RESEARCH ARTICLE

Leishmania DNA detection and species characterization within phlebotomines (Diptera: Psychodidae) from a peridomicile-forest gradient in an Amazonian/Guianan bordering area

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Abstract

In the border region between Brazil and French Guiana, American cutaneous leishmaniasis is a worrisome public health issue, and entomological studies are required there to better identify classical and putative emerging transmission patterns. The present study aimed to detect and characterize Leishmania DNA in the phlebotomine population of Oiapoque (Amapa State, Brazil). Phlebotomines were captured in anthropized and wild environments in the outskirts of Oiapoque municipality, using CDC light traps installed in vertical (ground/canopy level) and horizontal (peridomicile/extradomicile/forest-edge/forest) strata. Captured specimens were identified according to their morphology. Females were processed for Leishmania DNA detection and characterization using a multiplex polymerase chain reaction targeting kinetoplast DNA (kDNA) and the phlebotomine cacophony gene. The kDNA positive samples were characterized by cloning and sequencing the Leishmania 234 bp-hsp70 gene. Among the 3957 phlebotomine specimens captured, 26 pooled female samples were positive for Leishmania (Viannia) spp. DNA. Sequencing analysis allowed species-specific identification of L. (V.) braziliensis DNA in Trichophoromyia inini, Bichromomyia flaviscutellata, Nyssomyia umbratilis, and Evandro- myia infraspinosa, and L. (V.) guyanensis DNA in Ny. umbratilis. A pooled sample of Ny. umbratilis was positive for both L. (V.) braziliensis and L. (V.) guyanensis DNA. The present study provided additional information regarding ACL ecology in Oiapoque, highlighting the presence of L. (V.) braziliensis DNA in different phlebotomine species. The epidemiological implications of these findings and the determinant incrimination of L.
(V.) braziliensis as proven vectors in that region must be clarified. In this regard, studies on Leishmania spp. infection and suggestive anthropophilic behavior of associated phlebotomines need to be prioritized in entomological surveillance.

Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are insects of great medical importance owing to their capacity to transmit disease agents, such as Leishmania (Kinetoplastida: Trypanosomatidae) parasites, the causative agents of American cutaneous leishmaniasis (ACL)[1–3]. In nature, ACL causative agents are maintained in a complex series of transmission cycles involving a variety of vectors and reservoirs [4]. The impact of natural, ecological, or man-made pressures can further complicate these relationships and lead to the formation of new transmission cycles [5,6].

ACL is endemic to the Amazonian/Guianan region, where it is caused by five parasite species, namely: Leishmania (Viannia) guyanensis, L. (V.) braziliensis, L. (Leishmania) amazonensis, L. (V.) lainsoni, and L. (V.) naiffi [7]. Although L. (V.) guyanensis is considered to be the most important causative agent, potential emerging patterns of infection are worth investigation. For example, in the Oiapock basin, a natural border between the Brazilian state of Amapá and the Ultramarine Department of French Guiana, L. (V.) braziliensis is considered an emerging cause of ACL; reported to be linked to forest encroachment associated with the gold-mining industry [8]. Epidemiological data on the clinical profile of ACL patients in the Brazilian region revealed sporadic mucosal commitment presumed to be caused by this parasite species, drawing attention to a worrisome public health issue [9].

We recently studied ACL ecology in this region, providing comprehensive insights based exclusively on the investigation of the wild environment [6]. However, the lack of data regarding ACL in anthropized environments made us seek further information regarding horizontal stratification of the phlebotomine fauna associated with human dwellings. Therefore, present study aimed to fill some gaps about the eco-epidemiological knowledge of that bordering area, supplying our previous "dissection-based" data on natural infection with those of Leishmania DNA detection.

Materials and methods

Study area

The municipality of Oiapoque (03°49’29"N, 51°49’05"W) is the most important Brazilian socioeconomic link with the bordering Ultramarine Department of French Guiana. ACL is endemic in this region and is regarded as a "bi-national" issue, as approximately half of cases appear to be acquired on the Brazilian side, and half on the French Guianan side [9]. Two environments were selected to be surveyed, as follows:

1. Wild environment: Vila Vitória Road (03° 51’ 28.1” N, 51° 48’ 41.3” W), as described in our previous survey [6], a recently opened road that provides eastern access from Oiapoque to Vila Vitória. The forested area shows minimal evidence of human activity and is therefore considered to be well preserved.

2. Anthropized environment: Vila Vitória settlement (3°52’50.8”N, 51°47’35.7”W), a rural settlement surrounded by forest in the Brazilian border region of the Oiapoque River and approximately 6 km from the urban area of Oiapoque. Here, a randomly selected domicile
within an environment with known ACL cases was surveyed, taking into consideration to be at the pattern distance (200 to 500m) of the forested area, which is a buffer radius assumed for the preventive measures for ACL transmission by *L. (V.) guyanensis*, attributed to the apparently limited flight range of the vector *Nysomyia umbratilis*[10–13].

The Fig 1 shows the study area, located in Guianan Ecoregion Complex, South America (A), in the border area between Brazil and French Guiana (B), where in the outskirts of the Brazilian municipality of Oiapoque it can be observed two different environments, wild (D) and anthropized (E), supposed to be ACL foci.

**Sampling**

Site I was sampled using four CDC light traps installed approximately 100 m in-forest. Two were placed 20 m apart, 1.5m above ground (ground level) and two 20m apart, 20 m above ground (tree canopy level). Captures were performed from November 2016 to October 2017, four nights per month, totalizing 1152 h of sampling (12 h of operation for each CDC x two CDCs per site x four nights x 12 months) for each site.

Site II was sampled using four CDC light traps installed 1.5m above ground in a horizontal transect (from the direction of residence to forest), with the following vertical strata: peridomicile, extradomicile, forest-edge, and forest environments. Captures were performed from February to October 2016, four nights per month, totalizing 432 h of sampling (12 h of operation for each CDC x four nights per month x nine months) for each site.

Phlebotomines were immediately processed in the field laboratory and stored in 70% ethanol. They were identified using morphological characteristics in fresh conditions or processed for mounting on glass slides using Berlese fluid, according to Ryan [14]. Mounting of females was performed using only the last abdominal segments and head, as the thorax and abdomen...
were required for *Leishmania* DNA detection and characterization. Taxonomic criteria and nomenclature were adopted following those outlined by Galati et al. [15] and Galati [16].

**Leishmania** DNA detection and characterization

Procedures for DNA detection and characterization of *Leishmania* species from non-blood-fed phlebotomine females (stored in 70% ethanol) were based on the methodology proposed by Pita-Pereira et al. [17] and Araújo-Pereira et al. [18].

First, a total extract of the macerates of phlebotomine females of the same species without visible blood meal, individually processed or grouped in pools up to 20 specimens, and phlebotomine males (negative controls) were processed for DNA extraction. DNA was extracted using the commercial Wizard SV Genomic DNA Purification System kit (Promega, Madison, USA) according to the manufacturer’s specifications. Hot-start multiplex PCR was performed using two pairs of oligonucleotides as follows: The first pair targets a 120bp fragment of the constant region of the kinetoplast DNA (kDNA) mini-circle [oligonucleotide A (5’-GGC CCA TAC ACC AAC CCC-3’) and oligonucleotide B (5’-GGG GTA GGG GCG TTC TGC GAA-3’)] [19]. The second pair targets the IVS6 region of the phlebotomine cacophony gene [5Lccac (5’-GTG GCC GAA CAT AAT GTT AG-3’) and 3Llcac (5’-CCA CGA ACA AGT TCA ACA TC-3’)] [20]. The inclusion of the second pair of oligonucleotides confers reliability of DNA extraction from phlebotomine samples. The amplified products were resolved using 2% agarose gel electrophoresis and visualized by staining with Nancy-520 (Merck).

To optimize detection sensitivity, the amplification products were additionally subjected to dot-blot hybridization using a biotinylated probe specific for *Leishmania* (*Viannia*) (5’-TAA TTG TGC ACG GGG AGG CCA-3’) [21]. The hybridization reaction was developed using Luminol reagent (Santa Cruz Biotechnology, CA, USA). Positive controls consisted of a pooled sample of experimentally infected *Lutzomyia longipalpis* females, after 72h of feeding on rabbit blood containing 2 × 10⁵ *L. braziliensis* parasites/mL.

The DNA recovered from each *Leishmania*-positive sample was subjected to a second, semi-nested-PCR assay targeting the *hsp70* gene. This gene region validated for distinguishing different species of *Leishmania* present in Brazil, with broad coverage of the *L. (Viannia)* subgenus. In the first PCR step, a 234 bp fragment of *hsp70* was amplified using the oligonucleotides 5’-GGA CGA GAT CGA GCG CAT GGT-3’ and 5’-TCC TTC GAC GCC TCC TGG TTG-3’ [22]. In the second step, the same forward oligonucleotide is paired with the following reverse oligonucleotide: 5’-GGA GAA CTA CGC GTA CTC GAT GAA G-3’ [23] to amplify a 144 bp internal region of the 234bp fragment. The amplified fragments were purified and cloned into competent *Escherichia coli* DH5α cells using the pGEM T-Easy Vector kit vector (Promega), according to the manufacturer’s recommendations.

Sanger sequencing was performed with the RPT01A-PDTIS, Fiocruz-RJ sequencing platform (ABI 3730XL Applied Biosystem) [24], using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA, USA). The electropherograms were initially analyzed using the Phred program [25], and regions with good sequence resolution were submitted for assembly using the CAP3 program [26], Vector sequence removal was performed using the NCBI VecScreen program (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Sequences were compared to those available in the BLASTnucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN algorithm.

**Data analysis**

The Shannon-Wiener (H) diversity index was estimated and evaluated with a *t*-test to compare diversities between the different sites sampled with equal effort (i.e.: wild environment: 1,152 h
for each vertical stratum; anthropized environment: 432 h for each horizontal stratum), using the Past software version 3.22 (Øyvind Hammer, Oslo, Norway).[27]. Significance level was set at 5%.

Environmental issues
Capturing and processing invertebrate fauna (phlebotomines) were authorized by the Sistema de Autorização e Informação em Biodiversidade—SISBIO (Biodiversity Authorization and Information System), under protocol No. 44524.

Results
A total of 3957 phlebotomine specimens were captured, 1189 from the anthropized environment and 2768 from the wild environment. Thirty-one species were identified, including *Evandromyia infraspinosa*, *Nyssomyia umbratilis*, and *Trichophoromyia ininii* which were the most frequent, accounting for 2837 (71.7%) of the total number captured. Greatest diversity was observed, for the wild environment, in the ground level ($H = 1.907$) and, for the anthropized environment, in the peridomicile ($H = 1.809$) (Table 1). When comparing diversities within the anthropized environment, all horizontal strata, with exception of extradomicile x forest edge, were significantly different. Within the wild environment, both vertical strata (ground and canopy level) have diversity indexes significantly different (Fig 2).

Phlebotomine DNA extraction was considered successful in 551 samples that amplified the cacophony gene; and thus appropriate for *Leishmania* spp. DNA detection. Engorged phlebotomines were excluded from the infection assays due to the risk of detecting a non-established infection in flies that had recently consumed blood from an infected source.

Twenty-six samples were positive for *Leishmania* (*Viannia*) spp. DNA. In the anthropized environment, *Leishmania* (*Viannia*) spp. DNA was detected in forest-edge samples of *Psychozoon squamiventris maripaensis* (n = 1) and *Bichromomyia flaviscutellata* (n = 3), and in forest samples of *N. umbratilis* (n = 1) and *Th. ininii* (n = 2). In the wild environment, *Leishmania* (*Viannia*) spp. DNA was detected in *B. flaviscutellata* (n = 1) and *E. infraspinosa* (n = 2) caught at ground level, in *N. umbratilis* from ground (n = 4) and canopy (n = 9) levels, and in *P. s. maripaensis* from ground (n = 1) and canopy (n = 2) levels.

The minimum rate of DNA detection (number of positive samples / total samples tested x 100) was calculated for each the five species captured and positive for *Leishmania* spp. DNA: *P. s. maripaensis* (18.1%), *N. umbratilis* (5.8%), *B. flaviscutellata* (7%), *E. infraspinosa* (2.8%) and *T. ininii* (1.2%). *Leishmania* species identification was successful in 8 of the 26 positive samples, with coverage varying from 80–97% and identity from 88–100%. It was not possible to characterize the others due to cloning failures resulting from low DNA levels or incompatibility with the available sequences in the database.

Sequencing analyses are summarized in Table 2. BLAST analysis with sequences available from GenBank revealed the presence of *L. (V.) braziliensis* DNA in one *T. ininii* (10 specimens) sample from the forest-edge, one of *B. flaviscutellata* (6 specimens) from the forest, two of *N. umbratilis* (10 specimens, each) from canopy level, and two of *E. infraspinosa* (10 specimens, each) from ground level. Two samples of *N. umbratilis* (10 specimens each) from the canopy level were positive for *Leishmania* DNA, one was identified as *L. (V.) guyanensis* and the other contained DNA from both *L. (V.) braziliensis* and *L. (V.) guyanensis*.

Discussion
In Brazil, ACL presents three characteristic epidemiological patterns: 1) Sylvatic, where transmission occurs mainly in primary forest environments; considered a zoonosis of wild animals
with occasional human cases in areas of recent colonization due to the penetration of the man into the wild. 2) Occupational/recreational, associated with disordered forest exploration and with the clearing of forests for the construction of roads, hydroelectric power plants, village settlements, timber extraction, agricultural and military training activities, and ecotourism. 3) Rural and peri-urban in colonization areas, related to the migratory process, occupation of slopes and clusters in urban centers associated with secondary or residual forests [13]. Of

Table 1. Phlebotomine species composition and vertical/horizontal stratification in anthropized and wild environments of Oiapoque, Amapá, Brazil, bordering French Guiana.

| Species                                    | Environment          | Total | %  |
|--------------------------------------------|----------------------|-------|----|
|                                            | I       | II    | III  | IV   | V     | VI    |     |
| Evandromyia infraspinosa                   | 4       | 2     | 31   | 397  | 716   | 44    | 1194 | 30.2 |
| Nyssomyia unbratilis                       | -       | 2     | 7    | 15   | 203   | 899   | 1126 | 28.5 |
| Trichophoromyia innii                      | 5       | 27    | 249  | 141  | 90    | 5     | 517  | 13.1 |
| Psychodopygus ayrozai                      | -       | -     | -    | 1    | 133   | 148   | 282  | 7.1  |
| Trichopygomyia trichopyga                  | -       | 1     | 34   | 136  | 32    | 17    | 220  | 5.6  |
| Bichromomyia flaviscattellata              | 3       | -     | 1    | 40   | 109   | 7     | 160  | 4.0  |
| Scipoenyia sordelli                        | 3       | -     | 9    | 30   | 76    | -     | 118  | 3.0  |
| Psychodopygus squamiventris maripaensis    | -       | -     | 2    | 8    | 18    | 53    | 81   | 2.0  |
| Vianamyia furcata                          | 2       | 1     | -    | 5    | 18    | 12    | 38   | 1.0  |
| Micropygomyia rotaeensis                  | -       | -     | 1    | 1    | 18    | 6     | 26   | 0.7  |
| Evandromyia sp. of Baduel                  | 1       | -     | -    | -    | 13    | 10    | 24   | 0.6  |
| Scipoenyia fluviatilis                     | 1       | -     | 2    | 1    | 17    | 3     | 24   | 0.6  |
| Psychodopygus clausrei                     | -       | -     | -    | -    | 17    | 5     | 22   | 0.6  |
| Evandromyia williamsi                      | -       | -     | -    | 6    | 12    | 1     | 19   | 0.5  |
| Evandromyia monstroosa                     | -       | -     | -    | 6    | 9     | 1     | 16   | 0.4  |
| Psychodopygus davisi                       | -       | -     | -    | 2    | 3     | 8     | 13   | 0.3  |
| Psychododamy parapygus genicalatus         | -       | -     | -    | -    | 4     | 7     | 11   | 0.3  |
| Psathyromyia aragaoi                       | -       | -     | 1    | -    | 3     | 7     | 11   | 0.3  |
| Vianamyia tuberculata                      | -       | -     | 1    | 5    | 2     | 3     | 11   | 0.3  |
| Psychodopygus paraensis                    | -       | -     | -    | 1    | 1     | 6     | 8    | 0.2  |
| Psychodopygus hirsutus                     | -       | -     | -    | -    | 5     | 2     | 7    | 0.2  |
| Psathyromyia bigeniculata                  | -       | -     | -    | -    | 3     | 2     | 5    | 0.1  |
| Nyssomyia anduzei                         | -       | -     | -    | -    | -     | 5     | 5    | 0.1  |
| Nyssomyia pajoti                           | -       | -     | 2    | -    | 1     | 1     | 4    | 0.1  |
| Psathyromyia dendrophyla                   | -       | -     | -    | -    | -     | 3     | 3    | 0.1  |
| Psathyromyia inflata                       | -       | -     | 1    | -    | 2     | -     | 3    | 0.1  |
| Brumptomyia pintoi                         | -       | -     | -    | -    | 1     | 1     | 2    | 0.1  |
| Migonemyia migonei                         | -       | -     | -    | -    | -     | 2     | 2    | 0.1  |
| Pressatia choti                            | -       | -     | -    | -    | -     | 2     | 2    | 0.1  |
| Micropygomyia (Pilosa Series)              | -       | -     | -    | 1    | 1     | -     | 2    | 0.1  |
| Psathyromyia abonnenci                     | -       | -     | -    | -    | 1     | 1     | 1    | 0.0  |

Sampling effort on the anthropized environment: 432 h for each stratum; Sampling effort on the wild environment: 1152 h for each stratum; I: peridomicile; II: extradomicile; III: forest edge; IV: forest; V: ground level; VI: canopy level; (*) p< 0.05.

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these, the two former patterns are well recognized in the Amazon region, and support the hypothesis to be occurring in the studied area.

As hypothesized, the phlebotomine species composition observed in this study were highly similar to that recently presented in our previous study [6]. Therefore, to avoid redundancy, the present discussion here is focused on the results of horizontal stratification and *Leishmania* DNA detection within the phlebotomine population.

In the wild environment, diversity was significantly higher in the ground (*H* = 1.907; *p* < 0.05), where CDCs are usually set spatially congruent with the flight level of majority of phlebotomine species. On the other hand, canopy stratum comprise a ecological subsystem with fewer number of species with close relationship with arboreal vertebrates, such as occur with *N. umbratilis* and sloths and birds [6]. In the anthropized environment, differences on the diversity indexes were statistically significant when comparing between each stratum, with exception of extradomicile x forest edge. Similar environmental pressure and ecological niche between these sampling points may explain this apparent similarity. Greatest diversity found in the peridomicile (*H* = 1.809; *p* < 0.05) can be attributed to the search for shelter and offer of blood of domestic animals [13, 19].

Eight phlebotomine species were found in the extra-forest environments (peridomicile/extradomicile strata). Among these, it is important to highlight *B. flaviscutellata*, this species can be found in primary and secondary forests [28], and may be progressively adapting to environments modified by man [29,30]. Several studies have demonstrated the potential of peridomiciarization of this species in the Amazonian region [31–34]; however, the peridomiciary populations were usually small, compared to those in the forest stratum. The higher frequency of *Bi. flaviscutellata* in a forested environment, compared to the peridomiciary ecotope is consistent with the findings of other studies [30, 35–37]. Therefore, there is no concrete evidence herethat this species has established peridomiciary colonization. This assertion is further supported by a study conducted within the Bragança region of Pará, where pregnant *Bi. flaviscutellata* females were found only in the forest environment [38].

Molecular tools have been gradually replacing the traditional technique of phlebotomine dissection and provide several advantages, particularly with respect to sensitivity and specificity [39] allowing the detection of a single parasite [17]. However, it cannot be denied that...
Table 2. Samples of phlebotomines PCR-multiplex-positive for the cacophonous gene (Phlebotominae) and for the kDNA gene (*Leishmania* spp.) with descriptions of the environment where they were captured, number of specimens analyzed and sequencing results.

| IOC code       | Phlebotomine species                  | Environment         | N | *Leishmania* species (hsp70) | Blast analysis |
|----------------|--------------------------------------|---------------------|---|-----------------------------|---------------|
|                |                                       | Anthropized | Wild |                            |               |
| 1              | 5/16.4.mar. D26 Psychodopygus squamiventeris maripaiensis | forest       |     | 6                           | NI            |
| 2              | 5/16.4.fla. D27 Bichromomyia flaviscutellata | forest       |     | 10                          | NI            |
| 3              | 5/16.4.fla. D28 Bichromomyia flaviscutellata | forest       |     | 5                           | NI            |
| 4              | 5/16.co. umb.D30 Nyssomyia umbratilis | -            | CDC canopy | 10                          | NI            |
| 5              | 5/16.co. umb.D33 Nyssomyia umbratilis | -            | CDC ground | 10                          | NI            |
| 6              | 5/16.so. umb.D35 Nyssomyia umbratilis | -            | CDC ground | 3                           | NI            |
| 7              | 6/16.3.umb. D37 Nyssomyia umbratilis | border      |     | 5                           | NI            |
| 8              | 6/16.3.ini. D38 Trichophoromyia ininitii | border      |     | 10                          | L. (V.) braziliensis 96 98 GU368187.1 |
| 9              | 6/16.3.ini. D39 Trichophoromyia ininitii | border      |     | 10                          | L. (V.) braziliensis 91 89 XM_001566273 |
| 10             | 6/16.4.fla. D41 Bichromomyia flaviscutellata | forest       |     | 6                           | L. (V.) braziliensis 94 100 GU368187.1 |
| 11             | 6/16.co. umb.D46 Nyssomyia umbratilis | -            | CDC canopy | 10                          | NI            |
| 12             | 8/16.co.mar. D53 Psychodopygus squamiventeris maripaiensis | -            | CDC canopy | 2                           | NI            |
| 13             | 8/16.co. umb.D54 Nyssomyia umbratilis | -            | CDC canopy | 10                          | L. (V.) braziliensis 96 89 XM_001566273 |
| 14             | 8/16.co. umb.D55 Nyssomyia umbratilis | -            | CDC canopy | 10                          | L. (V.) braziliensis 91 89 XM_001566273 |
| 15             | 8/16.co. umb.D56 Nyssomyia umbratilis | -            | CDC canopy | 10                          | L. (V.) guyanensis/ L. (V.) braziliensis 89/97 94/99 H586362.1/ XM_001566273 |
| 16             | 8/16.co. umb.D57 Nyssomyia umbratilis | -            | CDC canopy | 10                          | L. (V.) guyanensis 97 100 H586362.1 |
| 17             | 8/16.co. umb.D58 Nyssomyia umbratilis | -            | CDC canopy | 10                          | L. (V.) guyanensis 97 100 H586362.1 |
| 18             | 9/16.co. umb.D60 Nyssomyia umbratilis | -            | CDC canopy | 10                          | L. (V.) guyanensis 97 100 H586362.1 |
| 19             | 9/16.co. umb.D61 Nyssomyia umbratilis | -            | CDC canopy | 10                          | L. (V.) guyanensis 97 100 H586362.1 |
| 20             | 9/16.so.mar. D62 Psychodopygus squamiventeris maripaiensis | -            | CDC ground | 1                           | NI            |
| 21             | 9/16.so.fla. D63 Bichromomyia flaviscutellata | -            | CDC ground | 4                           | NI            |
| 22             | 9/16.so. umb.D64 Nyssomyia umbratilis | -            | CDC ground | 10                          | NI            |
| 23             | 9/16.so. umb.D65 Nyssomyia umbratilis | -            | CDC ground | 10                          | NI            |
| 24             | 1/17.co.mar. D69 Psychodopygus squamiventeris maripaiensis | -            | CDC canopy | 3                           | NI            |
| 25             | 1/2.so.inf. D74 Evandromyia infraspinosa | -            | CDC ground | 10                          | L. (V.) braziliensis 95 88 XM_001566273.2 |

(Continued)
parasite isolation significantly extends the range of investigations which can be performed. Therefore, it is suggested that, in entomological studies, DNA detection techniques and molecular characterization of *Leishmania* spp. be combined with dissection and attempted parasite isolation. Present results support our previous “dissection-based” information about *Leishmania* parasites in the Oiapoque environment [6].

The minimum detection rate observed by the molecular method (4.7%) was higher than that demonstrated in our early experience in Oiapoque using the dissection method (0.78%) [6], which is apparently attributed to the recognized high sensitivity of PCR and its variants [17,39]. However, we recognize that, in hands of experienced team and in females without a visible bloodmeal, the microscopical examination of dissected midguts is equally sensitive as Q-PCR, as has been proven elsewhere [40]. In addition, there are several other possible explanations to these differences of infection/detection rates, like the effect of season, micro-location of trapping sites, that should not be neglected in such comparisons.

It is important to note that these values do not necessarily reflect the risk of human infection; other factors should be considered in assessing the risk of exposure to ACL agents, such as the degree of anthropophilia of their potential vectors [3].

The cloning and sequencing stages are important because phlebotomines present a complex biological material and although kDNA detection provides high sensitivity, it does not allow identification to species level. Using this method, identification is possible at the level of genus and subgenus and this is a particular problem with the subgenus *Viannia*. Unfortunately, the best targets for genotyping, such as *Hsp70*, are not good detection targets due to low copy number. Therefore, effective detection and identification of *Leishmania* spp. from phlebotomines requires a primary detection step targeting kDNA, followed by a subsequent genotyping step using another molecular target, such as *hsp70*. Even so, the efficiency is reduced, due to the low amount of target DNA, and then we consider some identity values lower than 90%. Although we were unable to characterize 18 of the 26 positive samples, the sequence of eight pools showed compatible coverage and identity to contemplate characterization at species-specific level; providing important information in the context of Amazonian/Guianan ACL.

*L. (V.) guyanensis* DNA was detected in several samples of *N. umbratilis*, supporting the whole context that this natural vector x parasite relationship that has been discussed in the course of our past experience in Oiapoque [6] and based on the extensive literature from the Amazon region [3,4,10,41–45]

*L. (V.) braziliensis* DNA was detected in four different phlebotomine species: *T. intiitii, B. flaviscutellata, E. infraspinosa*, and *N. umbratilis*. In Brazil, *L. (V.) braziliensis* transmission is associated with the highest number of known vectors, where there are 17 phlebotomine species with proven or potential links to its transmission; seven of which are only associated by

| IOC code | Phlebotomine species | Environment | N | *Leishmania* species (*hsp70*) | Blast analysis |
|----------|----------------------|-------------|---|--------------------------------|---------------|
| Anthropized | Wild | | | | |
| 26 | 1/2.so.inf. | - | CDC ground | 10 | *L. (V.) braziliensis* | 80 | 89 | XM_001566273.2 |

*: duplicate sample, since it generated clones of two different species of *Leishmania*;

N: number of specimens in the sample, ranging from individual samples to pools of up to ten specimens; NI: not identified at species level by sequencing. Minimal detection rate for positive phlebotomine species: *Psychodopygussquamiventrismaripaensis* (18.1%), *Nyssomyiaumbratilis* (5.8%), *Bichromomyiaflaviscutellata* (7%), *Evandromyiainfraspinosa* (2.8%) and *Trichophoromyiaininii* (1.2%).

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molecular findings [4]. The wide geographical distribution and genetic diversity of L. (V.) braziliensis strains [46] may contribute, in part, to the large list of its vectors. Furthermore, these transmission cycles may be very specific, as each leishmanian ecotope is reported to be spatio-temporally unique [6].

Interestingly, L. (V.) braziliensis DNA has not been found previously in any of these four phlebotomine species. Conversely, B. flaviscutellata and N. umbratilis play well-recognized roles in the transmission of the respectively associated parasites, L. (L.) amazonensis and L. (V.) guyanensis [2,4]. Leishmania sp. DNA was detected in Trichophoromyia ininii, considered a low-anthropophilic phlebotomine species, in Sabajo Heuvels, Suriname [47]. E. infraspinosa had previously been found in Oiapoque carrying promastigotes that were subsequently characterized as L. (V.) guyanensis from the residual material present on the slide [6].

The detection of L. (V.) braziliensis in four different phlebotomine species suggests concomitant contact of these insects with potential reservoirs of the parasite. Considering that rodents are the most likely animals to be involved in maintaining the enzootic cycle of L. (V.) braziliensis [48–51], it is rationally inferential that these phlebotomines are feeding and ingesting parasitic forms in a competent source of infection within a compatible spatio-temporal context. The potential feeding habits of three, of these four species of sand fly, is a corroborating factor for this statement. Rodentophilia is undoubtedly well recognized for B. flaviscutellata [52] and N. umbratilis has been found alternatively feeding on blood from rodents in an environmentally-impacted area close to Manaus, Amazonian Brazil [53]. Additionally, E. infraspinosa, which infrequently bites man in the bordering region of Jari, between the Brazilian states of Pará and Amapá, was relatively frequent species in Disney-trapped rodent trap catches [10]. Furthermore, Dasyprocta leporina DNA was recently detected in E. infraspinosa in Saint Georges de l’Oyapock, Guiana [54]. Still in regard of E. infraspinosa, in our past experience during captures in area I, this species has drawn medical attention for having been observed biting professionals during Shannon trap captures in same circumstances of having been found naturally infected by L. (V.) guyanensis [6]. Conversely, other populations of this fly species may present distinct feeding behavior. For example, previous studies of anuran try-panosomatids carried by this phlebotomine species in the western Amazon led to the hypothesis that it feeds on cold-blooded animals in this ecotope [55]. These findings stress the need for further investigation of E. infraspinosa with respect to L. (V.) braziliensis-associated ACL transmission; particularly as these phlebotomine populations may also be permissive to other Leishmania spp. and have suggestive anthropophilic behavior.

One pooled sample of ten females of N. umbratilis was concomitantly positive for L. (V.) braziliensis and L. (V.) guyanensis DNA; however, it cannot be ascertained whether these two parasites were within the same phlebotomine or in different specimens of that pool. In this study and in the literature [53–56] it is convergent with the possibility of N. umbratilis feed on different animals that act as potential reservoirs of Leishmania spp.

Conclusions

The results of the present study provide additional information on ACL ecology in Oiapoque, highlighting the presence L. (V.) braziliensis DNA in different phlebotomine species captured in ecologically distinct environments and strata in the study area. It should be noted, however, that these findings alone are not sufficient to conclude that there is a transmission cycle involving these phlebotomines and L. (V.) braziliensis. First, absence of visible blood cannot be interpreted as the possible survival of the parasite. Second, and more importantly, studies focused to this topic demonstrated that parasite DNA is detectable few days after Leishmania are killed in the non-natural vectors [57,58], reinforcing that PCR-based findings, when analyzed in
isolation, are not enough for vector incrimination. Considering established natural infection, the epidemiological importance of these potential transmission cycles still require further evidence. Quantification of the parasitic load and/or demonstration of infective forms would certainly present a further step towards improving knowledge of the potential vectors of \textit{L. (V.) braziliensis} in this region. Nonetheless, the known capacity of these species to harbor \textit{Leishmania} spp. combined with suggestive anthropophilic behavior present a need for prioritized entomological surveillance.

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