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Short Communication

Molecular diagnosis of cutaneous leishmaniasis in an endemic area of Acre State in the Amazonian Region of Brazil

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Abstract

Introduction: This study proposes to identify the *Leishmania* species found in the skin lesions of cutaneous leishmaniasis (CL) patients from Brasiléia municipality (Acre). **Methods**: Skin biopsy imprints or biopsy fragments were assayed via kDNA-PCR/RFLP and FRET-real-time PCR. **Results**: Of individuals with suspected CL, 18 were positive for *Leishmania* kDNA. *Leishmania (Viannia) braziliensis* (61.1%) and *Leishmania (Viannia) guyanensis* (5.5%) were identified in the positive samples. **Conclusions**: These results are congruent with the previous reports in Acre and Bolivia, revealing *L. braziliensis* as the most prevalent species. *L. guyanensis* identification also corroborates with the epidemiology of the disease in the Amazon Basin.

Keywords: Cutaneous leishmaniasis. Molecular diagnosis. Acre State.

Leishmaniasis is a tropical disease caused by protozoan parasites of the genus *Leishmania* and is characterized by a broad range of clinical manifestations classified as cutaneous and visceral leishmaniasis[1]. Cutaneous leishmaniasis (CL) is caused mainly by *Leishmania* (*Leishmania*) *major* and *Leishmania* (*Leishmania*) *tropica* in the Old World, and by parasites of the *Leishmania* (*Leishmania*) *mexicana* and *Leishmania* (*Viannia*) *braziliensis* complexes in Central and South America[2]. Epidemiological records indicate that CL is distributed worldwide, affecting at least 12 million people in 98 countries, with 90% of all cases occurring in only seven countries, including two from South America: Brazil and Peru[3]. In Brazil, CL is an important public health concern with 21,161 reported cases in 2015, the majority (69%) of these being in the Amazonian states[4].

In the New World, CL is a zoonotic disease caused by a variety of *Leishmania* species, transmitted to humans by female sandflies belonging to the *Lutzomyia* genus. Currently, CL is considered an emergent and re-emergent disease, and there is an increasing concern regarding the adaptation of its parasite transmission cycle to urban environments. In Brazil, the expansion of CL throughout the Amazon region appears to be facilitated by the development of agricultural frontiers, the emergence of mining areas, and the establishment of new urban centers in environments susceptible to disease transmission[5]. The Brazilian Amazonian State of Acre is an example of the driving effects of human activities on the epidemiology of leishmaniasis. Recent records from this state indicate that 3,538 confirmed CL cases were reported between 2010 and 2013. Moreover, up to 1,215 cases were reported exclusively in the municipality of Brasiléia, located on the Brazil/Peru/Bolivia tri-border, which stands out as the municipality with the second highest number of CL cases in Acre State[4]. This ongoing transmission in urban and peri-urban settings reinforces the need to better characterize the epidemiological patterns of CL in the state. A few studies have been conducted in the area, most of them in Rio Branco, the capital of Acre[6-8]. Furthermore, the difficulty of providing an accurate diagnosis may result in an underestimation of the incidence of leishmaniasis in Acre.

In this study, we aimed to assess the epidemiology of CL in the municipality of Brasiléia by applying molecular tools for the detection and genotyping of the existing *Leishmania* species. Our results will provide insights into the transmission of species responsible for leishmaniasis and could contribute to better surveillance and control strategies in the area.

In order to explore the epidemiology of CL in Brasiléia, we conducted a prospective study at the Brasiléia Health

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Center from September 2013 to February 2015. All patients provided written informed consent for collection of samples and subsequent analysis.

The City of Brasiléia is located in the Northern State of Acre, Brazil [11°00′S 68°44′W] (**Figure 1**) and had a population of 23,378 inhabitants in 2015. The region exhibits an equatorial tropical climate with annual temperatures between 22˚C and 33˚C and annual precipitation around 1**,**900mm.

All patients reside in Brasiléia, Acre and attended to the Fernando Azevedo Correia Center of Health. They presented with skin lesions with characteristics of CL and were submitted to Montenegro skin test. Between September 2013 and February 2015, patients with suspected or confirmed CL were invited to participate in the study after providing signed, written informed consent. Participants provided sociodemographic information, including sex, age and location (rural or urban area) during the medical appointment. In addition, skin biopsy imprints on filter paper (FTA® cards) were collected from the lesions, and in some cases, biopsy fragments were obtained from the border of lesions after administering a local anesthetic [2% lidocaine (xylocaine)]. These samples were stored in liquid nitrogen until processing. All recommended measures (e.g. the use of individual disposable blades for filter paper cutting and careful individual packaging of material) were taken to prevent cross contamination.

Deoxyribonucleic acid (DNA) was extracted from FTA® cards (skin biopsy imprints) and biopsy fragments as described by Marques et al.[9]. The kinetoplast DNA (kDNA)-polymerase chain reaction (PCR) was performed as previously described[10]. Following amplification, kDNA products were digested with HaeIII (Invitrogen®, USA) for 3h at 37°C, in accordance with the manufacturer's recommendations, and the resulting digestion fragments were analyzed on a 12% silver-stained polyacrylamide gel. A fluorescent resonance energy transfer (FRET) real-time

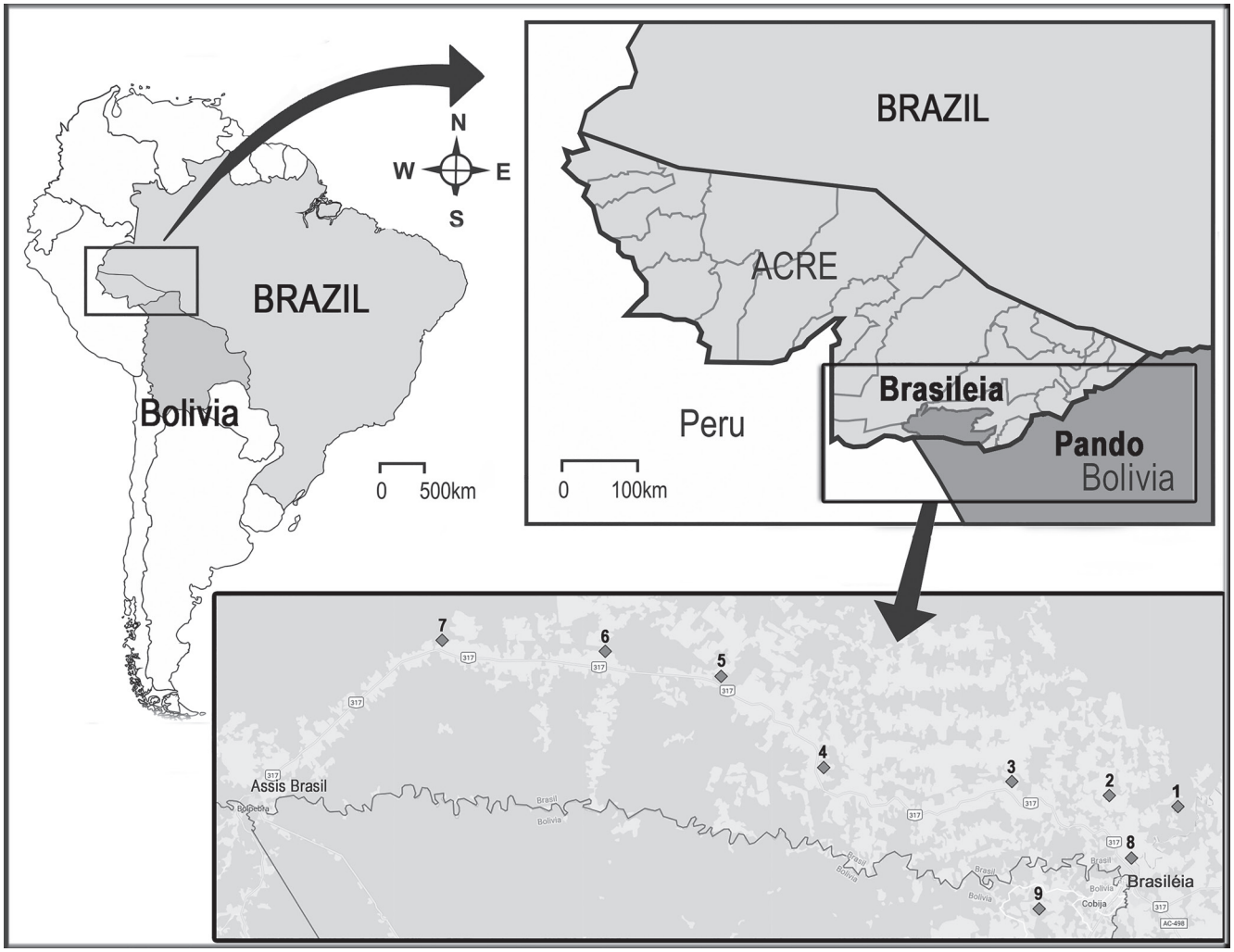

FIGURE 1: Brasiléia municipality, State of Acre, Brazil. Geographic distribution of the patients with clinically suspected cutaneous leishmaniasis from Brasiléia. **Rural areas** (transversely located along the Trans *Pacific* Federal Highway BR 317): **1.** Kilometer 4, *Ramal do Polo*; **2.** Kilometer 5, *Ramal do Jarinal* and *Filipinas extractive reserve*; **3.** Kilometer 18, *Guanabara extractive reserve*; **4.** Kilometer 52, *São Cristovão extractive reserve*; **5.** Kilometer 59, *Aputi*, *Tabatinga*, *Nova Linda*, *Etelhi* and *Nova Pinda extractive reserves*; **6.** Kilometer 67, *Guanabara extractive reserve*; **7.** Kilometer 84, *Guanabara* and *Amapá extractive reserves.* **Urban area: 8.** Center of Brasiléia City. **Imported case: 9.** Cobija, *Pando district - Bolivia.*

PCR based on *Leishmania* species-specific DNA polymorphisms in the genes encoding mannose phosphate isomerase (MPI) and 6-phosphoglucanate dehydrogenase (6PGD) was carried-out to support the identification of the *Leishmania* species in the kDNA-positive samples[11].

Twenty-two patients who provided clinical and sociodemographic information and presented positive results in the Montenegro skin test were included (**Table 1**). Of those, 18 lived in rural areas (transversely situated along the Federal BR317 Trans *Pacific* highway, named *Ramal or Ramais*, based on the distance from the center of Brasiléia); three patients were from urban areas (center of Brasiléia), and one represented an imported case from Cobija, Pando District, in Bolivia (**Figure 1** and **Table 1**). Most patients from rural areas (*n*=12) resided in isolated reserves located far away within the *Ramais*, in dense forest sites. The remaining patients from rural areas lived in kilometer 4 - *Ramal do Polo* (*n*=5) and kilometer 5 - *Ramal do Jarinal* (*n*=1), less isolated than the reserves, but still close to the dense forest (**Figure 1**). The houses were simple dwellings without basic sanitation, with many peridomiciliary animals, providing a favorable environment for the attraction and multiplication of insect vectors.

The patients positive for the Montenegro skin test presented with lesions that were clinically characterized as simple or multiple ulcer-crusted lesions with 59% of them presenting irregular borders and were mostly observed on the lower limbs (**Table 1**). Detection of *Leishmania* genus parasites via kDNA-PCR resulted in 18 positive samples out of 22 patients with clinically suspected CL. The 4 negative samples (three biopsy fragments and one imprint) were obtained from individuals living in kilometer 4 - *Ramal do Polo* (rural area; 3 males and 1 female) (**Table 1**). This negative result may probably be due to the lapse of time between lesion formation and the collection of clinical material, considering that parasite detection becomes more difficult as the number of parasites in the lesion decreases with the progression of the chronic granulomatous process[12]. Following HaeIII digestion of the kDNA amplified products from 18 positive samples, the restriction profile corresponding to *L.* (*V.*) *braziliensis* was identified in 11/18 samples (61.1%), namely the presence of two fragments of 40 and 80bp in 12% silver-stained acrylamide gel (data not shown).

FRET-based real-time PCR was used to confirm the identification of the species of *Leishmania* in the positive kDNA samples. This assay is based on the identification of

TABLE 1: Characteristics of the study group and diagnostic results of skin biopsy imprints and biopsy fragments from cutaneous lesions of patients with positive Montenegro test.

			Patients								
			Variable								
			sex		age				area		
Sampling			male	female	0-7	8-14	15-21	22-30	rural	urban	imported case
			10	12	2	6	7	7	18	3	1
Characteristics of the lesions	Distribution	Unique	7	9	1	5	5	5	14	1	1
		Multiple (2-7)	3	3	1	1	2	2	4	2	-
	Borders	Regular	5	4	1	2	2	4	8	1	-
		Irregular	5	8	1	4	5	3	10	2	1
	Diameter	cm	1-7	0.5-4	1-4	1-3	1-4	1-7	0.5-4	1.5-7	2
	Mean duration*	Days	15-120	30-120	15-120	30-120	15-120	30-60	15-120	60-120	30
	Localization	Upper limbs	X	-	-	X	X	-	X	-	-
		Lower limbs	X	X	X	X	X	X	X	X	X
		Thorax	X	-	-	-	-	X	-	X	-
		Face	X	X	-	X	-	-	X	-	-
Diagnostic results	Positive for KDNA**		7	11	1	6	6	5	14	3	1
	Leishmania species	*L.bra*	3	8	-	3	5	3	10	-	1
		L.guy	-	1	-	1	-	-	1	-	-
		NI	4	2	1	2	1	2	3	3	-

***L. bra*: *Leishmania braziliensis*; *L. guy*: *Leishmania guyanensis*; **NI**: not identified; **cm:** centimeters; **kDNA:** kinetoplast deoxyribonucleic acid.
*Based on the Montenegro skin test. **Positive samples for the presence of *Leishmania* kDNA.

mutations in the *6PGD* and *MPI* genes, yielding distinct melting peaks that are used to differentiate between five *Leishmania* (*Viannia)* species[11]. The method detects down to 60fg of parasite DNA, which is equivalent to less than five parasites per reaction[11]. **Figure 2** shows an example of the results obtained by the coupled analysis of 6PGD and MPI loci. Three samples (II, VI and VIII) identified as *L.* (*V.*) *braziliensis* or *Leishmania* (*Viannia*) *peruviana* by the analysis of *6PGD* gene (**Figure 2A**) were confirmed as *L.* (*V.*) *braziliensis* using the MPI locus (**Figure 2B**). The results of 6PGD analysis revealed infection by *L.* (*V.*) *guyanensis* (Floch 1954) in one sample (IX) (**Figure 2A**), whereas the same sample was identified

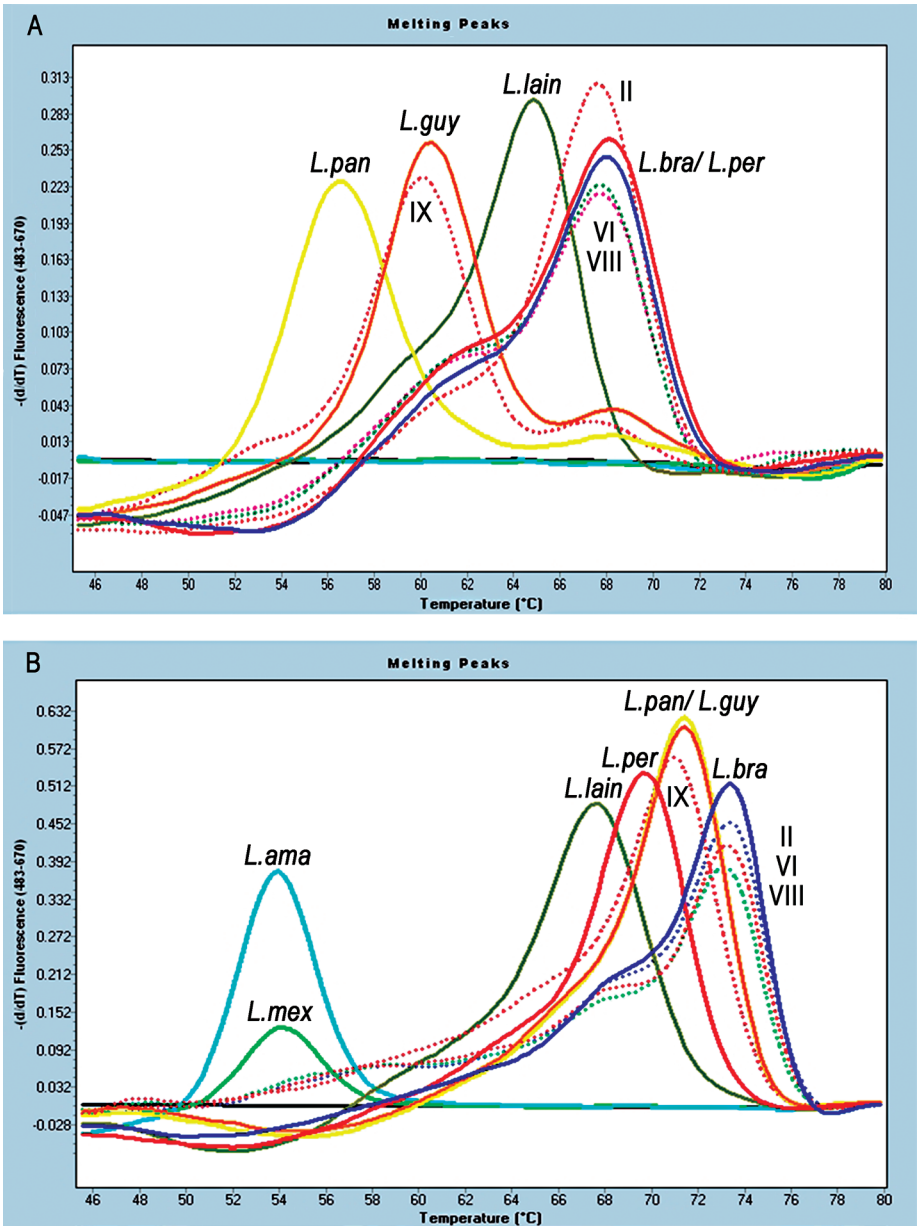

FIGURE 2: Example of melting curve analysis of FRET real-time PCR focusing on the 6PGD and MPI genes**.** Dotted lines represent cutaneous lesion samples from patients with suspected cutaneous leishmaniasis. Solid lines correspond to a set of New World *Leishmania* reference strains used as standard samples. *Leishmania* species were identified based on melting curves exhibiting the same dissociation temperature. **A**. **Melting curves corresponding to 6PGD-based amplification reactions**. Samples from three patients (II, VI and VIII) were identified as \ *braziliensis* or *L.* (*V.*) *peruviana*, and one sample (IX) revealed the same melting temperature attributed to *L.* (*V.*) *guyanensis* reference strain. **B**. **Melting curves corresponding to MPI-based amplification reactions**. Samples from patients (II, VI and VIII) were positioned at the same melting temperature corresponding to the *L.* (*V.*) *braziliensis* reference strain. Sample IX was identified as *L.* (*V.*) *panamensis* or *L.* (*V.*) *guyanensis*. ***L. pan:*** *Leishmania panamensis*; ***L. guy:*** *Leishmania guyanensis*; ***L. lain:*** *Leishmania lainsoni*; ***L. bra***: *Leishmania braziliensis*; ***L. per:*** *Leishmania peruviana*; ***L. ama:*** *Leishmania amazonensis*; ***L. mex:*** *Leishmania mexicana;* ***L.* (*V.*):** *Leishmania (Viannia)* **FRET PCR:** polymerase chain reaction; **6PGD:** 6-phosphogluconate dehydrogenase; **MPI:** mannose phosphate isomerase.

as *Leishmania* (*Viannia*) *panamensis* or *L.* (*V.*) *guyanensis* through the dissociation profile of the MPI amplification products (**Figure 2B**). Using FRET real-time PCR, we were able to confirm *L.* (*V.*) *braziliensis* infection in 11 out of 18 samples, corroborating the results of HaeIII digestion of kDNA-amplified products (PCR-RFLP- Restriction Fragment Length Polymorphism). It also allowed the detection of *L. (V.) guyanensis* in one sample that did not exhibit a restriction profile congruent with *L.* (*V.*) *braziliensis* in the PCR-RFLP.

Infection with *L. (V.) braziliensis* in 61.1% of the patients suggests that this species is the main etiological agent responsible for CL cases in the municipality of Brasiléia, which agrees with previous findings in Rio Branco and other areas in Bolivia and Peru, bordering the State of Acre[6-8,13]. This result was expected, considering that *L.* (*V.*) *braziliensis* is recognized as the predominant species associated with the evolution to the mucosal form of CL in Latin America[6]. Herein, it is important to note that an imported case of *L. (V.) braziliensis* was identified in a Brazilian individual who became infected in Cobija, Pando district, a border City of Bolivia (**Figure 1**). In spite of the proximity between Cobija and Brasiléia, it is worth mentioning that the construction of the Pacific Highway BR317 promoted an increase in migration across the Brazil/Peru/Bolivia borders. Together with the growth of tourism, this may have had a role in the dissemination of CL in the region[8].

We were able to identify *L. (V.) guyanensis* as the parasite responsible for the infection in a female 13 years old patient who resided in the *Guanabara extractive reserve,* kilometer 67). All *Leishmania* species implicated in the different clinical forms of CL in Brazil have been registered in the Amazon Basin[2]. *L. (V.) guyanensis* has been well characterized in Acre, Amapá, Roraima and Pará[14], and its main vector is *Nyssomyia umbratilis*, a sandfly recently observed at low density in Acre State[8]. In Peru, *L.* (*V.*) *guyanensis* is the third most common species associated with CL cases, following *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana*[15].

In 6 samples positive for the presence of *Leishmania* kDNA, identification of the parasite species by both methods was unsuccessful, raising the possibility that other *L.* (*Viannia*) species could be circulating in Brasiléia, as observed in other regions of the Brazilian Amazon[6,7]. The HaeIII/kDNA restriction profile was inconclusive, and the samples were not amplified following the 6PGD/MPI real-time PCR analysis. A possible explanation could be the differences in copy number when comparing *Leishmania* kDNA minicircles and the 6PGD/MPI loci. The first are represented as thousands of copies per parasite mitochondrial genome, whereas the MPI and 6PGD are present as single copy genes, which could explain the higher sensitivity of kDNA-PCR assay over the approach used in FRET real-time PCR[11]. Further studies are necessary to improve the molecular identification of other *Leishmania* spp. implicated in the epidemiology of CL in the State of Acre.

In conclusion, in this study, most of the individuals with clinically suspected CL live in rural areas (81.8%), suggesting that these local cases are associated with activities in a forest environment, despite the finding of three individuals infected with *L.* (*Viannia*) living in urban areas in the center of Brasiléia. The positivity for *Leishmania* kDNA was higher in women (91.7%) than men, and the ages of infected individuals varied from 3 to 30 years old (**Table 1**).

The study performed in the municipality of Brasiléia revealed the predominance of *L. (V.) braziliensis* in cutaneous lesions of individuals with clinically suspected leishmaniasis, and that cutaneous disease is probably associated with rural lifestyle, particularly with the activities in rubber extraction reserve sites. From an epidemiological aspect, there are a few studies carried-out in Acre, and this study is the first conducted in Brasiléia district despite the small number of individuals studied. Further investigation is needed, taking into account the vectors, reservoirs, the incidence of cutaneous disease, and its respective etiological agents, as well as surveillance activities, and the control of leishmaniasis in the State of Acre.

Ethical considerations

The study was approved by the Ethical Committee of the Oswaldo Cruz [*Instituto Oswaldo Cruz* (IOC)], Oswaldo Cruz Foundation [*Fundação Oswaldo Cruz* (Fiocruz)], Rio de Janeiro (RJ), Brazil; protocol CAE02765312.7.0000.5248.

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Conflict of interest

The authors declare that there is no conflict of interest.

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REFERENCES

1. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. Lancet. 2005;366(9496):1561-77.
2. Lainson R. The Neotropical *Leishmania* species: a brief historical review of their discovery, ecology and taxonomy. Rev Pan-Amaz Saude. 2010;1(2):13-32.
3. World Health Organization (WHO). World Health Statistics 2015. Global Health Observatory (GHO) data**.** Available from: http://www.who.int/gho/publications/world_health_statistics/2015/en/
4. Ministério da Saúde (MS). Sistema de Informação de Agravos de Notificação (SINAN). Brasilia: MS; 2015. Atualizado em 21 ago

2015; citado em 15 mai 2016. Disponível em: http://tabnet.datasus.gov.br/cgi/deftohtm.exe?sinannet/cnv/ltaac.def

5. da Silva-Nunes M, Cavasini CE, Silva NS, Galati, EAB. Epidemiologia da leishmaniose tegumentar e descrição das populações de flebotomíneos no município de Acrelândia, Acre, Brasil. Rev Bras de Epidemiol. 2008;11(2):241-51.
6. Tojal da Silva AC, Cupolillo E, Volpini AC, Almeida R, Romero GA. Species diversity causing human cutaneous leishmaniasis in Rio Branco, state of Acre, Brazil. Trop Med Int Health. 2006;11(9): 1388-98.
7. Teles CBG, Medeiros JF, Santos APA, Freitas LAR, Katsuragawa TH, Cantanhêde LM, et al. Molecular characterization of American cutaneous leishmaniasis in the tri-border area of Assis Brasil, Acre State, Brazil. Rev Inst Med Trop Sao Paulo. 2015;57(4):343-7.
8. Teles CBG, dos Santos APA, Freitas RA., Oliveira AFJ, Ogawa GM, Rodrigues MS, et al. Phlebotomine sandfly (Diptera: Psychodidae) diversity and their *Leishmania* DNA in a hot spot of American Cutaneous Leishmaniasis human cases along the Brazilian border with Peru and Bolivia. Mem Inst Oswaldo Cruz. 2016;111(7):423-32.
9. Marques MJ, Volpini AC, Genaro O, Mayrink W, Romanha AJ. Simple form of clinical sample preservation and *Leishmania* DNA extraction from human lesions for diagnosis of American cutaneous leishmaniasis via polymerase chain reaction. Am J Trop Med Hyg. 2001;65(6):902-6.
10. Pirmez C, Trajano VS, Paes-Oliveira Neto M, da-Cruz AM, Gonçalves-da-Costa SC, Catanho M, et al. Use of PCR in diagnosis of human american tegumentary leishmaniasis in Rio de Janeiro, Brazil. J Clin Microbiol. 1999;37(6):1819-23.
11. Tsukayama P, Nunez JH, De Los Santos M, Soberon V, Lucas CM, Matlashewski G, et al. A FRET-based real-time PCR assay to identify the main causal agents of new world tegumentary leishmaniasis. PLoS Negl Trop Dis. 2013;7(1):e1956.
12. Murback NDN, Hans-Filho G, Nascimento RAF, Nakazato KRO, Dorval MEMC. American cutaneous leishmaniasis: clinical, epidemiological and laboratory studies conducted at a university teaching hospital in Campo Grande, Mato Grosso do Sul, Brazil. An Bras Dermatol. 2011;86(1):55-63.
13. Garcia AL, Tellez T, Parrado R, Rojas E, Bermudez H, Dujardin JC. Epidemiological monitoring of American tegumentary leishmaniasis: molecular characterization of a peridomestic transmission cycle in the Amazonian lowlands of Bolivia. Trans R Soc Trop Med Hyg. 2007;101:1208-13.
14. Guerra JA, Prestes SR, Silveira H, Coelho LI, Gama P, Moura A, et al. Mucosal leishmaniasis caused by *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *guyanensis* in the Brazilian Amazon. PLOS Negl Trop Dis. 2011;5(3):e980.
15. Lucas CM, Franke ED, Cachay MI, Tejada A, Cruz ME, Kreutzer RD, et al. Geographic distribution and clinical description of leishmaniasis cases in Peru. Am J Trop Med Hyg. 1998;59(2):312-7.