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First description of *Migonemyia migonei* (França) and *Nyssomyia whitmani* (Antunes & Coutinho) (Psychodidae: Phlebotominae) natural infected by *Leishmania infantum* in Argentina

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ABSTRACT

Leishmania infantum is the etiological agent of the Visceral Leishmaniasis (VL) disease in America, with *Lutzomyia longipalpis* phlebotomine sandflies as its proven vectors in Argentina, and infected dogs as its main urban reservoir. In Puerto Iguazú City (Misiones province, Argentina), human and canine cases of VL were recorded. Additionally, in the rural area known as "2000 Hectáreas", less than 10 km away from the city, several human cases of Tegumentary Leishmaniasis (TL) were registered determining an endemic area with *Leishmania braziliensis* as the etiological agent. Because of this, several phlebotomine captures were done in this site showing that *Nyssomyia whitmani* is the most abundant sandfly followed by *Migonemyia migonei*. In this study, three of the sandflies captured were found infected whit *L. infantum* parasites, detected by PCR and sequencing. Two of them were *N. whitmani* and the other one was a *M. migonei* specimen, being this the first report of *L. infantum* natural infection for Argentina in these sandfly species. *N. whitmani* is the main vector of *L. braziliensis* in this area, and *M. migonei* has been suggested as a putative vector in other locations where human and canine cases of VL where reported with *L. longipalpis* apparently absent. In this context, we consider necessary further studies that could define the role of *M. migonei* and *N. whitmani* as specific or permissive vectors of *L. infantum,* their vectorial competence and capacity, and their actual role in the transmission of both Tegumentary and Visceral Leishmaniasis in the study area.

1. Introduction

Leishmaniasis are caused by protozoan species of *Leishmania* genus, obligated parasites of vertebrates macrophages, which are transmitted by female phlebotomines species (Diptera: Psychodidae: Phlebotominae) (WHO, 2010). The diseases clinic form in humans depends largely on the etiological agent involved, being *Leishmania infantum* responsible for Visceral Leishmaniasis (VL), while *Leishmania braziliensis*, *Leishmania amazonensis*, *Leishmania guyanensis*, and *Leishmania panamensis* are responsible for Tegumentary Leishmaniasis (TL) in Argentina (Marco et al., 2012; PAHO/WHO-SOPERJ, 2008; Salomón et al., 2008a). Additionally, each *Leishmania* species is transmitted by one or a few species of phlebotomine sandflies (specific or permissive vectors) (Volf and Peckova, 2007), being this parasite-sandfly specific interaction associated to the infectivity and virulence. The ecology, seasonality and behavior of the vector determine general characteristics of the transmission cycle; while the vector–host interaction and cultural patterns of the risk population determine the intensity of the zoonotic, anthropo–zoonotic or anthropic cycle (Salomón et al., 2008a).

In Argentina, while the incidence of TL oscillated between epidemic and interepidemic years, VL has increased sharply, accompanied by an expansion to new areas. Since 2006, when the first human case of VL was described (Posadas city, Misiones) (Salomón et al., 2008b), 141 cases have been reported in 4 provinces, two cases in Puerto Iguazu up to now during 2014 (Salomon, personal communication), being Misiones the one with the highest incidence (Gould et al., 2013). In this province the dis-

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ease has been associated to *L. infantum* infected dogs and *Lutzomyia longipalpis* sandflies (primary vector) within urban transmission cycles (Acardi et al., 2010; Lainson and Rangel, 2005; Salomón et al., 2011).

With respect to TL, the endemic zone in Argentina covers 9 provinces where several epidemic outbreaks occurred since the year 1985 (Salomón et al., 2006). Three epidemic scenarios were inferred from the study of these outbreaks: (1) wild cycle/wild transmission, (2) wild cycle/peridomestic transmission and (3) peridomestic cycle/peridomestic transmission (Salomón et al., 2008a). *Nyssomyia neivai* has been implicated as the main vector in most sites of the country, including the Puerto Esperanza outbreak (Córdoba-Lanús et al., 2006; Salomón et al., 2006, 2001) in Misiones province. While *Nyssomyia whitmani* is the most abundant specie followed by *Migonemyia migonei* in endemic areas of this province, related to epidemic scenarios 1 and 2 (Salomón et al., 2009). Indeed, the last outbreak of TL in Argentina, occurred in Puerto Iguazú City, Misiones, in a rural area known as "2000 Hectáreas", where *N. whitmani* and *M. migonei* have higher relative abundance than others species, particularly observed in animal shelters close to the ecotone of recent deforested patches (Fernández et al., 2012; Salomón et al., 2008a; Salomón et al., 2009).

The feasibility of characterize *Leishmania* species from reservoirs, putative vectors and patients through molecular techniques, constitutes a fundamental tool for understanding the medical entomology and epidemiology of the disease, allowing the construction of risk maps and prediction of disease expansion in endemo-epidemic areas. The use of molecular techniques, such as the polymerase chain reaction (PCR) and sequencing, for the detection of natural infected sandflies and genotyping of parasites involved contributes to identify potential Leishmaniasis vectors (Barker, 1989; Carvalho et al., 2010; Pita-Pereira et al., 2005; Rosa et al., 2012; Saraiva et al., 2010), whose abundance and distribution represent the mayor determinants and intensity modulators of the disease transmission (Salomón, 2009). The main advantages of PCR technique are its sensitivity and specificity, regardless of the number, location or stage of the infecting *Leishmania* in the digestive tract of the sandfly (Perez et al., 1994; Saraiva et al., 2010). There are 36 species of Phlebotomine sandflies described for Argentina (Casertano et al., 2015; Fernández et al., 2012; Salomón et al., 2010a), some of which have been found naturally infected with *Leishmania* spp. parasites: *N. neivai*, *N. whitmani*, *Micropygomyia quinquefer* and the *Cortelezzi* complex, the last one with *L. braziliensis* infection; while *L. infantum* was detected in *L. longipalpis* (Acardi et al., 2010; Córdoba-Lanús et al., 2006; Rosa et al., 2012; Salomón et al., 2009).

2. Material and methods

The capture site was located in Puerto Iguazú City (Misiones province, Argentina, 25° 36′S, 54° 35′W) in a farmland (rural area: "2000 Hectáreas", 25° 43′S, 54° 35′). CDC Mini Light traps (Sudia and Chamberlain, 1962) were placed during two consecutive nights at three locations, inside the henhouses, the swinery and the human dormitory (April 2014). Collected samples were processed by separating sandflies from other insects. Female Phlebotomine sandflies with presence of blood meal were subjected to abdominal segment dissection in order to visualize the spermathecaes helped with optical microscope; specific identification was performed according to Galati (2003) the sand-fly remainder parts were conserved individually in Eppendorf tubes at −80° C for DNA extraction purposes.

Total DNA extraction was done employing a commercial kit (Inbio HW AND Puriprep-S Cat *K1205-250*), followed by a DNA fluorometry quantitation (Qubit™, Invitrogen). As an extraction control, a constitutive gen of Phlebotominae was amplified using

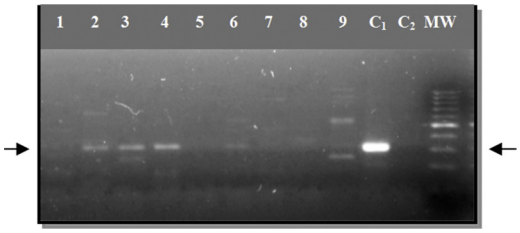

Fig. 1. 2% agarose gel electrophoresis of the PCR amplification of *ITS-1* region of *Leishmania* genome. The arrows indicate the expected 300–350 bp fragment. Lanes 1–9: phlebotomine samples. Lanes 1, 5–9: negative samples. Lanes 2–4: positive samples; C_1: positive control; C_2: negative control; MW: molecular weight marker (100 bp DNA Ladder).

specific primers targeted against the IVS6 region (*cacophony*), described by Lins et al. (2002). The presence of *Leishmania* was tested by the PCR-RFLP protocol described by Schönian et al. (2003); briefly, this protocol amplifies the ribosomal internal transcribed spacer 1 (*ITS-1*) using primers LITSR (5′-CTG GAT CAT TTT CCG ATG-3′) and L5.8S (5′-TGA TAC CAC TTA TCG CAC TT-3′) (El Tai et al., 2000, 2001), both with a 53 °C annealing temperature. These primers generate a 300–350 bp product, depending on the species of *Leishmania*. The PCR final volume was 50 μl, containing Buffer PCR 1X [200 mM Tris–HCl pH 8.0; 0.1 mM EDTA; 1 mM DTT; 50% (v/v) glycerol] (Invitrogen), 2.5 mM MgCl2 (Invitrogen), DMSO 2.5% (Sigma), 200 μM of each dNTP, 0.5 μM LITSR forward primer, 0.5 μM L5.8S reverse primer, and 1.4U of Taq polymerase (Invitrogen). PCR assays were performed in a thermocycler *MiniCycler PTC-0150*™ (MJ Research, Inc.; Waltham, USA), and the reaction products were resolved by electrophoresis (5 V/cm) in a 2% agarose gel, visualized with ethidium bromide (0.5 μg/ml) under ultraviolet light (305 nm). The evaluation of the electrophoretic bands sizes was done by comparison against a commercial molecular marker (CienMarker, Biodynamics®). As positive control, the OMS *L. braziliensis* HOM/BR75M2903 reference strain was used; negative control consisted of water molecular biology grade. For sequencing purposes, *ITS-1* PCR products were purified from the agarose gels with a commercial kit (Inbio HW AND Puriprep-GP). Sequence qualities were evaluated with *Codon Code Aligner*™ software (Version 2.0.6—LaBiMAp-FCEQyN-UNaM license), and *Leishmania* species identity by *BLASTm* accessible at *Genbank* (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

Thirty seven sand-flies with blood meal were captured at the henhouses (n 19) and the swinery (n 18). The species identified were *N. whitmani* (n 33), *M. migonei* (n 2), *N. neivai* (n 1) and *Pintomyia monticola* (n 1). The adequacy of Phlebotomine DNA extraction from all the samples was confirmed by the positive amplification of the *cacophony IVS6* gen in all samples.

Three sand-fly samples were positive for *ITS-1* PCR *Leishmania* protocol, all captured at the same experimental henhouse, showing the 300–350 bp expected product (Fig. 1). Two of them corresponded to DNA isolated from *N. whitmani* sandflies, and the third one belonged to a *M. migonei* specimen. Sequences obtained are available at *Genbank* with access numbers KR081260, KR081262 and KR081263 respectively. The sequence analysis of these three *ITS-1* positive samples showed 100% identity with *L. infantum* reference sequences already deposited at *Genbank*.

4. Discussion

Several reports had described natural infection caused by *L. infantum* in *L. longipalpis* (Acardi et al., 2010; Saraiva et al., 2010;

Savani et al., 2009) and other Phlebotomine sandflies, including: *L. cruzi* (Pita-Pereira et al., 2008), *Lutzomyia forattinii* (Pita-Pereira et al., 2008), *Lutzomyia almerioi* (Savani et al., 2009), *Nyssomyia antunesi* (Thies et al., 2013), *Nyssomyia intermedia* (Saraiva et al., 2010), *N. whitmani* (Saraiva et al., 2010), *M. migonei* (Carvalho et al., 2010), *Evandromyia cortelezzii* (Carvalho et al., 2008) and *Evandromyia termitophila* (Saraiva et al., 2010). Although *M. migonei* (Carvalho et al., 2010) and *N. whitmani* (Saraiva et al., 2010) have already been found with *L. infantum* infection, this is the first report of *L. infantum* natural infection for Argentina in these species.

M. migonei has been proposed as a potential vector of *L. infantum* in three VL epidemic foci where *L. longipalpis* was apparently absent; two of them located in Brazil, Pernambuco State (Carvalho et al., 2010), and Rio de Janeiro city (Souza et al., 2003) and the other in Santiago del Estero province, Argentina (Salomón et al., 2010b). The later one, considered as a low VL endemic focus, is where *M. migonei* has been suggested as a putative or secondary vector of an enzootic VL cycle with accidental transmission to humans, due to its prevalence around the VL cases, in peridomestic environments with plenty of domestic animals, and an increased supply of parasites provided by canines that migrated from other endemic areas (Salomón et al., 2010b). Additionally, in a few TL endemic areas, *M. migonei* has been suggested as a vector linking the enzootic and anthropo-zoonotic transmission cycles, due to its adaptation to manmade modified environments and its relative anthropophilic behavior (Chaves and Añez, 2004; Salomón et al., 2006, 2008c); these proposals are supported by PCR based molecular finding of *M. migonei* naturally infected by *L. braziliensis* in Rio de Janeiro, Brazil (Pita-Pereira et al., 2005).

In downtown Puerto Iguazu, 6 km afar from the trapping site, *L. longipalpis* is present, and canine cases were recorded showing that *L. infantum* is already circulating since 2010 (Salomón et al., 2011), and during 2014 two human VL cases were reported living in the city (Salomón, personal communication, April 2015). Due to our finding in the "2000 Hectáras" rural area, specifically at the henhouse close to the sylvatic area, it is epidemiologically relevant to give response to new issues related to the possible existence of a wild cycle of *L. infantum*, with perhaps other reservoirs than domestic dogs. Furthermore, the fact that this area is a TL transmission zone with *N. whitmani* as the main vector, questions regarding to possible new scenarios of transmission should be taking into account.

In spite of the results obtained, it is necessary to stress the difference between a positive sandfly by PCR-sequencing technique and a vector with proven competence and capability defined by Killick-Kendrick (Killick-Kendrick, 1990; Volf and Peckova, 2007). Reinforcing this concept, it is important to remark that the detection of *Leishmania* DNA itself is not enough to determine a Phlebotomine specie as a vector of a Leishmaniasis Cycle, mainly in areas where the animals that usually brings the blood source to Phlebotomines are infected; in this context we strongly consider the necessity of further investigations of natural and experimental infections, required to define the role of *M. migonei* and *N. whitmani* as specific or permissive vectors of *L. infantum,* their vectorial competence and capacity, and their actual role in the transmission of both Tegumentary and Visceral Leishmaniasis in the study area.

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