

Characterization of *Leishmania* spp. causing cutaneous leishmaniasis in Manaus, Amazonas, Brazil

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Abstract In the State of Amazonas, American tegumentary leishmaniasis is endemic and presents a wide spectrum of clinical variability due to the large diversity of circulating species in the region. Isolates from patients in Manaus and its metropolitan region were characterized using monoclonal antibodies and isoenzymes belonging to four species of the parasite: *Leishmania (Viannia) guyanensis*, 73% (153/209); *Leishmania (Viannia) braziliensis*, 14% (30/209); *Leishmania (Leishmania) amazonensis*, 8% (17/209); and *Leishmania (Viannia) naiffii*, 4% (9/209). The most prevalent species was *L. (V.) guyanensis*. The principal finding of this study was the important quantity of infections involving more than one parasite species, representing 14% (29/209) of the total. The findings obtained in this work regarding the parasite are further highlighted by the fact that

these isolates were obtained from clinical samples collected from single lesions.

Introduction

Epidemiologically, American tegumentary leishmaniasis (ATL) is considered an enzootic disease aggravated by several factors. In the State of Amazonas, Brazil, four species of *Leishmania* are responsible for the disease in humans, involving clinical presentation in the cutaneous and mucosal forms: *Leishmania (Viannia) guyanensis*, *Leishmania (Viannia) braziliensis*, *Leishmania (Viannia) naiffii*, and *Leishmania (Leishmania) amazonensis*.

In the last 12 years (1994 to 2005), an annual mean of 30,383 cases of tegumentary leishmaniasis have been registered. In 2006, 22,097 cases were notified and in 2007, 20,737 cases, which is an expressive decrease in the number of cases registered in previous years (<http://www4.ensp.fiocruz.br/Leishmaniose/situacao-da-lt-no-brasil/>). In the State of Amazonas, 12,005 cases were registered in the same period, with a mean incidence rate of 86.77; however, in 2003, the rate was 121.03, with 3,174 cases, 60.18% in the city of Manaus (Guerra et al. 2006). The cases are concentrated in the metropolitan region of Manaus, with around 600 cases per year, according to database of the Disease Notification Information System (*Sistema de Informação de Agravos de Notificação*, SINAN), run by the Secretary for Health and Safety (*Secretaria de Vigilância em Saúde*) of the Ministry of Health (<http://dtr2004.saude.gov.br/sinanweb/tabnet/tabnet?sinan/Ita/bases/ltabr.def>).

The classical taxonomic methods used for *Leishmania* classification and typing are based on a panel of specific monoclonal antibodies and an electrophoretic profile of isoenzymes (MLEE). Other methods based on DNA

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detection and sequencing are also used. Due to the abundance of the Amazon biodiversity, some authors have suggested that other species may exist in the region, including species not yet characterized that could also be responsible for infections in humans, given that flagellated forms of the parasite are frequently observed in mammals and phlebotomines in the region (Grimaldi et al. 1991; Silveira et al. 2004).

The human cases of ATL in this region are mostly verified in the adult population who are involved in work related to agricultural activities, such as the extraction of rosewood oil and cassava cultivation, as well as other subsistence crops like beans and corn. The majority of the autochthonous cases are attended at the Manaus Tropical Medicine Institute Foundation (*Fundação Instituto de Medicina Tropical de Manaus*). Precise categorical identification of *Leishmania* spp. is fundamental to understanding the epidemiology of the disease and improving current knowledge concerning its pathology, the use of chemotherapy, and for implementing control measures.

Specific monoclonal antibodies have been used for several years to identify *Leishmania* spp. (McMahon-Pratt et al. 1986; Grimaldi et al. 1987, 1991; Shaw and Lainson 1987, 1989; Barral-Neto et al. 1986; Barral 1988) and have demonstrated high and consistent specificity in the characterization of species of this parasite, unequivocally proving its identification (Grimaldi and Tesh 1993; Grimaldi and McMahon-Pratt 1996; Romero et al. 2002a, b, 2005; Abbas and Lichtman 2005).

The electrophoretic mobility of enzymes (multilocus enzyme electrophoresis, MLEE) is another tool for categorically characterizing this parasite, revealing polymorphisms that express phenotypes of population variations and taxonomically classify the different species of *Leishmania* (Cupolillo et al. 1994, 1998; Saravia et al. 1998).

In the last few years, polymerase chain reaction (PCR) has been widely used as a parasitological diagnostic test on clinical samples of patients with ATL, due to its high sensitivity (Barker et al. 1991; Degraeve et al. 1994) and to detect natural infection in phlebotomine vectors and reservoir hosts (Pita-Pereira et al. 2005; Brandão-Filho and Shaw 2006). Its use has demonstrated greater sensitivity in relation to the conventional method of diagnosis based on direct parasitological exam under an optic microscope (Isaza et al. 1999; Rodrigues et al. 2002; Weigle et al. 2002).

This study aimed to characterize the species of *Leishmania* isolated in patients with ATL originating from the city of Manaus and its metropolitan region, attended at the outpatient clinic of the Amazonas Tropical Medicine Foundation (*Fundação de Medicina Tropical do Amazonas, FMTAM*) in Manaus, relating patients' location of origin to the species of parasite detected.

Material and methods

Patients

The individuals included in the study spontaneously sought attendance at the FMTAM, a reference center for tropical diseases in the State of Amazonas, Brazil, to obtain a clinical and laboratorial diagnosis of ATL. The patients all originated from the city of Manaus and municipalities of the metropolitan region: Itacoatiara, Manacapuru, Novo Airão, Rio Presidente Figueiredo, and Preto da Eva. The study was submitted to and approved by the Ethics in Research Committee of the FMTAM, under no. 1830/2006. All the participants signed a term of free and informed consent.

Parasitological exam

All the patients involved in the study were submitted to direct exam by scarification of the margins of a single typical lesion, followed by Giemsa staining, in accordance with Ministry of Health directives (Brasil Ministério da Saúde and Fundação Nacional de Saúde 2000, 2002), to visualize the amastigotes under optic microscopy.

Isolation of *Leishmania* spp

Clinical samples obtained by fine needle aspiration biopsy of the margins of cutaneous lesions or fragments obtained by 3–4-mm punch biopsy, in accordance with the method described by Marzochi et al. (1993) and Romero et al. (2002a, b), were inoculated in Novy–Neal–Nicolle (NNN) culture medium, first described by Novy–Neal and Nicolle (1909) and modified by Shaw and Lainson (1981 and Shaw et al. (1989). The cultures were examined every 3 days for a maximum period of 30 days to detect promastigotes under optic microscopy.

Preparation of the parasitic mass for parasite characterization

The parasites were transferred from modified NNN culture medium to Schneider's *Drosophila* medium (S9895, Sigma) containing 20% fetal bovine serum (FBS) and antibiotics (50 mg/ml of streptomycin and 100 U/ml of penicillin or 80 mg/ml of gentamicin). They were observed for 3 to 5 days until they achieved the stationary growth phase. Once this occurred, an aliquot of the culture was added to 4% formaldehyde diluted 1:1,000 in phosphate-buffered solution (PBS), followed by parasite counts in a Neubauer chamber at concentrations between 1×10^5 and 1×10^7 . Next, they were washed twice in PBS, pH 7.2, and 0.01 M EDTA and centrifuged for 10 min at 2,500 rpm, in

accordance with Evans et al. (1984; Evans 1989; Brasil Ministério da Saúde and Fundação Nacional de Saúde 2000). The parasite mass was separated into aliquots, which were frozen and stored at -20°C while awaiting characterization.

Analysis of monoclonal antibodies (serodemes)

Preparation of the parasites for monoclonal typing was performed in accordance with laboratorial protocol L30/181/4 of the WHO Special Program for Research and Training on Tropical Diseases (WHO/TDR, 2002). Indirect immunofluorescence reaction on monoclonal antibodies was performed using the following panel of 14 specific monoclonal antibodies: *L. (V.) braziliensis* (D3-complex), *L. (V.) braziliensis* (B12,16,18), *L. (V.) guyanensis/panamensis* (B19,4,5,7,11), *L. (L.) amazonensis/venezuelensis* (M3,7,8,P9), and *L. (V.) naiffii* (B1). Series D and B react with species of the subgenus *Viannia* and series M and P react with species of the subgenus *Leishmania*. The following international standard strains were used as positive controls: *L. (V.) guyanensis* (MHOM 4147), *L. (V.) braziliensis* (MHOM 2903), *L. (L.) amazonensis* (Ph8 and MHOM 81889), and *L. (V.) naiffii* (MHOM 5533).

Characterization by isoenzyme electrophoresis

Analysis of electrophoretic mobility using isoenzymes (MLEE) was performed using a system consisting of seven enzymes. Electrophoresis was performed on agarose gel and the allelic variations were tested for the following enzymes: *isomerases*: 6-phosphogluconate dehydrogenase (6PGDH, E.C.1.1.1.44), mannose phosphate isomerase (MPI, E.C.5.3.1.8), glucophosphate isomerase (GPI, E.C.5.3.1.9); *transferases*: phosphoglucomutase (PGM, E.C.2.7.5.1); *oxidoreductases*: glucose-6-phosphate dehydrogenase (G6PDH, E.C.1.1.1.49), isocitrate dehydrogenase (IDH-NADP, E.C.1.1.1.42); and *lyases*: aconitase (ACON, E.C.4.2.1.3). The method was performed in accordance with the conditions described by Cupolillo et al. (1994, 1998). Each isolate was compared to the WHO reference species mentioned above (WHO/TDR, 2002).

DNA detection by polymerase chain reaction (PCR)

DNA isolation was performed using the Genomic Prep Cells and Tissue DNA Isolation kit (Amershan Biociences), in accordance with the manufacturer's recommendations. The PCR test for *Leishmania (Viannia)* spp. DNA was performed in accordance with De Bruijn and Barker (1992), using the kDNA minicircle region as the target and specific primers for the subgenus *Viannia*: B1-GGGGTTGGTGTAATATAGTGG 5' to 3' and B2-CTAATTGTGCACGGGGAGG 5' to 3'. For the subgenus *Leishmania (Leishmania)* spp., the target was

also the kDNA minicircle region and the primers were: RV1, (GC)(GC)(GC)CC(A/C)CTAT(A/T)TTACACCAACCCC 5' to 3' and RV2, GGGGAGGGGCGT 5' to 3', in accordance with Le Fichoux et al. (1999). The positive controls used were the same as the WHO reference strains used in the characterization by monoclonal antibodies and enzymes.

Results

A total of 209 *Leishmania* spp samples were isolated and characterized. The majority of the isolates, 61.2% (128/209), originated from patients who resided in Manaus, with 38.8% (81/209) residing in the metropolitan regions (Fig. 1). The direct parasitological exam by scarification was positive in 85.2% (178/209) of cases.

Men were predominant (77% (161/209)), and the mean patient age was 31.6 years old (Table 1).

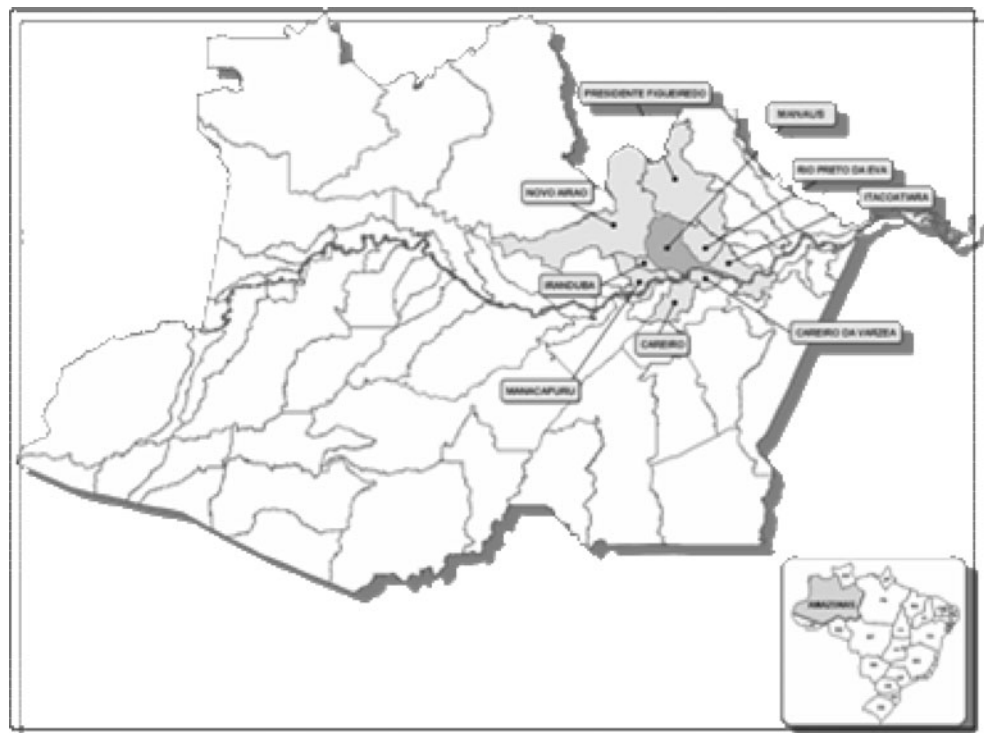
Four parasite species were identified: *L. (V.) guyanensis*, 73.2% (153/209); *L. (V.) braziliensis*, 14.4% (30/209); *L. (L.) amazonensis*, 8.1% (17/209); and *L. (V.) naiffii*, 4.3% (9/209). However, of the total, 13.9% (29/209) of patients presented mixed infections: 4.8% (10/209) involving *L. (V.) guyanensis* and *L. (V.) braziliensis*; 4.8% (10/209) involving *L. (V.) guyanensis* and *L. (V.) naiffii*; 1.4% (3/209) involving *L. (V.) guyanensis*, *L. (V.) braziliensis*, and *L. (V.) naiffii*; 1.4% (3/209) involving *L. (V.) guyanensis*, *L. (V.) braziliensis*, and *L. (L.) amazonensis*; 0.5% (1/209) involving *L. (V.) guyanensis* and *L. (L.) amazonensis*; 0.5% (1/209) involving *L. (V.) guyanensis*, *L. (V.) braziliensis*, and *L. (L.) amazonensis*; 0.5% (1/209) involving *L. (V.) guyanensis*, *L. (V.) naiffii*, and *L. (L.) amazonensis*; and 0.5% (1/209) involving *L. (V.) guyanensis*, *L. (V.) braziliensis*, *L. (V.) naiffii*, and *L. (L.) amazonensis* (Table 2).

Characterization by MLEE revealed a greater frequency of alleles of 6PGDH (45.0%, 94/209), followed by PGM (24.9%, 52/209), G6PDH (23.0%, 48/209), GPI (21.5%, 45/209), ACON (19.1%, 40/209), IDH-NADP (16.7%, 35/209), and MPI (1.9%, 4/209). Quality control was achieved by randomly testing 15% of the isolates, performed by the reference laboratory for *Leishmania* typing and *Leishmania* spp. collection (CLIOC/FIOCRUZ), and the results were in agreement.

Randomly selected samples from among the isolates characterized by monoclonal antibodies and MLEE (32.5%; 68/209) were submitted to the PCR test. Among these, 21.5% (45/209) were positive for the subgenus *Viannia* target and 11.0% (23/209) for the subgenus *Leishmania*.

The mean time of disease evolution was 33 days. No significant differences were observed among the patients who were identified with mixed infections compared to the disease evolution time in the remaining patients infected by a single species. The only patient typed as a carrier of four

Fig. 1 Locations of case occurrence in Manaus and its metropolitan regions



Leishmania spp. species was a male, 36 years of age, and a farmer, who presented five typical lesions on one leg, one hand, and on both arms.

Discussion

American tegumentary leishmaniasis is endemic in the State of Amazonas, where it presents a wide spectrum of clinical variability due to the diversity of species circulating in the region. The most prevalent species is *L. (V) guyanensis*, showing greatest concentration in the area of the city of Manaus and the surrounding metropolitan region.

The findings obtained in this study regarding the parasite are in agreement with those obtained by (Naiff 1998) and Romero et al. (2000, 2001a, 2002a, 2005).

Table 1 Distribution of ATL cases according to gender and age

Age group	M	F
0–10	11	9
11–20	61	15
21–30	69	26
31–40	43	17
41–50	38	5
51–60	24	5
61–70	5	2
>71	2	3
Mean	31.63	10.25

The most relevant finding of this work was the high percentage of patients who presented mixed infections, particularly when considering that the isolates were obtained from clinical samples taken from a single lesion. This further corroborates the reports of other researchers, including Shaw (1985), Shaw and Lainson (1987), Shaw et al. (1989), Grimaldi and McMahon-Pratt (1996), Strelkova et al. (1990a, b, 1997), Saravia et al. (1998), Vray (1998), Bastrenta et al. (2003), and Brito et al. (2009), which were conducted in several regions in Brazil and in other countries. It should be noted that the findings reported by Shaw (1985) were obtained from clinical samples collected from more than one lesion per patient.

These data also elucidate the diversity and heterogeneity of the disease and the complexity of the transmission cycles

Table 2 Distribution of cases of mixed infections according to *Leishmania* spp. species

Parasite species	Number of cases	Percentage of cases
<i>L. (V) guyanensis</i> e <i>L. (V) braziliensis</i>	10	4.78
<i>L. (V) guyanensis</i> e <i>L. (V) naiffi</i>	10	4.78
<i>L. (V) guyanensis</i> e <i>L. (L.) amazonensis</i>	1	0.48
<i>L.(V) guyanensis, L. (V) braziliensis</i> e <i>L. (V) naiffi</i>	3	1.44
<i>L. (V) guyanensis, L. (V) braziliensis</i> e <i>L. (L.) amazonensis</i>	3	1.44
<i>L. (V) guyanensis, L. (V) naiffi</i> e <i>L. (L.) amazonensis</i>	1	0.48
<i>L. (V) guyanensis, L.(V) braziliensis, L. (V) naiffi</i> e <i>L. (L.) amazonensis</i>	1	0.48

in this region in relation to the phlebotomines and reservoir hosts involved. The heterogeneity observed in mixed infections involving *L. (V.) guyanensis*, *L. (V.) braziliensis*, *L. (V.) naiffii*, and *L. (L.) amazonensis* and their capacity for concomitant survival while causing ATL, contrasts with the homogeneity of parasites isolated in other regions, particularly in the northeast and southeast of Brazil (Brito et al. 1993; Brandão-Filho et al. 2003; Cupolillo et al. 2003). Brito et al. (2009) verified 10 different zymodemes in isolates of *L. (V.) braziliensis* and *Leishmania (Viannia) shawii*, originating from the Atlantic Forest zone in Pernambuco, northeastern Brazil, confirming the coexistence of two species that divided the same ecosystem and interfered in the natural genetic polymorphism of the *Leishmania* spp. populations in this endemic region.

The results of typing using monoclonal antibodies were consistent with characterization by MLEE. Cases of cutaneous leishmaniasis can achieve cure spontaneously; however, in this study, all the patients were treated. Since disease notification is compulsory, all the cases were registered in the SINAN. Mixed infections have been reported in other diseases caused by different protozoans. In cases of malaria, up to 27.0% (Lorenzetti et al. 2008) of mixed infections have been verified in certain areas, demonstrating the coexistence of two species (*Plasmodium falciparum* and *Plasmodium vivax*) which contributes to increased virulence, transmissibility, and resistance to drugs due to intrahost competition (Havyliuk and Ferreira, 2009).

Characterization of the origin of the isolates provided detailed information regarding the distribution of the diversity of circulating species in Manaus, including the predominance of *L. (V.) guyanensis*. The municipalities of the metropolitan area that form AM010 (Rio Preto da Eva and Itacoatiara) presented a greater frequency of *L. (V.) braziliensis* and this species in association with other species. Although all the patients responded to treatment, even when successful, cutaneous leishmaniasis can still relapse or even evolve to the mucosal form. According to both Schubach et al. (1988) and Mendonça et al. (2004), the parasite can remain latent, so continuous follow-up is necessary in these patients.

Romero et al. (2001a, b) observed a casuistic of clinical characteristics in patients with ATL associated with *L. (V.) guyanensis* and correlated lesion size and numbers, which presented a larger number of lesions with smaller diameters than those who were infected by *L. (V.) braziliensis*. Multiple lesions can be explained by the occurrence of simultaneous bites by several infected insects and/or metastasis by hematogenic route (Naiff et al. 1999). Similar observations were made in this work, in agreement with Naiff et al. (1999), such that in patients presenting mixed infection, variations in lesion size and numbers also occurred.

Campbell-Lendrum et al. (2001) observed a change in the epidemiological pattern of leishmaniasis transmission

in several South American countries, with important domiciliation of the vectors in Venezuela, Peru, Bolivia, and Brazil. Analysis of these data demonstrates that disappearance of the disease in South America is not likely to occur, since domiciliation of the vector had already established and was currently evident in various Brazilian regions (Brandão-Filho et al. 1999).

The vectors *Lutzomyia umbratilis* and *Lutzomyia anduzei* are considered the principal and second most important transmitters in this region of Manaus, respectively (Guerra et al. 2006), while in a casuistic of 65 patients, Naiff et al. (1999) determined that 91% presented cutaneous lesions exclusively caused by *L. (V.) guyanensis*. They also verified an increase in the occurrence of cases in the rainy season (May–October) due to the high vector population density.

Further studies are required to confirm whether a tendency for urbanization exists in the expansion of the disease and in relation to the findings regarding mixed infections verified in this work. Characterizing the current status of the phlebotomine fauna and reservoir hosts responsible for the maintenance of the zoonotic cycle of American tegumentary leishmaniasis in the region is fundamental in order to assist in the adoption of measures directed at the surveillance and control of this important endemic disease in the State of Amazonas.

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