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FIRST DNA REPORT OF *LEISHMANIA INFANTUM* IN *EVANDROMYIA (COMPLEX) CORTELEZZII* AND *LUTZOMYIA LONGIPALPIS* IN ALTO PARANÁ, PARAGUAY

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ABSTRACT

Visceral leishmaniasis is one of the diseases with the greatest impact on public health. In Paraguay, it is transmitted mainly by *Lutzomyia longipalpis*, which is considered the main vector of *Leishmania infantum*. In this paper, we report for the first time the detection of *Leishmania spp.* DNA by real-time PCR in a specimen of *Evandromyia (complex) cortelezzii* in Alto Paraná-Paraguay. The PCR product was sequenced and showed 100% similarity with *Leishmania infantum*. *Lutzomyialongipalpis* is reported for the first time in the Alto Paraná department and 11 out 47 (23,4%) were positive for *Leishmania spp.* DNA. Besides it was identified for the first time *Mycropygomyia quinquefer* and *Brumptomyia cunhai* in this region and without natural infection. In areas where *Lutzomyia longipalpis* has not been reported or its abundance is very low, other alternative vectors may be involved in parasite transmission. Therefore, although the detection of *Leishmania* DNA does not incriminate these species as vectors, these results suggest a potential role of these and other phlebotominein the transmission of visceral leishmaniasis in the study area.

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INTRODUCTION

Visceral Leishmaniasis (VL) and Cutaneous Leishmaniasis (CL), are vector-borne neglected diseases transmitted by Phlebotomine (Psychodidae: Phlebotominae), both diseases are considered a public health problem since their mortality rate human cases are very significant (Maroli *et al*., 2012). The area of highest VLincidence is Asunción, the capital city, however, a number of cases have been identified in eastern Paraguay in the Alto Paraná department, located in the triple border area between Brazil and Argentina (Giménez-Ayala *et al.,* 2017). In Paraguay, cases of CL and mucocutaneous leishmaniasis (MCL) have decreased since 2004; however, Paraguay as well as Bolivia remain the countries with the highest number of cases of MCL (OPS, 2012). On the other hand, cases of VL increased in response to numerous canine reservoirsendemic to urban and peri-urban areas (Ministerio de Salud, 2011).

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Evandromyia cortelezzii has been incriminated as vector of CL due to its abundance in sporadic outbreaks and it was found naturally infected with *Leishmania braziliensis*in Minas Gerais, Brazil and in the Argentinian Chaco reinforcing the putative incrimination of the *cortelezzii* complex as vector of the CL disease (Carvalho *et al.,* 2008; Saraiva *et al.,* 2009, 2010; Rosa *et al.,* 2012). It has been described in border cities of Paraguay as Ponta Porá Brazil and Clorinda Argentina (de Andrade *et al.,* 2012, Gómez-Bravo *et al*., 2017).A total of 27 phlebotomine specieshave been identified in the country *(Torales et al., 2010)*. *Lutzomyia longipalpis* is a confirmed vector of VL (Torales *et al.,* 2004) and*Nyssomyia whitmani*that of CL(Hashigushi *et al.,* 1992). Other species such as *Nyssomyia neivai*, *Migonemyia migonei* and cortelezzii complex have been considered as potential vectors but have not been found naturally infected. However, these species were incriminated as vectors of CL in different provinces of Argentina (Quintana *et al*., 2012; Rangel and Lainson, 2009; Salomón *et al*., 2008). *Lu.longipalpis* is the most abundant vector related to canine and human cases infected with *L. infantum*in urban areas (Salomón *et al*., 2016). In fact, in the Alto Paraná department LV cases have been confirmed since

2008 (Giménez-Ayala *et al.,* 2017), however, *Lu. longipalpis* has not been identified before this study in this department where the species was highly abundant in urban areas. Other species of medical importance previously identified in the Alto Paraná department are: *Mg. migonei*, *Ny. neivai* and *Ny. Whitmani* (Inchaustti *et al*., 1990, Salomón *et al*., 2003, Torales *et al*., 2010). In order to detect infected phlebotomine species, to identify the role these vectors play in the transmission of the etiologic agent of VL and to elucidatethe dynamic transmission cycles that occur in urban-sylvatic spaces located in the border between Argentina and Brazil (Figure 1), a study at the Paraguayan side of this triple border was carried out and the presence of DNA of *L. infantum* was evaluated. In this context, it was possible to identify phlebotomine species with a potential role in the transmission cycles and their ability to maintain the local transmission (WHO, 2010). For this reason, we applied molecular techniques to identify *Leishmania spp*.

DNA in phlebotomine captured in two districts of the Alto Paraná department (Ciudad de Este and Presidente Franco) as activities of the IDRC #107577 research project (idrc.ca/en/project/addressing-emergence-and-spread-leishmaniasis-borders-argentina-brazil-and-paraguay).

MATERIALS AND METHODS

Phlebotomine were captured using REDILA traps (Fernández *et al*., 2015). Devices were located in peridomestic areas of randomly selected dwellings during three consecutive nights in the urban areas of Ciudad del Este and Presidente Franco districts of the Alto Paraná department, during different periods (October 2014, February, May and December, 2015 and May 2016). *In situ* taxonomical identification in the CIM Laboratory was carried out following the Galati (2003) keys. Females' identification was carried out by dissection of the abdominal segments and morphological analysis of the

Table 1. *Leishmania infantum* DNA detected according to phlebotomine species captured in the Alto Paraná department in Paraguay

Phlebotomine Species	DNA (+)	Percentage	DNA (-)	Total
Nyssomyia whitmani	0		38	38
Lutzomyia longipalpis	11	23,4%	36	47
Evandromyia cortelezzii	1	16,6%	5	6
Mycropygomyia quinquefer	0		4	4
Nyssomyia neivai	0		1	1
Brumptomyia cunhai	0		2	2
Total	12	12,2%	86	98

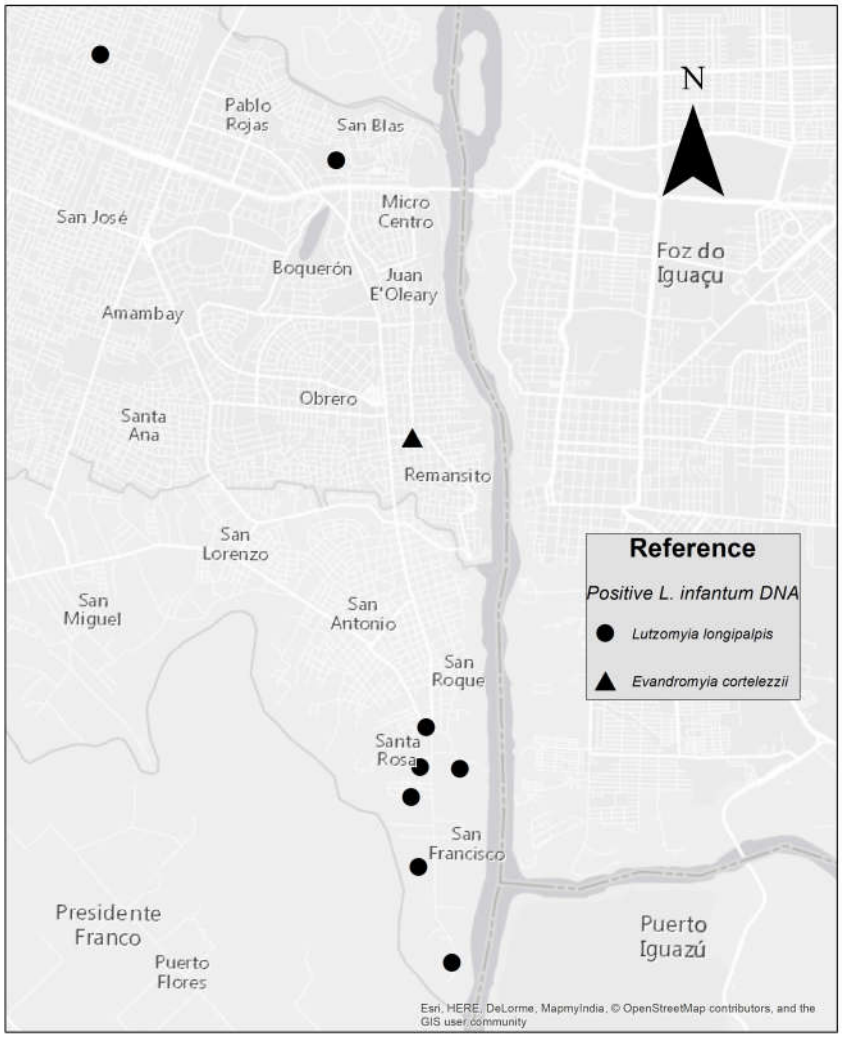

Figure 1. REDILA traps location with *Leishmania infantum* DNA positive phlebotomine species in Presidente Franco and Ciudad del Este districts, Alto Paraná in the Triple Border between Argentina, Brazil and Paraguay

spermathecae, while taxonomical keys identified males. Specimens were conserved individually in 1.5 milliliters microtubes and transported in dry ice to the CEDIC molecular lab, where they were conserved at -20°C until DNA extraction. Extraction and purification of genomic DNA was performed using the GeneJET Genomic DNA Purification Kit (# K0722 Thermo Scientific), following the manufacturer's instructions. The purity of the extracted genetic material was evaluated using a spectrophotometer (DeNovix DS-11FX +). For the detection of *Leishmania spp* DNA by real-time PCR the following primers that amplify the internal transcription space 1 of the ribosomal RNA (ITS-1) were used:

LSGITS1F.5'CATTTTCCGATGATTACAC 3 'and LSGITS1R: 5' CGTTATGTGAGCCGTTA 3 ' (de Almeida *et al* 2011, 2016). For the amplification reactions, the Maximum Mix Sybr Green qPCR (2X) (# K0251 Thermo Scientific ®) was used. The reactions were performed using the Rotor Gene 6000 (Qiagen) thermocycler in a final reaction volume of 25 µL with 5 µL of DNA at 20 ng/µL. In each of the amplification reactions positive and negative controls were added. The purified product was sent to Macrogen (Korea) for sequencing and the results were aligned with the BioEdit Sequence Alignment Editor software for analysis. Figure 1 was elaborated using ArcGIS Desktop versión 10.5.0.6491, Esri Inc., 2016.

RESULTS AND DISCUSSION

We analyzed 98 specimens of Phlebotomine that were captured in different geographic points of Ciudad del Este and Presidente Franco districts of Alto Paraná department. The most abundant species were *Lu. Longipalpis* (47,9%) and *Ny. whitmani* (38,8%) (Table 1). A total of 11 out of 47 *Lu.longipalpis* (23,4%) and one out of six (16,3%) *Evandromyia (complex) cortelezzii* were positive for DNA *Leishmania spp*. (Figure 1). The positive sample of *Evandromyia (complex) cortelezzii* was sent for sequencing. Alignment of the obtained sequences showed 90% similarity with *L.infantum*, (GenBanK access code: KX664454.1). Other species captured in this study were: *Mycropygomyia quinquefer, Nyssomyia neivai* and *Brumptomyia cunhai* (Table 1). This is the first report of natural infection in *Evandromyia (complex) cortelezzii* with *L. infantum* through DNA detection in Paraguayand the second one in *Lu. longipalpis*, in urban areas associated with positive canine cases, but it is the first report of this last species in Alto Paraná department, where it was not previously found, with a tendency of a vector colonization of ruro-urban areas. Other studies have shown that *Lu. longipalpis* started to appear in rural areas where it had not been found before (Salomón *et al*., 2016) and it is a widely distributed species in South America (Galati, 2003), including almost all Brazilian States (Araki *et al*., 2009; Salomón *et al*., 2010).This is also the first report identifying *Mycropygomyia quinquefer* and *Brumptomyia cunhai* in this region and in this ocassion without natural infection (Table 1). However, *My. quinquefer* was found naturally infected in Puerto Iguazu, Argentina in the triple border between Brazil and Paraguay (Salomón *et al.,* 2017). It is important to note that parasite genome detection in a phlebotomine that has been wildly caught does not confirm the vector competence of the species (Ready, 2013; Bates *et al*., 2015), however, detection through PCR and the following sequencing are an important tool to orient new assays on suspected positive species (Saraiva *et al*., 2010). New scenarios are emerging in the context of *Leishmania* transmission. *Leishmania infantum* DNA, not that of *L. braziliensis,* was detected in *Mg. migonei* and *Ny.whitmani*s and flies captured at a rural-sylvatic area, although there are reports that suggest previous CL transmission backgrounds in that area (Moya *et al*., 2015). Asimilar situation has occurred in Southeastern Brazil, where *L. infantum* DNA was detected in *Ny. whitmani* and *Ny. intermedia*, both recognized CL vectors (Saraiva *et al*., 2010). Thisdemonstrates the intensive exchange of meal access and sources of natural infections between sandflies species and sylvatic, ruro-urban and urban hosts. The fact that typical CL vectors such as *Evandromyia cortelezzi* in this study and other species in previous reports are being found naturally infected with *L. infantum* should serve as a great warning for vector control surveillance actions and public health programs. Further studies should be conducted in order to determine the vector capacity and role in *Leishmania* transmission of phlebotomine in the triple border of Argentina, Brazil and Paraguay.

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Conflict of interest: The authors declare that there is no conflict of interest.

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