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MICROBIOLOGY

Leishmania (Viannia) braziliensis in *Migonemyia migonei* and Cortelezzii complex (Diptera: Phlebotominae) from Chaco, Argentina

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Abstract: Chaco province is included in Argentinean ecoregions with human tegumentary leishmaniasis case records. During 2012-2014 in Pampa del Indio town an ecoepidemiological study was carried out including phlebotomine dynamics and its natural infection with *Leishmania* demonstrated by sand flies dissection, polymerase chain reaction and sequencing. The species recorded were: *Migonemyia migonei* (72.79%), *Nyssomyia neivai* (23.6%), *Evandromyia cortelezzii* (0.94%), *Evandromyia sallesi* (0.39%), Cortelezzii complex (1.61%), *Evandromyia aldafalcaoae* (0.05%), *Psathyromyia bigeniculata* (0.02%), *Brumptomyia brumpti* (0.6%) and Corumbaensis complex (0.01%). A total of 380 females sand flies (Cortelezzii complex, *Mg. migonei* and *Ny. neivai*) from peridomicile and extradomicile were individually dissected and no flagellates were observed in the intestinal tract. Later, these females were arranged in 38 pools for molecular analyses and *Leishmania braziliensis* DNA was amplified in 3 pools with a minimum infection rate of the total females of 0.8%, while specific rates were 0.5% for the Cortelezzii complex and 1.5% for *Mg. migonei*. In conclusion, our results would strengthen the hypothesis that, in the study area, these species are candidates to be incriminated as vectors, while further studies will be required to fulfill the criteria to characterize both species as proven vectors of *Le. braziliensis*.

Key words: Leishmaniasis, natural infection, phlebotomine sand flies, wild vector.

INTRODUCTION

Phlebotomine sand flies in Argentina includes 38 species distributed in 14 provinces, with greater abundance and richness in the Northeast (ANE), Northwest (ANW) and Chaqueña regions (Quintana et al. 2012, Szelag et al. 2017). In the latter, in the province of Chaco, 19 species distributed in the dry Chaco and wet Chaco bioregions were recorded (Szelag et al. 2017). *Nyssomyia neivai* and *Migonemyia migonei* were the most frequent and abundant species in wet Chaco and *Mg. migonei* and Cortelezzii complex in dry Chaco (Salomón et al. 2008a).

Nyssomyia neivai was the first species in Argentina involved as a probable vector of *Leishmania (Viannia) braziliensis* by amplification of the DNA of *Leishmania sp*. during the outbreak of tegumentary leishmaniasis (TL) in the province of Tucumán (ANW) (Córdoba Lanús et al. 2006). In other studies *Le. infantum* DNA was detected in *Ny. whitmani*, *Mg. migonei*, *Lutzomyia longipalpis* and *Micropigomyia quinquefer* in the province of Misiones (ANE) (Acardi et al. 2010, Moya et al. 2015, 2017) and *Le. braziliensis* in Cortelezzii complex in Chaco (Rosa et al. 2012). *Lutzomyia longipalpis* was incriminated as a vector of *Leishmania infantum*, the etiologic agent of

visceral leishmaniasis (VL) in Misiones province (Acardi et al. 2010). However, vector capacity is a biological definition referred to as potential in sustaining the transmission over time. It requires compliance with certain criteria in addition to a distribution of cases consistent with epidemiological data or presence of genetic material of the pathogen (Ready 2013).

Humans cases of TL increased in 22 of the 25 provincial departments of Chaco between 2010-2013, where the highest frequency was in Liberator General San Martin department from Pampa del Indio town, between 2012 and 2013. This led to the realization of ecoepidemiological studies in this locality through the active search of cases, studies of Phlebotominae dynamics and its natural infection or the detection of *Leishmania* DNA to define its role as a probable vector. In this context, it was proposed to determine the natural infection in sand flies captured in Pampa del Indio by observation of intestinal flagellates and the detection of *Leishmania* DNA by polymerase chain reaction (PCR) and sequencing.

MATERIALS AND METHODS

Study area

Province of Chaco, department General Liberator San Martín, Pampa del Indio (26°02'S 59°56'O) (Fig. 1). Pampa del Indio is located between the dry Chaco and wet Chaco bioregion divided by the 900mm isohyetal. The first is characterized by a semi-arid continental rainfall pattern between 500-800mm per year with the formation of xerophytic forests of quebracho/algarrobo (*Schinopsis*, *Aspidosperma,* and *Prosopis*)

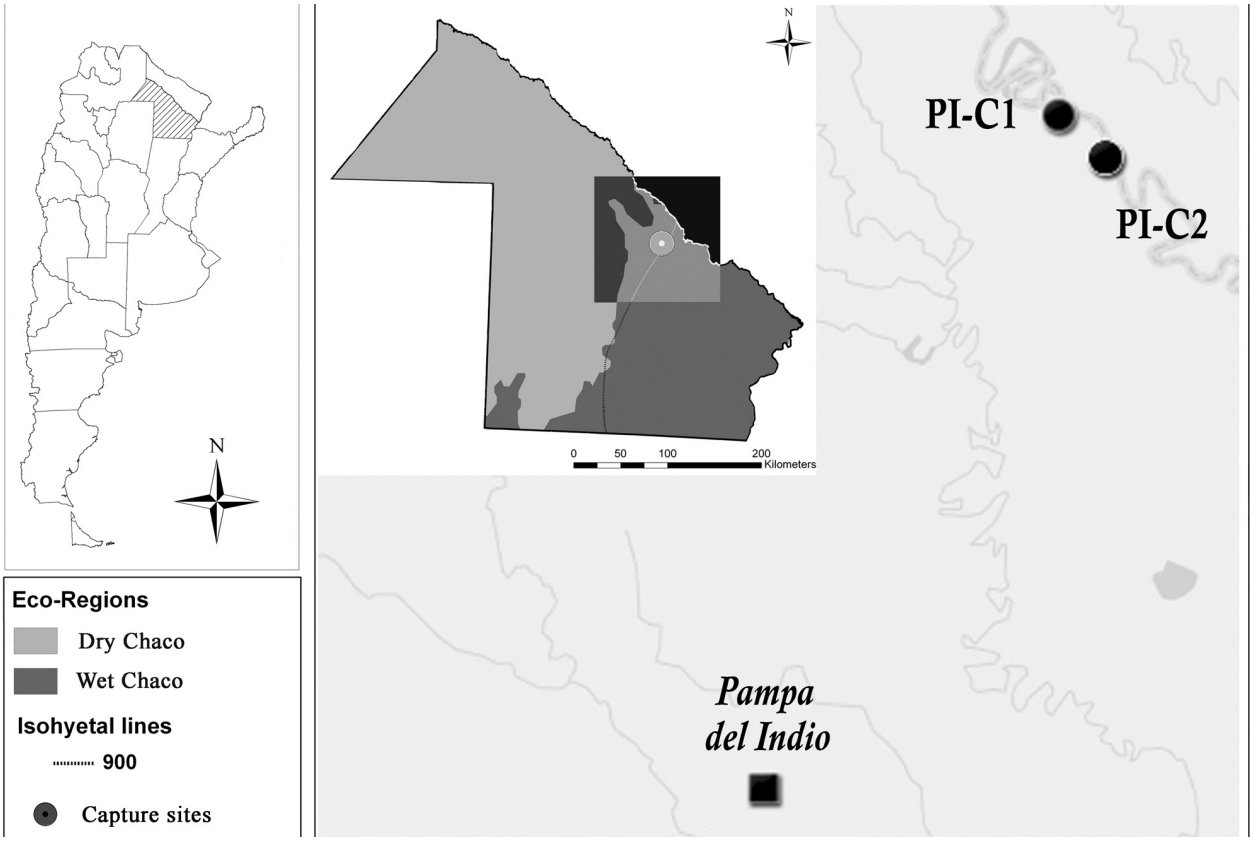

Figure 1. Study area and its location in Pampa del Indio, in Chaco biogeographical eco-region of Chaco Province, Argentina.

and quebracho/palo santo (*Schinopsis* and *Bulnesia*) and cacti. The wet Chaco bioregion has an Atlantic rainfall regime of 1,200mm per year, most frequently in summer and autumn, with the formation of parks and savannas (Cabrera 1971).

Sample site

Campo Cacique site. It corresponds to a protected area where Quom ethnic communities reside near to Bermejo River with areas of secondary forest of quebracho and palo santo, and an herbaceous stratum composed of bromeliads and grasses forming part of the gallery forests to the Bermejo River.

Phlebotominae capture

January 2013 to December 2014. In Campo Cacique, CI-PI (S25°53'W 059°49'S) and C2-PI (S25°51'W 059°51'S) were selected because they were referred as a probable site of leishmaniasis infection. Captures were carried out with CDC light traps (Sudia & Chamberlain 1962) with monthly frequency for three consecutive nights installed in the stratum base at 1.5m above the ground and the height stratum at 10m height. The first one included the domicile and galleries, the peridomicile up to 50m from the domicile with chicken coops, sheep and cattle corral, and the extradomicile in a tree base with a height greater than 10m in the secondary vegetation until 100m from the peridomicile. The stratum height refers to the canopy of the same extradomicile tree at 10m height.

Phlebotominae identification and detection of *Leishmania* sp.:

Sand flies were separated from other insects and females without blood content in the digestive tract collected in summer, spring and autumn 2013 and summer 2014 were selected. They were individually dissected according to the methodology described by Rioux (1986) with modifications oriented to the search for intestinal flagellates in phase contrast (40X). The identification of these females was made from the head mounted on an individual slide and the morphology of the spermathecae observed during dissection, while the rest of the sand flies were identified by the morphology of their entire bodies. The nomenclatures, abbreviations, and classification schemes were according to Galati et al. 2017 and Galati 2018. Cortelezzii complex refers to the females of *Ev. cortelezzii* and *Ev. sallesi* that are not differentiable based on morphological characters (Szelag et al. 2018). On the other hand, males of *Ev. cortlezzii* and *Ev. sallesi* are treated in this work as different species for diversity index analysis. The species richness (*S*), Shannon-Wiener diversity index (*H'*) and equitability of Pielou (*J*) were calculated to describe the communities where the sandflies with *Leishmania* DNA were captured. The selected females were grouped in lots of 10 females of the same species, site and date and kept at 4°C until molecular analysis at the National Institute of Tropical Medicine (INMeT-ANLIS), Puerto Iguazú, Misiones, Argentina. DNA extraction was performed by commercial DNA Puriprep-S Highway kit (Inbio, Argentina) from the unmacerated lots and with an incubation period of 1.45hs. For the detection of *Leishmania* DNA, the polymerase chain reaction (PCR) was performed with blank in the mini-exon gene belonging to the kinetoplast genome, using the Fme and Rme primers according to Marfurt et al. (2003), Gotaq green Master Mix (Promega, USA) and the reference strain MHOM/BR/75/M2903 as a positive control. The PCR products were separated by 1.5% agarose gel electrophoresis stained with Sybr Green (Invitrogen, USA) and visualized in 470nm blue light LED transilluminator. They were then purified and sent to sequence both ways (Macrogen Inc, Korea).

Sequences were evaluated by chromatogram observation and edition in MEGA v.7, the identity was confirmed with the Basic Local Alignment Search Tool (BLAST). Minimum infection rates were calculated according to Paiva et al. 2010 discriminating the values according to the total analyzed (MIRt) and according to the total of each species (MIRs).

RESULTS

Phlebotominae capture

In total 35,727 phlebotomine: *Mg. migonei* (72.79%), *Ny. neivai* (23.6%), *Evandromyia cortelezzii* (0.94%), *Ev. sallesi* (0.39%), Cortelezzii complex (1.61%), *Ev. aldafalcaoae* (0.05%), *Psathyromyia bigeniculata* (0.02%), *Brumptomyia brumpti* (0.6%) and Corumbaensis complex (0.01%) were recorded. The greatest richness in C1-PI was in base (*S*=8) and in C2-PI, it was in peridomicile (*S*=9). In height, both sites had the same richness (*S*=6). The greatest diversity and equitability were observed at domicile ($H^{'}$=0.8975; *J*= 0.4612) of C1-PI and, in base ($H^{'}$=0.8929; *J*=0.4294) of C2-PI. *Migonemyia migonei* and *Ny. neivai* predominated in peridomicile in both sites and periods (p <0.0001); in extradomicile, only *Mg. migonei* with no records of *Ny neivai* in 2014 (p <0.0001). Cortelezzii complex predominated in the base stratum in 2013. The highest abundance was observed in C1-PI, in 2014 and the lowest in C2-PI in 2013.

Leishmania spp. Detection

A total of 380 phlebotomine were individually dissected for observation of flagellated forms then, they were identified and arranged in 38 pools: Cortelezzii complex (21 pools), *Mg. migonei* (13 pools) and *Ny. neivai* (4 pools). No flagellates were observed in the intestinal tract. *Leishmania braziliensis* DNA was amplified in 3 pools from samples of March, November and January 2013 (1 pool each) in different ecotopes, one pool of Cortelezzii complex and two pools of *Mg. migonei* (Table I). Taking into account the total number of females analyzed, the minimum infection rate resulted in MIRt = 0.8%, while according to the species, it resulted in 0.5% and 1.5% for Cortelezzii complex and *Mg. migonei*, respectively. The three sequences obtained were edited and resulted in 105 bp (same haplotype) that showed 100% identity with those available in GenBank for *Le. braziliensis*.

DISCUSSION

The evidence involving one species of Phlebotominae as a vector of *Leishmania* sp. includes seven biological criteria, five of them were proposed by Killick-Kendrick (1990). Later,

Table I. Number of pools of 10 females of Phlebotominae distributed according to species, site and ecotope and DNA amplification.

	C1 - PI			C2- PI			Total	Total
	Peri	Base	Hei.	Peri	Base	Hei.		
Species	-/+	-/+	-/+	-/+	-/+	-/+	-/+	
Cortelezzii complex	1/1	7/0	2/0	3/0	4/0	3/0	20/1	21
Mg. migonei	2/0	2/1	0/0	4/0	1/1	2/0	11/2	13
Ny. neivai	2/0	2/0	0/0	0/0	0/0	0/0	4/0	4
Total	5/1	11/1	2/0	7/0	5/1	5/0	35/3	38

Ref .: Peri .: Peridomicile, Hei.: Height; +: Amplified; -: It did not amplify.

Bates (2007) and Ready (2013) incorporated mathematical criteria associated with epidemiological and ecological backgrounds. The Phlebotominae of the Old and the New World in which their role as vectors was demonstrated, meet the first four biological criteria (Ready 2013).

Microscopic examination of dissected phlebotomine females from Pampa del Indio did not show mobile flagellates in their digestive tract. The molecular PCR methodology, on the other hand, amplified *Leishmania* DNA in 0.8% of the total females analyzed considering at least one infected individual in each. The BLAST analysis showed 100% similarity with *Le. braziliensis* sequences obtained from reference strains (accession numbers KF633196-97) (Van der Auwera et al. 2014). These results are consistent in considering that the dissection has low sensitivity due to the difficulty of observation inherent to the practice of the procedure, the small number of flagellates, the migration capacity, the stage and the size of the protozoan. However, this method is still necessary to determine the location of parasites in the digestive tract and, after fixation and coloration, the determination of stage (criterion 2: Observation of the infectious stage in the anterior midgut and the stomodeal valve) (Ready 2013). Other conventional methods such as in vitro culture and inoculation in experimental animals generate uncertainty when interpreting the results. These observations were referred by Salomón (2002) in Argentina, not observing flagellates in the dissection of more than 3,000 females of *Lu. intermedia* (s.l) *Ny. neivai* (s.s.) and *Mg. migonei* captured during TL outbreaks in provinces of ANE and ANW region in 2004 (Córdoba Lanús et al. 2006). In this sense, the application of molecular biology techniques has greater sensitivity and specificity for the diagnosis and identification of species through amplification and sequencing of molecular markers (Saraiva et al. 2010, Van der Auwera 2014). However, the disadvantage is that it cannot determine the viability of the parasite or see its position in the digestive tract (criterion 2). These reasons make both methods complementary at the time of incriminating a species of phlebotomine as a probable vector (Bates 2007).

The first antecedents in Argentina of detection of natural infection were in Salta and Tucumán provinces with Phlebotominae captured in endemic areas between 1999 and 2003. *Nyssomyia neivai* was framed as a vector when *Le. (Viannia)* DNA was detected with MIRs=0.9%, it being the prevalent species with 96.5% relative abundance (Córdoba Lanús et al. 2006). *Migonemyia migonei,* widely distributed in South America, was framed as a *Le. braziliensis* vector in different regions of Brazil, Paraguay and Argentina (Salomón et al. 2008a, b). Later studies suggested *Mg. migonei* as a putative or secondary vector in the transmission of *Le. infantum* due to its relative abundance in VL epidemic outbreaks in ANW provinces in the absence of the primary vector *Lu. longipalpis* (Salomón et al. 2010). Later, *Le. infantum* DNA was reported in females of this species (Moya et al. 2015) until Guimaraes et al. (2016) proposed its role as a permissive vector by demonstrating its susceptibility in the development of *Le. infantum*.

Leishmania braziliensis DNA in Cortelezzii complex was detected in the dry Chaco bioregion in coincidence of space and time with the confirmation of human cases, with a minimum infection rate greater than that detected in this study, MIRs=4% (Rosa et al. 2012). The molecular detection of *Le. braziliensis* DNA in Cortelezzii complex was reported in Belo Horizonte, Minas Gerais, Brazil, with a MIRs of 3.2%, without having observed flagellate in the intestine (Saraiva et al. 2010). However, the highest MIRs for this

species with *Le. braziliensis* was recorded by Lana et al. (2015) in Jaboticatubas, Minas Gerais, Brazil with a value of 8.6% with relative abundance of Cortelezzii complex of 5.8%. These authors also reported Cortelezzii complex with *Le. infantum* infection (MIRs=1.9%). Also, in the same state of Brazil, Carvalho et al. (2008), using molecular techniques, detected *Le. infantum* DNA in *Ev. cortelezzii* (MIRs= 7.1%) and Saraiva et al. (2009) through dissection found *Le. infantum* in *Ev. sallesi* (MIRs=16.7%). Both, in the studies cited and in ours, the relative abundance of the Cortelezzii complex was less than 13%, but due to the recent colonization observed in the center of the country (province of Córdoba) (Visintin et al. 2016, Ontivero et al. 2018), its ability to adapt with broad thermo tolerance (Szelag et al. 2017), and the evidence discussed, we suggest that the species of the complex should be taken into account for future studies of vector competence.

In Pampa del Indio, Cortelezzii complex and *Mg. migonei* were recorded infected at domicile/peridomicile of C1-PI and in Base of C2-PI during the spring and summer months probably refer to their behavior in modified and little modified environments and to the food preference for synanthropic or wild animals. In the peridomicile of both sites, the presence of poultry (chickens and ducks), wool cattle (goats), equines and dogs, are probably attractive and a source of food for Phlebotominae females. In Argentina, there is a history of *Le. (Viannnia)* sp. DNA detection in *Aotus azarai azarai* monkeys in the province of Formosa (Acardi et al. 2013) and in sigmodontinal mammals (genus *Akodon* and *Euryoryzomys russatus*) infected with *Le. braziliensis* in Puerto Iguazú, Misiones, considered as potential wild reservoirs or incidental hosts (Fernández et al. 2017).

CONCLUSIONS

The eco-epidemiological map of the province of Chaco could include an intermediate or transition area between the bioregion of dry Chaco and wet Chaco with an overlap of common and autochthonous phlebotomine species of each subregion. In this geographical area and in the same period *Le. braziliensis* was amplified in human cases as in Cortelezzii complex and *Mg. migonei* applying criteria 1 and 4 (Strong strong ecological association and seasonality between phlebotomine, humans and reservoir animals). This would strengthen the hypothesis that, in the study area, these species are candidates to be incriminated as vectors, and further studies will be required to fulfill the criteria to characterize both species as proven vectors of *Le. braziliensis*.

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Juan Ramón Rosa is responsible for the development and execution of the research, fieldwork and laboratory activities. Sofía Lorián Moya performed the molecular and sequence analysis and Enrique Alejandro Szelag designed the figure and developed on field and laboratory activities. Authors Maria Gabriela Quintana and Oscar Daniel Salomón were advisors of the research project. All the authors contributed to the final manuscript and revision.