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A new focus of cutaneous leishmaniasis due to *Leishmania amazonensis* in a Sub Andean region of Bolivia

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Abstract

We detected a new outbreak focus with high incidence of cutaneous leishmaniasis in the Sub Andean region of La Paz. This area was never considered previously as an endemic zone of leishmaniasis. *Leishmania* stocks from human lesions were isolated: three stocks were explored by pulse field gradient electrophoresis, showing evidence for their affiliation to the *L. mexicana* complex. Eight stocks were submitted to isoenzyme electrophoresis and compared with five reference strains: *L. amazonensis*, *L. braziliensis*, *L. chagasi*, *L. mexicana* and *L. pifanoi*. Close genetic proximity was evidenced between newly isolated parasites and the reference stock of *L. amazonensis*, whereas high divergence was observed between them and either the *L. pifanoi*, *L. mexicana*, *L. braziliensis* and *L. chagasi* reference strains. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Cutaneous leishmaniasis; *Leishmania mexicana* complex; *L. amazonensis*; Multilocus enzyme electrophoresis; Orthogonal field alternated gel electrophoresis; Bolivia

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1. Introduction

In most parts of Bolivia, the main *Leishmania* species responsible for cutaneous leishmaniasis is *L. braziliensis*. Parasites belonging to *L. amazonensis* were exceptionally identified (Desjeux et al., 1986; O.M.S., 1990; Le Pont et al., 1992; Dedet, 1993). Two suspected cases presenting cutaneous diffuse leishmaniasis (CDL), without confirmation of the parasite identity, had been previously reported in the Yungas area (Prado Barrientos, 1948a,b; Valda Rodriguez, 1980). Parasites confirmed as *L. amazonensis* were occasionally reported in the lowlands of the Department of Santa Cruz, Bolivia (La Fuente et al., 1986; Dujardin et al., 1987; Grimaldi et al., 1987). The present study concerns human *Leishmania* parasite stocks from an outbreak focus located in the province Inquisivi, where cutaneous lesions seemed to occur without any apparent mucocutaneous complications.

The karyotype analysis assigned the parasites to the *L. mexicana* complex. An electrophoretic comparison between the eight parasite stocks isolated from patients and the five reference strains of *Leishmania* demonstrated that the parasites isolated corresponded to *L. amazonensis*. This is the first evidence of the presence of *L. amazonensis* in the province Inquisivi (Department of La Paz, Bolivia).

2. Materials and methods

2.1. Study area

The present investigation was carried out at a circumscript new focus with high incidence of cutaneous leishmaniasis involving Cajuata and surrounding communities in the province Inquisivi, at an altitude ranging from 1450 to 2100 m.a.s.l. in the southeast (67°15′W, 16°42′S) of the Department of La Paz, Bolivia (Fig. 1). Cultivation of citrus fruits, banana, coffee and coca and mining are the main local activities. Dwellings are dispersed between small areas of residual forest. The temperature during the dry season (May to October) is moderate between 20 and 25°C, reaching up to 30°C during the rainy season (November to April).

2.2. Clinical observations

Between 1995 and 1996, the area was visited monthly in order to detect the presence of patients with suspected lesions of leishmaniasis. Mucocutaneous lesions were sought on the basis of anamnestic and clinical data.

2.3. Parasite isolation and experimental infection of hamsters

Material from cutaneous ulcers of patients was aspirated into a syringe with saline solution and subcutaneously inoculated into the posterior legs of hamsters. Blood from hamsters developing granuloma at an inoculation site was aspirated into a syringe with sterile saline solution and then cultured in tubes of diphasic

medium (NNN and Schneider). The latter were stored at 24°C after changing from diphasic to monophasic medium (Schneider).

2.4. Karyotype analysis

Three stocks were characterized by OFAGE (Orthogonal field alternated gel electrophoresis). Procedures for cultivation, harvesting and preparation for OFAGE have been described elsewhere (Dujardin et al., 1993a,b). OFAGE electrophoresis was carried out using three time pulses of 45, 65 and 115 s. This allowed

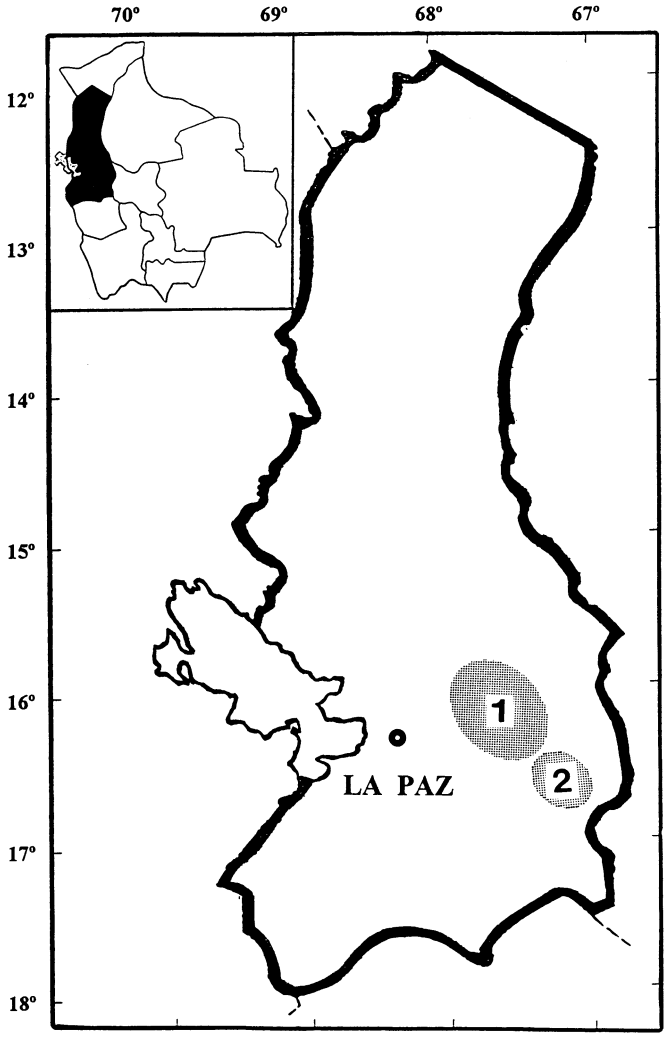

Fig. 1. Sketch map showing the location of the Cajuata focus in the Department of La Paz (Bolivia). 1, South Yungas; 2, Cajuata (Inquisivi).

resolution of the whole karyotype (Dujardin et al., 1993a,b). OFAGE-resolved chromosomes were transferred to nylon filters (HYBOND-N, Amersham (R)), processed and hybridized according to the manufacturer's instructions with a [^{32}P]dCTP random prime labeled beta-tubulin probe (pTbBETA-Tc2, Tomashow et al., 1983).

2.5. Isoenzyme electrophoresis

Eight stocks derived from human lesions were compared with five reference strains: *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903), *L. chagasi* (MHOM/BR/74/PP75), *L. mexicana* (MNYC/BZ/62/M379) and *L. pifanoi* (MHOM/VE/57/LV135).

Parasite stocks were submitted to Multilocus Enzyme Electrophoresis (MLEE) on cellulose acetate plates (Helena, Beaumont, TX). Running conditions and revelation techniques were derived from Dujardin et al. (1996). Each sample was mixed with a hypotonic enzyme stabilizer, maintained during 30 min on ice, centrifuged for 2 min at $3500 \times g$ and then immediately run for electrophoresis. Each 16 μl aliquot allowed the survey of as many as 12 different enzyme systems, including additional analyses for control and further verifications. The following 12 enzyme systems were assayed: Aconitase (EC 4.2.1.3, ACON), Glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PD), Glucose phosphate isomerase (EC 5.3.1.9, GPI), alfa-glycerophosphate dehydrogenase (EC 1.1.1.8, aGPD), Isocitrate dehydrogenase (EC 1.1.1.42, IDH), Malate dehydrogenase (EC 1.1.1.37, MDH), Peptidase 1, substrate L-leucyl-leucine (EC 3.4.11, PEP 1), 6-Phosphogluconate dehydrogenase (EC 1.1.1.44, 6PGD), Phosphoglucomutase (EC 2.7.5.1, PGM), Malic enzyme (EC 1.1.1.40, ME), Mannose phosphate isomerase (EC 5.3.1.8, MPI) and Fructose diphosphatase (EC 3.1.3.11, FDP).

2.6. Numerical analysis

The proportion of loci with 'fixed differences' (unshared alleles) was estimated between stocks (Richardson et al., 1986). From these distances, an UPGMA (Unweighted Pair Group Method of Analysis) tree was constructed.

3. Results

3.1. Clinical observations

After 1 year of active detection of cases, 172 people were found with active lesions typical of cutaneous leishmaniasis, 11 with scars. No one showed clinical evidence of mucocutaneous lesions.

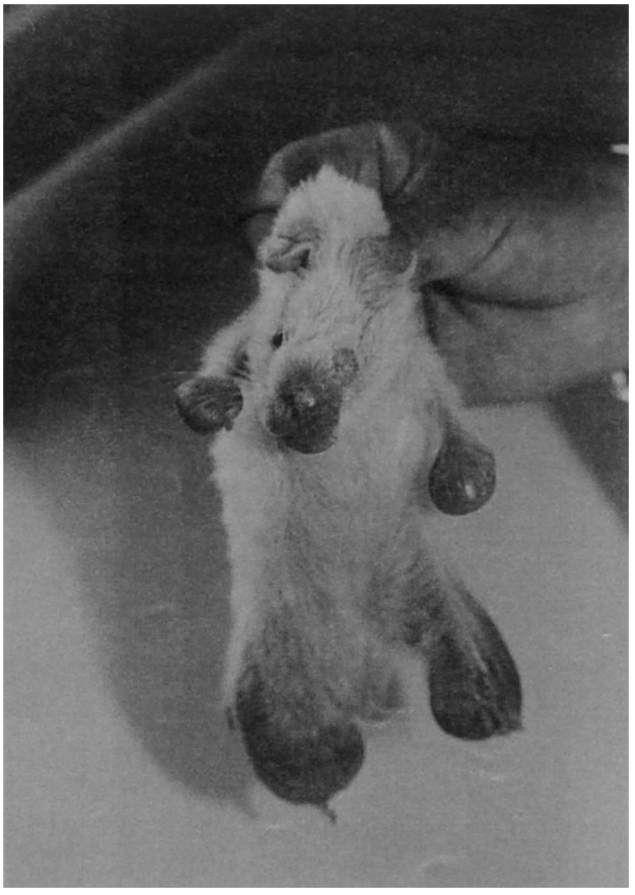

Fig. 2. Hamster infected by a human parasite, and presenting typical lesions of *L. mexicana* complex parasite (see leg extremities). Metastases are observed on ears, nose and mucocutaneous zones.

3.2. Parasite isolation and experimental infection of hamsters

The stocks isolated from patients developed in the culture media generally after 48 h. Four to six weeks after inoculation into the hind legs, the experimentally infected hamsters developed nodular lesions without ulcerations which progressively increased and gave metastatic peripheral lesions (fore legs, nose, ears, tail and mucocutaneous zones) after 6–8 months of evolution (Fig. 2). Samples obtained from these lesions showed abundant free parasites as well as many vacuolated histiocytes containing parasites.

3.3. Molecular karyotyping

The whole karyotype analysis (after ethidium bromide staining) revealed very close similarity between the three stocks examined (Fig. 3A). After hybridization with a beta-tubulin probe, the stocks were shown to present two hybridizing chromosomes, of 1640 and 760 kb respectively, a typical feature of the *L. mexicana* complex (Fig. 3B).

3.4. Isoenzyme electrophoresis

Out of the 12 enzyme systems used, 10 were retained for their good reproducibility and conditions of histochemical revelation on the gels. Since one of these systems (MPI) systematically produced two bands, a total of 11 loci were estimated (Fig. 4). The reference strain of *L. chagasi* shared no allele at 11 loci (i.e. there was 100% of 'fixed differences'), with the patient stocks, and so did *L. braziliensis* except for one human stock at one locus. Also, strong genetic divergence (69% of fixed difference) was evidenced with the reference strain of *L. pifanoi* (MHOM/VE/57/LV135) and *L. mexicana* (MNYC/BZ/62/M379). Contrasting with these high levels of differences, an average of 30% of fixed differences was found between patient parasite stocks and the reference strain of *L. amazonensis* (IFLA/BR/67/PH8) (Fig. 5).

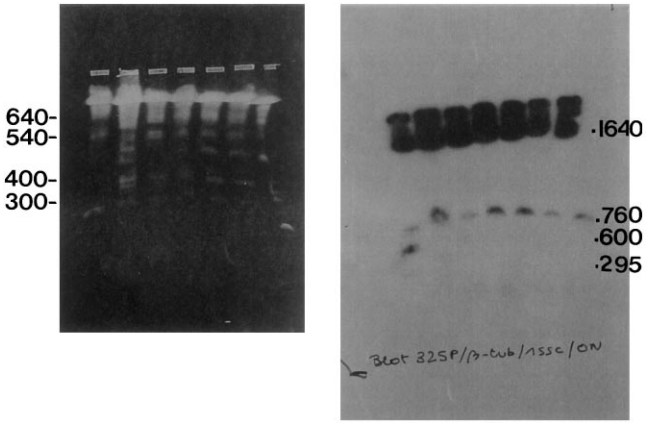

Fig. 3. (A) OFAGE analysis, pulse 45 s, size expressed in kb. (B) Hybridization of a previous OFAGE gel (pulse 115 s) with a β-tubulin probe. Lane 1: *Leishmania brazilienzis* (MHOM/BR/75/M2903); lane 2: *L. amazonensis* (MPRO/BR/77/M1845); lane 3: human stock originating from patient infected at a distant site from Cajuata in the province Inquisivi; lanes 4, 5, 6: different patient's stocks from Cajuata; lane 7: *L. mexicana* (MNYC/BZ/62/M379).

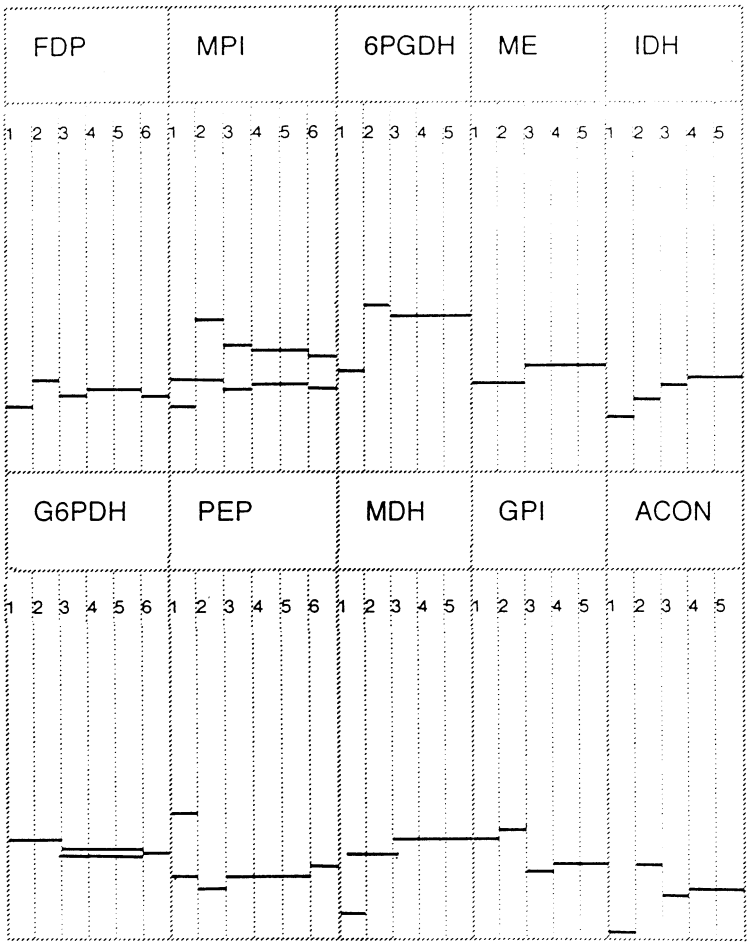

Fig. 4. Isoenzyme gel patterns for 10 enzymes. Electrophoretic migration goes upward. Lane 1: *L. braziliensis* (MHOM/BR/75/M2903); lane 2: *L. chagasi* (MHOM/BR/74/PP75); lane 3: *L. mexicana* (MNYC/BZ/62/M379) and *L. pifanoi* (MHOM/VE/57/LV135); lane 4: *L. amazonensis* (IFLA/BR/67/PH8); lanes 5 and 6: human stocks. Abbreviations: see text.

3.5. Numerical analysis

The UPGMA dendrogram based on Richardson's distances illustrates the close proximity between strains isolated from patients and *L. amazonensis*, as well as their strong differences with the *L. braziliensis*, *L. chagasi*, *L. mexicana* and *L. pifanoi* reference strains (Fig. 5).

4. Discussion

Four arguments converged to the identification of *L. amazonensis* circulating in humans at Cajuata: (i) the absence of mucocutaneous complication; (ii) the aspect of lesions observed after experimental infection in hamsters, suggesting a *L. mexicana* complex parasite infection; (iii) the rapid development of the parasites in culture media, and (iv) the molecular data (karyotype and isoenzymes).

Karyotype hybridization with tubulin (Fig. 3) assigned the parasites to the *L. mexicana* complex (Dujardin et al., 1987). Interestingly, the analysis of the whole karyotype showed unusual homogeneity among the stock patterns, suggesting that transmission could be microfocal.

Isoenzymes provided more accurate characterization, identifying the parasite circulating at Cajuata as *L. amazonensis* (Figs. 4 and 5). Rowton et al. (1992) showed that five loci could distinguish the main species of *Leishmania*. With 11 loci, i.e. more than used in many previous studies (Rioux et al., 1984; Hashiguchi et al., 1991), the clear-cut differences found between the field isolates and the reference strains of *L. braziliensis*, *L. chagasi*, *L. mexicana* and *L. pifanoi* are probably relevant.

Our study confirmed the presence of *L. amazonensis* at a higher altitude (between 1450 and 2100 m in the Department of La Paz) than previously reported (in the

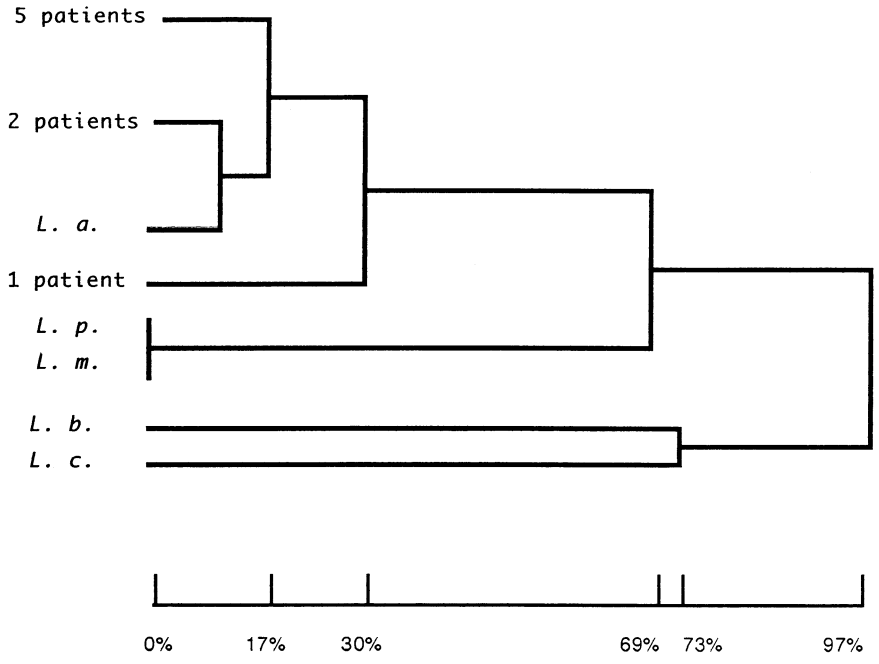

Fig. 5. UPGMA tree derived from Richardson's distances (see horizontal bottom line). *L.a.* = *Leishmania amazonensis*; *L.p.* = *L. pifanoi*; *L.m.* = *L. mexicana*; *L.b.* = *L. braziliensis* and *L.c.* = *L. chagasi*.

lowlands of Bolivia, see La Fuente et al., 1986; Dujardin et al., 1987; Grimaldi et al., 1987). The reported case of CDL (Prado Barrientos, 1948a,b), which was described from La Plazuela (Yungas-Inquisivi frontier), could be attributed to *L. amazonensis* and would indicate a focus of transmission older than expected. Up to now the province Inquisivi was unknown as an endemic zone and seems to represent a very active focus of cutaneous leishmaniasis transmission.

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