Zymodeme and Serodeme Characterization of *Leishmania* Isolates Obtained from Costa Rican Patients

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Thirty-four Leishmania *isolates obtained from Costa Rican patients, from different geographical areas, were characterized by isoenzyme electrophoresis and indirect immunofluorescense with monoclonal antibodies. Thirty-two were characterized as* L. panamensis *strains and two were* L. braziliensis *variants. We confirm the evident predominance of* L. panamensis *as the main etiological agent of leishmaniasis in Costa Rica and the existence of* L. braziliensis *in the country.*

Key words: leishmaniasis - Costa Rica - *Leishmania panamensis* - characterization - etiological agent

Human leishmaniasis is widespread throughout Central America and constitutes an important public health problem. Annual incidence is estimated to be about 20,000 cases (Carreira et al. 1995), of which 4,000 to 5,000 correspond to Costa Rica (Zeledón 1992). The disease exists under different clinical manifestations and in a variety of ecological and epidemiological patterns (Zeledón 1985) making it a complex problem that is difficult to manage.

The identity of the *Leishmania* species involved is a factor that largely determines the clinical manifestations of the disease (Lainson & Shaw 1987, Alexander & Russell 1992) and its response to treatment (Navin et al. 1992, Grogl et al. 1992). The World Health Organization (WHO 1990) recommends isoenzyme electrophoresis and the use of specific monoclonal antibodies as appropriate methods for characterizing *Leishmania*.

In Costa Rica, although it is known that *L. panamensis* species is the principal etiological agent of leishmaniasis (Zeledón 1992), there are no detailed and extensive taxonomic studies of the implicated parasites in different areas of the country. Here, we report the identification by isoenzyme electrophoresis and indirect immunofluorescence (IFA) with monoclonal antibodies of 34 *Leishmania* strains, isolated from different geographical and ecological areas of Costa Rica, from patients with cutaneous leishmaniasis.

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MATERIALS AND METHODS

Reference strains - The WHO *Leishmania* reference strains used in this study are shown in Table I.

Parasite isolation and cultivation - The primary isolation was made from patients with cutaneous lesions by inoculation of material obtained by needle aspiration in biphasic Senekjie's medium with Locke's solution (Tobie & Rees 1948). Promastigotes were expanded in enriched Schneider's *Drosophila* liquid medium (Gibco, Grand Island, NY) suplemented with 20% fetal bovine serum (FBS), and incubated at 27ºC. All strains were stored in liquid nitrogen in Schneider' medium contanining 30% FBS and 7.5% dimethylsulfoxide.

Preparation of extracts for electrophoresis - Cultured *Leishmania* were harvested during exponential growth by centrifugation at 1,000 x g at 4ºC for 10 min. The pellet was washed three times in phosphate buffered saline (PBS), pH 7.2 and resuspended in an equal volume of membrane buffer (14 parts distilled H2O: 1 part 0.1 M Tris/ Maleic Acid/EDTA/$MgCl_2$, pH 7.4). The material was stored at -70ºC until use. For electrophoresis, the pellets were lysed by three cycles of freezing and thawing in liquid nitrogen.

TABLE I

Reference strains of *Leishmania*

Species	Geographical origin	International codes
L. panamensis	Panamá	MHOM/PA/71/LS94
L. braziliensis	Brazil	MHOM/BR/75/M2903
L. guyanensis	Brazil	MHOM/BR/75/M4147
L. chagasi	Brazil	MHOM/BR/74/M2682
L. mexicana	Belize	MHOM/BZ/82/BEL21
L. amazonensis	Brazil	IFLA/BR/67/PH8

Isoenzyme electrophoresis - Electrophoresis was carried out in cellulose acetate as described by Kreutzer and Christensen (1980) and Kreutzer et al. (1983).

The following eight enzymatic systems, allowing a good separation of all reference strains, were used: 6-phosphogluconate dehydrogenase (E.C.1.1.1.44 6-PGDH), phosphoglucomutase (E.C.2.7.5.1 PGM), glucose phosphate isomerase (E.C.5.3.1.9 GPI), proline iminopeptidase (E.C.3.4.11.5 PEP-D), alanine aminotransferase (E.C.2.6.1.2 ALAT), aspartate aminotransferase (E.C.2.6.1.1 ASAT), mannose phosphate isomerase (E.C.5.3.1.8 MPI), and nucleoside hydrolase (E.C.3.2.2.1 NH).

Indirect immunofluorescence with monoclonal antibodies - The monoclonal antibodies, and their respective reactivity, used in this study are shown in Table II. Promastigotes, washed in PBS, pH 7.2 and standardized to a concentration of $1x10^6$ parasites/ml, were air dried, in 10 µl volumes, on twelve well microscope slides. The slides were submerged in blocking solution (PBS, pH 7.2 + 5% FBS) during 15 min, washed with PBS, and air dried. Each well was covered with 10 µl of the monoclonal antibodies diluted in PBS (1:1000) for 30 min (McMahon-Pratt et al. 1984). The unreactive protein was removed by three washes in PBS and 10 µl/well of anti-mouse IgG labeled with fluorescein and diluted 1:50 was added. Incubation was continued for 30 min at room temperature, unreactive conjugate was removed by three washes in PBS and the slides were mounted with buffered glycerol and observed with a Olympus fluorescence microscope.

TABLE II

Monoclonal antibodies and their specificity

Monoclonal antibody	Specificity	Titer
B-16 (XIII-3E6-B11)	*L. braziliensis*	1×10^{-4}
B-19 (XLIV-5A2-B9)	*L. guyanensis*	6×10^{-4}
B-4 (VI-2A5-A4)	*L. panamensis*	1×10^{-5}
B-12 (XIII-3H6F9E3)	*L. braziliensis* complex	2×10^{-5}
M-3 (IX-5H9-C10)	*L. amazonensis*	2×10^{-5}
M-8 (LXVIII-4D8-E3)	*L. mexicana*	5×10^{-5}
M-7 (LXVIII-1D7-B8)	*L. mexicana* and *L. amazonensis*	3×10^{-5}

L.: *Leishmania*

RESULTS

Isolation of strains - Thirty-four strains from a total of 104 patients with suspected cutaneous leishmaniasis from the main endemic areas of Costa Rica were isolated. The details of patients from whom isolates originated as well as the identification obtained by enzyme electrophoresis and monoclonal antibodies are presented in Table III.

Isoenzyme analysis - All of the 34 isolates showed isoenzyme patterns similar to members of the subgenus *Viannia*. Thirty of them had identical profile to *L. panamensis* (Table III, Fig. 1). Two strains (CCS3 and CBR3) showed slight variations in the migration of 6-PGDH, PGM, and MPI as compared with *L. panamensis* reference strain (Fig. 1). The two remaining isolates had a distinct profile from *L. panamensis* and were closer to *L. braziliensis*. One of them (CBR2) shared 25% of the alleles with *L. braziliensis* and the other (CHA9) shared 75%.

Indirect immunofluorescence - The reactivities of the eight different monoclonal antibodies were tested with 34 *Leishmania* strain of Costa Rica. Monoclonal antibody B-4 specific for *L. panamensis* species reacted with 32 of the 34 isolates. Twenty-seven of them reacted strongly whereas five reacted weakly with this antibody. Monoclonal antibody B-16, which reacts selectively with *L. braziliensis*, reacted with only two of the 34 Costa Rican strains, although the reactivity was weak in comparison with the reactivity of the *L. braziliensis* reference strain (M-2903). None of the monoclonal antibodies specific for the *L. guyanensis* (B-19) or *L. mexicana* complex (M-3, M-8, M-7) reacted with the strains isolated in this study.

DISCUSSION

Previous studies on leishmaniasis in Costa Rica have revealed usefull information about the epidemiology of the disease. It is known that sloths, *Bradypus griseus* and *Choloepus hoffmanni*, are the main reservoirs of *L. panamensis* both in the Caribbean and Pacific basins (Zeledón et al. 1975). It also has been isolated from a specimen of the rodent *Heteromys dermarestianus* (Zeledón et al. 1977). Futhermore, *L. panamensis* has been isolated from the sandflies *Lutzomyia ylephiletor* and *Lu. trapidoi* (Zeledón & Alfaro 1973, Zeledón et al. 1977). Taxonomic studies of the parasite implicated have been limited to a few areas, confirming the predominance of *L. panamensis* (Zeledón 1992). Grimaldi et al. (1987) included five Costa Rican strains in a study of characterization with monoclonal antibodies and all were *L. panamensis*. In the present study, we confirm *L. panamensis* as the main etiological agent of cutaneous leishmaniasis in all the endemic areas of the country. We also found *L. panamensis* enzymatic variants and we confirm the existence of *L. braziliensis* enzy-

TABLE III

Characteristics of the patients and identification of 34 *Leishmania* strains from Costa Rica

Patient No.	Code No.	Age (Years)	Sex	Geographical origin (County-Province)	Characterization Isoenzyme	Characterization Monoclonal
1	MHOM/CR/95/CCS1	14	M	Acosta, San José	*L. panamensis*	*L. panamensis*
2	MHOM/CR/94/CCS2	28	M	Acosta, San José	*L. panamensis*	*L. panamensis*
3	MHOM/CR/91/CCS3	19	M	Acosta, San José	*L. panamensis*	*L. panamensis*
4	MHOM/CR/90/CCS4	1	F	Acosta, San José	*L. panamensis*	*L. panamensis*
5	MHOM/CR/92/CCS5	19	M	Acosta, San José	*L. panamensis*	*L. panamensis*
6	MHOM/CR/91/CCS6	38	M	Acosta, San José	*L. panamensis*	*L. panamensis*
7	MHOM/CR/91/CCS7	11	M	Acosta, San José	*L. panamensis*	*L. panamensis*
8	MHOM/CR/95/CBR1	22	F	Pérez Zeledón, San José	*L. panamensis*	*L. panamensis*
9	MHOM/CR/95/CBR2	56	M	Pérez Zeledón, San José	*L. braziliensis*	*L. braziliensis*
10	MHOM/CR/95/COC1	6	F	San Ramón, Alajuela	*L. panamensis*	*L. panamensis*
11	MHOM/CR/95/COC2	25	M	San Ramón, Alajuela	*L. panamensis*	*L. panamensis*
12	MHOM/CR/95/CHN1	14	F	San Carlos, Alajuela	*L. panamensis*	*L. panamensis*
13	MHOM/CR/95/CHN2	25	M	San Carlos, Alajuela	*L. panamensis*	*L. panamensis*
14	MHOM/CR/96/CHN3	14	M	Los Chiles, Alajuela	*L. panamensis*	*L. panamensis*
15	MHOM/CR/96/CCN1	44	M	Sarapiquí, Heredia	*L. panamensis*	*L. panamensis*
16	MHOM/CR/95/CBR3	11	M	Buenos Aires, Puntarenas	*L. panamensis*	*L. panamensis*
17	MHOM/CR/95/CBR4	93	M	Puerto Jiménez, Puntarenas	*L. panamensis*	*L. panamensis*
18	MHOM/CR/95/CCE1	10	M	Turrialba, Cartago	*L. panamensis*	*L. panamensis*
19	MHOM/CR/95/CCE2	12	M	Turrialba, Cartago	*L. panamensis*	*L. panamensis*
20	MHOM/CR/95/CHA1	½	F	Guácimo, Limón	*L. panamensis*	*L. panamensis*
21	MHOM/CR/95/CHA2	30	F	Guácimo, Limón	*L. panamensis*	*L. panamensis*
22	MHOM/CR/95/CHA5	42	M	Cariari, Limón	*L. panamensis*	*L. panamensis*
23	MHOM/CR/95/CHA3	61	F	Guápiles, Limón	*L. panamensis*	*L. panamensis*
24	MHOM/CR/95/CHA8	9	M	Guápiles, Limón	*L. panamensis*	*L. panamensis*
25	MHOM/CR/95/CHA9	39	M	Guápiles, Limón	*L. braziliensis*	*L. braziliensis*
26	MHOM/CR/95/CHA10	19	M	Guápiles, Limón	*L. panamensis*	*L. panamensis*
27	MHOM/CR/95/CHA11	15	F	Guápiles, Limón	*L. panamensis*	*L. panamensis*
28	MHOM/CR/95/CHA6	7	M	Siquirres, Limón	*L. panamensis*	*L. panamensis*
29	MHOM/CR/95/CHA7	35	F	Pocora, Limón	*L. panamensis*	*L. panamensis*
30	MHOM/CR/95/CHA12	1	M	Central, Limón	*L. panamensis*	*L. panamensis*
31	MHOM/CR/95/CHA13	48	M	Central, Limón	*L. panamensis*	*L. panamensis*
32	MHOM/CR/94/CHA4	40	F	Talamanca, Limón	*L. panamensis*	*L. panamensis*
33	MHOM/CR/96/CHA14	16	M	Sixaola, Limón	*L. panamensis*	*L. panamensis*
34	MHOM/CR/96/CHA15	22	M	Sixaola, Limón	*L. panamensis*	*L. panamensis*

matic variants in two important foci of leishmaniasis in Costa Rica, one in the Pacific and another in the Caribbean side (Fig. 2). The presence of strains with some genetic (enzyme) polymorphism, suggests the existence of other local populations but, as stated by Kreutzer (1996), probably belonging to "a single reproductive population". The electrophoretic mobilities of our *L. panamensis* and *L. braziliensis* variants are different from the *L. panamensis/L. braziliensis* hybrid as characterized by Belli et al. (1994) in Nicaragua.

In Costa Rica, mucocutaneous leishmaniasis (MCL) occurs in 3 to 5% of the cases (Zeledón 1992) and three isolates obtained in cultures directly from the mucosal lesions by biopsy were identifyied by zymodemes as *L. panamensis.* The only other species of *Leishmania* recorded from Costa Rica is *L. infantum* from cutaneous lesions detected in an outbreak in the north-west part of the country (Zeledón et al. 1989). A previous report of *L. mexicana* from the same area originated from a mislabeled culture (Zeledón 1992).

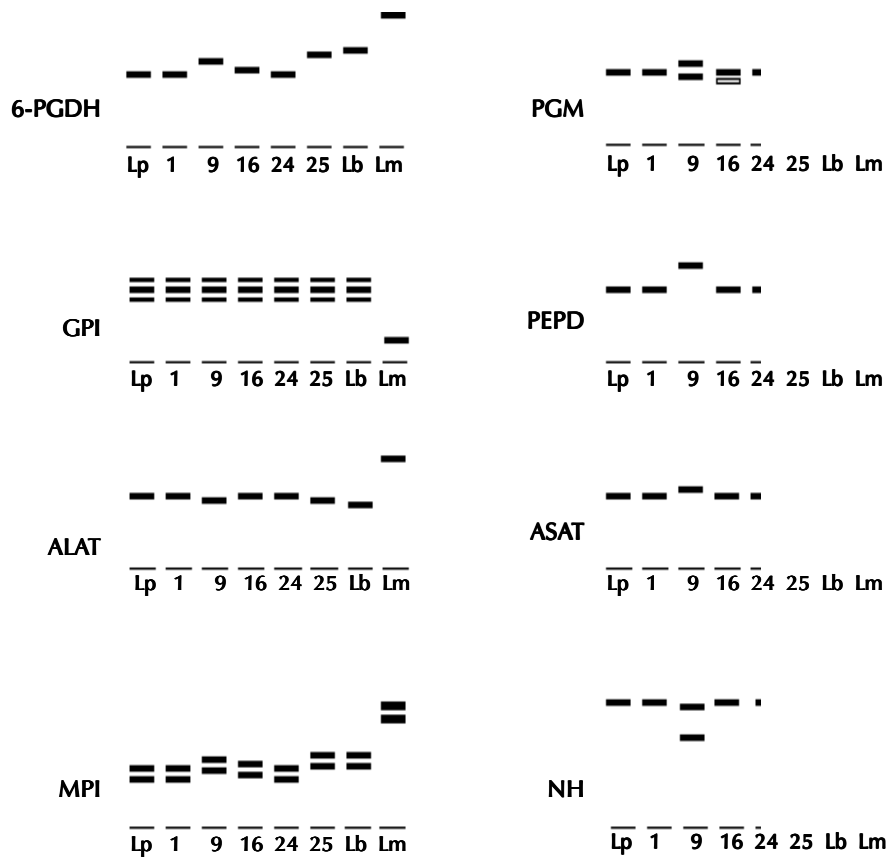

Fig. 1: diagramatic representation of the isoenzyme variation observed in eight enzymatic patterns. Lp: *Leishmania panamensis* reference strain (MHOM/PA/71/LS94); 1: Costa Rican strain (MHOM/CR/95/CCS1); 9: Costa Rican strain (MHOM/CR/95/CBR2); 16: Costa Rican strain (MHOM/CR/95/CBR3); 24: Costa Rican strain (MHOM/CR/95/CHA8); 25: Costa Rican strain (MHOM/CR/95/CHA9); Lb: *L. braziliensis* reference strain (MHOM/BR/75/M2903); Lm: *L. mexicana* reference strain (MHOM/BZ/82/BEL21). (Of the strains not shown in the figure, one has pattern similar to track 16 and the rest have patterns similar to tracks 1 and 24).

Fig. 2: geographical distribution of strains. (●) *Leishmania panamensis*; (▲) *L. panamensis* variant; (O) *L. braziliensis* variant.

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