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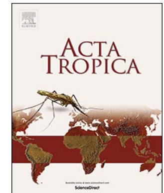

Short communication

Leishmania infantum DNA detected in phlebotomine species from Puerto Iguazú City, Misiones province, Argentina

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ABSTRACT

In Puerto Iguazú City, Argentina, human and canine Visceral Leishmaniasis cases have been recorded since the year 2010, with *Leishmania infantum* as the etiological agent and *Lutzomyia longipalpis* as its main vector. In the present study, polymerase chain reaction and sequencing were used to detect *L. infantum* DNA in 3.9% of the female sandflies captured in Puerto Iguazú City. This is the first report of *L. infantum* DNA detection in *Micropygomyia quinquefer*, and the second one in *Lu. longipalpis* and *Nyssomyia whitmani* for Argentina. Although the detection of *Leishmania* DNA itself is not enough to determine a Phlebotomine species as a vector, these results are significant in setting the direction of further investigations of vectorial competence and capacity, necessary to define the roles of different sandflies species as specific or permissive vectors in the transmission VL cycle.

Phlebotomine (Psychodidae: Phlebotominae) are insects of medical relevance because of the role of many of its species in the transmission of Visceral Leishmaniasis (VL) and Cutaneous Leishmaniasis (CL), both considered as vector-borne neglected diseases, the last with significant mortality rates in human cases (Maroli et al., 2012). *Leishmania infantum*, the etiological agent of VL in Argentina, has caused 140 human cases in four provinces during the period from 2006 to 2015 (Salomón et al., 2016), being Misiones the one with the highest incidence (Gould et al., 2013). Meanwhile, CL cases were recorded in epidemic focuses distributed in ten provinces since 1980 (Salomón et al., 2016), with *L. braziliensis*, *L. amazonensis*, *L. guyanensis* and *L. panamensis* as the etiological agents (Bates et al., 2015; Salomón et al., 2008a). Of the 36 species of phlebotomine distributed through 14 provinces of the country (Quintana et al., 2012a; Szelag et al., 2016), *Lutzomyia longipalpis* is a proven vector of VL, while *Nyssomyia neivai* (Córdoba-Lanús et al., 2006; Salomón et al., 2001, 2008a), *Ny. whitmani* (Salomón et al., 2009), *Migonemyia migonei* and *Cortelezzi* complex (Rosa et al., 2012) are incriminated as vectors of CL agents in different provinces (Quintana et al., 2012b; Rangel and Lainson, 2009; Salomón et al., 2008a). *Migonemyia migonei* has epidemiological outstanding relevance because it was suggested also as a putative of *L. infantum* vector due to its high abundance, concomitantly with the absence of *Lu. longipalpis* in epidemics foci (Guimarães et al., 2016; Salomón et al., 2010a). Particularly at Puerto Iguazú City, surrounded by protected natural areas of subtropical forest, the entomological registry has shown a high abundance of *Lu. longipalpis* in areas where canine cases and at least three human VL cases were recorded, showing the fact that *L. infantum* is circulating within urban transmission cycles since 2010 (Salomón et al., 2016). Otherwise, CL incidence showed only scattered cases after the last outbreak (2009) at a rural area located at southwest of Puerto Iguazú City, close to the ecotone of recent deforested patches, where the main CL vector and most abundant specie is *Ny. whitmani*, followed by *Mg. migonei* (Fernández et al., 2012).

In this scenario, the possibility of identifying at the species level potential *Leishmania* vectors from others phlebotomine species constitutes a valuable tool to orientate and address vector competence studies and to determine their role in local transmission cycles (Kato et al., 2010; WHO, 2010). In this sense, molecular techniques like polymerase chain reaction (PCR) and DNA sequencing allow the detection and characterization of *Leishmania* species from putative vectors with high sensitivity and specificity, regardless of the number, location or the biological cycle stage of the parasite in the digestive tract of the sandfly (Barker, 1989; Saraiva et al., 2010). In the present study, DNA of *L. infantum* was identified in field-caught phlebotomine from Puerto Iguazú City as a part of scheduled sampling procedures belonging to the IDRC #107577 research project (idrc.ca/en/project/addressing-

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emergence-and-spread-leishmaniasis-borders-argentina-brazil-and-paraguay).

Phlebotomine were capture using REDILA traps (Fernández et al., 2015) during three consecutive nights (March 2015) in the urban area of Puerto Iguazú City (Misiones Province, Argentina, 25° 36′ S, 54° 35′ W). In order to identify phlebotomine at species level, not engorged females were subjected to abdominal segment dissection and microscope morphological analysis of the spermathecae. Species identification was performed in accordance with the key by Galati (2003), and the generic abbreviations proposed by Marcondes (2007). The remnant parts of each specimen were conserved individually in 1.5 mL microtubes at −20 °C until total DNA extraction process (commercial kit Inbio HW DNA Puriprep-S Cat K1205-250). PCR was conducted following the RV1-RV2 protocol as described by Lachaud et al. (2002), which targets the highly repetitive kinetoplast (mitochondrial) DNA of *L. infantum* and generates an amplicon of 145 bp. The PCR assays were carried out with: 5 μl of DNA template in a final reaction volume of 50 μl containing 1 X PCR buffer [100 Mm Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol] (Invitrogen™), 2.5 mM $MgCl_2$ (Invitrogen™), DMSO 2.5% (Sigma™), 200 μM of each dNTP, 0.5 μM RV1 forward primer, 0.5 μM RV2 reverse primer and 1.4 U of Taq polymerase (Invitrogen™). The cycle profile employed was: an initial denaturalization step of 94 °C for 4 min, followed up by 40 cycles of a denaturalization step of 94 °C for 30 s, an hybridization step of 56 °C for 30 s and an extension step of 72 °C for 30 s, with a final extension step of 72 °C for 10 min. The cycle procedure was performed in a thermocycler MiniCycler PTC-0150™ (MJ Research, Inc.; Waltham, USA). As positive control a confirmed sample of *L. infantum* was employed for the RV1-RV2 assay; negative controls were also included (water and the reference strain WHO *L. braziliensis* HOM/BR75M2903). In addition, the extraction process was evaluated by a PCR assay targeted against the constitutive gen IVS6 (cacophony) of Phlebotominae, giving an expected 220 bp product (Lins et al., 2002). All PCR products were separated through a 2% agarose gel electrophoresis (1 h at 5 V/cm), and visualized with SyberGreen® (Invitrogen™). For sequencing purposes, PCR products were purified by a commercial kit (Inbio HW DNA Puriprep-GP) from agarose gel. Sequence qualities were evaluated with Codon Code Aligner™ software (V 2.0.6-LaBiMAp-FCEQyN-UNaM license), and *Leishmania* species identity was confirmed by Blast Nucleotide Standard software (blast.ncbi.nlm.nih.gov/Blast).

One hundred and twenty seven female sandflies were captured, belonging to five phlebotomine species: *Ny. whitmani* (n = 98, 77% of whole sample), *Lu. longipalpis* (n = 24, 19%), *Micropygomyia quinquefer* (n = 2), *Brumptomyia* spp. (n = 1), *Pintomyia monticola* (n = 1) and *Psathyromyia bigeniculata* (n = 1). The adequacy of phlebotomine DNA extraction from all samples was confirmed by positive amplification of the cacophony IVS6 region. Five phlebotomine were positive for *L. infantum* (3.9% of the females) by visualization of the 145 bp PCR amplicon and sequencing confirmation by an identity of 99% with *L. infantum* reference sequences deposited at Genbank. Three of them were *Lu. longipalpis*, one was *Ny. whitmani* and one was *Mi. quinquefer*.

Given that the abundance and geographical distribution of competent vectors represent a main determinant of disease transmission (Salomón et al., 2009), the identification of the parasites and vectors species in new foci would contribute to epidemiological studies, enabling the characterization of new transmission scenarios. However, as the incrimination of a species as a vector is based on a series of criteria (Killick-Kendrick, 1990; Volf and Peckova, 2007), just the detection of the genome of the parasite in a phlebotomine wild caught specimen does not imply the confirmation of the species vectorial competence (Bates et al., 2015; Ready, 2013), because it may be due to the recent ingestion of blood meal from an infected mammal. Nevertheless, the use of PCR and sequencing techniques has become an useful tool to identify putative vectors, allowing the detection and typing of *Leishmania* DNA in females of different phlebotomine species (Freitas-Lidani et al., 2014; Saraiva et al., 2010). Further, the presence in non-engorged females, as it was tested in this study, suggest at least persistent infection.

A study carried out by our group in the year 2008 at rural environments of Puerto Iguazú City reported *Ny. whitmani* and *Mi. quinquefer* females with *Leishmania* DNA, (without parasite typing); their abundances were 91.8 and 0.008% respectively (Salomón et al., 2009). In 2014 *Leishmania infantum* DNA was detected in *Mg. migonei* and *Ny. whitmani* sandflies captured at a rural-forest ecotone area, even though the expected parasite to be detected should have been *L. braziliensis*, due to the phlebotomine species records for this area and CL transmission antecedents (Moya et al., 2015). A similar situation was recorded in Southeastern Brazil, where *L. infantum* DNA was detected, besides the expected in *Lu. longipalpis*, also in *Ny. whitmani* and in *Ny. intermedia,* although the last two are the main vectors of *L. braziliensis* (Saraiva et al., 2010).

The present study constitutes the second report for Argentina of *L. infantum* DNA detection in *Lu. longipalpis* and *Ny. whitmani*, with relatives abundances of 40 and 59% respectively, at an urban scenario. These results are noteworthy because, as discussed by us previously, surveys from recent years show a vector colonization of *Ny. whitmani* to urban areas while *Lu. longipalpis* started to appear in rural areas where it had not been found before (Salomón et al., 2016), additionally, detection of *L. infantum DNA* in *Ny. whitmani* was formerly recorded at a rural scenario in Puerto Iguazú (Moya et al., 2015). Both species present a wide spread geographical distribution in South America (Galati, 2003), that includes almost every Brazilian States, showing populations that have been recognized as species complexes (Araki et al., 2009; Costa et al., 2007; Hoyos et al., 2012; Salomón et al., 2010b), so sibling species could have different vector competence or capacity (Bauzer et al., 2007; Lanzaro and Warburg, 1995). *Lutzomyia longipalpis* was recorded in the Argentinian provinces of Salta, Formosa, Chaco, Entre Rios, Corrientes and Misiones; while *Ny. whitmani* was found in Corrientes,Tucumán and Misiones (Quintana et al., 2012a).

Regarding *Mi. quinquefer*, this is the first report of *L. infantum* DNA detection in this species, even though its low abundance. A previous study reported *Mi. quinquefer* associated with *L. braziliensis* through molecular methods in Mato Grosso State, Brazil. It worth noting that as in the present study, the proportion of positives sandflies with *Leishmania* DNA was also high (one out of 5), while in Brazil as well as in the previous report in the rural environment of Puerto Iguazú where the only pool (n = 12) analyzed was positive (Paiva et al., 2010; Salomón et al., 2009). In Argentina, *Mi. quinquefer* has been recorded at the provinces of Chaco (Rosa et al., 2012; Salomón et al., 2008b), Salta (Quintana et al., 2012b), and the Misiones localities of Puerto Esperanza (Salomón et al., 2006), Guarani and Candelaria associated to CL focuses (Salomón et al., 2016). In Brazil, its records include thirteen States belonging to Northeast, Central-West, Southeast Regions and Paraná State in the South Region, bordering with Misiones province (Galati, 2003); while in Bolivia it was recorded in the departments of Beni, Santa Cruz, La Paz and Tarija, whose populations have been found to represent incipient species due to quantitative morphological evidence (Dujardin et al., 1999).

The urbanization as a biological event contributes to the finding of *L. infantum* in new phlebotomine species in different ways. The urban canine VL generates spatial-time clusters of dogs with high reservoir capacity, so the offer of parasites in this ‘hot spots’ could be very high and PCR would detect *L. infantum* DNA parasites in any hematophagous. On the other hand, the vector populations in these peri-urban mixed and dynamic landscapes, show a decrease in diversity but an increase in abundance of the species capable to colonize or in trend of colonization anthropic modified environments, so increasing the opportunity of vector-reservoir contact of this species. Therefore, in these scenarios, as the one described in this paper, just the finding of the parasite DNA is not enough to incriminate a species as a vector and the other criteria of vector competence assessment should be strengthen. However, for the same reason, furthermore when the parasite DNA was

found in females without visible digested blood, an eventual role in the transmission could not be discarded. Hence, reiterative reports of infection in one species is a clue to encourage further studies in vector competence, capacity, epidemiological weight in actual transmission settings, and on the probability of replacement of the primary vector under control interventions.

Conflict of interest

The authors declare that there is no conflict of interest.

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