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Isoenzyme characterization of *Leishmania braziliensis braziliensis* isolates obtained from Bolivian and Peruvian patients

S. Revollo, L. Dimier-David, C. David, P. Lyevre, C. Camacho and **J. P. Dedet** *Instituto Boliviano de Biología de Altura, c/o Embajada de Francia, Casilla 717, La Paz, Bolivia*

Abstract

Thirty-four *Leishmania* isolates obtained from Bolivian and Peruvian patients infected with mucocutaneous leishmaniasis were characterized by isoenzyme electrophoresis using 10 enzymatic markers; all belonged to the subspecies *L. b. braziliensis*. Three isolates showed marked variation compared with the reference strain with respect to 5 or 6 enzymes. These variant isolates originated from patients with forms of the disease which were unresponsive to treatment.

Introduction

Mucocutaneous leishmaniasis (MCL) is widespread throughout South and Central America, affecting numerous countries between Argentina and Guatemala (LAINSON & SHAW, 1987; WALTON, 1987), and there have been frequent reports of MCL in the Andean countries (BALCAZAR, 1946; DESJEUX *et al.*, 1987; LUMBRERAS & GUERRA, 1985; ROMERO *et al.*, 1987; WALTON & VALVERDE, 1979).

In spite of long-standing knowledge of the disease and its wide territorial extension, the parasite responsible has scarcely been studied or characterized biochemically. Characterization of as many isolates as possible, obtained from patients of diverse geographical origin, is a priority for studying this particular type of leishmaniasis.

In the present paper, we report isoenzyme characterization of 34 isolates obtained from Bolivian and Peruvian patients, showing the existence of intraspecific variation within the taxon *Leishmania braziliensis braziliensis*.

Materials and Methods

Isolation of the parasite

The isolates were obtained from biopsies and/or scrapings of human cutaneous and/or mucous lesions. The cultures were grown in NNN biphasic medium with Schneider's *Drosophila* medium (Gibco Bio-cult, Paisley, UK) supplemented with 10% (v/v) inactivated foetal calf serum (Serva, Heidelberg, Germany) as liquid phase. Culture tubes were incubated at 28°C and subcultured every 4 d.

Preparation of the samples

When adapted to culture, the parasites were mass cultivated in 200 ml plastic boxes (Corning, New York, USA) in Schneider's liquid medium. The parasites were centrifuged at 3000 rpm, 4°C, for 10 min and the pellets preserved at −70°C. For electrophoresis, the pellets were resuspended in an equal volume of hypotonic stabilizing solution containing 2 mM ε-aminocaproic acid, ethylenediaminetetraacetic acid and dithiothreitol.

Enzyme electrophoresis

Electrophoresis was carried out on cellulose acetate, following the technique of TIBAYRENC & LE RAY (1984), adapted from LANHAM *et al.* (1981).

The following 10 enzymatic systems were examined: malate dehydrogenase (MDH) EC 1.1.1.37, malic enzyme (ME) 1.1.1.40, isocitrate dehydrogenase (ICD) EC 1.1.11.42, glucose-6-phosphate dehydrogenase (G6PD) EC 1.1.1.49, 6-phosphogluconate dehydrogenase (6PGD) EC 1.1.1.44, glutamate dehydrogenase NAD^+ (GDH-NAD^+)EC1.2.1.2, glutamate dehydrogenase $NADP^+$ (GDH-$NADP^+$) EC 1.4.1.2, phosphoglucomutase (PGM) EC 2.7.5.1, peptidase: L-leucyl-leucyl-leucine (PEP) EC 3.4.11, and glucose phosphate isomerase (GPI) EC 5.3.1.9.

Seven strains of American subspecies of *Leishmania* (see LAINSON & SHAW, 1979), were used as reference strains (Table 1).

Table 1. Reference strains of *Leishmania*

Subspecies[a]	Geographical origin	International codes
L. b. braziliensis	Brazil (Pará)	MHOM/BR/75/M2903
L. b. panamensis	Panama	MHOM/PA/67/Boynton
L. b. guyanensis	Brazil (Pará)	MHOM/BR/75/M4147
L. b. peruviana	Peru	MHOM/PE/84/LC26R43
L. d. chagasi	Brazil (Bahia)	MHOM/BR/74/PP75
L. m. amazonensis	Brazil (Pará)	MHOM/BR/73/M2269
L. m. mexicana	Belize	MHOM/BZ/82/BEL21

[a]All isolates from human hosts.

Results

Isolation of stocks

Thirty-four isolates of *Leishmania* were obtained, 5 from Peruvian patients (originating from the Madre de Dios department) and 29 from Bolivian patients (originating from Yungas Valleys, Alto Beni and Chaparé regions). They were isolated from 9 cutaneous and 25 mucous lesions, in patients with confirmed clinical and immunological diagnoses of leishmaniasis.

All the characteristics of these patients, including epidemiological and clinical aspects and treatment, were fully documented. The details of patients from whom isolates originated are presented in Table 2.

Isoenzyme electrophoresis

The isoenzyme electrophoresis showed that all of the 34 isolates tested were closely related to *L. braziliensis braziliensis*. Seven of them showed slight variations in the migration of one or more enzymes: 3 in 6PGD only, 2 in GPI only, 1 in G6PD and MDH and 1 in GPI, 6PGD, G6PD and ME.

Three of the isolates, however, exhibited marked variations compared with the *L. b. braziliensis* reference strain. Two of them, of Peruvian origin, showed marked variations in 4 enzymes (MDH, 6PGD, G6PD and PEP) and a slight variation in two others (ME and GPI in one, GDH-$NADP^+$ in the second). They were similar to the *L. b. braziliensis* reference strain in all 5 other enzymes, and differed in many enzyme patterns from the other *L. braziliensis* subspecies, with the exception of *L. b. peruviana* which is known to be similar to *L. b. braziliensis* (Figure).

The third variant isolate, of Bolivian origin, showed marked variations in the following 6 enzymes: MDH, 6PGD, G6PD, GPI, PGM and GDH-NAD^+, but again was more closely related to the *L. b. braziliensis* reference strain than to the reference strains of other species of the *L. b. braziliensis* complex (Figure).

These three variant isolates originated from 2 patients with mucous and one with cutaneous leishmaniasis, who were resistant to treatment (meglumine antimonate [Glucantime®], amphotericin B, pentamidine).

Discussion

Our results confirm the complete predominance of the subspecies *L. b. braziliensis* as the causative organism of

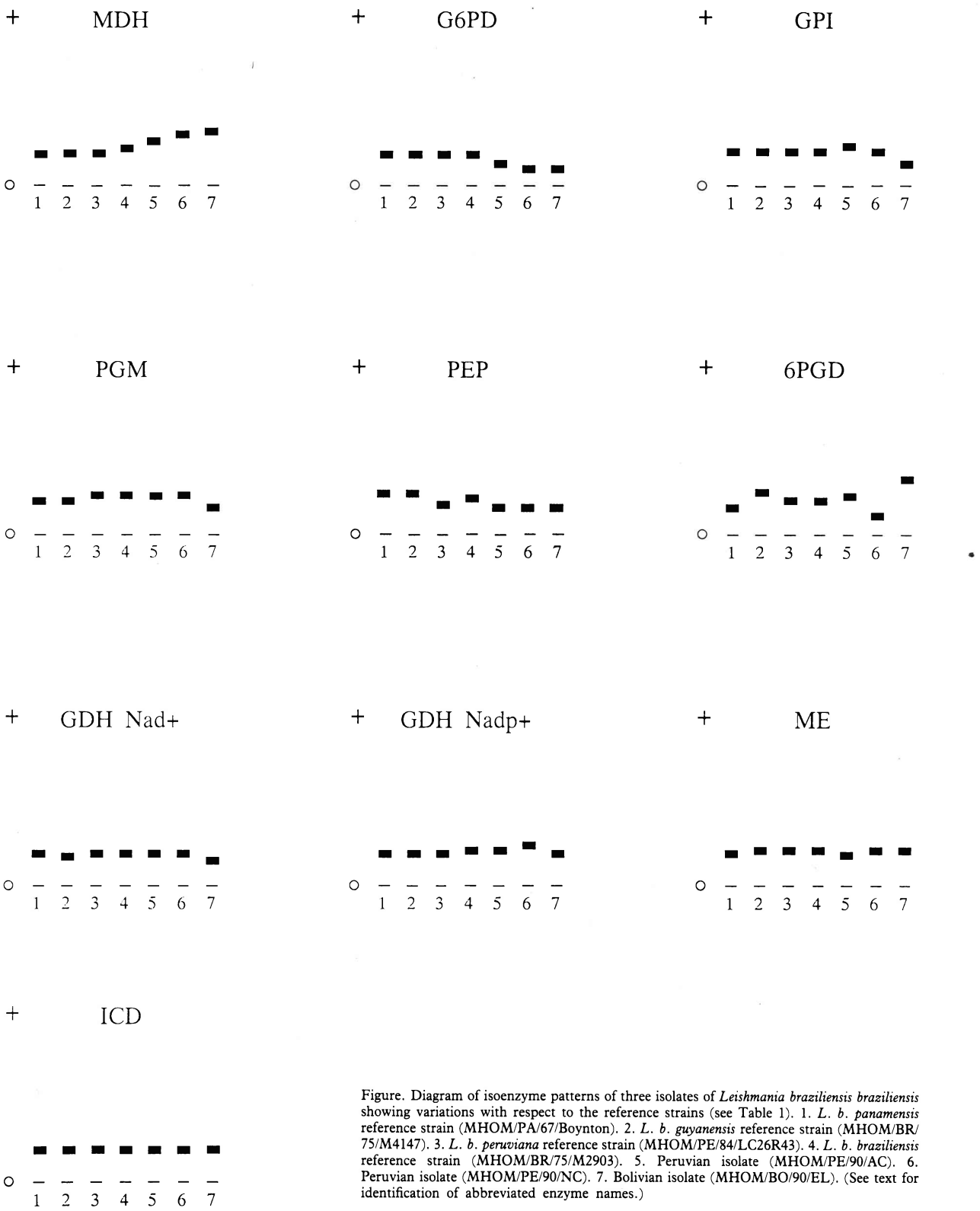

Figure. Diagram of isoenzyme patterns of three isolates of *Leishmania braziliensis braziliensis* showing variations with respect to the reference strains (see Table 1). 1. *L. b. panamensis* reference strain (MHOM/PA/67/Boynton). 2. *L. b. guyanensis* reference strain (MHOM/BR/75/M4147). 3. *L. b. peruviana* reference strain (MHOM/PE/84/LC26R43). 4. *L. b. braziliensis* reference strain (MHOM/BR/75/M2903). 5. Peruvian isolate (MHOM/PE/90/AC). 6. Peruvian isolate (MHOM/PE/90/NC). 7. Bolivian isolate (MHOM/BO/90/EL). (See text for identification of abbreviated enzyme names.)

cutaneous leishmaniasis in Bolivia, as previously reported by DESJEUX *et al.* (1986, 1987).

The diversity of the geographical origin of the isolates we tested extends the previous reports. Our isolates originated not only from middle altitude areas (2000 m) with a subtropical climate, such as the Yungas and Chapare in Bolivia, but also from low altitude (250–600 m) areas with a humid tropical climate, such as Alto Beni in Bolivia and Madre de Dios in Peru. This illustrates the adaptative capability of *L. b. braziliensis* to diverse ecological conditions within its large endemic area, and during its historical spread.

The existence of different zymodemes within the same *Leishmania* taxon has been previously reported in the New World parasites *L. mexicana amazonensis* (MILES *et al.*, 1979) and *L. b. guyanensis* (DESJEUX & DEDET,

Table 2. Particulars of the patients from whom *Leishmania* stocks were obtained

Patient	Age (years)	Sex[a]	Geographical origin	Skin test[b]	Indirect immunofluorescence titre	Type of lesion	Treatment Drug	Treatment Effect	Code no.
1	33	M	Alto Beni	5	1/80	Mucous	Glucantime	Cured	MHOM/BO/89/SB
2	28	M	Yungas	7	1/40	Mucous	Amphotericin B	Cured	MHOM/BO/89/BM
3	47	M	Yungas	N	1/40	Mucous	Glucantime	Relapsed	MHOM/BO/89/RMo
4	28	M	Alto Beni	10	1/80	Mucous	Amphotericin B	Cured	MHOM/BO/89/PM
5	49	M	Yungas	5	1/160	Mucous	Glucantime	Relapsed	MHOM/BO/89/EQ
6	45	M	Alto Beni	4	1/40	Mucous	Glucantime	Cured	MHOM/BO/89/CM
7	20	M	Yungas	5	1/80	Mucous	Amophotericin B	Cured	MHOM/BO/89/VC
8	20	M	Yungas	10	1/320	Mucous	Glucantime	Cured	MHOM/BO/89/EC
9	23	M	Yungas	9	1/40	Mucous	Glucantime	Cured	MHOM/BO/90/DG
10	30	M	Yungas	10	1/40	Mucous	Glucantime	Cured	MHOM/BO/90/TC
11	25	M	Chapare	10	1/160	Mucous	Glucantime	Cured	MHOM/BO/90/EN
12	58	M	Alto Beni	N	1/160	Mucous	Glucantime	Relapsed	MHOM/BO/90/GM
13	27	M	Beni	9	1/160	Mucous	Glucantime	Cured	MHOM/BO/90/NT
14	30	M	Madre de Dios	N	1/80	Cutaneous/mucous	Amophotericin B	Failed	MHOM/PE/90/AC[c]
15	18	M	Yungas	15	1/80	Cutaneous	Pentacarinat	Cured	MHOM/BO/90/JP
16	1	M	Yungas	8	1/80	Mucous	Glucantime	Cured	MHOM/BO/90/AN
17	3	F	Yungas	5	1/80	Cutaneous	–	–	MHOM/BO/90/CS
18	11	F	Yungas	N	1/40	Cutaneous	Glucantime	Cured	MHOM/BO/90/SL
19	37	F	Yungas	N	1/40	Cutaneous	Pentacarinat	Failed	MHOM/BO/90/EL[c]
20	18	M	Yungas	5	1/80	Cutaneous	Pentacarinat	Failed	MHOM/BO/90/LD
21	32	M	Yungas	8	1/80	Cutaneous	Pentacarinat	Failed	MHOM/BO/90/HL
22	29	M	Yungas	–	1/40	Cutaneous	Glucantime	–	MHOM/BO/90/CG
23	33	M	Yungas	5	1/40	Cutaneous	Glucantime	–	MHOM/BO/90/JM
24	36	M	Alto Beni	12	1/160	Mucous	Glucantime	Cured	MHOM/BO/90/MQ
25	34	M	Alto Beni	5	1/80	Mucous	Glucantime	Cured	MHOM/BO/90/PG
26	61	M	Alto Beni	6	1/80	Mucous	Glucantime	Cured	MHOM/BO/90/RP
27	35	M	Alto Beni	8	1/40	Mucous	Glucantime	Cured	MHOM/BO/90/VO
28	50	M	Madre de Dios	N	1/160	Mucous	Amphotericin B	Cured	MHOM/PE/90/FY
29	60	M	Madre de Dios	N	1/160	Mucous	Glucantime	Cured	MHOM/PE/90/TP
30	26	M	Madre de Dios	6	1/80	Cutaneous/mucous	Glucantime	Failed	MHOM/PE/90/NC[c]
31	45	M	Madre de Dios	12	1/80	Mucous	Glucantime	Cured	MHOM/PE/91/GP
32	13	M	Yungas	20	1/40	Cutaneous	Pentacarinat	Cured	MHOM/BO/91/RF
33	52	M	Yungas	12	1/160	Mucous	Glucantime	Suspended	MHOM/BO/91/RM
34	50	M	Yungas	8	1/40	Mucous	Glucantime	Cured	MHOM/BO/91/AO

[a]F=female, M=male.
[b]Diameter of reaction in mm; N=negative.
[c]Isolates showing marked variation from reference strains.

1989). Similarly, LANOTTE *et al.* (1981) and LE BLANCQ *et al.* (1986). reported the existence of various zymodemes in several Old World species: *L. major, L. tropica, L. aethiopica* and *L. donovani s.l.* Such intra-specific variation could be related to genetic exchange, according to MAAZOUN *et al.* (1981) and LE BLANCQ *et al.* (1986).

Two publications have reported the existence of slight variations in *L. b. braziliensis* affecting the enzymes NH, ES, MPI and PEP-D (SARAVIA *et al.*, 1985) and MDH and ICD (DESJEUX *et al.*, 1986), as we have observed with other enzymatic markers.

The existence of marked variations in the isolates tested can be interpreted differently depending on whether they are considered as variant zymodemes of *L. b. braziliensis* or as enzymic phenotypes distinct from this taxon. But whatever the interpretation, it may be significant in relation to the large geographical range of the taxon. This finding emphasizes the need to study a large number of stocks from distinct geographical origins, in order to understand better the biochemical particularities of such a species, the territorial extension of which extends from latitude 20°S to 18°N.

In our sample, 30 patients received correct dosage treatment using Glucantime®, amphotericin B or pentamidine (Pentacarinat®). Twenty-two were cured by the end of the treatment, while 3, who were initially cured, later relapsed. The treatment was a complete failure in 5 of the 30 cases (Table 2). The 3 isolates with marked enzymic variations originated from 3 of these patients unresponsive to treatment.

The occurrence of unresponsive or drug resistant cases is well known in various forms of leishmaniasis: East African kala-azar (WIJERS, 1971), Indian kala-azar (World Health Organization, unpublished information, 1990), and South American mucocutaneous leishmaniasis (MARSDEN *et al.*, 1985).

The biological basis of such a phenomenon remains unknown. The evidence of a relation between particular zymodemes and drug resistance could suggest a tentative explanation, independent of any effect of the host.

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Announcements

President's Fund

Council has decided to launch an appeal to raise money with the aim of establishing a fund to sponsor prospective Fellows from developing countries, who are at present unable to become Fellows of the Society because of their country's fiscal rules which prevent them from paying.

The fund will be known as *The President's Fund for Overseas Fellows in Developing Countries* and will be used to sponsor deserving candidates for full Fellowship of the Society for a trial period of three years.

The Society will make an initial donation of £2000 from its reserves to launch the Fund, and will then rely on donations from Fellows (including Honorary and Life Fellows) of the Society, and other, perhaps commercial, sources to build up the fund.

Any Fellows willing to donate to the President's Fund in order to help sponsor a Fellow from a developing country, are asked to write to Manson House.

Residential Meeting, Edinburgh, July 1993

The second 'residential meeting' of the Royal Society of Tropical Medicine and Hygiene (to include the Annual General Meeting) and other European Societies of Tropical Medicine will be held at the Royal College of Physicians of Edinburgh, Scotland from Monday 5th to Wednesday 7th July 1993. Accommodation will be on the University campus or alternatively in hotels near the City centre. Full social programme including reception and banquet. Further details are available from the Administrator, Royal Society of Tropical Medicine and Hygiene, Manson House, 26 Portland Place, London, W1N 4EY (Tel: 071 580 2127; Fax: 071 436 1389).