

## MOLECULAR CHARACTERIZATION OF AMERICAN CUTANEOUS LEISHMANIASIS IN THE TRI-BORDER AREA OF ASSIS BRASIL, ACRE STATE, BRAZIL

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### SUMMARY

In this study, *Leishmania* species were identified by Polymerase Chain Reaction (PCR). The epidemiology of patients suspected of having American Cutaneous Leishmaniasis in the municipality of Assis Brasil, Acre State, located in the Brazil/Peru/Bolivia tri-border was also investigated. By PCR, the DNA of *Leishmania* was detected in 100% of the cases (37 samples) and a PCR-Restriction Fragment Length Polymorphism (RFLP) of the *hsp 70* gene identified the species in 32 samples: *Leishmania (Viannia) braziliensis* (65.6%), *L. (V.) shawi* (28.1%), *L. (V.) guyanensis* (3.1%) and mixed infection *L. (V.) guyanensis* and *L. (Leishmania) amazonensis* (3.1%). This is the first report of *L. (V.) shawi* and *L. (L.) amazonensis* in Acre. The two predominant species were found in patients living in urban and rural areas. Most cases were found in males living in rural areas for at least three years and involved in rural work. This suggests, in most cases, a possible transmission of the disease from a rural/forest source, although some patients had not engaged in activities associated with permanence in forest areas, which indicate a possible sandflies adaptation to the periurban setting.

**KEY WORDS:** *Leishmania*; Acre State; PCR.

### INTRODUCTION

American Cutaneous Leishmaniasis (ACL) is an infectious-parasitic disease caused by different species of protozoa of the *Leishmania* genus that is transmitted to humans by phlebotomine flies.<sup>19</sup> Although there was a decreased incidence of ACL in Brazil, due to Brazilian socioeconomic improvement and increased environmental monitoring over the last 15 years, providing a decrease of people exposed to the vectors<sup>26</sup>, the disease still persists in the country with a high number of cases. Between 2010 and 2012, 68,855 occurrences of ACL were recorded in Brazil. The disease occurs in several endemic areas, 40% of which are found in the North, the area of higher endemicity. Acre was the unit of the federation with the highest incidence of ACL, with 141.64 cases per 100,000 inhabitants in 2010<sup>4</sup>.

In spite of the high incidence of ACL in Acre, the published clinical and demographic studies about this disease are based on the data provided by the Ministry of Health<sup>16,22,23</sup>. The only exception is an epidemiological study in Rio Branco, in which *Leishmania* species were characterized using molecular biology techniques<sup>27</sup>.

The identification of *Leishmania* species from endemic areas is an important step in our understanding of the epidemiology of ACL that could improve its diagnostic and prognostic, and also contribute to the

implementation of control and epidemiological surveillance measures<sup>20,27</sup>. The Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) of samples obtained from leishmaniasis-like lesions - can distinguish the different pathogenic species and this method is more sensitive than the conventional method, which is a parasitological examination performed with an optical microscope<sup>7,20</sup>.

In the majority of Acre municipalities, the reports of ACL are based on non-specific tests, such as the Montenegro's intradermal-reaction, direct microscopic examination and clinical-epidemiological findings<sup>16,22,23</sup>. In Acre, the epidemiological profile study of ACL and its control must be considered in a special context, because this is a region that borders with two endemic countries for ACL: Peru/*Madre de Dios*, with 380 cases/100,000 inhabitants from 2000 to 2012 and Bolivia/*Pando*, with 659 cases/100,000 inhabitants in 2012<sup>3,18</sup>.

Assis Brasil/Brazil is located in the microregion of Brasileia, which, together with the microregion of Rio Branco, is an endemic area of epidemiological relevance for ACL in the Acre State<sup>22,23</sup>. Between 2007 and 2010, a total of 227 cases were reported in the municipality, which corresponds to a mean detection coefficient of 1,027 ACL cases per 100,000 inhabitants.<sup>4</sup> This was the first study performed in the municipality that used molecular methods to characterize the *Leishmania* from ACL patients.

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The diagnostic of ACL was performed on 37 patients that spontaneously attended the Basic Urban Health Unit of Assis Brasil between August 2012 and December 2013 with cutaneous lesions raising the suspicion of ACL. The study was approved by the Ethics Committee in Human Research of the *Instituto de Ciências Biomédicas - USP* (n° 77885/2012). Each patient underwent a clinical examination. A biopsy was collected and sent to the Genetics Laboratory of *Fiocruz Rondônia* for the PCR assay.

During the medical appointment, the following clinical-epidemiological data were collected: sex, age, address (rural or urban), type of lesion (cutaneous, cutaneous-mucosal, and diffuse cutaneous), time since the lesion was detected, if it was a recurring lesion, what type of treatment had been used (which drug) and whether there had been any progress towards the clinical cure of the lesion. Additional information on the patients' occupations was also gathered in the following categories: rural (such as farmers, herdsmen, professors and students from rural areas), non-rural (military personnel, journalists and drivers) and others (housewives, retired workers and children).

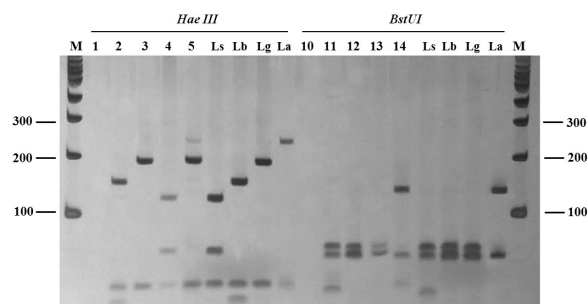
The DNA was extracted from the biopsies according to the recommendations of the manufacturer (PureLink Genomic DNA Mini Kit™; Invitrogen® Carlsbad, CA, USA). To identify the *Leishmania* genus, PCR was performed as described by OLIVEIRA *et al.*<sup>16</sup>. This PCR targeting a conserved region of the *Leishmania* kDNA minicircle (PCR *mkDNA*) was performed with primers 5'-GGG(GT)AGGGGCGTTCT(G/C)CGAA-3' and 5'-(G/C)(G/C)(G/C)(A/T)CTAT(A/T)TTACAC CAACCCC3'. The final volume of the PCR reactions was 25 µL (18.7 µL of Milli-Q water; 2.5 µL of Buffer Green; 0.75 µL of MgCl<sub>2</sub> (2 mM final); 0.38 µL of each *mkDNA* primer (1 µmol final); 0.50 µL of dNTPs (0.2 mM final); 0.25 µL of Taq Polymerase (1.25 U); 2 µL of DNA). The amplification conditions were: 94 °C denaturation for five minutes, 40 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 45 seconds, and a final extension at 72 °C for 10 minutes.

In order to identify the *Leishmania* species from the human biopsies, a fragment of the *hsp70* gene was amplified (PCR *hsp70*) with primers *hsp 70cF* 5'-GGACGAGATCGAGCGCATG GT-3' and *hsp 70cR* 5'-TCCTTCGACGCTCCTGGTTG-3' (adapted from GRAÇA *et al.*)<sup>8</sup>. The PCR mix composition was: 36.25 µL of Milli-Q water, 5.0 µL of Buffer Green; 1.5 µL of MgCl<sub>2</sub> (2 mM); 1.0 µL of each primer *hsp70* (1 µmol final); 2.0 µL of dNTPs (0.2 mM final); 0.5 µL of Taq Polymerase (1.25 U); 5.0 µL of DNA, for a final volume of 52.25 µL. The amplification conditions were: 94 °C denaturation for four minutes, 33 cycles at 94 °C for 15 seconds, 58 °C for 30 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes.

The *mkDNA* (120 pb) *hsp70* (234 pb) amplicons were analyzed on a 2% agarose gel stained with GelRed and examined under UV light.

For the digestion of the *hsp70* amplicons, the restriction enzymes *HaeIII* (Invitrogen®, USA) and *BstUI* (Bio Labs®, New England) were used in independent reactions according to the recommendations of the manufacturer. The resulting products of the restriction digestion were analyzed on a silver-stained 12% polyacrylamide gel (Fig. 1). As a positive control for the PCR and RFLP reactions, DNAs of reference strains of the *Leishmania* collection from the *Instituto Oswaldo Cruz*

(*CLIOC*) were used, namely: *L. (V.) braziliensis* (IOCL 566), *L. (V.) guyanensis* (IOCL 565), *L. (V.) lainsoni* (IOCL 1023), *L. (V.) naiffi* (IOCL 1365), *L. (V.) shawi* (IOCL 1545) and *L. (L.) amazonensis* (IOCL 575). As negative controls, a PCR reaction mix without DNA was used.



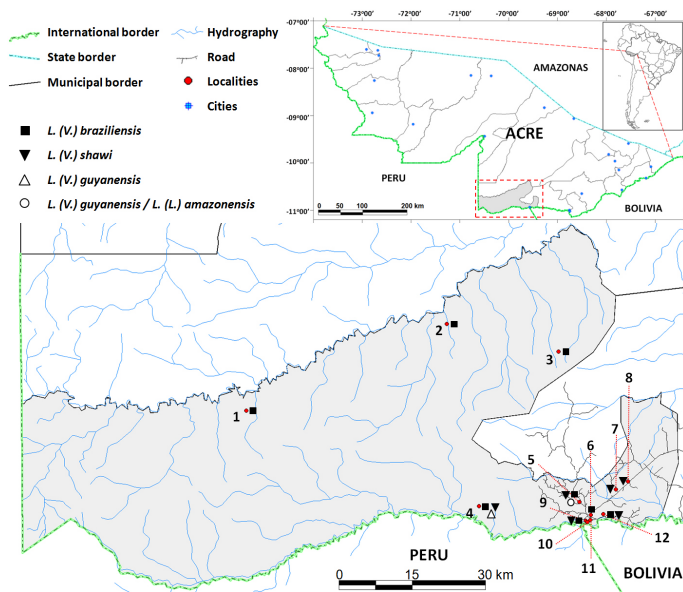
**Fig.1** - Amplicons of *hsp70* (234 pb) after digestion with *Hae III* and *BstUI* from samples of patients suspected of having ACL. Silver-stained 12% polyacrylamide gel. Lanes 1 and 10: negative control; Lanes 2 and 11: sample from patient 01; Lanes 3 and 12: sample from patient 02; Lanes 4 and 13: sample from patient 03; Lanes 5 and 14: sample from patient 04 with mixed infection; Ls: *L. (V.) shawi*; Lb: *L. (V.) braziliensis*; Lg: *L. (V.) guyanensis*; La: *L. (L.) amazonensis*. M: 100 pb molecular weight marker (Invitrogen®).

Measures of central tendency (mean, Md = median, standard deviation, and Q = quartiles) were used for the descriptive analysis, and to calculate the proportions and the confidence interval (IC 95%) for each variable.

PCR for the genus *Leishmania* was positive in the 37 samples. The *hsp70* PCR identified four species in 32 (86.4%) samples: *Leishmania (V.) braziliensis* (21/32; 65.6%), *L. (V.) guyanensis* (1/32; 3.1%), *L. (V.) shawi* (9/32; 28.1%) and a mixed infection (1/32; 3.1%) by *L. (V.) guyanensis* and *L. (L.) amazonensis*; notably, this is the first report of *L. (V.) shawi* and *L. (L.) amazonensis* in Acre. In the microregion of Rio Branco, besides *Leishmania (V.) braziliensis* and *L. (V.) guyanensis*, *L. (V.) lainsoni* and *L. (V.) naiffi* have also been identified<sup>27</sup>. Except for *L. (V.) shawi*, the species that have been described in Acre were also found in other regions of Occidental Amazonia: in *Rondônia* State, species *Leishmania (V.) braziliensis*, *L. (V.) lainsoni*, and *L. (V.) guyanensis*<sup>21</sup> were reported; and in Amazonas State, *L. (L.) amazonensis*, *L. (V.) braziliensis*, *L. (V.) guyanensis* and *L. (V.) naiffi* have been detected<sup>1,9,25</sup>, *L. (V.) guyanensis* being the most frequent species<sup>6,10</sup>.

*L. (V.) braziliensis* was the most common species in this study, similar to observations in the microregion of Rio Branco and other areas in Bolivia and in the Peruvian State of *Madre de Dios*<sup>8,27,28</sup>. It is possible that the high number of *L. (V.) braziliensis* in Assis Brasil/Brazil and its broad geographical spreading in the municipality (Fig. 2) was related to the several phlebotominae species adapted to both the rural/forest and peridomicile environments. *Lu. wellcomei*, *Lu. migonei*, *Lu. carrerai*, *Lu. davisii* and *Lu. whitmani* were the potential *L. braziliensis* vectors<sup>19</sup>, the latter two notable for their relative abundance in Acre State<sup>2,24</sup>.

The identification of *L. (V.) braziliensis* as the predominant species is a relevant epidemiological factor, given that this species is the mainly responsible for the evolution of the mucosal form of ACL in Latin America<sup>11,27</sup>. In Assis Brasil/Brazil, from 2003 to 2010, there were 53.5% confirmed cases of the cutaneous form, 17% confirmed cases



**Fig. 2** - Geographic distribution of the *Leishmania* spp. from patients attended in Assis Brasil (AC) suspected of having ACL. Each symbol represents one or more specimens collected in a given region. 1: *Mamoadate* Indigenous reserve; 2: *Icuriã* Extractive rubber reserve; 3: *Guanabara* Extractive rubber reserve; 4: *São Francisco* Extractive rubber reserve; 5: *Paraguçu* Extractive rubber reserve; 6: Federal highway BR 317 km 2; 7: Federal highway BR 317 km 10; 8: Federal highway BR 317 km 13; 9: Centro Neighborhood; 10: Cascata Neighborhood; 11: Bela Vista Neighborhood; 12: *Santa Quitéria* Extractive rubber reserve.

of the mucosal form, and 29.5% for which there was no information of the clinical form<sup>16</sup>. Besides its association with the mucosal form of the disease, *L. (V.) braziliensis* is also considered a therapeutic challenge<sup>14</sup>. The study found three recurring cases infected by that species, and from the six clinical cases that underwent two cycles of treatment with meglumine antimoniate (glucantime) for lesions heal, five were infected with *L. (V.) braziliensis*.

*L. (V.) shawi*, the second most common species in this study, was recently identified in cutaneous lesions from patients of *Madre de Dios*/Peru<sup>13</sup>, sporadically in cases of the Brazilian Amazonas region, namely in *Pará*, where it was isolated in cutaneous lesions<sup>12</sup>, and recently was also reported in Pernambuco<sup>5</sup>, Northeastern Brazil. The presence of this species could be linked to the presence of the phlebotomine *Lu. whitmani*<sup>19</sup>.

The identification of *L. (V.) guyanensis* and *L. (L.) amazonensis* has important epidemiological and clinical implications, because both species are associated with the development of metastases and a high failure rate of treatment with meglumine antimoniate<sup>9,11</sup>. *L. (V.) guyanensis* has been well characterized in the Amazon Basin, including Acre, *Amapá*, *Roraima* and *Pará*, with higher prevalence in the Amazonas State, where the parasite is associated with edentate animals and marsupials, and its main vector is *Lu. umbratilis*<sup>9,19</sup>. The same vector has been reported in Acre/Brazil by AZEVEDO *et al.*<sup>2</sup>. In Peru, *L. (V.) guyanensis* is one of the species more frequently found in cases of ACL, whereas *L. (L.) amazonensis* has a broad geographic distribution in Brazil, associated with different clinical forms, including the diffused form; its main reservoirs are rodents and marsupials, and the *Lu. flaviscutellata* and *Lu.*

*olmea* phlebotomines are the main vectors<sup>19</sup>. Although the finding of mixed infections by more than one species of *Leishmania* spp. obtained from clinical samples from a single lesion is rare, which was also observed in 14% of skin lesions characterized in Manaus/Brazil by COELHO *et al.*<sup>6</sup>. For these authors, abundance and diversity of infected vectors in an endemic area may contribute to an increased reporting of cases with mixed infection.

The average duration of the cutaneous disease was  $55.9 \pm 40.7$  days (Q1 = 30.0, Q3 = 90 days, Md = 33 days and n = 72.9%). The evolution of the disease in the studied group had a healing rate of 97.3% (36/37), the percentage of patients that abandoned treatment was 2.7% (1/37) and 16.2% (6/37) of the patients (five infected with *L. (V.) braziliensis* and one with *L. (V.) shawi*) were administered two cycles of treatment with meglumine antimoniate. Antimonial chemotherapy studies in Latin America have shown different healing rates (26% - 100%), including healing failure. Several factors can influence the outcome of treatment, such as evolution, number, size and location of the lesions, as well as the *Leishmania* species causing the infection<sup>14,15</sup>. The early diagnosis in the majority of the population studied, the sensitivity of the diagnostic tests (100% for the *mKDNA* PCR and 86.4% for the *hsp70* PCR) to confirm disease and the access to the correct ACL treatment may explain the overall treatment success. These conditions may also reduce the risk of developing a mucosal lesion, which could appear years after the primo-infection.

Most of the ACL patients reported in this work have lived in a rural area (75.7%) for at least three years (70.3%), which suggests that these local cases are linked to activities in a forest environment (59.4%; 22/37). A higher percentage of male individuals was also observed (81.1%) and the age of the infected individuals varied from five to 46 years (Median = 23 years, Mean =  $21.6 \pm 10.6$  years), with a higher percentage of young individuals in their productive years (15 - 40 years) (Table 1). Together, these data suggests that the transmission occurred outside of the residence during the working hours of the population studied. Such epidemiological profile has been observed, in Acre as in other regions of the Brazilian Amazon, which shows that ACL is an occupational disease, since most of the patients are men from rural areas engaged in activities associated with the permanence in the forest<sup>16,22,23,25</sup>. On the other hand, SILVA & MUNIZ<sup>22</sup> reported a different epidemiological leishmaniasis profile in the microregion of Rio Branco, with increasing numbers in recent years of infected women and individuals living in urban areas, and with non-rural occupations. Although in that study the majority of positive cases are from rural areas, particularly *Seringal Paraguçu*, *Seringal Icuriã*, *Seringal São Francisco* and *Seringal Guanabara*, which showed the highest indexes of ACL (13.5%, n = 5; 13.5%, n = 5; 11%, n = 4; 8%, n = 3, respectively), thus suggesting the classical rural/forest transmission, it should be noticed that several of those localities are in the periphery of the municipality urban center (Fig. 2). This is a matter of concern, because such proximity could favor the adaptation of infected vectors to intra and peridomicile in the municipality urban area.

These data revealed a predominance of *L. (V.) braziliensis* in the ACL cases and that probably this disease is linked to rural life, particularly rural work. The presence of several *Leishmania* species supports a scenario of heterogeneous transmission, which calls for a broader epidemiological study that takes into consideration the vectors, reservoirs, virulence of the parasites and incidence of clinical forms.

**Table 1**

Epidemiological profile of patients examined in Assis Brasil (AC) with cutaneous lesions used in the diagnostic of *Leishmania* by PCR

Variables		Positive	% (CI 95%)
Sex	Male	30	81.1 (66.1-91.3)
	Female	7	18.9 (8.7-33.8)
Age	0 - 11	7	18.9 (8.7-33.8)
	12 - 21	13	35.1 (21.1-51.4)
	22 - 31	9	24.3 (12.5-39.9)
	32 - 41	7	18.9 (8.7-33.8)
	≥41	1	2.7 (0.1-12.6)
Profession	Rural	22	59.4 (43.2-74.3)
	Non-Rural	7	18.9 (8.7-33.8)
	Others	8	22.6 (10.6-36.9)
Area	Rural	28	75.7 (60.0-87.4)
	Urban	9	24.3 (12.5-39.9)
Localities	Seringal <i>Paraguaçu</i>	5	13.5
	Seringal <i>Icuriã</i>	5	13.5
	Seringal <i>São Francisco</i>	4	11
	Seringal Guanabara	3	8
	Federal highway BR 317	3	8
	Urban area	9	24
	Others	8	22

## RESUMO

### Caracterização molecular da leishmaniose tegumentar americana em área de tríplice fronteira, Assis Brasil, Estado do Acre, Brasil

O presente estudo caracterizou as espécies de *Leishmania* pela Reação em Cadeia da Polimerase (PCR). Também descreveu os aspectos epidemiológicos de pacientes com suspeita de leishmaniose tegumentar americana no município de Assis Brasil, Estado do Acre, Brasil, localizado na tríplice fronteira Brasil/Peru/Bolívia. A PCR detectou DNA de *Leishmania* em 100% dos casos (37 amostras) e a PCR-Restriction Fragment Length Polymorphism (RFLP) do gene *hsp 70* identificou as espécies em 32 amostras: *Leishmania (Viannia) braziliensis* (65,6%), *L. (V.) shawi* (28,1%), *L. (V.) guyanensis* (3,1%) e infecção mista *L. (V.) guyanensis* e *L. (Leishmania) amazonensis* (3,1%). Esse é o primeiro registro de *L. (V.) shawi* e *L. (L.) amazonensis* no Acre. As duas espécies predominantes foram encontradas em indivíduos residentes em áreas rurais e urbanas. O maior número de casos foi notificado entre indivíduos de áreas rurais, sexo masculino, de ocupação rural e tempo de residência maior que três anos. Esses dados sugerem possível transmissão da doença em ambiente rural/florestal na maioria dos casos, no entanto alguns pacientes não tinham envolvimento com atividades relacionadas com a permanência na floresta, indicando possível adaptação de flebotômicos no ambiente periurbano.

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## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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