Research note

Surveillance of cutaneous leishmaniasis in clinical samples: distribution of *Leishmania guyanensis* in the state of Amapá, Brazil, 2018*

doi: 10.5123/S1679-49742020000100007

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Abstract

Objective: to investigate *Leishmania* species in a series of autochthonous cutaneous leishmaniasis (CL) cases in Amapá State, Brazilian Amazon. **Methods:** this was a descriptive ecological study carried out from January-October/2018 at a reference center for CL diagnosis in Amapá; individuals with CL receiving care from January-May/2018 were recruited; clinical data and skin biopsies were obtained; from extracted DNA (phenol-chloroform) we amplified the *hsp70-234* gene region (PCR) for nucleotide sequencing (Applied Biosystems: ABI3500XL). **Results:** 38 individuals were interviewed, examined and diagnosed; men predominated (28/38; mean age=32.5±11.3); lesions (most ulcers: 37/38) measuring 0,4-10mm (34/38) and ≥11mm (4/38) were multiple in 20/38 individuals; diagnosis of *L. braziliensis* (1), *L. naiffi* (1), *L. infantum* (1), *L.* (*Viannia*) sp. (1), *L. amazonensis* (2) and *L. guyanensis* (32); individuals infected with *L. guyanensis* (32/38) lived in 9/10 municipalities represented in the sample, and 17/32 of these had multiple lesions. **Conclusion:** presence of *Leishmania guyanensis* predominated and was frequently associated with multiple lesions.

Keywords: Leishmaniasis, Cutaneous; *Leishmania guyanensis*; Molecular Epidemiology; Amazonian Ecosystem; Epidemiology, Descriptive.

*This research received financial support from the Amapá State Research Support Foundation (FAPEAP) – Process No. 250.203.039/2016 – and a doctoral Research Grant from the Pará State Research Support Foundation (FAPESPA) – Process No. 003/2014.

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Introduction

Leishmaniasis is a neglected tropical disease caused by protozoa of the *Leishmania* genus and has two main clinical forms: tegumentary leishmaniasis (TL), which can be cutaneous and/or mucousal; and visceral leishmaniasis (VL).[1]

Between 2007 and 2015, 6,801 cases of cutaneous leishmaniasis (CL) were registered in the state of Amapá.[2] CL can progress to severe although not fatal forms. Notwithstanding, between 2017 and 2018, the Amapá State Health Surveillance Superintendency recorded four deaths of patients with CL being treated with Glucantime®[3] (meglumine antimoniate) and 19 cases of canine VL.[4] Also in 2018 the first autochthonous case of human VL in the state was confirmed: a riverside dweller aged 82 who was infected in the municipality of Mazagão and died from complications caused by cirrhosis of the liver.[4]

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Surveillance of VL, which is caused by the etiological agent *Leishmania infantum*, favors early diagnosis and treatment, thus reducing risk of death. Nevertheless, it is uncommon for people with CL to die and death usually results from comorbidities exacerbated by the toxicity of Glucantime®.[5] Among the agents causing CL, *Leishmania braziliensis* and, principally, *Leishmania guyanensis*, show resistance to Glucantime®, the prolonged use of which increases the risk of adverse events.[6] *L. guyanensis* occurs in the North of Brazil[1,7,8] but its frequency and distribution in Amapá are unknown.

The objective of this study was to investigate *Leishmania* species in a series of autochthonous cutaneous leishmaniasis cases in Amapá in 2018.

Methods

We conducted a descriptive study of a series of CL cases. The Brazilian state of Amapá has 670,000 inhabitants, distributed over 16 municipalities.9 One side of the state faces the Atlantic Ocean, while to the North it borders with Suriname and French Guiana, and to the south it borders with the Brazilian state of Pará. In 2018, the CL detection coefficient in Amapá was 68.7/100,000 inhabitants, with autochthonous cases in 14 of the state's 16 municipalities.[3] Phlebotomine insects, which are potential CL vectors, are abundant in this Amazon subregion,[8,10,11] where CL is endemic.[2,3]

In order to collect data, we recruited individuals attending the Amapá Tropical Diseases Reference Center between January and May 2018. The inclusion criteria were: males and females over 12 years old, with primary lesions suggestive of CL for at least two weeks. Those with medical contraindication to anesthesia and/or biopsy or with some other laboratory diagnosis were excluded.

The *Leishmania* species identified in the study were assessed in relation to the following variables:

a) sociodemographic variables
- sex;
- municipality of infection;
- mean age; and
- age groups;

b) clinical variables
- number of cases;
- type of lesion (ulcer; verrucous);
- diameter of lesion;
- site of lesions on the body; and
- predominant clinical manifestations (crusting, secondary infection, satellite lesions etc.).

Leishmania variable measurement and diagnosis were based on leishmaniasis surveillance criteria defined in the Ministry of Health technical manual,1 as well as being based on molecular techniques.[12,13]

Lesion exudates were used to confirm infection, obtained via swabs for smearing on glass slides, using Giemsa stain to examine for amastigotes under the microscope.[1] *Leishmania* were characterized using DNA extracted from the skin (phenol-chloroform). Skin was collected via lesion biopsy using local anesthesia (2% lidocaine). The sample was kept in a flask containing 0.5mL of NET (NaCl 0.15mM; EDTA 50mM; Tris-HCl 0.1M/pH 7.5). In order to ensure the quality of the extracted skin/DNA samples, refrigerator temperature was controlled daily (2-8ºC). Polymerase chain reaction (PCR) was performed with subsequent sequencing of the 234 base pair target, i.e. *Leishmania* DNA heat shock-protein 70 gene coding region (*hsp70-234*).[12]

The following assays were performed:

a) Polymerase chain reaction – PCR

PCR was based on *Leishmania hsp70-234*, highly sensitive for protozoan DNA detection and species discrimination.12 Assay conditions: 50mL Mix (Taq DNA polymerase 0.03U/µL; MgCl2 1.5mM; Invitrogen® KCl buffer; dNTPs 0.25mM; F- 5' GGA CGA GAT CGA GCG CAT GGT 3' e R- 5' TCC TTC GAC GCC TCC TGG TTG 3' initiators, 0.2pM each; DNA 3.0µL); denaturation, annealing and extension (1x 94°C/5'; 32x 94°C/0,5', 61ºC/1' and 72ºC/1'); and final extension (72°C/10'). A positive result confirms the presence of *Leishmania* DNA in the lesion, since it signifies amplification of the *hsp70-234* gene fragment.[12]

b) DNA sequencing

This procedure identifies the sequence of nucleotide bases of the *hsp70-234* gene region, confirming the distinction between *Leishmania* species by comparing the sequence with well characterized sequences available in the GenBank. The PCR-*hsp70-234* products were therefore purified (illustra ExoProStar; GE Healthcare Life Science) and sequenced (forward and reverse) in the automatic ABI3500XL DNA analyzer, using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). BioEdit and Basic Local Alignment Search Tool (BLAST) were used to analyze sequences.13 Control samples were comprised of DNA from *L. braziliensis* (MHOM/BR/1975/M2904), *L. guyanensis* (MHOM/BR/1975/M4147), *L. lainsoni* (MHOM/BR1881/M6426), *L. shawi* (MCEB/BR/1984/M8408), *L. naiffi* (MDAS/BR/1979/M533) and *L. amazonensis* (IFLA/BR/1968/PH8).

We used BioStat 5.0 to produce frequency tables and to calculate means and standard deviations (available at: www.mamiraua.org.br).

The study project was approved by the Federal University of Amapá Research Ethics Committee (Certification of Submission for Ethical Appraisal [CAAE] No. 57036716.0.0000.0003, approved on 22/08/2016) and by the Evandro Chagas Institute Research Ethics Committee (CAAE No. 57036716.0.3001.0019, approved on 03/10/2016).

Results

Thirty-eight individuals with CL were included. They were 28 males and 10 females aged between 13 and 81 with a mean age of 34.4 years (standard deviation: 14.2), resident in ten municipalities in the state of Amapá. The largest number of cases (n=13) came from the municipality of Mazagão. The most frequent etiological agent among the participants was *Leishmania guyanensis* (32/38). Clinical/demographic aspects and etiology of the CL cases are shown in Table 1.

With regard to the number of lesions, 20 individuals had multiple lesions while 18 had a single lesion. In 37/38 cases, lesions were of the ulcer type, mostly with a 5-6cm diameter, predominantly located on the upper limbs (n=9) and lower limbs (n=11). Predominant clinical manifestations were crust, secondary infection and satellite lesions. The characteristics of the lesions of the individuals with CL and respective etiological agents identified following sequencing and analysis of the *Leishmania hsp70-234* gene region are described in Table 2.

This study contains the first report of *L. infantum* associated with CL in Amapá: a 35year-old woman infected in the municipality of Porto Grande, with multiple varied size verrucous cutaneous lesions on her body, including her thighs and legs, without visceral disease

Discussion

The most frequent etiological agent among the five species identified in individuals with CL in Amapá was *L. guyanensis*: 32 of the 38 cases analyzed. The *hsp70-234 Leishmania* marker discriminates six pathogens in the Amazon.[12,14] The specificity of *hsp70-234* has not been previously evaluated for L. lindenbergi, the seventh pathogen that causes human CL in the Amazon.[8] Some species identified in this study are worthy of note: (i) *Leishmania amazonensis*, in addition to localized CL it can cause diffuse (anergic) leishmaniasis which is rarely curable; and (ii) *Leishmania guyanensis* and *L. braziliensis*, which induce cutaneous/mucous lesions that are hard to deal with,[1,15] having a high degree of hypersensitivity and which may appear months or years after clinical cure of the primary lesion.[1] Only long-term medical follow-up will ensure effective diagnosis and treatment of relapses.[15] Even in the absence of mucous involvement, knowing what the pathogen is enables a prognosis to be defined.

Those who had CL caused by *L. braziliensis* and *L. naiffi* had a single ulcer. *Leishmania braziliensis* is widely distributed in Brazil.[1] *L. naiffi* infection in humans is limited to the states of Amazonas, Acre, Pará, Rondônia and Mato Grosso,[16] with no complications

having been reported until our study revealed the first case of CL caused by *L. naiffi* in Amapá.

L. infantum is phylogenetically close to *Leishmania donovani*, the agent that causes VL and post-kala-azar dermal leishmaniasis in the Old World (Europe);[17] occurrence of cutaneous lesions caused by *L. infantum* in immunocompetent individuals is uncommon in the Americas, especially in Brazil.[14,18]

L. infantum has been isolated in the skin of Africans with CL, producing a lesion histologically distinct from that caused by L. major.[19] Since 2015, cases of CL caused by *L. infantum* have been recorded in different Brazilian states.[14,18] In Africa and the Americas, the clinical characteristics of CL vary according to the etiological agent.[1,19] Our study highlights the pleomorphism of CL caused by *L. infantum*, alerting

Table 1 – Sociodemographic aspects and etiology of cutaneous leishmaniasis in males and females in a series of leishmaniasis cases in the state of Amapá, Brazil, 2018

Variables	Sex		Total
	Male	Female	
	n=28	n=10	n=38
Age group (in years)			
12|— 20	3	3	6
20|—30	9	1	10
30|—40	9	1	10
40|—50	5	3	8
50|—60	2	1	3
60|—81	–	1	1
Mean age (standard deviation)	32.5 (11.3)	39.9 (19.9)	34.4 (14.2)
Municipality of infection			
Mazagão	10	3	13
Porto Grande	3	4	7
Serra do Navio	5	–	5
Tartarugalzinho	3	–	3
Laranjal do Jari	3	–	3
Santana	-	2	2
Oiapoque	2	–	2
Ferreira Gomes	1	–	1
Pedra Branca do Amapari	1	–	1
Calçoene	–	1	1
Molecular diagnosis (*Leishmania* species)[a]			
Leishmania (Viannia) guyanensis	23	9	32
Leishmania (Leishmania) amazonensis	2	–	2
Leishmania (Viannia) braziliensis	1	–	1
Leishmania (Viannia) naiffi	1	–	1
Leishmania (Leishmania) infantum	-	1	1
Leishmania (Viannia) sp	1	–	1

a) Molecular diagnosis based on polymerase chain reaction (PCR) and *Leishmania hsp70-234* gene region sequencing.

Table 2 – Characteristics of lesions found in individuals with cutaneous leishmaniasis (n=38) and respective etiologic agents, identified following sequencing and analysis of *Leishmania hsp70-234* gene region, Amapá, Brasil, 2018

Variables	Leishmania Species						
	Leishmania guyanensis	*Leishmania amazonensis*	*Leishmania braziliensis*	*Leishmania naiffi*	*Leishmania infantum*	*Leishmania (Viannia)* sp	Total (n)
Number							
Single	15	1	1	1	–	–	18
Multiple	17	1	–	–	1	1	20
Type							
Ulcer	32	2	1	1	–	1	37
Verrucous	–	–	–	–	1	–	1
Diameter (in cm)							
0.4|—2	5	1	–	1	–	1	8
2|—4	4	–	1	–	–	–	5
4|—6	9	1	–	–	–	–	10
6|—8	5	–	–	–	1	–	6
8|—10	5	–	–	–	–	–	5
10|—	4	–	–	–	–	–	4
Site							
Head/face	–	–	–	1	–	–	1
Torso	2	–	–	–	–	–	2
Upper limbs	7	1	1	–	–	–	9
Lower limbs	11	–	–	–	–	–	11
Head and torso	1	–	–	–	–	–	1
Head, upper and lower limbs	2	–	–	–	–	–	2
Head, torso and lower limbs	1	–	–	–	–	–	1
Torso and upper limbs	1	–	–	–	–	–	1
Torso and lower limbs	4	–	–	–	–	–	4
Upper and lower limbs	3	1	–	–	1	1	6
Predominant clinical manifestations (types)[a]							
Crust	16	–	–	1	–	1	18
Secondary infection	9	–	–	–	–	1	10
Satellite lesions	5	1	–	–	–	–	6
Lymphangitis	5	–	–	–	–	–	5
Lymphadenopathy	2	–	–	–	–	–	2
Fibrosis	1	–	–	–	–	–	1
Headache	1	–	–	–	–	–	1
Erysipelas	1	–	–	–	–	–	1

a) One or more types of associated manifestations may have occurred in the same individual with LC.

as to the need for early diagnosis[20] and, in this sense, the importance of surveillance for preventing also human VL in Amapá.

CL hotspots have been identified in Amapá, principally in areas of agricultural, plant extractivism and prospecting/mining settlements,[2] where males of productive age are most affected. The majority of the individuals we analyzed had multiple primary lesions, suggesting diverse vector bites. This is a recognized behavior of *Nyssomyia umbratilis*, the agent that transmits *L. guyanensis*.[1,8] In these cases, prolonged treatment with Glucantime® would be necessary; however, there is considerable morbidity and even deaths associated with the toxicity of this chemotherapy.[3,5,21,22]

Although Glucantime® is the first drug option for treating CL in Brazil, Pentamidine Isethionate® is recommended where *L. guyanensis* is predominant.[1]

Study participants infected with *L. guyanensis* came from nine of the ten municipalities considered in the sample, suggesting widespread distribution of this species in Amapá, where vector fauna are abundant.[8,10,11]

L. braziliensis and *L. guyanensis* infection caused by *Leishmania* RNA virus subtype 1, found in Peru, Bolivia, French Guiana, Suriname and Brazil, would explain the high virulence and resistance of these species to the drug.[23] Biological factors of CL need to be monitored, especially in frontier regions.

With regard to treatment, with effect from 2017 Pentamidine Isethionate® has been considered preferential to Glucantime® for systemic treatment of CL in areas where *L. guyanensis* is predominant in Brazil. In cases of (i) kidney, heart and liver failure, (ii) people who have had kidney, heart and liver transplants, (iii) pregnant women and people more than 50 years old, the use of liposomal Amphotericin B® is recommended. Glucantime® would be the first option only for intralesional use in these areas.[1] The results found by our study support the new guidelines for treatment of CL in Amapá adopted with effect from 2018.[24]

Our study confirmed the predominance of *L. guyanensis* infection in Amapá, frequently associated with multiple lesions, as was as providing the first reports of the presence of *Leishmania naiffi* and *L. infantum* in clinical samples of CL in the state of Amapá.

Acknowledgements

Our thanks go to Dr. Inês Celeste Ribeiro Martins, Director of the Amapá Tropical Diseases Reference Center (CRDT-AP), and to Dr. Aldo Aparecido Proietti Júnior, Coordinator of the Federal University of Amapá Special Applied Microbiology Laboratory (UNIFAP), for their support for our study, making available the necessary space and conditions for our work at CRDT/AP and for proper storage of samples at UNIFAP.

Authors' contributions

Almeida ANF, LCS Nascimento LCS, Sousa ESMM, Oliveira AJD, Sena MG, Resende BM, Chaves RCG and Garcez LM contributed substantially to all stages of the study: conception, fundraising, collection, analysis and interpretation of the results, writing and critically reviewing the intellectual contents of the manuscript. All the authors have approved the final version and are responsible for all aspects of the study, guaranteeing its accuracy and integrity.

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Received on 09/02/2019
Approved on 29/06/2019

Associate editor: Maryane Oliveira Campos – orcid.org/0000-0002-7481-7465