health Institute of Lima. The characterization was made by isoenzyme analysis, restriction kDNA analysis using different restriction endonucleases, and single-step multiplex PCR assay. In the same way, we have included for the PCR assay those isolates characterized in the previous work (Rodríguez-González *et al.*, 2006).

## Materials and methods

### Parasite isolation and *in vitro* culture

The 10 stocks were isolated from different areas of Peru. The stock that we call LP5 was isolated from a male 20 years of age who presented a cutaneous jungle lesion in the year 2001. Stocks LP6 and LP7 were also isolated from males having cutaneous lesions, LP8 being from a male 47 years of age with a cutaneous lesion on the back in the year 2002. These four isolates came from the Peruvian jungle region. Stocks LP9 and LP10 came from males of the Andes area of Peru (Department of Huanuco) bearing cutaneous lesions and were isolated in the year 2002. Stock LP11 came from the same region, but was isolated from a male 73 years of age with a cheek lesion (verrucosa form) and an ulcerous lesion on the leg of 5 months development. Stocks LP12, LP13 and LP14 were from males living on the border between Colombia and Ecuador, all presenting cutaneous lesions on the right and left temple. All these isolates were sent to our laboratory from the Lima National Health Institute (Peru). The stocks (from LP5 to LP14) isolated were cloned and cultured *in vitro* in MTL medium plus 10% inactivated foetal bovine serum kept in an air atmosphere at 28 °C (Sánchez-Moreno *et al.*, 1995).

Stocks LP1–LP4, LM1–LM3 and LS1, their isolation, and origin are described in Rodríguez-González *et al.* (2006). For reference strains, we used *L. (L.) donovani* (LCR-L 133, Leishmania Reference Center Jerusalem, Israel) isolated in a human case of kala-azar in Begemder (Ethiopia), *L. (V.) peruviana* (MHOM/PE/1984/LC26), two strains of *L. (L.) infantum* I and II, characterized as MCAN/ES/2001/UCM-10 and MCAN/2000/UCM-1, respectively isolated in Spain, *L.* (*L.*) *amazonensis* (MHOM/BR/1973/M1845) and *L.* (*V.*) *brazilensis* (MHOM/BR/1975/M2904). The reference strains have been maintained for several years in our laboratory by successive passes in cultures of NNN medium modified with a liquid phase of minimal essential medium (MEM) plus 10% inactivated foetal bovine serum kept in an air atmosphere at 28 °C. To maintain infectivity, a subculture was made approximately every 2 weeks and at was inoculated in the Syrian golden hamster, *Mesocricetus auratus*, least every 6 months, and parasites were isolated from the spleen 30–45 days postinoculation. The isolates from hamsters were cloned and cultured *in vitro* as previously described (Sánchez-Moreno *et al.*, 1995).

### Isoenzyme characterization

Crude homogenates were obtained from 300 mL of culture medium containing $2 \times 10^7$ cells $mL^{-1}$. Cells were harvested by centrifugation at $1500\,\boldsymbol{g} \times 10$ min, washed twice in a phosphate-buffered saline (pH 7.4), and resuspended in a hypotonic enzyme stabilizer solution containing 2 mM dithiothreitol, 2 mM E-aminocaproic acid, and 2 mM EDTA (Fernández-Ramos *et al.*, 1999). The samples were frozen at −80 °C for 15 min and thawed at 25 °C. After several freezing–thawing cycles, cell lysates were centrifuged at $8000\,\boldsymbol{g} \times 20$ min at 4 °C, and the supernatants were stored

in liquid nitrogen until use. The protein concentration was determined using the Bradford method and stored at a final concentration of 1 mg $mL^{-1}$ of protein.

The enzymes were separated by isoelectric focusing in a PhastSystem apparatus, using Phast-gel 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), phosphoglucomutase (PGM), 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 $2 \times 10^7$ cells $mL^{-1}$, after about 5 days. They were washed twice in 50 mL of 0.15 M NaCl, 0.015 M sodium citrate, and once with SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0). kDNA was obtained according to the procedure described by Gonçalves *et al.* (1984).

### Restriction-enzyme digestion and electrophoresis analysis

The kDNA extracts (3 μg $mL^{-1}$) were completely digested with restriction endonucleases (HaeIII, BamHI, HinfI and EcoRI, and MspI) 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). The gels were stained with ethidium bromide (10 μg $mL^{-1}$ for 10 min) and photographed under UV light with a Polaroid camera (665 film).

### Primer design and PCR amplification

Sequences for the SL RNA region of the New World *Leishmania* species were aligned with GENEJOCKEY II software (Bosoft, Ferguson, MO) to identify potential sites for genus-specific as well as complex-specific PCR priming (Harris *et al.*, 1998). We chose oligonucleotide primers that were either conserved in all *Leishmania* species (LU-5A) or specific to each New World complex (LB-3C, LM-3A, and LC-3L). The primers and their sequences are as follows: LU-5A, 5′-TTTATTGGTATGCGAAATTC-3′; LB-3C, 5′-CGT(C/G)CCGAACCCCGTGTC-3′; LM-3A, 5′-GCACCG CAC CGG(A/G)CCAC-3′; and LC-3L, 5′-GCCCGCG (C/T)GTCACCACCAT-3′. Oligonucleotides were synthesized by Thermo Electron Corporation (Germany).

We prepared a 50-μL reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM of each deoxynucleotide triphosphate, 15 mM $MgCl_2$, 10.5% dimethyl sulphoxide, 50 mM tetramethylammonium chloride, 0.4 μM 5′ primer LU-5A, 0.2 μM of each 3′ primer (LB-3C, LM-3L, and LC-3L), 0.04 U of Taq DNA polymerase (Promega), and 10 ng of kDNA preparation. An initial denaturing step of 95 °C for 5 min was followed by 35 cycles of 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR products were electrophoresed in a 1.3% agarose gel and stained with ethidium bromide.

### 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 ($R_k$ 0.7355), average linkage among groups ($R_k$ 0.7518), average linkage ($R_k$ 0.7726), centroid method ($R_k$ 0.7586), median method ($R_k$ 0.7204), and the Ward method ($R_k$ 0.7570). The cophenetic coefficient ($R_k$) 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 $R_k$ of Rand, which is an index of the similarity between classifications. This analysis was made with the STATGRAPHICS program, version 5.0.

## Results

*Leishmania* isolates, cultured *in vitro* in MTL medium plus 10% inactivated foetal bovine serum, reached cell densities on the order of $2 \times 10^7$ cells $mL^{-1}$. Figure 1 shows isoenzymatic profiles of the isolates analysed by seven enzyme systems (EM, G6PDH, IDH, MDH, GPI, PGM, and SOD). The isoenzymatic pattern of some of the isolates studied appears to reveal variations in the number of bands and their isoelectric points. This was the case of *L.* (*V.*) *braziliensis*, for which the profile with the seven systems studied, in general, differed from the rest of the stocks (Fig. 1c–e and g, lane 1); *L.* (*L.*) *amazonensis* was differentiated from the rest of the new isolates with the enzymes IDH and MDH (Fig. 1c and d, lane 2). The new isolate LP11 presented a similar profile for the enzyme MDH with *L.* (*L.*) *amazonensis* (Fig. 1d, lane 9). This same new isolate presented great differences with respect to the other isolates with the enzymes IDH and SOD (Fig. 1c and g, lane 9). Stocks LP6, LP13, and LP14 shared the same profile as *L.* (*L.*) *amazonensis* for malic

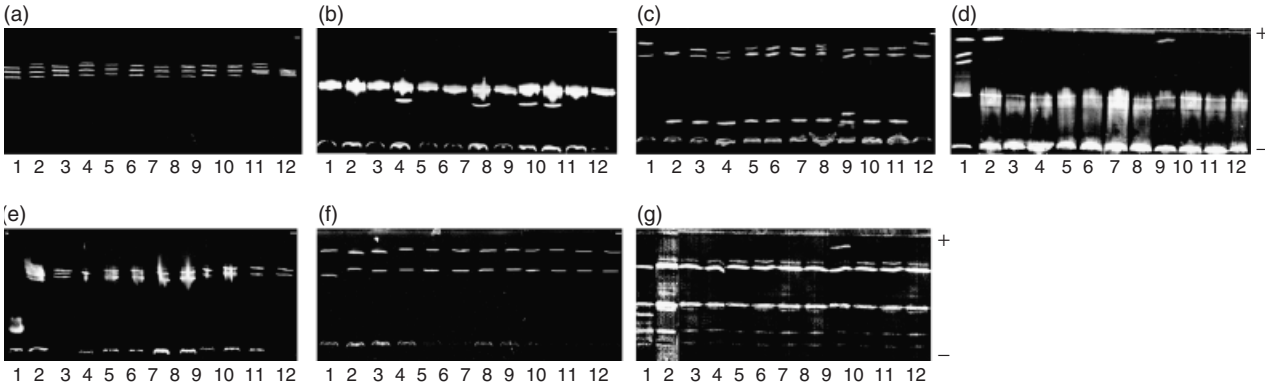


**Fig. 1.** Isoenzyme profiles of *Leishmania* isolates. Organisms: (1) *L. (V.) brazilensis*; (2) *L. (V.) amazonensis*; (3) isolate LP5; (4) isolate LP6; (5) isolate LP7; (6) isolate LP8; (7) isolate LP9; (8) isolate LP10; (9) isolate LP11; (10) isolate LP12; (11) isolate LP13; and (12) isolate LP14. 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) phospho-glucomutase (PGM); and (g) superoxide dismutase (SOD).

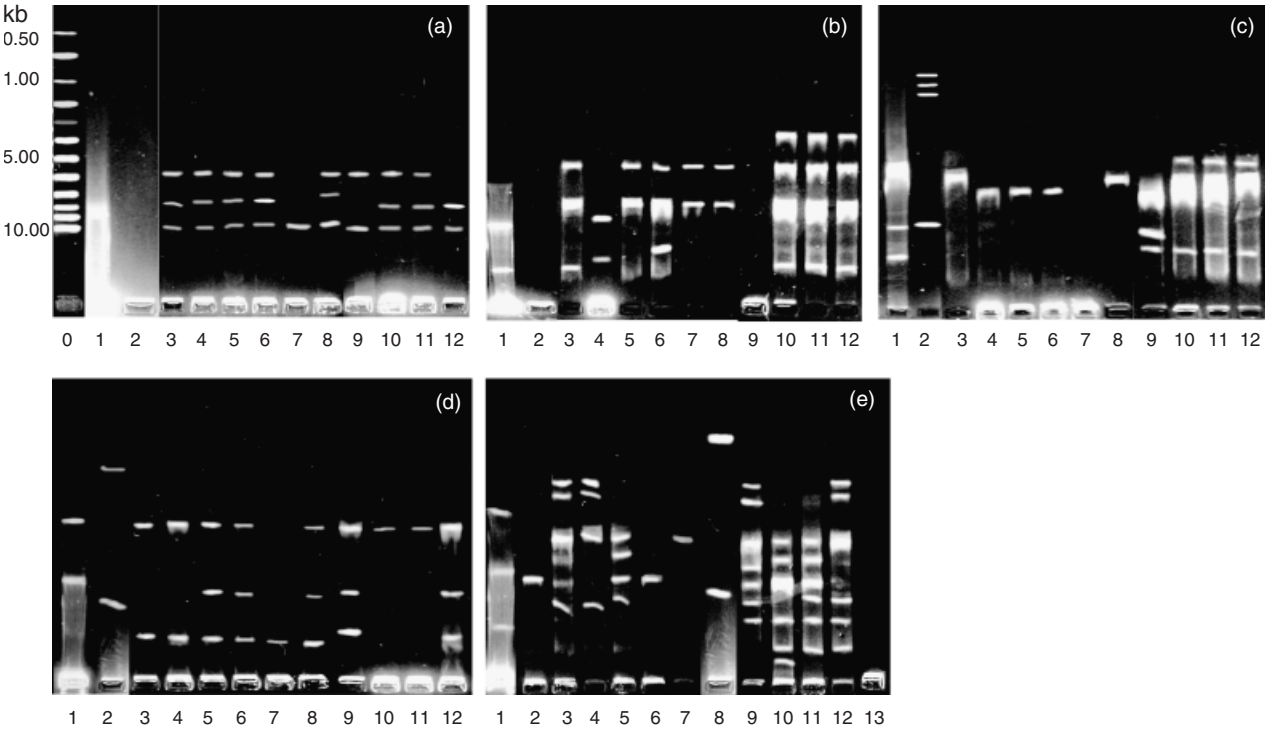


**Fig. 2.** Restriction endonuclease analysis of kDNA of *Leishmania* isolates. (a) kDNA+BamHI; (b) kDNAs+HinfI; (c) kDNAs+HaeIII; (d) kDNA+EcoRI; and (e) kDNA+*Msp*I. Lane (1) *L. (V.) brazilensis*; (2) *L. (V.) amazonensis*; (3) isolate LP5; (4) isolate LP6; (5) isolate LP7; (6) isolate LP8; (7) isolate LP9; (8) isolate LP10; (9) isolate LP11; (10) isolate LP12; (11) isolate LP12; (12) isolate LP14; and Lane 13: Intact kDNA *L. (V.) brazilensis*. Size markers are 1 Kb DNA ladder fragments (Lane 0).

enzyme (Fig. 1a, lanes 2, 4, 11, and 12). Stocks LP6, LP9, LP11, and LP12 with glucose 6-phosphate dehydrogenase enzyme were clearly differentiated from the two reference strains and the rest of the isolates (Fig. 1b, lanes 4, 7, 9, and 10). The enzyme PGM did not enable the detection of any significant difference between any of the isolates (Fig. 1f).

The kDNA of the 10 isolates and the two reference strains were purified and then digested with five restriction enzymes (BamHI, HinfI, HaeIII, EcoRI, and MspI). Electrophoresis using agarose gel revealed that while intact kDNA was not able to penetrate the gel (Fig. 2e, lane 13), the smaller kDNA restriction fragments that resulted after

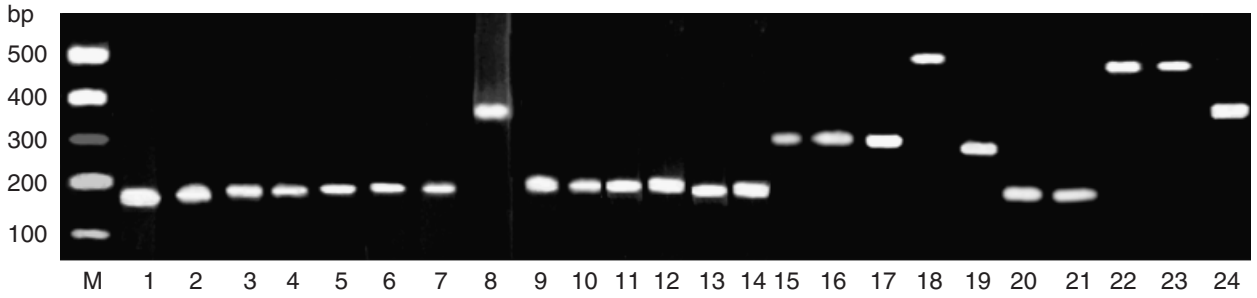


**Fig. 3.** Amplification products of the PCR multiplex assay from different *Leishmania* species and isolates using the primers LU-5A, LB-3C, LM-3A, and LC-3L. In each lane 10 μL of sample was placed. Lanes 1–14, LP1–LP14, respectively. Lane 15, isolate LM3; lane 16, isolate LM2; lane 17, isolate LM1; lane 18, isolate LS1; lane 19, *L. (V.) amazonensis*; lane 20, *L. (V.) peruviana*; lane 21, *L. (V.) brazilensis*; lane 22, *L. (L.) infantum* I; lane 23, *L. (L.) infantum* II; lane 24, *L. (L.) donovani*. Lane M, 100-bp DNA ladder; the lower band shown is 100 bp.

digestion did penetrate the gel, and a complete cleavage of kDNA by the endonucleases was characterized by a total disappearance of DNA from the top of the gel (Fig. 2). Not all the enzymes were capable of completely digesting the kDNA of the different isolates; for example, the enzymes BamHI (Fig. 2a) and HinfI (Fig. 2b) were incapable of completely digesting the kDNA of *L.* (*V.*) *brazilensis*, *L.* (*L.*) *amazonensis,* and the kDNA of the isolates LP6 and LP11.

The restriction-cleavage patterns of the reference strains *L.* (*V.*) *brazilensis* and *L.* (*L.*) *amazonensis* were completely different from each other with the five restriction enzymes, and both differed from the rest of the stocks. The isolates LP5–LP10 presented very homogeneous profiles with the enzymes BamHI, HinfI, and EcoRI (Fig. 2a, b and d, lanes from 3 to 8, both inclusive). Meanwhile, stock LP11 showed marked differences in the fragments caused by the enzymes HinfI, HaeIII, and MspI (Fig. 2b, c and e, lane 9). The isolates LP12–LP14 presented homogeneous restriction profiles that differed from the other isolates (Fig. 2a–d, lanes 10–12).

Figure 3 presents the results corresponding to the amplification of the kDNA by multiplex PCR assay of the 10 new isolates, of the two reference stocks, and of the isolates characterized previously by isoenzyme analysis and by restriction enzymes (Rodríguez-González *et al*., 2006). An amplification band of 370–390 bp was visible in the case of the reference stock *L.* (*L.*) *donovani* (Fig. 3, lane 24) and for the isolate LP7 (Fig. 3, lane 8). The two reference stocks *L.* (*L.*) *infantum I* and *II* and the stock called LSI presented a band of 530–550 bp as a product of the PCR. The three stocks from Mexico (LM1–LM3) amplified a band of 250–270 bp common to the three isolates. The reference stocks *L.* (*V.*) *peruviana* and *L.*(*V.*) *brazilensis* and the stocks LP1–LP14, excepting the isolate LP7, had similar kDNA fingerprints, with a band of 160–170 bp (Fig. 3, lanes 20, 21, from 1 to 7 and from 9 to 14). The reference stock *L.*(*L.*) *amazonensis* gave a band of 250-bp as the PCR product (Fig. 3, lane 19).

## Discussion

The prognosis of human leishmaniasis depends in part on the species of the infecting parasite. In field situations the isolation of *Leishmania* cultures for this species characterization is not always possible, especially as very few parasites are often present in lesions, blood, skin, organs, and sandflies. Moreover, isolation and culture protocols can select for particular clones from a heterogeneous population. It is highly desirable to develop sensitive molecular tools that are able to identify and type *Leishmania* directly (Breniere *et al*., 1999).

By isoenzymatic analysis, 10 *Leishmania* isolates were characterized from different areas of Peru: the isolates LP5–LP8 from the Peruvian jungle, stocks LP9–LP11 from the Andes area, and stocks LP12–LP14 from the border with Colombia and Ecuador. The characterizations were made with seven enzyme systems that can accurately identify parasites in the genus *Leishmania* (Shamsuzzaman *et al*., 2000). The statistical study was performed on the basis of the results from the isoenzyme-pattern comparisons, including in this study other previously characterized isolates from Peru: LP1–LP4 and the reference strain *L.* (*V.*) *peruviana* (Rodríguez-González *et al*., 2006). The different isolates were grouped again into three clusters: Cluster 1 with stocks LP1, LP2, LP4, and the reference stock *L.* (*V.*) *brazilensis*; Cluster 2 with *L.* (*V.*) *peruviana*, *L.* (*L.*) *amazonensis*; and the stocks LP5, LP7, LP8, LP13, and LP14; and Cluster 3 with the rest of the stocks LP3, LP6, and LP9–LP12.

When the statistical study was made on the basis of the restriction cleavage patterns, the isolates were regrouped into four clusters: Cluster 1 included the reference stock *L.* (*V.*) *peruviana* together with the three isolates from the border between Peru, Colombia, and Ecuador (LP12–LP14); Cluster 2 held *L.* (*L.*) *amazonensis*; Cluster 3 was composed of *L.* (*V.*) *brazilensis* and the isolates LP1–LP11, excepting LP9, which made up Cluster 4.

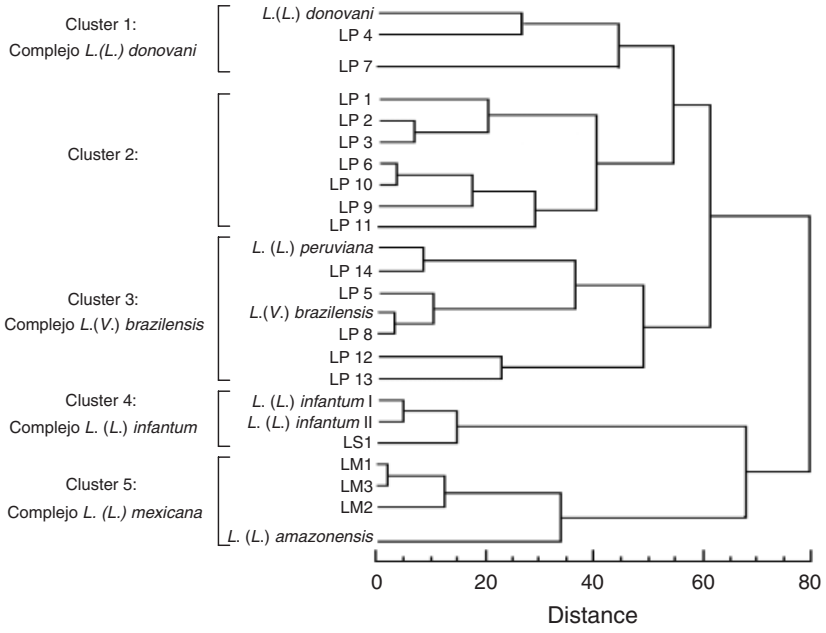


**Fig. 4.** Dendograma based on individual hirarchical cluster analysis (Program STAT-GRAPHICS version 5.0).

A multiplex PCR assay was developed and it uses a single conserved 5′ primer common to all *Leishmania* species plus three distinct 3′ primers, each of which is specific to a different complex (Harris *et al*., 1998). Thus, the identification of the *Leishmania* complexes was based on both the sizes of the products and the sequence specificities of the primers. For the *L.* (*L.*) *donovani*, products ranged in size from 370 to 390 bp, as in the case of the isolate LP7; for the three isolates from Mexico (LM1–LM3), the products ranged in size from 250 to 270 bp, and for *L.* (*V.*) *brazilensis* and the isolates LP1–LP6 and LP8–LP14, products ranged in size from 150 to 170 bp.

The PCR was expected to be specific only for the stocks belonging to the complexes *L.* (*L.*) *donovani*, *L.* (*V.*) *brazilensis*, and *L.* (*L.*) *mexicana*; however, with the PCR evaluation with stocks *L.* (*L.*) *infantum* I and II and the isolate called LS1, a common band was amplified to the three of 530–550 bp. This behaviour was presumably because the amplified regions were highly conserved in all the species, with some divergences, favouring the hybridization of the oligos (Cáceres & Montoya, 2002). The multiplex assay distinguished *Leishmania* from *Trypanosoma cruzi* and generated no product (data not shown). This is an important feature, because mixed infections of *Leishmania* and *T. cruzi* can occur and are subject to antibody cross-reactivities to related antigens in the two parasites (Harris *et al*., 1998).

When the statistical treatment is applied to these results, the isolates are grouped into three large Clusters. The first, in turn, is divided into two subclusters: (a) is *L.* (*L.*) *donovani* and isolate LP7, which may indicate that this isolate is *Leishmania* (*L.*) *chagasi*; (b) is comprised of *L.* (*L.*) *amazonensis* and the three Mexican stocks. The second large cluster was composed of two stocks of *L.* (*L.*) *infantum* I and II and the isolate LSI, which we can now identify as *L.* (*L.*) *infantum*. The third Cluster was made up of the reference stocks *L.* (*V.*) *brazilensis* and *L.* (*V.*) *peruviana* together with the rest of the isolates.

The most appropriate identification and phylogenetic relationship was achieved with the analysis of all the data pooled, including those from the previous study (Rodríguez-González *et al*., 2006). The following grouping of five clusters resulted according to the statistical method (Fig. 4). The first group (Cluster 1) contained *L.* (*L.*) *donovani* together with the isolates LP4 and LP7, which would comprise the *donovani* complex, these surely being species belonging to the New World visceral form (*L.* (*L.*) *chagasi*). Cluster 2 would be made up of LP1, LP2, LP3, LP6, LP10, LP9, and LP11; these would be phylogenetically intermediate between the *donovani* complex (Cluster 1) and the *brazilensis* complex (Cluster 3), or they could be hybrids, as is known to occur on the American continent (Hernández *et al*., 1991). This hybridization would not be surprising as these strains were isolated in the same geographical area and present similar chronic cutaneous lesions, compromising the mucosal ones, while the strain *L.* (*L.*) *donovani* is phylogenetically closer to the Peruvian strains (Rodríguez-González *et al*., 2006). Cluster 3 could be divided into two subgroups, one formed by *L.* (*V.*) *peruviana*, together with the isolates LP14 and LP5, and the second subgroup composed of *L.* (*V.*) *brazilensis* and the isolate LP8, these two subgroups forming part of the *brazilensis* complex (Laison & Saw, 1987). *L.* (*V.*) *peruviana* is known to be ecogenetically heterogeneous, and thus *L.* (*V.*) *peruviana* from the Department of Huancabamba (Piura) differs from

species originating in the south (Departments of Ancash, Lima, and Ica). There is a close relationship between the evolution of the species *L.* (*V.*) *peruviana* and *L.* (*V.*) *brazilensis*, although the latter is karyotypically more homogeneous than *L.* (*V.*) *peruviana*. Meanwhile, *L.* (*V.*) *brazilensis* and *L.* (*V.*) *peruviana* are genetically closer, as they can be distinguished only by the locus of one enzyme (Dujardin *et al.*, 1995).

Cluster 4 would be formed by the stocks *L.* (*L.*) *infantum* [*L.* (*L.*) *infantum* I and II and LSI]. Finally, Cluster 5 would include the three Mexican stocks (LM1–LM3) forming a subgroup, and another subgroup would have *L.* (*L.*) *amazonensis*, these two subgroups forming the *mexicana* complex. All these groupings establish a certain correlation between the geographical distribution of the isolates with the identity of the species.

New World *Leishmania* species of different complexes can be found in the same types of clinical specimen; for example, *L.* (*L.*) *mexicana* and *L.* (*L.*) *chagasi* are found in cutaneous nodules (Neva *et al.*, 1997), while *L.* (*V.*) *brazilensis* and *L.* (*L.*) *amazonensis* are found in lesions of cutaneous leishmaniasis (Grimaldi & Tesh, 1993), among others. Other studies suggest that the aetiology of the American visceral leishmaniasis in the New World may be more complex than currently believed. Most of the New World *Leishmania* species appear capable of producing a spectrum of disease manifestations (Hernández *et al.*, 1991). The different species of *Leishmania* are morphologically indistinguishable, and thus the taxonomy of this genus bears special medical importance. It is very difficult to establish the number of species existing in Peru for the continuous discovery of new species, including, in the last few years, hybrid forms (Mita, 2001; Flores *et al.*, 2002). Furthermore, near Brazil, Venezuela, Colombia, Chile, Bolivia, and Ecuador, a mixture of species has resulted from human migrations, urbanization, deforestation, etc., but much more from the animals that migrate through the Amazon, carrying parasites previously unknown in these areas (Davies *et al.*, 2000; Desjeux, 2004; Alcántara de Castroa *et al.*, 2005). Moreover, New World cutaneous and mucocutaneous leishmaniasis are increasingly diagnosed among European travellers returning from Latin American countries and the disease is usually acquired in rural or jungle areas (Schwartz *et al.*, 2006). Information on the incidence of cutaneous leishmaniasis in travellers is scarce for a number of reasons: (1) it is not a notifiable disease in most industrialized countries; (2) the number of exposed people (i.e. the total number of travellers to endemic areas) is often unknown; (3) the disease is frequently misdiagnosed; and (4) spontaneous self-healing is possible (Scope *et al.*, 2003). Latin America was the primary source of infection (Zeegelaaer *et al.*, 2005). The differentiation and characterization of these parasite populations are vital in order to establish better diagnosis, treatment, prognosis, and control of the parasitoses, as well as to understand the influence that intraspecific variations may have in the epidemiology of these diseases. In this work, we have characterized a series of human isolates of different stocks or species of *Leishmania* that are currently circulating throughout Peru using isoenzymatic characterization, by kDNA analysis by restriction enzymes, and PCR multiplex assay, which enables the phylogenetic study of these stocks and contributes to a rapid identification of *Leishmania* complexes for epidemiological purposes in areas where leishmaniasis is endemic. Today, PCR is the diagnostic method of choice since it has a high sensitivity and gives a species-specific diagnosis (Santamaría *et al.*, 2005; Schwartz *et al.*, 2006). Recently, several studies in Ecuador have found that PCR-based methods are more sensitive (85.4% on average) than classical diagnostic techniques, called slit smears (45.4%), culture (57.2%), and histopathology (34.7%) (Calvopina *et al.*, 2004).

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