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Identification and biochemical characterization of *Leishmania* strains isolated in Peru, Mexico, and Spain

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Abstract

Eight *Leishmania* promastigotes were isolated from different geographical areas: three (LP1, LP2, and LP3) from the provincial department La Libertad and the fourth (LP4) from the department of Cajamarca (northern Peru); another three (LM1, LM2, and LM3) in the province of Campeche (Mexico); and the last (LS1) from a clinical case of a dog in Madrid (Spain). The isolates were characterized by carbohydrate cell-surface residues using agglutinations with four purified lectins, by isoenzyme analysis using different isoenzymes, by analysis of kinetoplast DNA (kDNA) restriction fragment length polymorphism using four different restriction endonucleases and by the final metabolite patterns after in vitro culture. These isolates were compared with four reference strains and typified as: *Leishmania* (*Leishmania*) *donovani*, two strains of *L.* (*L.*) *infantum*, and one species of *L.* (*Viania*) *peruviana*. According to our results and the statistical study, the Peruvian isolates represent three different strains: one would be *L.* (*V.*) *peruviana*, another the strain isolated in Cajamarca (LP4) and the third would include the three strains from the department of La Libertad (LP1, LP2, and LP3), these latter three isolates being phylogenetically closer to the reference strain *L.* (*L.*) *donovani*. Meanwhile, the three isolates from Mexico form a group with close phylogenetic relationships to each other. The isolate from Spain belongs to the species *L.* (*L.*) *infantum*. Thus, a close correlation was drawn between the identity of each strain and its geographical origin.

Index Descriptors and Abbreviations: *Leishmania* species; In vitro culture; Lectin agglutination: Con A, lectin from *Canavalia ensiformis*; VV, lectin from *Vicia villosa*; WGA, lectin from *Triticum vulgaris*; PNA, lectin from *Arachis hypogae*; Isoenzyme electrophoresis: ME, malic enzyme [EC 1.1.1.40]; MDH, malate dehydrogenase [EC 1.1.1.37]; IDH, isocitrate dehydrogenase [EC 1.1.1.42]; GPI, glucose phosphate isomerase [EC 5.3.1.9]; PGM, phosphoglucomutase [EC 2.7.5.1]; SOD, superoxide dismutase [EC 1.15.1.1]; kDNA restriction pattern: kinetoplast deoxyribonucleic acid; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; MEM, minimal essential medium; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; DNA, deoxyribonucleic acid

1. Introduction

Human leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, constitutes a serious public health problem in several countries, according to the World Health Organization (W.H.O., 1997). In human hosts, the clinical profile of different *Leishmania* species can vary from a single cutaneous lesion, which may undergo spontaneous cure, to mucocutaneous lesions that can become grossly disfiguring. Severe diffuse cutaneous lesions, that is, extremely difficult to treat, can also occur. Moreover, the disease can evolve to visceral forms that are lethal in most cases (Ferreira et al., 2003).

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Clinicians are confronted with steadily higher numbers of leishmaniasis patients not only in countries where the disease is endemic but also in countries where these parasites are not endemic. This increased incidence is partly due to geographical expansion of the disease, changing patterns of international travel and population migration, non-immune people moving into endemic regions, or infected people moving into non-endemic regions (Desjeux, 2001).

Leishmania species are morphologically very similar and species identification is possible using standard biochemical methods (lectin agglutination, isoenzyme analysis, analysis of kDNA restriction fragment using different restriction endonuclease, etc.). As has been demonstrated in many works where these techniques have been satisfactorily used to characterize *Leishmania* isolates (Andrade and Saraiva, 1999; Belhadj et al., 2003; Sampali et al., 2003; Shamsuzzaman et al., 2000), 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 co-exist. Identification of the infecting species based on clinical symptoms can be problematic, since several species cause both cutaneous and mucocutaneous disease, while others cause visceral and cutaneous disease (Schönian et al., 2003).

In the present work, we characterized eight *Leishmania* isolates from different areas of Latin America (Peru and Mexico) as well as from the Mediterranean region (Spain), using interaction of the parasites with lectins together with electrophoretic analysis of their isoenzyme profiles and analysis of kDNA restriction fragment. Morphologically, all these have been considered to be members of the genus *Leishmania*. For comparison, we used four isolates from human cases characterized as: *L.* (*L.*) *donovani*, two strains belonging to *Leishmania* (*L.*) *infantum* and another characterized as *Leishmania* (*Viania*) *peruviana*. In addition, we made a comparative study of the major end-products excreted into the culture medium by the parasites.

2. Materials and methods

2.1. Parasite isolation and in vitro culture

The eight *Leishmania* were isolated from different areas of Peru, Mexico, and Spain. Three of these isolates were from cutaneous cases in the central zone (LP1 and LP2) and north-eastern part (LP3) of the department of La Libertad (Peru); a fourth isolate (LP4) from a mucosal lesion was isolated in the zone of Cajamarca, in north-eastern Peru (near the border of Ecuador and Colombia). Three isolates (LM1, LM2, and LM3) were from cutaneous lesions in Campeche (Mexico) during the period 2000–2002. The final isolate (LS1), from the area of Madrid (Spain), was taken from a dog. For comparison, we also included: *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/84/LC26) and two strains of *L.* (*L.*) *infantum*: I and II, characterized as MCAN/ES/2001/UCM-10 and MCAN/2000/UCM-1 isolated in Spain, respectively. (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 approximately every two weeks and at least every six months, was inoculated in the Syrian golden hamster, *Mesocricetus auratus*, and parasites are isolated from the spleen 30 to 45 days post-inoculation.

The isolates from hamsters were cloned and cultured in vitro as previously described (Sánchez-Moreno et al., 1995). Different monophasic cultures were tested: RPMI-1640, MTL, and TC medium (Gibco, Karlsruhe, Germany) and MEM (Sigma, St. Louis, MO).

The epimastigote forms of *Trypanosoma cruzi* strain Maracay were cultured in vitro using Grace's medium (Sigma) (Sánchez-Moreno et al., 1995).

2.2. Lectin-agglutination test

Lectins from *Canavalia ensiformis* (Con A), *Vicia villosa* (VV), *Triticum vulgaris*, a wheat-germ agglutination (WGA), and *Arachis hypogaea* (PNA) were used (Sigma, St. Louis, MO). Parasites were washed three times with phosphate-buffered saline, pH 7.4, and collected by centrifugation ($600g \times 10$ min, 4 °C), and resuspended in phosphate-buffered saline, pH 7.4, containing 0.5% inactivated foetal bovine serum. Tests were performed in duplicate in 96-well plates (Flow Laboratories). The parasite suspension (50 μl) was incubated with equal volumes of lectins at different concentrations for 1 h at room temperature (final lectin concentrations of 1, 10, 20, 50, 100, 150, 500, 750, and 1000 μg/ml). Agglutination was determined by microscopic observation according to (Zubiaur and Alonso, 1985). Controls for specific agglutination were performed using 0.1 M α-D-glucosyl for Con A, 0.1 M *N*-acetyl-D-galactosamine for VV, 0.1 M β-galactose for PNA, and 0.1 M *N*-acetyl-D-glucosamine for WGA.

2.3. Isoenzyme characterization

Crude homogenates were obtained from 250 ml of culture medium containing 2×10^7 cells/ml. Cells were harvested by centrifugation at $1500g \times 10$ 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 (Fernandez-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

8000g × 20 min at 4 °C, and the supernatants were stored in liquid nitrogen until used. The protein concentration was determined using the Bradford method and stored at a last concentration of 1 mg/ml 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 (ME), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), and superoxide dismutase (SOD). The staining procedures are described in Fernandez-Ramos et al. (1999).

2.4. Kinetoplast-DNA isolation, restriction enzyme digestion, and electrophoresis analysis

Promastigotes were collected by centrifugation of 300 ml of culture medium, after about 5 days. When their concentrations had reached about 2×10^7 cells/ml. 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). Kinetoplast DNA was obtained according to the procedure described by Gonçalves et al. (1984).

The kDNA extracts (3 μg/μl) were digested to completion with restriction endonucleases (*Hae*III, *Bam*HI, *Hin*fI, and *Eco*RI) according to the manufacturer's prescribed buffer conditions (Boehringer–Ingelheim, Barcelona, Spain). The digestion products were electrophoresed in 1.5% agarose slab gels as described (Riou and 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 μg/ml for 10 min) and photographed under UV light with a Polaroid camera (665 film).

2.5. ^1H NMR spectroscopy and metabolite identification

For the spectroscopic studies, 5 ml of a 5-day-old culture of each isolate in MEM was centrifuged at 1500g for 10 min at 4 °C. The pellet was discarded, and the parasite-free supernatant was stored at −20 °C until used.

The ^{1}H NMR spectra were determined according to a previous described method (Fernandez-Ramos et al., 1999). The chemical displacements used to identify the respective metabolites were consistent with those of Sánchez-Moreno et al. (1995).

2.6. 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 R and, which is an index of the similarity between classifications. This analysis was made with the StatGraphics program, version 5.0.

3. Results

Leishmania isolates were cultured in vitro, assaying different liquid media. In general, all the isolates grew satisfactorily in MEM (Fig. 1), reaching cell densities on the order of 6.5×10^6–1×10^7 cells/ml, depending on the isolate.

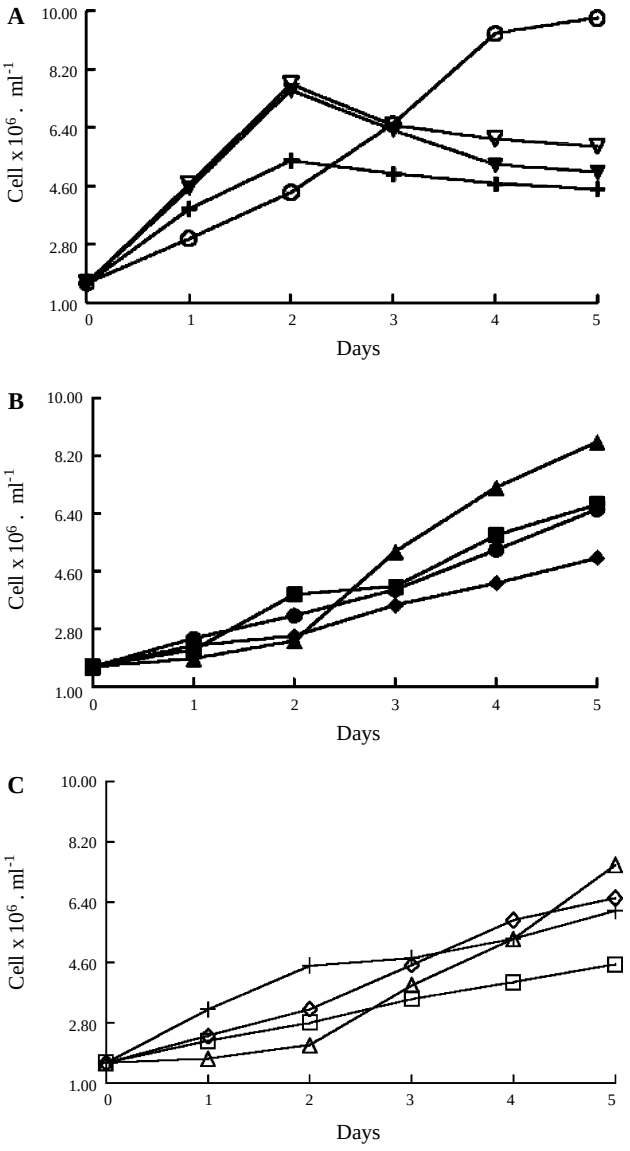

Fig. 1. Growth curves of *Leishmania* isolates in Latin America and Spain. (A) *L.* (*L.*) *donovani* (○), *L.* (*L.*) *infantum* I (▼), *L.* (*L.*) *infantum* II (▽), and LS1 (+). (B) *L.* (*V.*) *peruviana* (▲), LP1 (◆), LP2 (■), and LP3 (●). (C) LP4 (△), LM1 (+), LM2 (◇), and LM3 (□).

Table 1 presents the results for lectin-agglutination tests. All the isolates showed agglutination with the lectins Con A, but with different degrees of agglutination and minimum concentrations required to agglutinate. The reference strain *L.* (*V.*) *peruviana* weakly agglutinated at a concentration of 1 μg/ml; the three isolates from Spain (*L.* (*L.*) *infantum* I and II and LS1) agglutinated at 5 μg/ml, while the rest of the isolates and *L.* (*L.*) *donovani* needed a greater concentration of lectins (20 μg/ml), although for the isolates from Peru (LP1, LP2, and LP3) and Mexico (LM1) the agglutination was very strong.

Only the isolates from Peru agglutinated with the *V. villosa* lectin at the maximum concentration assayed. With the WGA lectin at a concentration of 5 μg/ml, only the reference strain *L.* (*V.*) *peruviana* agglutinated. The three isolates from Spain and the LP4 isolate also agglutinated with WGA, but agglutinated, despite that the concentrations were close to the maximum assayed. *A. hypogaea* lectin agglutinated with all the isolates, but with differences in the concentration required for agglutination.

Fig. 2 shows the isoenzymatic profiles of the isolates analyzed by six enzyme systems (GPI, IDH, MDH, ME, SOD, and PGM). 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.* (*L.*) *donovani*, for which the profile with the six systems studied, in general, differed from the rest. The isolate LP4 was also different from the rest of the strains.

The pattern of *L.* (*V.*) *peruviana* (Fig. 2, lane 1), differed from the other isolates from Peru (Fig. 2, lanes 3, 7–9); nevertheless, the isolates LP2 and LP3 showed profiles very similar to the enzymes IDH, ME, and SOD (Figs. 2B, D, and E, lanes 7 and 9, respectively).

The isolates from Mexico had isoenzymatic profiles similar to each other, although the variations between LM2 and the other two isolates in the number of bands was apparent in the enzyme GPI (Fig. 2A). The isolates LM1 and LM3 were similar in the enzymes IDH, ME, SOD, and PGM (Figs. 2B, D–F). The isolate LM2 was similar to LM1 and LM3 only in SOD. Variations are observed among all isolates in GPI and MDH (Figs. 2A and C).

The DNA of the kinetoplast of the eight isolates and the four reference strains were purified and then digested with four restriction enzymes (*Hae*III, *Hin*fI, *Bam*HI, and *Eco*RI). Electrophoresis using agarose gel revealed that while intact kDNA was not able to penetrate the gel (Fig. 3E, lane 17), the smaller DNA restriction fragments that resulted after 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. 3). Only *Hin*f proved incapable of digesting all the kDNA of the four reference strains and the eight new isolates (Fig. 3B).

The restriction cleavage patterns of the reference strains *L.* (*V.*) *peruviana* and *L.* (*L.*) *donovani* were completely different from each other and from the strains *L.* (*L.*) *infantum* I and II as well as from the eight new isolates, with the 4 endonucleases (Figs. 3A–D, lanes 1 and 2). The *L.* (*L.*) *infantum* strains and the LS1 isolate presented homogeneous restriction profiles, although different from the other isolates (Figs. 3A–D; lanes 4–6); the same was true of the three Mexican isolates (Fig. 3, lanes 10–12), although the enzyme *Bam*HI was not able to digest the kDNA of these isolates.

The Peruvian isolates LP2, LP3, and LP4 presented very heterogeneous profiles. LP3 and LP1 were found to be homogeneous in sequence for the restriction enzymes tested (Fig. 3, lanes 7 and 8). The LP2 isolate somewhat resembled the LP3 and LP1 isolates, with the enzymes (*Hae*III and *Bam*HI) (Figs. 3A and C, lane 9), but more closely resembled the profile presented by the LP4 isolate, when its kDNA was digested by the enzyme *Eco*RI (Fig. 3D, lane 9).

The metabolites excreted by the parasite during its in vitro growth are shown in Fig. 4. All the isolates excreted pyruvate and acetate as main metabolites, although quantitative differences were evident between the isolates. For example, for LP4 and LM1 (Figs. 4B and G) the main metabolite was acetate, followed by pyruvate, while for the rest of the isolates the main metabolite was pyruvate followed by acetate (Figs. 4C–F). Some isolates,

Table 1
Agglutination activity of lectins for eight *Leishmania* isolates

Leishmania	Minimal concentration required to agglutinate (μg/ml)[a]			
	Concavalina ensiformis (Con A)	*Vicia villosa* (VV)	*T. vulgaris* (WGA)	*Arachis hypogaea* (PNA)
L. (*V.*) *peruviana*	1 (+)	1000 (+)	5 (+)	1 (+)
L. (*L.*) *donovani*	20 (++)	—	—	10 (+++)
L. (*L.*) *infantum* I	5 (+)	—	500 (+)	1000 (+)
L. (*L.*) *infantum* II	5 (+)	—	1000 (+)	1000 (+)
LS1	5 (+)	—	1000 (+)	1000 (+)
LP1	20 (+++)	1000 (+)	—	50 (+)
LP2	20 (+++)	1000 (+)	—	10 (+++)
LP3	20 (+++)	1000 (+)	—	1 (+)
LP4	20 (+)	—	1000 (+)	10 (+)
LM1	20 (+++)	—	—	1000 (+)
LM2	20 (+)	—	—	1000 (+)
LM3	20 (+)	—	—	1000 (+)

[a] Agglutination was scored from – (no agglutination at 1000 μg/ml) to (+++) (virtually complete agglutination). Average of three experiments.

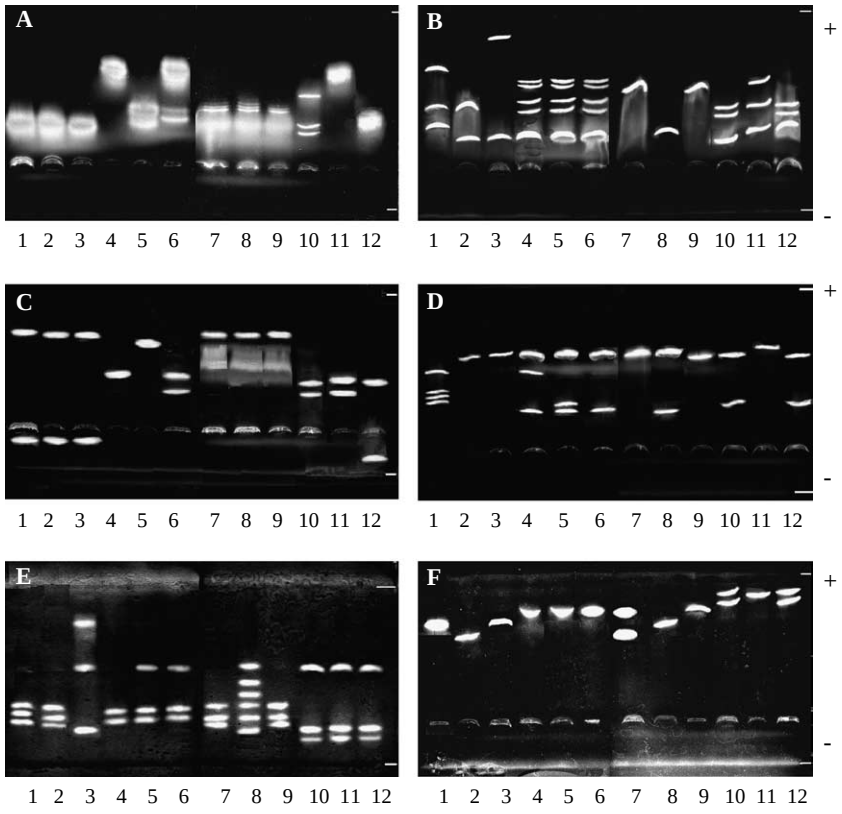

Fig. 2. Isoenzyme profiles of *Leishmania* isolates. Organisms: (lane1) *L.* (*V.*) *peruviana*; (lane 2) *L.* (*L.*) *donovani*; (lane 3) isolate LP4; (lane 4) *L.* (*L.*) *infantum* I; (lane 5) *L.* (*L.*) *infantum* II; (lane 6) isolate LS1; (lane 7) isolate LP3; (lane 8) isolate LP1; (lane 9) isolate LP2; (lane 10) isolate LM1; (lane 11) isolate LM2; and (lane 12) isolate LM3. Enzymes: (A) Glucose phosphate isomerase (GPI). (B) Isocitrate dehydrogenase (IDH). (C) Malate dehydrogenase (MDH). (D) Malic enzyme (ME). (E) Superoxide dismutase (SOD), and (F) Phosphoglucomutase (PGM).

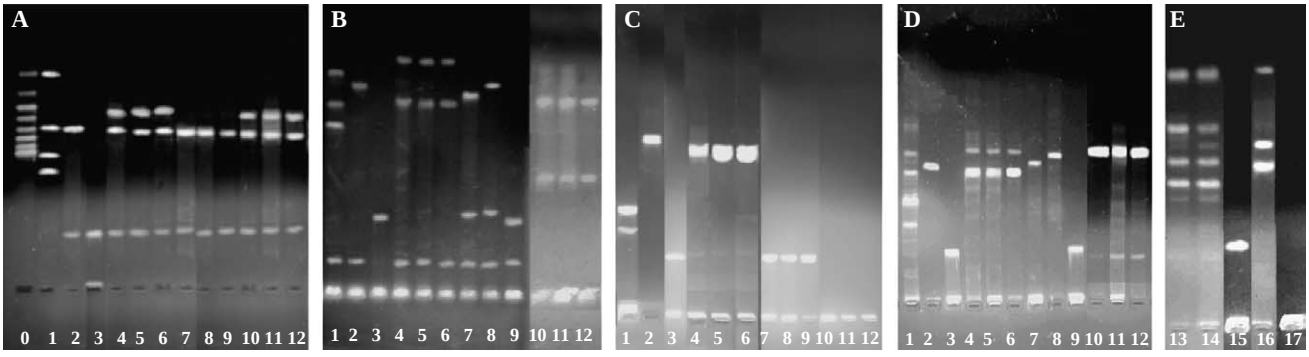

Fig. 3. Restriction endonuclease analysis of kDNA of *Leishmania* isolates. (A) kDNAs + *Hae*III. (B) kDNAs + *Hin*fI. (C) kDNA + *Bam*HI. (D) kDNA + *Eco*RI. Lane 1, *L.* (*V.*) *peruviana*; lane 2, *L.* (*L.*) *donovani*; lane 3, isolate LP4; lane 4, *L.* (*L.*) *infantum* I; lane 5, *L.* (*L.*) *infantum* II; lane 6, isolate LS1; lane 7, isolate LP3; lane 8, isolate LP1; lane 9, isolate LP2; lane 10, isolate LM1; lane 11, isolate LM2; and lane 12, isolate LM3. (E) Controls: kDNA of *Trypanosoma cruzi* strains *maracay*. Lane 13, kDNAs + *Hae*III; lane 14, kDNAs + *Hin*fI; lane 15, kDNA + *Bam*HI; lane 16, kDNA + *Eco*RI; and lane 17, Intact kDNA *L.* (*L.*) *donovani*. Size markers are 100-bp DNA ladder fragments (lane 0).

apart from excreting L-alanine as a secondary metabolite (Figs. 4B–C and F–G); (*L.* (*L.*) *donovani*, LP1, and LM1), also excreted a certain quantity of succinate. The three isolates from Spain showed some very similar spectra (data not shown), and a similar situation occurred with the isolates from Peru, as *L.* (*V.*) *peruviana* resembled the isolate LP4, and the rest of the Peruvian isolates were very similar to each other, differing only in the heights of the peaks (data not shown).

4. Discussion

To prevent variability derived from the culture medium, all the isolates were cultured in the same type of medium. The isolates were cultured in different cultured media having been assayed to identify the most adequate one. The MEM gave the highest growth density for most of the isolates and was selected to grow the isolates.

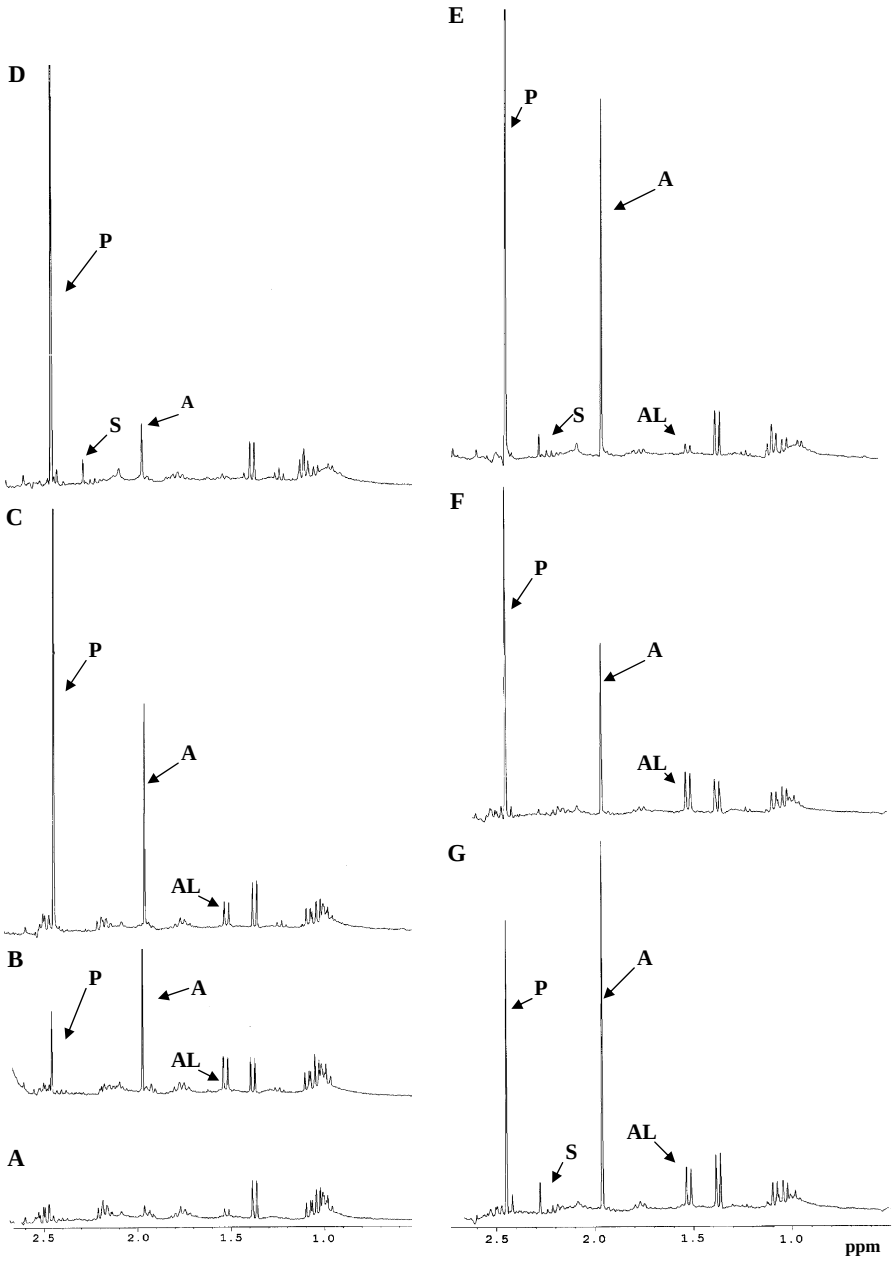

Fig. 4. ^{1}H NMR spectra of the *Leishmania* culture medium. (A) Fresh culture medium before inoculation of cells, (B) isolate LP4, (C) *L.* (*L.*) *infantum* I, (D) *L.* (*L.*) *donovani*, (E) isolate LP1, (F) isolate LM2, and (G) isolate LM1. **P**, pyruvate; **A**, acetate; **AL**, L-alanine; and **S**, succinate.

After determining the most suitable medium, we characterized all the isolates which identified to the genus *Leishmania*, by agglutination tests with lectins, by isoenzymatic analysis, by kDNA analysis and by the metabolic strategies of *Leishmania* in degrading energy substrates.

Cell-surface carbohydrates of *Leishmania* play a key role in parasite entry and survival inside the macrophage and in the digestive tract of the sandfly vector. Lectins are useful tools to study the topography, fate, distribution, and function of glycoconjugates on and inside parasites due to their specific and well-characterized sugar-binding properties (Andrade and Saraiva, 1999).

Cell-surface carbohydrates of different *Leishmania* species have been studied by a number of authors, taking advantage of their lectin-binding characteristics (Jacobson, 1994; Jacobson and Doyle, 1996). In the present study, all the isolates were agglutinated by Con A, at different concentrations, and results are in agreement with Dwyer (1977). The binding of WGA by *L.* (*L.*) *donovani* reported by Bandyopadhyay et al. (1991), was not observed in this study and other studies (Andrade and Saraiva, 1999; Sacks et al., 1995), although agglutination was observed with the Spanish *Leishmania* isolates as well as the LP4 and *L.* (*L.*) *peruviana* isolates. Our data coincide with those reported by different other authors (Andrade and Saraiva, 1999; Shottelius, 1982) in that all the isolates agglutinate with the PNA lectin.

According to the statistical analysis of Group Average Method, the Euclidean distance of the lectin-agglutination

test grouped the different isolates into four clusters: first *L.* (*L.*) *donovani*, the isolates from Mexico (LM1, LM2, and LM3) together with the Peruvian isolate LP4; second, the isolate from Spain (LS1), and the two reference strains of *L.* (*L.*) *infantum;* third, the isolates from Peru (LP1, LP2, and LP3); and fourth, the reference strain *L.* (*V.*) *peruviana* (data not shown).

Also, the isolates were characterized with six enzymes that can accurately identify parasites in the genus *Leishmania* (Shamsuzzaman et al., 2000). When the statistical study was made on the basis of the results from the isoenzyme-pattern comparisons, the different isolates were grouped again in four clusters: cluster I with *L.* (*V.*) *peruviana*; cluster II with *L.* (*L.*) *donovani* and the Peruvian isolates (LP2, LP3, and LP4); cluster III with the Spanish strain *L.* (*L.*) *infantum* I, *L.* (*L.*) *infantum* II and the isolate LS1 together with the Mexican isolates and cluster IV with Peruvian isolate LP1.

When the statistical study was made on the basis of the restriction cleavage patterns, the isolates were grouped into four clusters. Cluster 1 contained *L.* (*V.*) *peruviana*, cluster 2 the LP4 isolate, and cluster 3 *L.* (*L.*) *donovani*; cluster 4 could be divided into three subclusters, the first included the two *L.* (*L.*) *infantum* strains and the LS1 isolate, the second included the LP1, LP2, and LP3 isolates, and the third included the three Mexican isolates.

It is known that members of the family Trypanosomatidae are incapable of completely degrading carbohydrates even in the presence of oxygen, producing CO_2 and dicarboxylic acids. The relative proportion of these end-products varies among subspecies (Miralles et al., 2002; Urbina et al., 1993).

The ^{1}H NMR study indicated that the main metabolites by all the isolates excreted were pyruvate and acetate. Some isolates the secondary metabolites were succinate and in others alanine. The Group Average Methods statistical treatment grouped the isolates into three clusters: first, *L.* (*V.*) *peruviana* and LP4; second, *L.* (*L.*) *donovani*; and third, the rest of the isolates (data not shown).

The most appropriate identification and phylogenetic relationship was achieved with the analysis of the all data pooled, providing the following grouping according to the statistical method used (Fig. 5): the cluster analysis gave two clear groups that in turn could be broken down into subgroups. The first large group (cluster 1) was composed of three subgroups: the first would be *L.* (*V.*) *peruviana* alone; the second would be the Spanish strains, two of which had already been classified as belonging to *L.* (*L.*) *infantum*, and our data indicate that the third strain (LS1) also belongs to one species; and the third subgroup would be from Mexico, which according to our data would belong to the same strain. The second large cluster is formed by the Peruvian strains (LP1, LP2, and LP3), this not being surprising as these strains were isolated in the same geographical area and present similar chronic cutaneous lesions, compromising the mucosa ones, and the strain *L.* (*L.*) *donovani* is phylogenetically closer to the Peruvian strains.

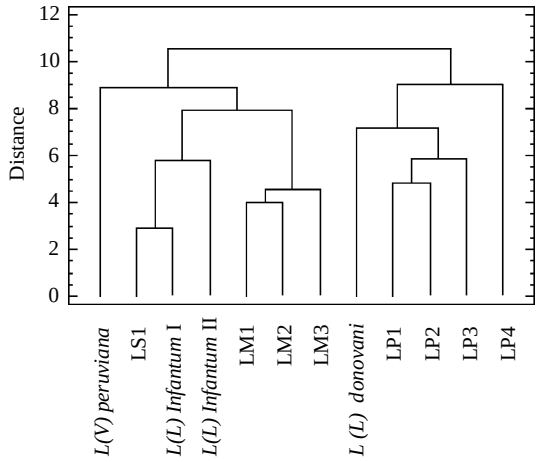

Fig. 5. Dendrogram based on individual hierarchical cluster analysis (Program Stat-Graphics version 5.0).

Finally, farther removed and forming a different subgroup is the LP4 isolate of the department of Cajamarca, which would belong to a different strain from *L.* (*V.*) *peruviana* and from the other three Peruvian isolates, and presumably for their geographic location would be closer to an Ecuadorian or Colombian strain.

All these groupings establish a certain correlation between the geographical distribution of the isolates with the identity of the species. Nevertheless, it is necessary to probe further into this matter in order to establish whether the Peruvian isolates present three different species: one would be *L.* (*V.*) *peruviana*, another the strain isolated in Cajamarca (LP4) (department bordering Ecuador and Colombia); and the third would include the three strains isolated from the department of La Libertad, which would be considered a different strain or subspecies, also with different clinical manifestations. In addition, it is necessary to include the reference strain *L.* (*L.*) *mexicana* to confirm that the three isolates from Mexico belong to it or are very closely related to it.

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