RESEARCH NOTE

A New Enzymatic Variant of *Leishmania* (*Leishmania*) *forattinii* Isolated from *Proechimys iheringi* (Rodentia, Echimydae) in Espírirto Santo, Brazil

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Key words: *Leishmania* - *Leishmania* (*Leishmania*) *forattinii* - taxonomy - monoclonal antibodies - isoenzyme electrophoresis - DNA analysis

Taxonomic studies of leishmanial isolates from the New World indicate tremendous diversity within this genus (E Cupolillo et al. 1994 *Am J Trop Med Hyg 50*: 296-311). A number of new *Leishmania* species from sylvan areas of the Neotropics have been described recently. Some of those parasites are associated with disease in humans; others appear to be restricted to lower orders of mammals, such as rodents and edentates (G Grimaldi et al. 1989 *Am J Trop Med Hyg 41*: 687-725). However, some of the latter parasites may yet be shown capable of causing human disease, particularly in persons with altered cellular immune responses.

During a survey of potential reservoir hosts of *Leishmania* done in the State of Espírito Santo, Brazil (1982-1985), three leishmanial parasites (designated with stock codes MPRO/BR/82/RV203, MPRO/BR/82/RV228, and MPRO/BR/85/RV260) were isolated from skin lesion samples taken from spiny-rats, *Proechimys iheringi* (Rodentia, Echimydae), captured in a secondary forest located in the municipality of Viana (A Falqueto et al. 1985 *Mem Inst Oswaldo Cruz 80*: 497). In studies using an indirect radioimmune binding assay (RIA) and a large panel of monoclonal antibodies (Mabs) derived for selected species of *Leishmania*, the isolates were characterized as members of the *L. mexicana* complex. These strains were different from those species infective to humans, in that they reacted with either the *L.*(*L.*) *amazonensis* or *L.* (*L.*) *mexicana* specific MAbs. Furthermore, this was also the case with cloned organisms from two stocks showing that they were not mixed populations of *Leishmania* (G Grimaldi et al. 1987 *Am J Trop Med Hyg 36*: 270-287). This group of distinct parasites presented, however, similar serodeme patterns in relation to the new recently described species of the *Leishmania* subgenus, *L.* (*L.*) *forattinii* (E Yoshida et al. 1993 *Mem Inst Oswaldo Cruz 88*: 397-406).

The latter parasite was isolated from sylvatic reservoir hosts (*Didelphis marsupialis aurita* and *P. iheringi denigratus*) captured on regions endemic for American cutaneous leishmaniasis in Brazil, respectively in the municipalities of Conchas, São Paulo (E Yoshida et al. 1979 *Rev Inst Med Trop S Paulo 21*: 110-113) and Três Braços, Bahia (A Barreto et al. 1985 *Rev Soc Bras Med Trop 18*: 243-246). The general morphology and the growth characteristics *in vitro* of the new species were similar to those of other *L. mexicana* complex parasites.

Here we have further studied the Espírito Santo isolates using additional techniques for the characterization of *Leishmania*. The molecular procedures used for typing the strains (isoenzyme electrophoresis, blot enzyme binding assay using MAbs, restriction endonuclease fragment patterns of k-DNA, and molecular karyotype analysis) have been described in detail in previous publications (Yoshida et al. 1979, 1983 *loc. cit.*, Cupolillo et al. 1994 *loc. cit.*, A Franco et al. 1997 *Mem Inst Oswaldo Cruz 92*: 63-68).

Results of electrophoretic analysis were based on the following enzymatic loci, namely: glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDHNADP), 6-phosphogluconate dehydrogenase (6PGDH), nucleotidase (NH1 and NH2), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), proline dipeptidase (PEPD), leucine peptidase (PEP2), malic enzyme (ME), mannose phosphate isomerase (MPI), and aconitate hydratase (ACON). The enzyme profiles of the selected isolated signature MPRO/BR/82/RV228, which represents the poorly defined group of parasites from

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Received 29 April 1998
Accepted 28 July 1998

Espírito Santo, shared more electromorphs (bands of enzyme activity as revealed by electrophoresis) with *L.* (*L.*) *forattinii* and fewer with other members of the *L. mexicana* group (data not shown). The enzymes useful in distinguishing the new strain variant of *L.* (*L.*) *forattinii* are G6PDH, MDH, IDHNADP, PEPD, PEP2, and MPI. In addition, for most of the 13 enzyme loci examined, the profiles of this zymodeme (IOC-68) were different from those of all other species complexes of *Leishmania*.

The taxonomical position of strain MPRO/BR/82/RV228 in relation to other selected species complexes of *Leishmania* (Table) was defined by phenetic numerical analysis of the enzyme data (Cupolillo et al. 1994 *loc. cit.*). Affinities between zymodemes were calculated using the Jaccard´s similarity coefficient and were transformed into a phenogram, by the unweighted paired-group method using arithmetical avarage (UPGMA). As shown (Fig. 1), the two zymodemes classified as *L.* (*L.*) *forattinii* (IOC-11) and enzymatic variant (IOC-68) form distinct phenetic groups, clustered within the *L. mexicana* species complex.

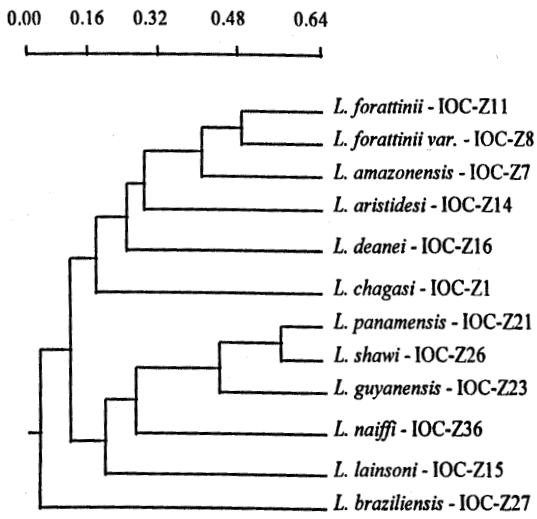

Fig. 1: phenogram showing the Jaccard´s coefficient of similarities between groups and/or species of *Leishmania* and the two strains (L67 and L182) of *L.* (*L.*) *forattinii* from Brazil.

TABLE

Origin and identification of reference strains and selected *Leishmania* species or strain variants employed in this study

Stock	Designation[a]	Species	Zymodeme[b]	Geographic origin
L67	MDID/BR/77/Conchas[c]	*L. forattinii*	IOC-11	São Paulo, Brazil
L168	MHOM/BR/73/MT(D)	*L. amazonensis*	IOC-7	Amazonas, Brazil
L181	MPRO/BR/82/RV203	*L. forattinii* var	IOC-68	Espírito Santo, Brazil
L182	MPRO/BR/82/RV228	*L. forattinii* var	IOC-68	Espírito Santo, Brazil
L289	MPRO/BR/83/P584/585	*L. forattinii*	IOC-11	Bahia, Brazil
L561	MHOM/BZ/82/BEL21[c]	*L. mexicana*	IOC-6	Belize, Belize
L562	MHOM/PA/71/LS94[c]	*L. panamensis*	IOC-21	Canal Zone, Panama
L564	MORY/PA/68/GML3[c]	*L. aristidesi*	IOC-14	Darien, Panama
L565	MHOM/BR/75/M4147[c]	*L. guyanensis*	IOC-23	Pará, Brazil
L566	MHOM/BR/75/M2903[c]	*L. braziliensis*	IOC-27	Pará, Brazil
L567	MCAV/BR/45/L88[c]	*L. enriettii*	IOC-13	Paraná, Brazil
L568	MHOM/VE/74/PMH17[c]	*L. venezuelensis*	IOC-9	Lara, Venezuela
L569	MHOM/BR/73/M2269[c]	*L. amazonensis*	IOC-7	Pará, Brazil
L575	IFLA/BR/67/PH8[c]	*L. amazonensis*	IOC-7	Pará, Brazil
L577	MNYC/BZ/62/M379[c]	*L. mexicana*	IOC-6	Cayo, Belize
L583	MHOM/VE/57/LL1[c]	*L. pifanoi*	IOC-6	Venezuela
L584	MHOM/VE/76/JAP78[c]	*L. garnhami*	IOC-8	Merida, Venezuela
L615	MHOM/BR/85/MNB	*L. amazonensis*	IOC-7	Bahia, Brazil
L750	MPRO/BR/85/RV260	*L. forattinii* var	IOC-68	Espírito Santo, Brazil
L1023	MHOM/BR /81/M6426	*L. lainsoni*	IOC-15	Pará, Brazil
L1365	MDAS/BR/79/M5533[c]	*L. naiffi*	IOC-36	Pará, Brazil
L1545	MCEB/BR/84/M8408[c]	*L. shawi*	IOC-26	Pará, Brazil

a: designation code. Host (M: mammalia; CAV: *Cavia porcellus*; CEB: *Cebus apella*; DAS: *Dasypus novemcinctus;* DID: *Didelphis marsupialis*; HOM: *Homo sapiens*; NYC: *Nyctomys sumichrasti*; ORY: *Oryzomys capito*; PRO: *Proechimys iheringi*; I: Insecta; FLA: *Lutzomyia flaviscutellata*)/country of origin/year of isolation/original code; *b*: *Leishmania* zymodeme classified by enzyme electrophoresis according to their enzyme patterns (Cupolillo et al. 1994 *loc. cit.*); *c*: *Leishmania* reference strain (G Grimaldi et al. 1992 *Mem Inst Oswaldo Cruz 87*: 221-228, Yoshida et al. 1993 *loc. cit.*).

Western blot studies were performed using the *L. mexicana* complex specific Mabs (D McMahon-Pratt et al. 1985 *J Immunol 134*: 1935-1940) to further group/differentiate among these parasites. Antigens of each of the strains tested were prepared as promastigote homogenates containing protease inhibitors (Franco et al. *loc. cit.*). As shown (Fig. 2A), Mabs M9 and M11 recognized multiple molecular components with apparent relative mobility (Mr) values ranging from less than 14 to 200-kDa. Differences occurred between *Leishmania* species in either the Mr, intensity or number of components recognized by these Mabs. *L.* (*L.*) *amazonensis* fell into one subgroup (G1a). *L.* (*L.*) *aristidesi* showed as a distinct parasite (G1b), with an intermediary pattern between G1a and another subgroup (G1c), representing *L. forattinii* strains (which produced a similar profile, regardless of geographic distribution). In contrast, these antigenic determinant were not recognized in the remainder *L. mexicana* complex species analyzed. Although the banding patterns of these subgroups showed some similarities when tested with M4 and M6 (Fig. 2B), they were distinct from the other main group representing *L.* (*L.*) *mexicana* (G2). The main facts to emerge from this study are: (a) the presence of two major antigenically distinct groups in the *L. mexicana* complex; and (b) the apparent antigenic similarity within the group of the species (*L.*) *amazonensis*/*L.* (*L.*) *garnhami*, as well as the species *L.* (*L.*) *mexicana*/ *L.* (*L.*) *pifanoi*.

Comparisons of kDNA restriction enzyme fragments profiles from stocks representing species complexes of New World *Leishmania* and the selected strains of *L.* (*L.*) *forattinii* from São Paulo (stock L67) and Espírito Santo (stock L182) were carried out by polyacrylamide gradient gel electrophoresis. Variation between the two strains of *L. forattinii* was demonstrated by their different schizodeme profiles with the enzymes *Hinf* I (Fig. 3A), *Msp* I (Fig. 3B), *Hae* III and *Mbo* I (data not shown). In addition, these profiles were distinct from those of all strains examined (Fig. 3).

In previous molecular karyotype analysis (Yoshida et al. 1983 *loc. cit.*), although *L. forattinii* and other species of the *L. mexicana* complex showed a specific number of size-concordant DNA molecules, clear karyotypic differences exist among these parasites. Moreover, chromosomes were polymorphic in both the number and size of bands between the two strains (L67 and L182) of *L. forattinii* analyzed in this study (data not shown).

To date, no human infection with *L.* (*L.*) *forattinii* has been identified in the study areas. The

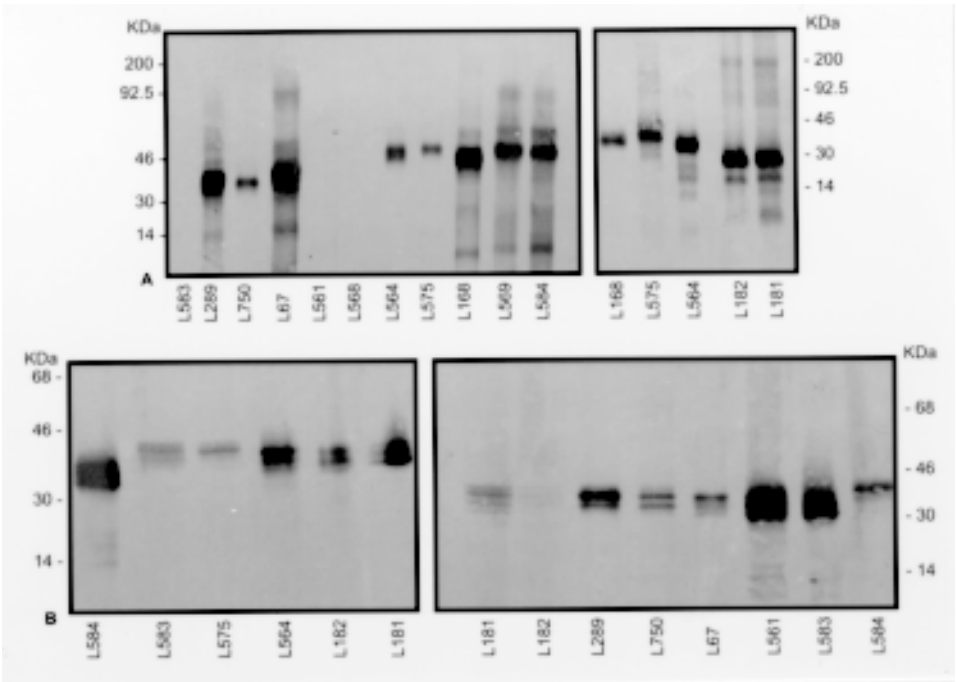

Fig. 2: western blot analyses of promastigotes homogenates of members of the *Leishmania mexicana* complex using monoclonal antibodies (A) M9 (XLV-2B5-H7) and M11 (XLV-1D11-E11), and (B) M4 (IX-1F9-D8) and M6 (LXVII-4C7-B8). The stocks codes of the strains analyzed are indicated below the lanes, and their origins are shown in the Table. Molecular weights are indicated in kDa besides the figure.

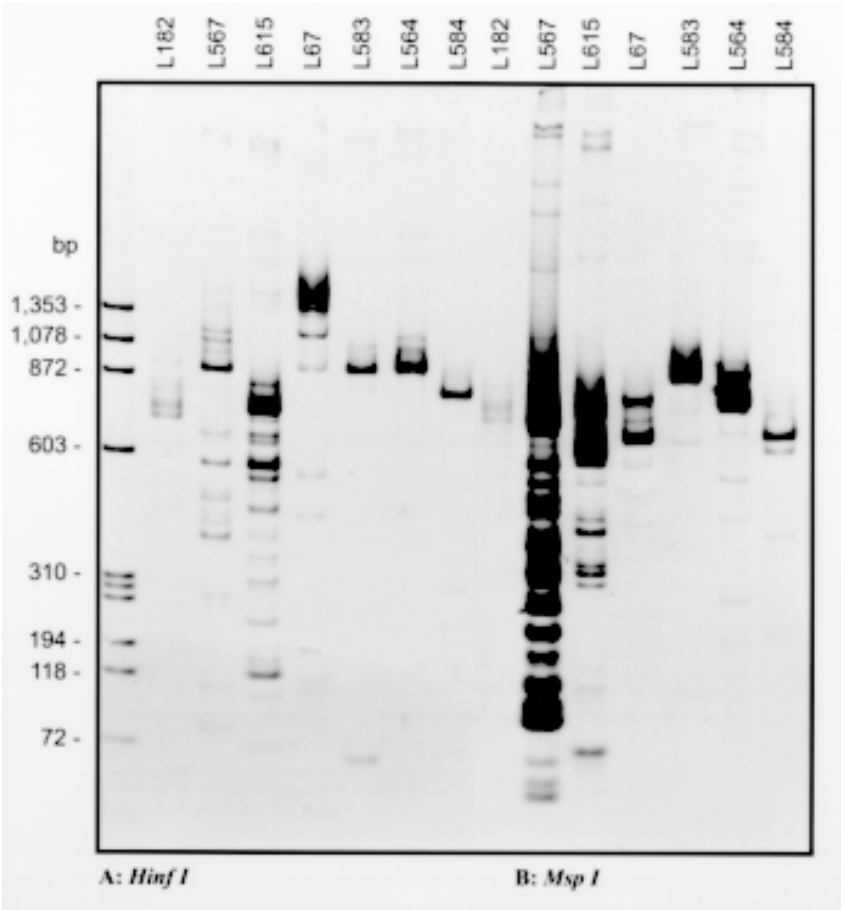

Fig. 3: acrylamide gradient (3.5-12%) gel electrophoresis comparison of kDNA fragment patterns, generated with the restriction enzyme *Hinf* I (A) and *Msp* I (B), among selected species complexes of *Leishmania* and the two strains (L67 and L182) of *L.* (*L.*) *forattinii* from Brazil. The identification of the *Leishmania* strains analyzed is given in the Table. Molecular weights of k-DNA fragments are indicated in basepairs (bp) besides the gel.

species definitely associated with human and canine infections in Espírito Santo are (a) *L.* (*Viannia*) *braziliensis*, causing cutaneous ou mucosal leishmaniasis; and (b) *L.* (*L.*) *chagasi*, responsible for cases of visceral leishmaniasis (Grimaldi et al. 1989 *loc. cit.*, A Falqueto et al. 1991 *Mem Inst Oswaldo Cruz 86*: 499-500). Here we have demonstrated that other leishmanial strains with similar characteristics to *L.* (*L.*) *forattinii* were recovered from *Proechimys* captured in the same region.

Moreover, we have been investigating on the feeding habits of the phlebotomine sandflies in relation to human and *Proechimys*, in the forest area where the infected rodents were caught. Of 320 sandflies, 314 (98.1%) were identified as *Lu. gasparviannai* Martins, Godoy & Silva, 1962, indicating this species as the probable vector of the parasite among the rodents. No specimens of *Lu. gasparviannai* were found, however, among 355 phlebotomines caught feeding on humans (Falqueto et al. 1991 *loc. cit.*). These data suggest that the parasite here identified as a new enzymic variant of *L.* (*L.*) *forattinii* is not usually transmitted to humans. However, the pathogenicity of the new parasite was indistinguishable from other *L. mexicana* complex species, based on its virulence and development in laboratory animals (Yoshida et al. 1979, 1983 *loc. cit.*).