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Epidemiological and Genotypical Mapping of Human *Leishmania (Viannia) braziliensis* in Paraguay

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ABSTRACT

Twenty *Leishmania (Viannia) braziliensis* isolates recovered from patients living in three distinct endemic areas in Paraguay and presenting single or multiple cutaneous lesions were analyzed. Alto Paraná and Canindeyú located near the boundary of Paraná State (Brazil) present epidemiological patterns compatible with areas of ancient transmission of the disease. The area of San Pedro located at the oriental shore of the Paraguay River is considered to be of recent transmission, with mucocutaneous clinical forms being present.

Three different polymorphic markers were used in order to characterize and to provide a preliminary molecular mapping of the circulating strains. Restriction fragment length polymorphisms of kDNA analysis revealed the presence of three different schizodemes circulating in the endemic areas. Isoenzymatic variants were also detected in six out of the sixteen strains studied. However, no correlation was found between enzyme polymorphisms and a specific type of schizodeme.

Our results demonstrate the intraspecific heterogeneity of the *Viannia* species circulating in Paraguay suggesting a more complex epidemiological situation in some endemic foci. Furthermore, the finding of genotypic polymorphisms among isolates of the same species raises questions regarding parasite virulence in the American Tegumentary Leishmaniases.

INTRODUCTION

In the New World thirteen different species of *Leishmania* are responsible for American Tegumentary Leishmaniases (ATL) (Grimaldi et al. 1989). The species are transmitted to silvatic and domestic animal reservoirs by the bite of phlebotomine sandflies. The ATL is considered as a zoonosis and the man an accidental host. Although the human infection is widespread in tropical and subtropical regions of the Americas (Grimaldi et al. 1989), the epidemiology is quite complex. In Brazil, environmental changes have induced the appearance of an another epidemiological profile evolving beyond the man, domestic animals and sandflies with high capability to adapt to modified environments (Lainson 1983).

In Paraguay, since 1976 the occurrence of individuals suffering from cutaneous and mucocutaneous leishmaniases is increasing in the majority of the Departments form the east side of Paraguay River, being endemic in Alto Paraná, Canindeyú, San Pedro and Caaguazú (Hashiguchi et al. 1991; 1992a). Up to now three species of *Leishmania: L. (Viannia) braziliensis, L. (Leishmania) amazonensis* and *L. (Leishmania) major*-like (Grimaldi et al. 1989; Yamasaki et al. 1994) have been identified in Paraguay. Such species were isolated from human cases in a newly established community in the southeast region just near the Brazilian border.

According to cases reported in Paraguay, the clinical manifestations of the disease differ markedly from single cutaneous lesions to disfiguring and non-healing mucocutaneous ones (Boggino and Insaurrald 1945; Hashiguchi et al. 1992). The impact of the disease is dramatic causing great suffering, especially because of the social and psychological trauma associated with disfigurement for life, usually from a young age.

The different clinical forms may be due to the *Leishmania* involved (Blackwell and Alexander 1960), the polyclonality of the initial inoculum (Pacheco et al. 1990) as well as the degree of susceptibility of the human host (Blackwell and Alexander 1960). On the other hand, the plasticity observed in parasites of the *Vianna* subgenus can also be resultant from changes in the environmental condition associated to the introduction of new human and animal populations probably due to the attempts in establishing new biocenose between the parasites and its hosts (Blackwell and Alexander 1960).

Intraespecific variation in the *Vianna* subgenus has been demonstrated at nuclear and mitochondrial levels (Lopes et al. 1984; Pacheco et al. 1986; Saravia et al. 1990; Cupollilo et al. 1994; Pacheco et al. 1995). Although the biological meaning of such polymorphisms in mitochondrial DNA remains an enigma, the maintenance of minicircle heterogeneity may offer a selective advantage to the

parasites and has been incriminated as a source of genetic information as stressed by Borst (1991).

The recent paper reports the finding of intraespecific genetic polymorphisms in both nuclear and mitochondrial genomes in *L. (V.) braziliensis* isolated from patients proceeding from the States of Alto Paraná, Canindeyú and San Pedro.

MATERIALS AND METHODS

Localities and Leishmania *isolates:*

The study was carried out in Limoy (State of Alto Paraná) and Naipi (State of Canindeyú), both at the Brazilian border. In Pancholo (State of San Pedro), located at the eastern side of Paraguay River, within the low land areas of the "chaco austral", limited at the west side by Argentina and Bolivia (Fig. 1).

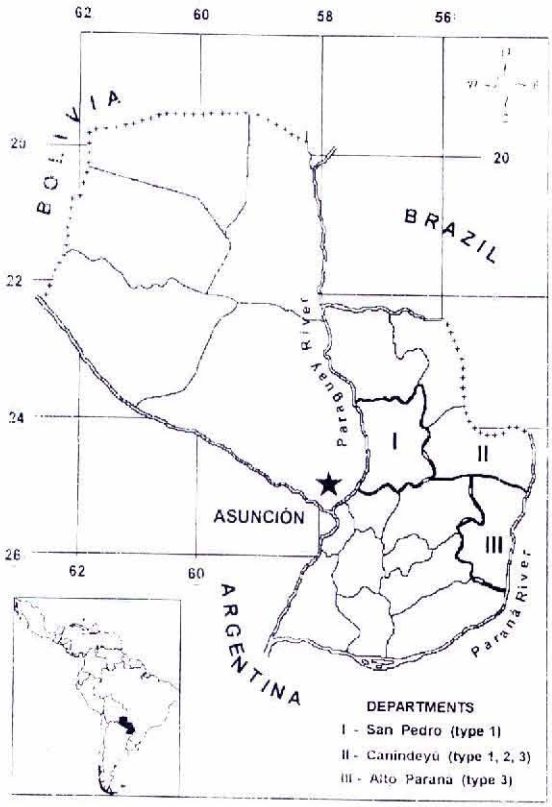

Figure 1. Map of Paraguay showing the endemic areas studied and the three genotypic profiles found.

Twenty isolates from single and multiple lesions of patients with cutaneous leshmaniasis were analyzed. Table 1 summarizes all information regarding demographic data, clinical conditions, origin and identification of the isolates.

Table 1. Patients studied, origin and characterization of the isolates.

Patients	Sex	Age	Number of lesions	Origin	Molecular characterization		
					Zymodeme	Schizodeme	Hybridization
Y.M.S.	M	6	3	A. Paraná	*L. (V.) b.* §	Type 3	ND
J.P.S.	M	63	2	A. Paraná	*L. (V.) b.*	Type 3	ND
R.P.	F	13	1	A. Paraná	*L. (V.) b.*	ND	+
A.R.	M	54	1	A. Paraná	*L. (V.) b.* §	Type 3	+
B.C.A.	M	15	1	A. Paraná	*L. (V.) b.* §	Type 3	+
J.C.D.	M	6	3	A. Paraná	*L. (V.) b.*	ND	+
I.S.C.	M	29	6	A. Paraná	*L. (V.) b.* §	Type 3	+
M.M.C.	M	16	2	A. Paraná	*L. (V.) b.*	ND	+
I.D.	M	48	1	Canindeyú	*L. (V.) b.*	Type 2	+
R.R.S.	F	1	1	Canindeyú	*L. (V.) b.* †	Type 2	+
J.A.	M	38	2	Canindeyú	*L. (V.) b.*	Type 2	+
V.O.	M	18	1	Canindeyú	*L. (V.) b.*	Type 1	+
J.E.G.	M	35	5	Canindeyú	*L. (V.) b.*	Type 2	+
G.M.	M	25	1	Canindeyú	ND	Type 3	+
S.G.P.	F	30	1	S. Pedro	ND	Type 1	+
C.A.O.	F	28	1	S. Pedro	ND	Type 1	+
B.P.C.	M	63	1	S. Pedro	*L. (V.) b.*	Type 1	+
B.G.N.	F	25	1	S. Pedro	*L. (V.) b.* §	Type 1	+
N.I.V.	F	25	1	S. Pedro	*L. (V.) b.*	Type 1	+
C.S.	M	-	1	S. Pedro	ND	Type 1	+

ND: not done, §: variant A, †: variant B.

Leishmania parasites obtained from biopsies of cutaneous lesions were cultured in Schneider's Drosophila medium (Sigma) supplemented with 10% of fetal bovine serum (WL-Immunoquimica) at 26°C. The parasites in the stationary phase were harvest by centrifugation at 4,000 rpm for 10 min at 4°C and washed several times with sterilized phosphate buffered saline, pH 7.2. *L. (V.) braziliensis* (MHOM/BR/75/M2903), *L. (L.) guyanensis* (MHOM/BR/75/M4147) and *L. (L.) amazonensis* (IFLA/BR/67/PH8) were used as reference strains in zymodeme, shizodeme and hybridization analyses.

Multilocus enzyme electrophoresis (zymodeme) analysis:

Suspensions were by homogenizing 10^8 promastigotes in phosphate buffer, pH 7.0. Analysis was performed using agarose gel as described by Momen et al. (1985). Nine different enzymes were used: 6-fosfogluconate dehydrogenase (6PGDH E.C.1.1.1.43), Glucose phosphate isomerase (GPI E.C.5.3.1.9), Isocitrate dehydrogenase (IDH E.C.1.1.1.42), Glucose-6-phosphate-dehydrogenase (G6PDH E.C.1.1.1.1.49), Malato dehydrogenase (MDH E.C.1.1.1.37), Nucleosidase (NH E.C.3.2.2.1), Phosphoglucomutase (PGM E.C.1.4.1.9), Peptidase 3 (PEP3 E.C.3.4.11) and Proline aminopeptidase (PEP-D E.C.3.4.13.9).

Restriction fragment length polymorphism (schizodeme) of kDNA analysis:

The kDNA extraction and restriction enzyme analysis were carried out according to the method previously described (Goncalves et al. 1984; Pacheco et al. 1986). The kDNA preparations were digested with the restriction enzymes Msp I and Rsa I at 37°C for 3 hrs in the appropriate buffers. kDNA fragments were separated by electrophoresis in 5-10% linear polyacrylamide gradient gels and stained with ethidium bromide.

Molecular hybridization:

Purified kDNA (20 ng) from the isolates were prior to application in nylon membranes (Zeta probe, BioRad), denatured in 0.4 N NaOH and applied under vacuum using a manifold (Schleicher & Schwell Inc., Keene, NH, USA).

A cloned minicircle of *L. (V.) braziliensis* (M2903) was radiolabeled with alpha ^{32}P dATP according to the protocol previously described (Pacheco et al. 1994), to a specific activity of about 10^9 dpm/μg. Hybridization conditions were as follows: membranes were presoaked for 2 hrs in 0.6 M NaCl, 0.12 M Tris, 4 mM EDTA, 0.75% non-fat-milk and 0.15% SDS at room temperature, and then in hybridization solution (the same components above plus 50% formamide and 6% PEG) for 40 min at 42°C prior to the addition of the denatured probe. Hybridization was carried out at 42°C overnight and the membranes were washed initially in 2× SSC for 20 min at room temperature and then 3 times in 0.1× SSC, 0.5% SDS at 60°C for 30 min.

A synthetic oligonucleotide subgenus Viannia specific (Fernandes et al. 1995) was radiolabeled with gamma ^{32}P ATP and T4 polynucleotide kinase and also used as probe in dot blot experiments. Autoradiograph was done overnight with an intensifying screen at -70°C.

RESULTS AND DISCUSSION

The Departments of Alto Paraná and Naipi are deforested areas with change to subsistence culture specially soy and wheat. San Pedro Department is a boggy region and a large area for cattle raising and plantation of banana, cassava, tea and tabacco. The media temperature of the country is about 23°C (Fig. 1).

Eight human isolates out of the twenty analyzed proceeded from Alto Paraná, six from Canindeyú and the remaining from San Pedro. Fourteen patients were male and six female with ages ranging from one to sixty three years old. Single and multiple lesions were observed in individuals living in Alto Paraná and Canindeyú. However patients from San Pedro presented a single cutaneous lesion. The biochemical characterization using three different molecular techniques confirmed the identification of the isolates as *Leishmania (V.) braziliensis*. Enzyme electrophoresis using nine enzymatic loci showed zymodeme homogeneity. Similar electromorphic profiles were found in the majority of the isolates analyzed when compared to a reference strain (M2903) of *L. (V.) braziliensis*. Eight out of the nine enzyme tested displayed no polymorphism and were represented by a single electromorph. Enzymatic polymorphism was only found for the enzyme PEP-3 in six isolates. Results are shown in Table 2.

Table 2. Electromorphs observed in *Leishmania (V.) braziliensis* isolates from Paraguay.

Isolates	Enzymes								
	6PG	GPI	IDH	NH	MDH	PGM	G6P	PEP-D	PEP-3
L(V)b	1	1	1	1, 4	1	1	2	2	1
YMS	1	1	1	1, 4	1	1	2	2	1, 3
JPS	1	1	1	1, 4	1	1	2	2	1
RP	1	1	1	1, 4	1	1	2	2	1
AR	1	1	1	1, 4	1	1	2	2	1, 3
BCA	1	1	1	1, 4	1	1	2	2	1, 3
JCD	1	1	1	1, 4	1	1	2	2	1
ISC	1	1	1	1, 4	1	1	2	2	1, 3
MMC	1	1	1	1, 4	1	1	2	2	1
ID	1	1	1	1, 4	1	1	2	2	1
RRS	1	1	1	1, 4	1	1	2	2	1, 2, 3
JA	1	1	1	1, 4	1	1	2	2	1
VO	1	1	1	1, 4	1	1	2	2	1
JEG	1	1	1	1, 4	1	1	2	2	1
BPC	1	1	1	1, 4	1	1	2	2	1
BGN	1	1	1	1, 4	1	1	2	2	1, 3
NIV	1	1	1	1, 4	1	1	2	2	1

Biochemical characterizations using kDNA restriction profiles and hybridization have also identified the isolates as *L. (V.) braziliensis*. Genotypic heterogeneity was detected among the isolates. Polymorphisms in kDNA minicircle regions were observed after digestion with the restriction enzyme Rsa I, indicating the presence of three distinct profiles of *L. (V.) braziliensis* circulating in endemic areas in Paraguay (Fig. 2).

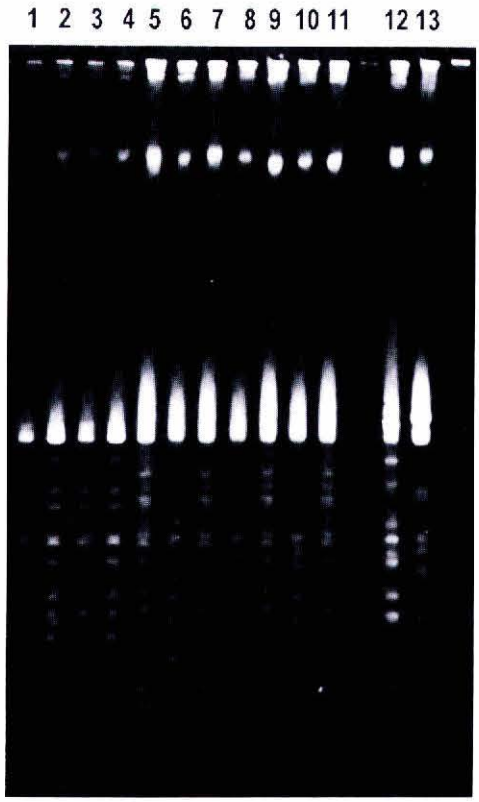

Figure 2. Linear polyacrylamide gradient gel (5-10%) showing fragments of kDNA minicircles after digestion with the restriction enzyme Rsa I. Three distinct genotypic profiles (schizodemes) can be observed. Lanes 1-4: schizodeme type I, lanes 5, 7, 9 and 11: schizodeme type II, lanes 6, 8 and 10: schizodeme type III: lane 12: *L. (V.) braziliensis* reference strain (M2903), lane 13: *L. (V.) guyanensis* reference strain (M4147).

The restriction profile type I was observed in the kDNA from parasites isolated from San Pedro's patients. Besides the type I pattern, two other variants, type II and III were observed in samples from Canindeyú and the type III was only detected in isolates from Alto Paraná.

The hybridization analysis has validated the results of zimodeme and schizodeme analyses by showing strong hybridization signals in all isolates with both DNA probes. The specificity of our probes was confirmed by the absence of cross-hybridization with kDNA from *L. (L.) amazonensis* (Fig. 3).

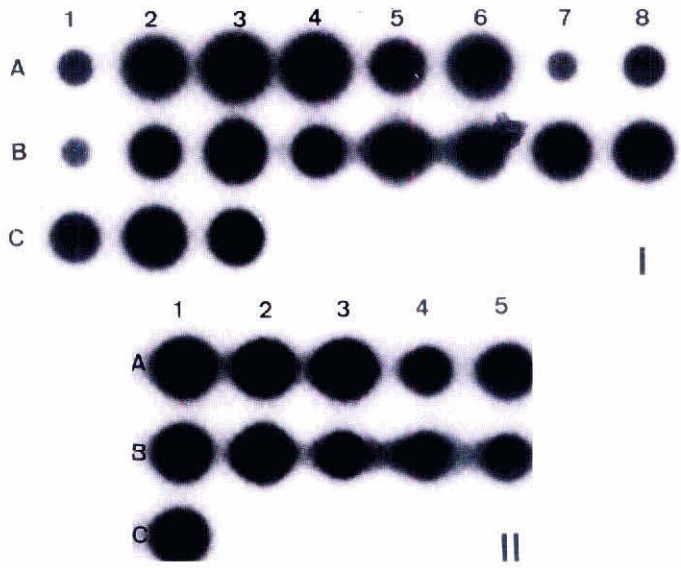

Figure 3. Autoradiographs showing dot-blot hybridizations. I: purified kDNAs from the isolates belonging to the three genotypic patterns against a cloned minicircle of *L. (V.) braziliensis* as probe, C3: *L. (V.) braziliensis* strain M2903 as positive control, C4: *L. (L.) amazonensis* strain PH8 as negative control. II: selected kDNAs related to the three schizodemes against the oligonucleotide *Viannia* specific as probe, C1: *L. (V.) braziliensis* strain M2903 as positive control, C2: *L. (L.) amazonensis* strain PH8 as negative control.

In this study we have reported the molecular characterization of twenty Paraguayan *Leishmania* isolates from patients living in Limoy (Alto Paraná), Naipi (Canindeyú) and Pancholo (San Pedro).

Among the patients studied the majority was male (70%), except in San Pedro where it was also verified that the age was above 25 years old, suggesting that the transmission of the disease might be occurring far from the houses. In Pansholo the patients examined displayed only one cutaneous lesion. In Limoy and Naipi there was no difference in age prevalence suggesting the occurrence of a peridomiciliar transmission, and it was possible to detect children and adult presenting one or more lesions. The Departments of Alto Paraná and Canindeyú located near the boundary of Paraná State (Brazil) present epidemiological patterns compatible with areas of ancient transmission, as observed in Brazil (Marzochi and Marzochi 1994). The area of San Pedro located at the oriental shore of the Paraguay River is considered to be of recent transmission (Marzochi and Marzochi 1994).

Hashiguchi et al. (1992b) listed five species of sandfly captured by human bait and/or Shannon trap in Paraguay eastern localities. All species were

carried out in the same regions it was demonstrated the prevalence of *Lu. withmani* among the sandfly fauna caught surrounding the houses in the Itaipu dike areas (Villar 1993). On the other hand, in Pancholo *Lu. withmani* was captured only in silvatic areas. Although both sandflies are found in anthropic environment, it seems reasonable to suppose that there are differences between types of transmissions in the localities studied, suggesting a peridomiciliar spreading in Limoy and Naipi. In Pancholo (San Pedro State) the transmission probably occurs for from the houses, in the forest environment.

All the isolates analyzed were characterized as *L. (V.) braziliensis* by phenotypic and genotypic methods as recommended to distinguish from other species found in Paraguay (Yamasaki et al. 1994). The molecular hybridizaiton was used in this study in order to strengthen the results obtained by isoenzyme and schzodeme analyses. High copy number of homologous sequences was detected in the kDNA from all isolates when tested against a cloned minicircle of *L. (V.) braziliensis* and an oligonucleotide Vianna specific used as probes.

The restriction fragment length polymorphisms of kDNA (schizodeme) analysis is considered as a powerful tool in epidemiological investigations being efficient for studying the natural distribution of *Leishmania* strains in different endemic foci (Pacheco et al. 1986; Lopes et al. 1984). In this study the schizodeme analysis provided an additional contribution by revealing that three distinct genotypic profiles of *L. (V.) braziliensis* are circulating in humans in Paraguay. The polymorphism observed in kDNA minicircle regions from some isolates can be regarded as genetic microheterogeneities. However, as patients living in endemic areas are constantly exposed to the bite of phlebotomines, the accumulation of multiple independent infections can be a possibility. Hence, genotypic differences found in human isolates from the same species can be attributable to such mechanism.

Interestingly, variant zymodemes were more predominantly found in isolates from Alto Paraná (4/8) belonging to the schizodeme type III. Nevertheless, no correlation was observed between isoenzymatic variants and an specific type of schizodeme. Isoenzymatic variants were also detected in the schizodeme type I from a patient resident in San Pedro and in the schizodeme type II from a patient living in Canideyú. According to isoenzyme results two *L. (V.) braziliensis* populations are present in Paraguay.

Genotypic profiles type I, II and III were found in Canideyú's isolates and type III in Alto Paraná. Both Departments are near two Brazilian states where ATL occurs (Grimaldi et al. 1989; Teodoro et al. 1991). Canindeyú and Alto Paraná are newly established Brazilian communities, even though all patients were Paraguayan.

Only one genotypic pattern (kDNA type I) was observed circulating in patients from San Pedro suggesting a more simple epidemiological situation. The region of San Pedro is distinguishable from the others studied, by the demographic aspects with the recent Paraguayan colonization and the absence of critical environmental changes.

These molecular techniques together with further clinical and epidemiological information will surely provide new insights into the natural history of the American Tegumentary Leishmaniases in Paraguay.

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