Detection of Leishmania infantum DNA in Pintomyia evansi and Lutzomyia longipalpis in an endemic area of Non-ulcerative cutaneous leishmaniasis from Honduras

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DOI: https://doi.org/10.21203/rs.3.rs-36505/v1

Keywords: Leishmania infantum, Pintomyia (Pifanomyia) evansi, Visceral Leishmaniasis, Non-Ulcerative Cutaneous Leishmaniasis,
Abstract

Background The two most abundant sand flies species in Honduran Pacific coast are Lutzomyia (Lutzomyia) longipalpis and Pintomyia (Pintomyia) evansi. Both species are proved vectors of the agent of for visceral leishmaniasis in South America. Although, visceral and cutaneous non-ulcerated leishmaniasis are endemic of the Central American Pacific, being the Non-ulcerative cutaneous leishmaniasis the most frequent manifestation. In this study, we evaluate the circulation of Leishmania spp in sand flies species in the El Tigre Island, an endemic area of Visceral and Non-ulcerative cutaneous leishmaniasis in Honduras.

Results Six sand flies species were identified, being Lu. longipalpis (81%) and Pi. evansi (16%) the more abundant species. Leishmania (Leishmania) infantum DNA was found in 9 of the 96 specimens analyzed, 7 of these specimens was identify as Lutzomyia (Lutzomyia) longipalpis and 2 as Pintomyia (Pifanomyia) evansi, with an infection rate of 9.4% and 2.7% respectively.

Conclusion Our results present the first record of Leishmania (L.) infantum DNA in Pintomyia (Pintomyia) evansi in a Non-ulcerative cutaneous leishmaniasis endemic region from Central America. Considering the natural infection of Lu. longipalpis our results suggest that Pintomyia (Pifanomyia) evansi might be a secondary vector of Leishmania (L.) infantum and probably involved in the disease's transmission cycle. Undoubtedly, the detection of natural infections of in this region contributes to the understanding of the L. (L.) infantum infection epidemiology in Honduras.

Background

Leishmaniasis is a vector-borne disease caused by parasite species of the genus Leishmania (Trypanosomatidae: Trypanosomatidae). The geographic distribution occurs mainly in the tropical and subtropical regions, and in 98 countries throughout Europe, Africa, Asia, and America [1]. More than 1,000 sand fly species have been described worldwide, from which 530 species are present in the Americas [2, 3] and at least 30 species are considered as Leishmania vectors [4]. In Honduras, 29 sand fly species have been reported [2, 5–7]. Lutzomyia (Lutzomyia) longipalpis and Pintomyia (Pifanomyia) evansi are the most abundant species in the endemic area for Visceral Leishmaniasis (VL) and Non-ulcerative cutaneous leishmaniasis (NUCL) in the southern region of Honduras [6, 8]. However, Lutzomyia (Lutzomyia) longipalpis is the only specie reported as vector of Leishmania (L.) infantum [9]. Nevertheless, the role of Pintomyia evansi as vector of Leishmania (L.) infantum in Honduras is unknown, although evidences about it vector competence has been reported in South America [10]. For that reason, the aim of this study was to evaluate the DNA circulation of Leishmania (L.) infantum in Lutzomyia (Lutzomyia) longipalpis and Pintomyia (Pifanomyia) evansi by on a Mesoamerican Pacific island.

Methods
**Study area and sand flies collections**

The study was carried out in Amapala municipality (N13 15.618, W87 37.463), Valle department, with an area of 80.7 km$^2$. The municipality comprises two islands, Zacate Grande and El Tigre, located in the Gulf of Fonseca in the southern Honduras. Sand flies were sampled for five consecutive nights in May 2018 on five localities (Las Pelonas, Punta Honda, Tigüilotada, Islitas and Playa Grande). Samplings was carried out to 18:00 at 6:00 h, using automatic CDC miniature light traps (model 512; John W. Hock Co., Gainesville, FL, USA) in neighborhoods selected by the presence of active cases of Non-ulcerative or atypical cutaneous leishmaniosis (NUCL). The traps were installed in peri-domiciliary environment, mainly near of decomposing organic matter, or next to the latrines. The specimens were separated and processed the day after the capture.

**Taxonomic identification of Sand fly species**

The phlebotomine sand flies were mounted for morphological identification, following the procedures outlined by Mejía et al., 2018 [6]. The sand fly species were identified according to Young and Duncan, 1994 [5], and the genera and species classification is presented according Galati´s key, 2017 [11].

**Genomic material extraction and polymerase chain reaction (PCR)**

Genomic DNA was extracted only from the bowel dissection of individual female sand flies using Chelex® 100 (Bio-Rad Lab Inc., Hercules, California, USA). As an internal control of the extraction of DNA, the cacophony IVS6 gene present in the genome of sand flies was amplified [12]. For the detection of genus *Leishmania* we use the primers Leish1: 5′- AACTTTTCTCTGGTCCTCCGGGTAG-3′ and Leish2: 5′- ACCCCCATGCTCTGGTACATC 3′ in order to amplify a ≈120-base-pair fragment of the *Leishmania* kinetoplast DNA minicircle [13]. Amplifications were performed using a commercial kit (Master Mix 2X -Promega). Each reaction was performed by adding 4 µL of target DNA and 0.6 µmol/L of each primer in a final volume of 20 µL. The PCR reactions were done in a Applied Biosystem 2770 Thermal Cycler (ThermoFisher Scientific/USA), under the following conditions initial denaturation cycle at 94°C for 5 minutes, followed by 35 cycles alt 94°C for 15s, 60°C for 20s and 72°C for 60s, and final extension of 72°C for 10 min. The amplification products were analyzed by electrophoresis in a 1.5% agarose gel.

To characterize the *Leishmania* species, PCR-RFLP was performed which amplifies a specific region of the hsp70 gene [14]. Primers used were hsp70 sense (5 ′GACGCTGCCTGCTACTTCAA 3′) and hsp70 antisense (5 ′CCGCCCATGCTCTGGTACATC 3′). Reaction mixture was prepared in a final volume of 50 µL with 25 µL of Master Mix 2X (Promega), 5 µL of target DNA and 0.6 µmol/L of each primer. The PCR reactions were done under the following conditions initial denaturation at 94°C for 5 min, followed by 37 cycles at 94°C for 30 s, 61°C for 1 min and 72°C for 3 min, and a final extension cycle at 72°C for 10 min.
The amplification products were analyzed by electrophoresis in a 2% agarose gel. To perform the restriction of PCR products, the enzyme Hae III (Promega) was used, adding 5 µL of amplified DNA to the reaction and incubated at 37°C for 3 h. The profiles of each species were observed using a 2% agarose gel subjected to electrophoresis as described by Montalvo [15].

**Result And Discussion**

A total of 222 specimens were collected in, being predominant males (66%). The number of sand flies according sex and species is presented in Table 1. Six species were identified through morphological characters, with predominance of *Lutzomyia (Lutzomyia) longipalpis*, followed by *Pintomyia (Pifanomyia) evansi*. Other species captured were *Micropygomyia (Micropygomyia) cayennensis cayennensis*, *Micropygomyia (Coquillettimyia) chiapanensis*, *Dampfomyia (Coromyia) beltrani* and *Lutzomyia (Tricholateralis) gomezi*. *Lutzomyia (Lutzomyia) longipalpis* has been previously studied in the region and incriminated as the vector of *Leishmania (L.) infantum* [9]. Additionally, behavioral characteristics of this species in the study area were described by Carrasco et al. [7]. Recently, Mejía et al. [6] described aspects of the feeding preference of sand flies in the Pacific Honduran area. These authors observed the predominance of *Pintomyia (Pifanomyia) evansi* and *Lutzomyia (Lutzomyia) longipalpis*, but couldn’t determine the presence of *Leishmania (L.) infantum* in those species [6]. Similar results were described by Zeledón et al., (1984) and Raymond et al., (2010) who reported those species as the two most common species in areas endemic to NUCL in Costa Rica and Nicaragua, respectively.
Table 1
Sand flies species captured by locality and detected with *Leishmania* spp and *Leishmania (L.) infantum*.

| Locality (n) | Species | M | F | %  | Specimens detected with *Leishmania* spp DNA n (%) | Females detected with *Leishmania (L.) infantum* DNA n (%) |
|--------------|---------|---|---|----|-----------------------------------------------|--------------------------------------------------|
| Playa Grande (175) | *Lutzomyia (Lutzomyia)* longipalpis | 95 | 35 | 79.2 | 26 (35.13) | 7 (9.45) |
| | *Pintomyia (Pifanomyia) evansi* | 10 | 24 | 20.8 | 11 (14.8) | 2 (2.70) |
| Las Pelonas (16) | *Lutzomyia (Lutzomyia)* longipalpis | 12 | 2 | 87.5 | - | - |
| | *Micropygomyia (Micropygomyia) cayennensis* | 1 | 1 | 12.5 | - | - |
| Punta Honda (29) | *Lutzomyia (Lutzomyia)* longipalpis | 24 | 4 | 96.5 | - | - |
| | *Micropygomyia (Coquillettidomyia) chiapanensis* | 0 | 1 | 3.5 | - | - |
| Islitas (13) | *Lutzomyia (Lutzomyia)* longipalpis | 6 | 2 | 61.5 | - | - |
| | *Dampfomyia (Coromyia) beltrani* | 0 | 2 | 15.4 | - | - |
| Locality (n) | Species                      | M | F | %  | Specimens detected with Leishmania spp DNA n (%) | Females detected with Leishmania (L.) infantum DNA n (%) |
|-------------|------------------------------|---|---|----|-----------------------------------------------|--------------------------------------------------|
|             | *Pintomyia* (*Pifanomyia*) evansi | 0 | 1 | 7.7| -                                             | -                                                |
|             | *Micropygomyia* (*Micropygomyia*) cayennensis cayennensis | 0 | 1 | 7.7| -                                             | -                                                |
|             | *Lutzomyia* (*Tricholater alis*) gomezi | 0 | 1 | 7.7| -                                             | -                                                |

The PCR trial showed positive results to the *Leishmania* genus in 37 of 96 analyzed female specimens analyzed (Fig. 1). Nine samples showed presence of *Leishmania* (*L.*) *infantum* DNA, 7 from *Lutzomyia* (*Lutzomyia*) *longipalpis* and 2 from *Pintomyia* (*Pifanomyia*) *evansi*. The *Leishmania* (*L.*) *infantum* infection rate was 9.4% for *Lutzomyia* (*Lutzomyia*) *longipalpis* and 2.7% for *Pintomyia* (*Pifanomyia*) *evansi*. All the analyzed samples, threw an amplified product of 220 pb corresponding to a *Lutzomyia* spp. constitutive gene (cacophony), confirming the insect DNA preparation integrity and the absence of PCR inhibitors. Our results are the first report of the presences of *Leishmania* (*L.*) *infantum* DNA in *Pintomyia* (*Pifanomyia*) *evansi* females in Central America. In Colombia, the natural infection of *Leishmania* (*L.*) *infantum* in endemic areas of VL associated with *Pintomyia* (*Pifanomyia*) *evansi* has been recorded [10, 16] with an infection rate of 0.10 and 0.34%. Meanwhile in *Lu. longipalpis* the natural infection rates between 0.5 and 1.1%, when done with direct observation on intestine dissection [9, 17] or using intestine dissection followed by PCR [18–20]. We reported 9.4% infection of *Leishmania* (*L.*) *infantum* in *Lutzomyia* (*Lutzomyia*) *longipalpis*, which agree with other reports [19, 20]. Although the *Leishmania* DNA detection in sand flies not represent the ability of a specie to transmit the parasite, our results present evidence of contact between the specie *Pintomyia evansi* and natural host of *Leishmania* (*L.*) *infantum* in the study area. Considering that the vector competence of this specie was previously described (Travi et al. 1990). They could be involved with this parasite specie transmission in south Honduras.

**Conclusion**
Our results present the first finding of the presence of *Leishmania (Leishmania) infantum* DNA in *Pintomyia (Pifanomyia) evansi* by *Leishmania (L.) infantum* in a Non-ulcerative cutaneous leishmaniasis endemic region in Central America. Considering the natural infection of *Lu. longipalpis* our results suggests that *Pintomyia (Pifanomyia) evansi* might be a secondary vector of *Leishmania (L.) infantum* and probably involved in the disease's transmission cycle. Undoubtedly, the detection of natural infections in this region contributes to the understanding of the *Leishmania (L.) infantum* infection epidemiology in Honduras.

**Abbreviations**

PCR
Polymerase Chain Reaction
bp
Base pairs
RFLP
Restriction Fragment Length Polymorphism
VL
Visceral Leishmaniasis
NUCL
Non-ulcerative cutaneous leishmaniasis

**Declarations**

• **Ethics approval and consent to participate:**
  • Not applicable

• **Consent for publication:**
  • Not applicable

• **Competing interest:**
  • The authors declare that they have no competing interest.

• **Funding:**
  
  This study was financially supported by the Research Directorate of the National Autonomous University of Honduras (DICU-UNAH).
• Authors’ contributions:

Conceptualization: MDL, FG, WSO; methodology, WSO, FG, JV, YL, GVAF, CMSP, CZ, GS; formal analysis, WSO, FG, MDL; writing -original draft preparation WSO, FG, MDL.; All authors have read and agreed to the published version of the manuscript.

• Acknowledgments:

• We thank to Ministry of Health of Honduras for allowing and assisting the work in the endemic area and Jessica Cardenas for the perfect technical support.

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Figures

Figure 1

PCR to determine Leishmania spp. infection using primers Leish1 -Leish2 to target conserved DNA regions of the kinetoplast DNA from Leishmania spp. (120 pb). Line M: molecular weight marker (100 bp DNA ladder). Line 1- 16 female sand fly DNA (lanes 1, 2-10, Lutzomyia (Lutzomyia) longipalpis, positive female; line 11, 13-16, Pintomyia (Pifanomyia) evansi, positive female). Lane 17: PCR positive control (DNA extracted from a mixture of male insect pool containing Leishmania (L.) infantum DNA), Line 18: amplification reaction without added DNA (PCR negative control).