Diversity patterns, *Leishmania* DNA detection, and bloodmeal identification of Phlebotominae sand flies in villages in northern Colombia

Camila González1*, Cielo León1, Andrea Paz1a,b, María López1, Gisell Molina1, Diana Toro1, Mario Ortiz1, Juan Manuel Cordovez2, María Claudia Atencia3, Germán Aguilera1, Catalina Tovar4

1 Centro de Investigaciones en Microbiología y Parasitología Tropical, CIMPAT, Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá, Colombia, 2 Grupo de Biología Matemática, BIOMAC, Departamento de Ingeniería Biomédica, Facultad de Ingeniería, Universidad de Los Andes, Bogotá, Colombia, 3 Facultad de Ciencias de la Salud. Universidad del Sinú, Montería, Córdoba, Colombia, 4 Grupo de Enfermedades Tropiclas y Resistencia Bacteriana, Facultad de Ciencias de la Salud. Universidad del Sinú, Montería, Córdoba, Colombia

*a* Current address: Department of Biology, City College of New York, New York, NY, United States of America

*b* Current address: Department of Biology, Graduate Center, City University of New York, New York, NY, United States of America

*e.gonzalez2592@uniandes.edu.co*

Abstract

Leishmaniases are neglected tropical diseases exhibiting complex transmission cycles due to the number of parasite species circulating, sand fly species acting as vectors and infected mammals, including humans, which are defined in the New World as accidental hosts. However, current transmission scenarios are changing, and the disease is no longer exclusively related to forested areas but urban transmission foci occur, involving some species of domestic animals as suspected reservoirs. The aim of this study was to determine the transmission cycles in urban environments by evaluating sand fly diversity, detection of *Leishmania* DNA, and bloodmeal sources through intra and peridomestic collections. The study was carried out in Colombia, in 13 municipalities of Córdoba department, implementing a methodology that could be further used for the evaluation of vector-borne diseases in villages or towns. Our sampling design included 24 houses randomly selected in each of 15 villages distributed in 13 municipalities, which were sampled in two seasons in 2015 and 2016. Sand flies were collected using CDC light traps placed in intra and peridomestic habitats. In addition to the morphological identification, molecular identification through DNA barcodes was performed. A total of 19,743 sand flies were collected and 13,848 of them (10,268 females and 3,580 males) were used in molecular procedures. Circulation of two known parasite species—*Leishmania infantum* and *Leishmania panamensis* was confirmed. Blood source analyses showed that sand flies fed on humans, particularly in the case of the known *L. infantum* vector, *P. evansi*; further analyses are advised to evaluate the reservoirs involved in parasite transmission. Our sampling design allowed us to evaluate potential
transmission cycles on a department scale, by defining suspected vector species, parasite species present in different municipalities and feeding habits.

Introduction

Leishmaniases belong to the group of neglected tropical diseases affecting mainly low-income populations in Africa, Asia and Latin America. Transmission of the disease is strongly related to socioeconomic factors such as malnutrition and population displacement, among others [1]. Every year, 700,000 to one million new cases are recorded, 50,000 to 90,000 of visceral leishmaniasis (VL), and 20,000 to 30,000 deaths. For the cutaneous form of the disease (CL), the most common, one million cases are reported annually [1]. A mucocutaneous (MCL) form of the disease has also been described, which develops as a late reaction to previously untreated cutaneous lesions and may affect nasal cavities, the septum and the palate [2,3]. Urbanization of the leishmaniases has been recorded, particularly regarding the visceral form caused by Leishmania infantum [4,5,6] as well as variation in species’ spatial distribution and vector-parasite interactions [7,8].

The ecology of leishmaniases transmission is complex, due to the number of parasite species circulating (20 known to infect humans, 10 with public health importance), sand fly species acting as vectors (47 reported as proven in the New World) and mammals infected [7,9–11]. There is a lack of information regarding species acting as reservoirs in transmission foci. Although in America several groups of both wild and domestic mammals (rodents, marsupials, primates, canids, bats, and others) have been found to be infected with the parasites [12], their incrimination as reservoirs requires further research and is hard to accomplish [13,14].

Regarding vector species incrimination, procedures used for species identification such as clearing, make it difficult to perform molecular analyses for parasite detection. Establishment of vector feeding habits through bloodmeal source identification is also difficult because specimens must be processed within 96 hours of feeding to avoid blood digestion [15]. These difficulties impose important challenges from the perspective of disease ecology, since the identification of Leishmania parasite species infecting vectors and mammals is a critical step in the definition of transmission cycles, and their threat to humans [16].

In 2015, Colombia was among the ten countries recording the highest prevalence of leishmaniases in the world [1], while 10,743 cases of leishmaniasis were reported in 2016, 98.3% of them were of CL [7]. Leishmaniasis is an endemic disease commonly located in rural areas in the country, but can also be found in urban and peri-urban areas [17,18]. The disease is widely spread in the country, while VL foci are principally identified in the Magdalena River Valley region and the Caribbean coast. One important VL transmission focus is located in the department of Córdoba, in the municipality of San Andrés de Sotavento. In 2015 and 2016, Córdoba had the second highest number of VL cases in the country with seven and five cases reported each year respectively. Nevertheless, the greatest number of cases reported in the area corresponds to CL, with 218 cases recorded in 2015 and 105 in 2016. The locality of Tierralta contributed to more than half of these cases, however there is no local transmission in the municipality and all the cases seem to belong to soldiers of the Colombian Army performing surveillance in forested areas [19]. Parasites known to be circulating in the department are Leishmania braziliensis and Leishmania panamensis [20], while 15 species of phlebotomine with medical importance have been reported: Migonemyia migonei [21], Lutzomyia gomezi [7,22,23], Psychodopygus panamensis [7,22,23], Pintomyia evansi [7,24,25], Micropygomyia
cayennensis [22,23], Pintomyia rangeliana [22], Micropygomyia trinidadensis [23,25], Evandro-
myia dubitans, Psathyromyia carpenteri, Pressatia camposi, Pressatia dysponeta, Micropygomyia
micropyga, Nyssomyia yuilli yuilli, Micropygomyia atroclavata and Psathyromyia shannoni [23].
In spite of the high number of cases occurring in the department, it has been described as one
of the regions with the lowest sand-fly collection records [7]. The lack of eco-epidemiological
studies contributing to the understanding of disease transmission is remarkable, possibly due
to the political instability of the region that has always posed a challenge to fieldwork studies.

From this perspective, the aim of this study was to determine the transmission cycles in
urban environments by evaluating phlebotomine sand fly diversity, Leishmania parasite DNA
detection, and bloodmeal sources through intra and peridomestic collections. With this
approach, we wish to provide an overview of urban transmission cycles using a sampling
methodology for zoonoses and vector borne diseases that could be implemented in eco-epide-
miological studies in villages in other regions.

Materials and methods

Study site

The department of Córdoba, in northwest Colombia, has an elevational range between 260
and 2200 m.a.s.l. Fifteen localities within 13 municipalities out of the 30 present in the depart-
ment were selected to perform entomological collections, based on accessibility and security
(Fig 1, S1 Table). Within each selected locality a total of 24 houses were randomly sampled. In
localities for which satellite images with enough resolution to distinguish houses were available
in Google Earth [Google Earth (Version 7.1.8.3036) [Software]. Mountain View, CA: Google
Inc. (2009). Available from https://earth.google.com/] we spatially randomized house selection
(Fig 1). We created a fishnet of 25m² 25m covering all the area with houses in ArcMap 10.1
(ESRI, Redlands, CA, USA) and randomly selected 24 cells for sampling (Fig 1). Seven locali-
ties did not have available satellite images for sampling design (Villa Lucia-Sahagún, San Juan-
Puerto Libertador, Hoja Ancha-San Andrés De Sotavento, Punta Verde-Planeta Rica, Alto-
mirar-Moñitos, Guaimaro abajo-Los Córdobas and Pica Pica-Montelibano) so an initial
reconnaissance trip was made to georeference all the houses. Posteriorly, all houses were num-
bered and 24 houses were randomly selected for sampling using R (R Core Team (2015) R: A
language and environment for statistical computing).

To evaluate the transmission cycles in urban environments, we performed adult collections
in eight houses each night during three consecutive nights (for a total of 24 houses). In each
house a CDC light trap was located intradomiciliary and one in peridomiciliary areas. Traps
were activated at 6:00 pm and recovered at 6:00 am (sampling effort:12 hours/ night per
house) for immediate sample processing; insects were sacrificed by putting the collection jars inside a
bag containing triethylamine, and posteriorly sorted for each procedure. Each locality was vis-
ited twice and all fieldwork was conducted between August 21st 2015 and October 11th 2016.
Once sand flies were separated, they were placed in eppendorf tubes containing 70% ethanol.
All the collection tubes were taken for taxonomic identification and molecular processing to
the Center for Research in Microbiology and Tropical Parasitology (CIMPAT for its initials in
Spanish) at Universidad de Los Andes in Bogotá. Sand flies were sorted in groups using exter-
nal characteristics; 88% of the males and 6% of the females were then clarified and used for
species identification following Young and Duncan [26] and Galati [27], and to establish the
reference collection. Once the Phlebotominae fauna present in the collection sites was estab-
lished, the remaining females were identified following external characteristics such as wing
venation patterns, pigmentation, and length of the palpomeres, avoiding the clarification pro-
cess to optimize molecular procedures. When necessary, females were dissected without
clarification to complete species identification based on the spermatheca. The identified females were pooled per house with up to 20 individuals per pool to perform Leishmania DNA detection as described below; when only one individual was captured, DNA extraction was performed individually. Females containing blood in their abdomens were kept individually to perform blood source analyses. For all processed females, species confirmation was performed using barcode.

Fig 1. Sampling localities in the Cordoba department in Colombia. a. Córdoba department location in the country. b. Fifteen sampled localities in 13 municipalities of the Cordoba department. Colors correspond to the distribution of Leishmania species, in red *Leishmania infantum*, in yellow *L. panamensis* and in black localities with no parasites detected. c. Gridded sampling design with a 25m² 25m resolution built using satellite images to identify houses. On the grid 24 random cells were selected and the houses present inside were sampled in each locality. This grid is an example that corresponds to the Vereda el Vidrial, Cordoba, Colombia.

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Parasite detection and blood meal analyses

Polymerease chain reaction amplification. To detect sand fly natural infection with *Leishmania* parasites, DNA was extracted from pools with a maximum of 20 females, using ZR Tissue & Insect DNA Miniprep kit (Zymo CA, USA). Conventional PCR was performed with previously described primers specific for *Leishmania* heat-shock protein 70 gene (HSP70) from Hernández et al. [28] and rRNA intergenic region (ITS) from El Tai et al. [29]. The reaction was carried out in a volume of 25 μl using paired primers (10 μM each) and 2x GoTaq Green Master Mix (Promega WI, USA). After an initial denaturation at 95˚C for 5 min, PCR amplification was performed with 40 cycles of denaturation (95˚C, for 1 min), annealing (60˚C, for 1 min), and polymerization (72˚C, 1 min), followed by a final extension at 72˚C for 10 min. The PCR products were resolved by agarose gel electrophoresis, generating a product with a length of 300–350 bp.

High Resolution Melting HRM. Real-time PCR was coupled with HRM analysis using the HSP70 and ITS1 genes in a Real-time PCR system 7500 (Applied Biosystems, Inc., CA, USA) with 21 μl amplification reactions. The reaction mix contained 1X of Master Mix MeltDoctor HRM (Applied Biosystems, Inc., CA, USA), a 5 μM solution of each primer HSP70F (5’ AGG TGA AGG CGA CGA ACG 3’) and HSP70R (5’ CGC TTG TCC ATC TTYGCG TC 3’) (with one variation in one nitrogenous base) for the amplification of HSP70 and for ITS1, LITSR (5’ CTG GAT CAT TTT CCG ATG 3’) and L5.8S (5’ TGA TAC CAC TTA TCG CAC TT 3’) and 10 ng/μL of DNA template. Real-time PCR cycle conditions were adjusted to the protocol described by Hernadez et al [28] with variation in denaturation time (95˚C during 30 sec) and High Resolution Melting at 95˚C for 45 seconds.

Blood source analysis. To analyze the feeding preferences in each environment (intra and peridomicile), blood-fed females were stored in individual eppendorfs with 70% ethanol. Blood source identification was carried out using cytochrome b sequences. All DNA templates were tested with two primer pairs, mammals C described by Molaei et al [30] and a primer pair for avian species (GCCAAATATCATTTCTTAGGCCG[f], GGGCAATAGAAAAATATCATTTGTGG[r], 410 bp) described by Ferro et al [7]. Controls were used during extraction and amplification; the negative control for amplification consisted of a pool of sand fly legs, and the positive was DNA from dogs and humans. The amplification products were purified, sequenced and identified by comparing the DNA sequence to GenBank databases using Blast (National Center for Biotechnology Information).

DNA barcoding. To confirm species identification, a subsample of the collected specimens was DNA barcoded. The DNA barcode region from the COI gene was PCR-amplified from individual sand flies of both sexes. DNA extracts were prepared from a small sample of leg tissue or abdomen tissue in individuals lacking appendages and extraction was performed using the ZR Tissue & Insect DNA Miniprep kit (Zymo CA, USA). A PCR reaction, 1 μl total DNA were mixed with the following reagents: 12.5 μl of 2x GoTaq Green Master Mix, 10 μM of forward and reverse primers LCO1490 (5’ - GGTCAACAAATCATAAAGATTGG-3’) and HCO2198 (5’ - TAAAACCTCAGGGTGACCAAATATCA-3’) [31] to a final reaction volume of 25μl. The thermo cycling conditions consisted of one cycle of 1 min at 94˚C, 40 cycles of 40 s at 94˚C, 40 s at 52˚C, and 1 min at 72˚C, and finally 5 min at 72˚C, the size of the specific PCR product was 658 bp. All sand fly individuals that were *Leishmania* positive through HRM were confirmed by sequencing of the parasite CytB, and therefore only the infected specimens with an elevated identification percentage (97% or higher) in Blast were accepted as positive [20].
Eco-epidemiological and spatial analyses

Spatial and temporal distribution. To evaluate patterns of species’ spatial distribution, we tested for spatial aggregation of all vector species, *P. evansi* alone and all parasites separately using Moran’s I index for spatial autocorrelation in ArcMap 10.2 [ESRI 2011. ArcGIS Desktop: Release 10.2. Redlands, CA: Environmental Systems Research Institute]. Community analyses for sandfly composition were done using the vegan package for R [R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/], we computed the Bray-Curtis dissimilarity between sites and a posterior cluster analysis for visualization as implemented in the vegan package for R [R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/]. The same analysis was repeated for peridomiciliary and intradomiciliary samples separately and we computed the average distance between the two sampled communities using the Bray-Curtis and Jaccard indexes.

Epidemiological information. To evaluate infestation rates per house and identify infection patterns, a household survey designed for this study was performed. For each sampled house, we recorded the number of inhabitants, the presence of animals (divided in 11 categories) and the use of insecticides. We measured the correlation between the abundance of sand flies and the number of inhabitants and animals with a spearman correlation test. We also evaluated the relation between the use of insecticides and the abundance of insects. Analyses were performed separately for *L. infantum* and *L. panamensis* vectors.

Additionally, the relation between presence of parasite in sand flies and occurrence of human cases in the municipality was assessed. To establish infection rates since results were obtained by pool, we calculated the minimum infection rate assuming at least one infected sand fly per positive pool. The number of cases was recorded by the National System of Public Health Vigilance (SIVIGILA by its Spanish acronym) and were obtained for each municipality during the time of the study. All statistical analyses were done in R [R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/].

Results

Adult collection and species identification

A total of 19,743 phlebotomine sand flies were captured (15,527 females and 4,216 males), and 70% of the collected individuals (10,268 females and 3,580 males) were processed for species identification and molecular analyses. The remaining individuals belonged to a single municipality, San Andrés de Sotavento (5,945 individuals) and to a single species, *P. evansi*, but were not processed due to time limitation (Table 1).

Species morphologic identification following the clarification process was performed in 6% of the collected females (n = 618), and 90% of the males (n = 3,140). Non-clarified females were identified using external characters as described in the methods section, and 20% of them were dissected for confirmation using the spermatheca. All the non-clarified females were processed for molecular analyses. The confirmation of morphological identification was performed in 101 individuals using DNA barcoding, excluding blood fed females (Table 2). In total, eleven species were identified; *Pintomyia evansi* was the most abundant species (71% of all the identified sand flies) followed by *Micropygomyia cayennensis* 9.01% and *Lutzomyia gomezi* 7.09%.

Regarding species richness, at the scale of sampling localities, *M. cayennensis*, *P. rangeliana* and *L. gomezi* were collected in the 15 sampled localities, while *P. camposi*, was the rarest species, with only one individual present in San Juan (Puerto Libertador).
Nueva Unión, Los Córdobas and Tierralta were the sites with the greatest species richness, since nine sand fly species were present out of the eleven identified. The localities of Martinez and El Vidrial, on the other hand, had only three species of sandflies. Abundances in La Doc-trina and Martinez were very low with a total of 13 and 10 sand flies for each site, while the localities with the highest number of captured sand flies were Nueva Unión (n = 4,820) and Hoja Ancha (n = 4,465), which are both located in the township of San Andres de Sotavento. Summing the latest two localities, *P. evansi* accounted for more than 80% of the species presence in the whole department. Furthermore, this species was collected in every sampled house in ten localities. In Nueva Unión, a single house had 10% of the collections made for this species in the whole study (1,048 individuals, 784 of them collected intradomiciliary) (Fig 2).

### Table 1. Total number of collected sand flies by species in each of 15 localities in the Cordoba department in Colombia.

| Sand fly Species/ Village code | CGA | CM | LD | LMC | MAL | MBE | MPP | MV | PL | PV | SHA | SNU | SVL | TNU | VSR | TOTAL | Percent |
|-------------------------------|-----|----|----|-----|-----|-----|-----|----|----|-----|-----|-----|-----|-----|-----|-------|---------|
| *E. dubitans*                 | 77  | 2  | -  | 14  | 50  | 13  | -   | -  | -  | 6   | 96  | 158 | -   | 3   | 6   | 425   | 3.07%   |
| *M. cayennen sis*            | 96  | 7  | 4  | 2   | 228 | 162 | 165 | 18 | 10 | 111 | 99  | 107 | 192 | 27  | 21  | 1249  | 9.02%   |
| *L. gomelzi*                 | 459 | 4  | 1  | 59  | 61  | 88  | 29  | 3  | 42 | 15  | 55  | 64  | 27  | 60  | 16  | 983    | 7.10%   |
| *M. trinidadensis*           | 4   | -  | -  | 13  | 16  | 15  | -   | 3  | -  | 1   | 12  | -   | 6   | 1   | 13  | 84     | 0.61%   |
| *P. evansi*                  | 3   | -  | 1  | 407 | 593 | -   | -   | -  | -  | 32  | 4156| 4360| 573 | 1   | 2   | 10128  | 73.14%  |
| *P. rangeliana*              | 18  | 2  | 3  | 3   | 25  | 43  | 11  | 1  | 1  | 9   | 6   | 43  | 33  | 1   | 1   | 200    | 1.44%   |
| *P. camposi*                 | -   | -  | -  | -   | -   | -   | -   | -  | -  | -   | -   | -   | -   | -   | -   | 1      | 0.01%   |
| *P. dysponeta*               | -   | -  | -  | -   | 35  | -   | -   | -  | -  | -   | -   | -   | -   | 3   | 6   | 44     | 0.32%   |
| *P. carpenteri*              | 33  | -  | -  | 49  | 15  | 1   | 2   | -  | -  | -   | -   | -   | -   | 4   | -   | 104    | 0.75%   |
| *P. panamensis*              | 19  | -  | 1  | 3   | 29  | 20  | -   | 59 | 7  | 52  | 75  | 1   | 136 | 226 | 628 | 453    | 4.53%   |
| *P. shannoni*                | 1   | -  | -  | -   | -   | -   | -   | -  | -  | 1   | -   | -   | -   | 2   | -   | 0.01%  |
| **TOTAL**                    | 710 | 13 | 10 | 66  | 800 | 980 | 303 | 22 | 117| 182 | 4465| 4820| 839 | 236 | 291 | 13848  |         |
| **Percent**                  | 5.13%| 0.09%| 0.07%| 0.48%| 5.78%| 7.08%| 2.19%| 0.16%| 0.84%| 1.31%| 32.24%| 34.81%| 6.06%| 1.70%| 2.10%|         |         |

Species known as proven or suspected vectors.

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### Table 2. Results from DNA barcode analyses for species identification confirmation.

Samples were collected in 24 houses in each of 15 localities in the Cordoba department in Colombia a subset of the collected specimens were DNA barcoded using standard protocols and primers. The table shows the species identity according to morphology-based taxonomy and the reference sequence genbank number and ID for that species. All the samples belonged to single female specimens except for *P. evansi* specimens that were a pool of 10 individuals. We show the percentage of barcodes produced for each nominal species that matched the reference sequence, in parenthesis the number of matching nucleotides and total number of produced barcodes. References correspond to publications of the reference sequences.

| Species determination by taxonomy | GenBank Access number | Query cover | % Identity | Species | Reference |
|----------------------------------|-----------------------|-------------|------------|---------|-----------|
| *Psychodopygus panamensis*       | GU909460.1            | 99%         | 98% (669/683) | *Lutzomyia panamensis* | [32] |
| *Micropygomyia cayennensis*      | GU909472.1            | 96%         | 99% (679/682) | *Lutzomyia cayennensis cayennensis* | [32] |
| *Pintomyxia evansi*              | GU909458.1            | 100%        | 98% (659/662) | *Lutzomyia evansi* | [32] |
| *Lutzomyia gomezi*               | KC291248.1            | 98%         | 98% (685/699) | *Lutzomyia gomezi* | [33] |
| *Lutzomyia shannoni*             | GU909469.1            | 95%         | 98% (234/238) | *Lutzomyia shannoni* | [32] |
| *Micropygomyia trinidadensis*    | GU909498.1            | 97%         | 100% (658/681) | *Lutzomyia trinidadensis* | [32] |
| *Pintomyxia rangeliana*          | GU909493.1            | 98%         | 99% (675/681) | *Lutzomyia rangeliana* | [32] |
| *Evandromyia dubitans*           | GU909446              | 97%         | 99% (677/682) | *Lutzomyia dubitans* | [32] |
| *Psathyromyia carpenteri*        | GU909444.1            | 99%         | 98% (668/683) | *Lutzomyia carpenteri* | [32] |
| *Presatia dysponeta*             | GU001732.1            | 71%         | 97% (302/314) | *Lutzomyia dysponeta* | [34] |

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In general, 68% of the collected specimens were found peridomiciliary and 31.2% intradomiciliary, however, *M. cayennensis* was predominantly collected intradomiciliary and *P. shannoni* was collected in equal proportions in traps placed inside and outside of the households. Regarding localities, Punta Verde was the only one where more insects were collected inside households (53.3%), and equal proportions were obtained for El Vidrial.

**Parasite detection and blood meal analyses**

From the total sample of identified females, 90% (9,293) were processed to detect infection, and to identify the parasite species involved. Infection was analyzed by location, taking into account the number of houses where phlebotomine sand flies were identified as positive for *Leishmania* parasites (S2 Table).

After sequencing positive samples, two parasite species *Leishmania infantum* and *Leishmania panamensis* were identified in 24 pools containing from a single sand fly up to 20. *Leishmania infantum* was found in 16 pools from five households in three localities: Nueva Unión (three households, 13 positive pools) and Hoja Ancha (one household, two positive pools) in San Andres de Sotavento, and Villa Lucia in Sahagun (one household, one positive pool). Species identified as suspected vectors for the transmission of *Leishmania panamensis* based on their natural infection were *L. gomezi*, *M. cayennensis* and *P. panamensis*. Seven houses in five localities had infected sand flies with *Leishmania panamensis*. In tree localities (Moñitos, Los Cordobas and Lorica), only *L. gomezi* was found infected, while one pool of 11 *M. cayennensis* was found infected in Montelibano, and one pool of 16 *P. panamensis* females was found naturally infected in Valencia (Fig 1, Table 3). Regarding the minimum infection rate, the lower were obtained for *P. evansi* in Hoja Ancha and Villa Lucia and for *L. gomezi* in Moñitos. The highest infection rates were obtained for *L. gomezi* in Lorica because only 16 individuals were collected.

**Blood source analysis**

In total, 50 fed females were found and blood source species identification was performed. Half of the processed samples were identified as *P. evansi* (n = 26), followed by *L. gomezi* (n = 9), *P. panamensis* (n = 7), *P. rangeliana* (n = 4), *M. cayennensis* (n = 3) and *M. trinidadensis* (n = 1). Species confirmation was performed trough barcode, and no misidentifications were found (Table 4). To further confirm the absence of contamination with human DNA, we performed an additional experiment amplifying a 268bp fragment of the human beta globin gene (primers GH20/PC04) on 35 original sand flies DNA extracts. We managed to obtain amplification in 80% of the samples, confirming the presence of human DNA before any procedure. The remaining 20% (7 individuals) could be either degraded or contaminated during the process.

The most common blood source was human (72%), followed by chicken (12%) and dog (8%). Other blood sources were present in one specimen of *P. evansi* from Altomirar, which fed on pig, and two species feeding on birds: *M. cayennensis* on ducks and *M. trinidadensis* on *Cantorchilus leucotis*. The only specimen found to be feeding on wild mammals was one *P. panamensis* fed on *Marmosa robinsoni*.

*Pintomyia evansi* was the species with highest number of blood fed females and was found feeding on humans, chickens, dogs and pigs. In San Andres de Sotavento, *P. evansi* predominantly fed on humans (90% of the samples), one specimen in Hoja Ancha fed on *Gallus gallus* and one in Nueva Unión fed on dog (Table 4).

In other localities where sand flies infected with *L. panamensis* were detected and blood-fed females were captured, all the specimens were positive for human blood source: two *L. gomezi*, one in Bellacohita and one in Guaimaro Abajo, and two *P. panamensis* in San Rafael.
Fig 2. Sand fly species diversity and community composition in each of the 15 sampled localities in the Cordoba department in Colombia. Diversity was computed as total number of sand flies captured by locality (grouped from 24 houses, sampled both intra and peridomiciliary). Different colors represent different species of sand flies identified with traditional morphological techniques and confirmed via DNA barcoding.

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Eco-epidemiological and spatial analyses

Spatial and temporal distribution

Considering sand fly spatial distributions, tests of spatial aggregation showed that insects are randomly distributed in the sampling areas (Moran’s I = -0.066, Z-score = -0.32 and P-value = 0.75), a conclusion that holds when studying *P. evansi* only (Moran’s I = -0.122, Z-score = -1.11 and P-value = 0.27). The distribution of vectors infected with parasites is also random at the department scale (Moran’s I = -0.076, Z-score = -0.45 and P-value = 0.65).

Community similarity measures based on sand fly composition showed that towns are not clustered geographically (Fig 3). For example, Bellacohita is more closely related to Villa Lucia than to Altomirar although it is only a few kilometers away from the last one. Another example is that of Mata de Caña (in the north of the Department) and San Juan, the southernmost site sampled, that cluster together according to species composition. That is, presence of certain community assemblages might be driven by other factors such as land cover in the particular site.

Furthermore, community composition is different between intra and peridomiciliary samples with them sharing around half of the vector species (Bray Curtis = 0.4209348, Jaccard = 0.592475). When measuring community similarity based on either intra domiciliary samples or peridomiciliary samples alone, results in slightly different clustering are obtained. Two sites, Mata de Caña and Guaimaro Abajo have particularly unstable relations when changing the studied community (between intra and peridomiciliary communities). Regarding spatial distribution of infected houses, there was no spatial aggregation or any other identifiable pattern that could explain their distribution in each locality (Fig 3).

Epidemiological information

Regarding the number of cases recorded in Cordoba during the time of the study, 194 cases from 17 known municipalities were notified to the SIVIGILA. Seven of the VL, three of MCL and 184 of CL. Six of the municipalities that recorded cases were not included in our study and five were positive for sand fly presence but negative for parasite infection. The remaining
Table 4. Blood sources detected in sand fly specimens in each locality. Blood fed females were processed individually, identified based on external characters, and species confirmation was performed using DNA barcode procedures.

| Municipality           | Total screened (individually) | Morphological species identification | Species confirmation using barcodes | GenBank Access number | Blood source                  |
|------------------------|-------------------------------|--------------------------------------|-------------------------------------|-----------------------|-------------------------------|
| Cereté                 | 1                             | L. gomezi                            | L. gomezi                           | KC921254.1            | Gallus gallus                 |
| Lorica                 | 1                             | L. gomezi                            | L. gomezi                           | KC921254.1            | Canis lupus familiaris        |
|                        | 1                             | M. cayennensis                       | No amplification                    |                       | Canis lupus familiaris        |
|                        | 1                             | M. cayennensis                       | No amplification                    |                       | Anas platyrhynchos domesticus |
|                        | 2                             | P. evansi                            | P. evansi                           | GU909458.1            | Homo sapiens                  |
|                        | 1                             | P. rangeliana                        | No amplification                    |                       | Homo sapiens                  |
| Los Córdobas           | 1                             | L. gomezi                            | L. gomezi                           | KC921254.1            | Homo sapiens                  |
|                        | 1                             | P. panamensis                        | P. panamensis                       | GU909460.1            | Marmosa robinsoni             |
| Montelíbano            | 1                             | L. gomezi                            | L. gomezi                           | KC921254.1            | Homo sapiens                  |
| Montería               | 1                             | M. cayennensis                       | No amplification                    |                       | Homo sapiens                  |
| Moñitos                | 1                             | L. gomezi                            | No amplification                    | KC921247.1            | Gallus gallus                 |
|                        | 1                             | L. gomezi                            | L. gomezi                           | KC921254.1            | Homo sapiens                  |
|                        | 1                             | L. gomezi                            | No amplification                    | KC921254.1            | Sus scrofa domestica          |
|                        | 1                             | P. evansi                            | P. evansi                           | GU909457.1            | Homo sapiens                  |
|                        | 1                             | P. rangeliana                        | No amplification                    |                       | Homo sapiens                  |
| Planeta Rica           | 1                             | L. gomezi                            | L. gomezi                           | KC921254.1            | Homo sapiens                  |
|                        | 1                             | P. evansi                            | P. evansi                           | GU909457.1            | Homo sapiens                  |
|                        | 1                             | P. rangeliana                        | No amplification                    |                       | Gallus gallus                 |
| Puerto Libertador      | 1                             | P. panamensis                        | P. panamensis                       | GU909460.1            | Homo sapiens                  |
| Sahagún                | 1                             | P. evansi                            | P. evansi                           | GU909458.1            | Homo sapiens                  |
|                        | 1                             | P. evansi                            | P. evansi                           | GU909458.1            | Gallus gallus                 |
|                        | 1                             | P. rangeliana                        | No amplification                    |                       | Canis lupus familiaris        |
|                        | 1                             | M. trinidadensis                     | No amplification                    |                       | Cantorchilus leucotis         |
| San Andrés de Sotavento| 2                             | L. gomezi                            | L. gomezi                           | KC921247.1            | Homo sapiens                  |
|                        | 1                             | P. evansi                            | P. evansi                           | GU909458.1            | Canis lupus familiaris        |
|                        | 1                             | P. evansi                            | P. evansi                           | GU909458.1            | Homo sapiens                  |
|                        | 1                             | P. evansi                            | No amplification                    |                       | Gallus gallus                 |
|                        | 1                             | P. panamensis                        | P. panamensis                       | GU909460.1            | Homo sapiens                  |
|                        | 1                             | P. panamensis                        | P. panamensis                       | GU909460.1            | Gallus gallus                 |
| Tierra Alta            | 1                             | P. panamensis                        | P. panamensis                       | GU909460.1            | Homo sapiens                  |
| Valencia               | 2                             | P. panamensis                        | P. panamensis                       | GU909460.1            | Homo sapiens                  |

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six municipalities where cases were reported also had records of infected sandflies. San Andrés de Sotavento recorded 12 CL cases and two VL cases, however we detected only *L. infantum* present in sand flies. On the contrary, Sahagún didn’t record human cases but one positive pool was detected for *L. infantum*; in Moñitos one VL case was recorded but only *L. panamensis* was isolated from sand flies. Regarding the cutaneous and mucocutaneous forms, Lorica reported one MCL case and in this municipality 2/24 households were positive for *L. panamensis* in *L. gomezi*. Los Córdobas recorded one CL case and we detected 2/24 households positive for *L. panamensis* in *L. gomezi*. Lastly, Valencia recorded seven CL cases and *L. panamensis* was detected in *P. evansi*, while in Montelíbano 4 CL cases were reported and *M. cayennensis* was found infected with *L. panamensis*.

In general, there was no correlation between the number of infected pools and the local abundances of *P. evansi* collected; however, in Nueva Unión, the single household that
accounted for 10% of the collected *P. evansi* in the whole study, had ten pools positive for *L. infantum*. As reported for *L. infantum* infection, no correlation was found between the number of infected pools and sand fly local abundances.

The abundance of vectors for both parasites *L. infantum* and *L. panamensis*, was moderately correlated with the number of animal categories present in the household (\(\rho = 0.257\), p-value = \(8.2 \times 10^{-7}\) and \(\rho = 0.20\), p-value = 0.00015 respectively). There was no significant correlation between abundance of vectors for either parasite and inhabitants of the household (\(\rho = 0.076\), p-value = 0.152 and \(\rho = 0.087\), p-value = 0.149 respectively). No relation was found between insecticide use and the abundance of vectors (p-value = 0.604 and p-value = 0.381 respectively).

**Discussion**

In this study, we identified key factors related to *Leishmania* transmission in urban localities: by using different approaches and an important sampling effort we were able to collect and identify phlebotomine sand flies, while also detecting parasite species and blood sources in an accurate way. Our study design can be applied to other areas where the disease exhibits similar occurrence patterns and the acquisition of eco epidemiological information is needed.

Two parasite species were identified: *L. infantum*, present in three villages in two municipalities, all located in the northeast, and *L. panamensis*, known to cause MCL and CL, in five municipalities, with sparse distribution. However, infection rates in sand flies didn’t reflect the recorded occurrence of cases in the sampled municipalities; possibly infected sand flies are not...
reaching the threshold necessary to produce human cases, or underreporting of cases from infected localities are missing in the national epidemiological reports. However, we could identify infected sand flies and human blood sources in municipalities where transmission is occurring, providing important information to health authorities, as we will discuss further.

The use of DNA barcodes allowed the confirmation of morphologic identifications for 101 sand fly individuals and flagged only four individuals as misidentifications, reinforcing the reliability of our results. In all cases, the identity of the generated barcodes with the reference database was higher that 97%. In this way, the technique constitutes an important tool to process simultaneously samples both for species identification and parasite detection without compromising the DNA integrity in the clearing process.

Parasite detection and species identification from sand flies was successful in this study, however the HRM technique, with parasite DNA extracted from sand flies did not perform as expected [28]. Therefore, sequencing of the parasite’s Cytb was the best alternative to perform adequate species identification. Although HRM is promising, it needs to be further validated with field samples to achieve a consensus in the algorithm used for species discrimination and the melting temperature ranges for each *Leishmania* species.

All the sand fly species that we found infected have been previously reported in the country as species with medical importance, they have anthropophilic habits, and are widely distributed, characteristics that reinforce their potential to act as vectors [23]. *Micropygomyia cayennensis* is a species widely distributed in Central and South America and has been found infected with unidentified promastigotes in Cordoba, Tolima and Cundinamarca [35]; this species is commonly collected with the primary vectors *P. evansi* or *L. longipalpis* in VL transmission foci [36,37]. It is known to feed on cold-blooded vertebrates [26] but has also been collected inside households [21] and in high abundances, being the second most abundant species after *P. evansi* in Montes de María [26,38]. In high densities, it has been reported biting humans [35]. Regarding *P. panamensis* and *L. gomezi*, they are known vectors of *Leishmania panamensis* in Panamá [39] and *Leishmania braziliensis* in Venezuela [40,41] and in Colombia both species have been found infected with *L. panamensis* in the Boyacá Department [42]. In Colombia, *L. gomezi* is distributed in different ecosystems, from moist and dry forest [37, 43, 44] to urban areas where it is known to be highly anthropophilic [45,46]. The importance of this species as a vector is increasing due to its ability to adapt to transformed ecosystems.

Infection rates detected in our study, varied for *L. infantum* from 0.06% in Hoja Ancha to 0.42 in Nueva Unión, both located in San Andrés de Sotavento. In the same municipality, Montoya-Lerma et al [47], established *P. evansi* infection rates with *L. infantum* in 0.05%, while in Venezuela it was 0.23% [47].

Regarding *P. panamensis*, infection rates varied from 0.15% to 0.74% in those localities where more than 100 sand flies were processed, and reached 6.25% in Lorica where only 16 individuals were analyzed. In a well-characterized transmission focus in Chaparral, infection rates of *Pintomyia longiflora* infected with *L. guyanensis* were 0.2% [8]. Insect’s local abundances were not related to infection rates; it is necessary to perform further analysis aiming to define the key parameters involved in parasite presence or prevalence, for example infection of vertebrate species present in households.

The fact that insects were collected in similar proportions inside and outside households suggest that insects attracted to light can easily encounter humans. Although they can be breeding outside, their bloodmeals can occur inside, as the high proportion of human blood source detected in our samples suggests. All the infected sand fly species fed mostly on humans except for *M. cayennensis* that was found equally feeding on humans, dogs and birds.

*Pintomyia evansi*, the most abundant species, has been incriminated as the vector of *L. infantum* in San Andrés de Sotavento, and dogs and *Didelphis marsupialis* have been identified...
as the zoonotic reservoirs [48, 49]. A study developed by Montoya-Lerma et al [48], aiming to confirm *P. evansi*’s anthropophilic behavior in San Andrés de Sotavento, demonstrated a clear preference of *P. evansi* towards human blood over dogs and opossums, and no significant difference in preference between the last two. Nevertheless, preference distinction depended on sand fly abundances; when low densities where identified no significant host preferences were determined but when high density was identified there was a marked preference for humans; in the present study, human blood source was detected in most of the *P. evansi* sand flies in localities with overwhelmingly high abundances [48]. It would be important to follow the appearance of cases in these localities since perhaps this scenario can be used as an alert to predict an outbreak. High local abundances of vector species can push a switch in feeding habits increasing the contact between competent vectors and humans; the role of humans as reservoirs has not been established yet, however, in Colombia, there is evidence of *Leishmania* being present in human skin samples in the absence of lesions [50].

A study developed in Sucre, found that dogs are less attractive to sand flies in the presence of other animals like cattle, pigs and donkeys [51]. Pigs and chickens raised close to households constitute other species with epidemiological relevance, since their habitat provides breeding sources for sand flies in peridomestic habitats [52–54]. Birds are refractory to *Leishmania* transmission, but are an attractive blood source for sand flies. Here we detected domestic (chickens and ducks), but also sylvatic (*Thryothorus leucotis*) avian blood sources. On the other hand, avian blood sources could promote a dilution effect, making it likely that in areas where sand flies feed on them, parasite prevalence in insects and mammals, including humans, is low [55]. Nevertheless a study developed by Morrison et al [56] demonstrated a clear preference of *L. longipalpis* towards cows and pigs over chickens, and hen houses can actullay be playing the role as attractants to various other reservoirs, including dogs used as protectors, therefore enabling *Leishmania* cycle development [11]. The number of engorged sand flies that we could detect is not enough as to accomplish further analysis on this direction; however the evidence they provide is important and poses important questions to be further addressed. Future work is required to evaluate which could be the main reservoir in the region.

Considering spatial distribution analyses, infected houses do not show spatial patterns of distribution and infection is not correlated to the number of sand flies present in each house either. Studies on breeding sites could contribute exceptional information in order to detect the key factors influencing sand fly local abundances.

From the epidemiological perspective, our sampling design cannot be correlated with the occurrence of cases in the department. Sampling at a local scale can provide useful information, however in order to be able to describe a proper transmission scenario, this kind of study should be established in localities were human cases have been identified so that the link between all the variables and the risk of parasite transmission to humans can be determined.

Although we are detecting natural infection and blood sources, our results are not sufficient to determine variables leading to differential sand fly local abundances and infection. Further analyses are required to better understand how these variables relate to human cases and which species could be acting as reservoirs for the disease.

**Supporting information**

S1 Table. Localities included in the study.
(DOC)

S2 Table. Comparison of identification methods for *Leishmania* species.
(DOC)
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Author Contributions

Conceptualization: Camila González, Mario Ortiz, Juan Manuel Cordovez, Catalina Tovar.

Data curation: Cielo León, Andrea Paz, Marla López, Gisell Molina, Diana Toro, María Claudia Atencia, Germán Aguilera.

Formal analysis: Camila González, Cielo León, Andrea Paz, Marla López, Gisell Molina, Diana Toro, Mario Ortiz, María Claudia Atencia, Germán Aguilera.

Funding acquisition: Camila González, Catalina Tovar.

Investigation: Camila González, Cielo León, Andrea Paz, Marla López, Gisell Molina, Diana Toro, Mario Ortiz, Juan Manuel Cordovez, María Claudia Atencia, Germán Aguilera, Catalina Tovar.

Methodology: Camila González, Andrea Paz, Gisell Molina, Diana Toro, Juan Manuel Cordovez, María Claudia Atencia.

Project administration: Camila González, Cielo León, Catalina Tovar.

Resources: Catalina Tovar.

Supervision: Camila González, Mario Ortiz, Juan Manuel Cordovez, Catalina Tovar.

Validation: Cielo León, Germán Aguilera.

Visualization: Andrea Paz.

Writing – original draft: Camila González, Cielo León, Andrea Paz, Marla López, Gisell Molina, Diana Toro, Mario Ortiz, Juan Manuel Cordovez.

Writing – review & editing: María Claudia Atencia, Catalina Tovar.

References

1. World Health Organization. Fact sheet. Updated April 2017. Available from http://www.who.int/mediacentre/factsheets/fs375/en/. Cited 14 September 2017.
2. Velez ID, Hendrickx E, Robledo SM, Agudelo S del P. Leishmaniiasis cutánea en Colombia y género. Cad Saude Publica. 2001; 17(1):171–80. PMID: 11241940
3. González M, Benito F, García L, Iglesias A. Mucocutaneous leishmaniasis: an imported illness with ENT repercussions. Acta Otorrinolaringol espanhola. 2009; 60(4):298–300.
4. Acosta L, Díaz R, Torres P, Silva G, Ramos M, Fattore G, et al. Identification of Leishmania infantum in Puerto Iguazu, Misiones, Argentina. Rev Inst Med Trop Sao. 2015; 57(2):175–6.
5. González C, Paz A, Ferro C. Predicted altitudinal shifts and reduced spatial distribution of Leishmania infantum vector species under climate change scenarios in Colombia. Acta Trop [Internet]. 2014 Jan [cited 2014 Sep 28]; 129(83–90. https://doi.org/10.1016/j.actatropica.2013.08.014 PMID: 23988300
6. Ferro C, López M, Fuya P, Lugo L, Cordovez JM, González C. Spatial distribution of sand fly vectors and eco-epidemiology of cutaneous leishmaniasis transmission in Colombia. PLoS One. 2015; 10(10):1–16.

7. Harhay M, Olliaro P, Costa D, Nery C. Urban parasitology: visceral leishmaniasis in Brasil. Trends in Parasitology. 2011; 27(9):403–09. https://doi.org/10.1016/j.pt.2011.04.001 PMID: 21596622

8. Ferro C, Marín D, Góngora R, Carrasquilla MC, Trujillo JE, Rueda NK, et al. Phlebotomine vector ecology in the domestic transmission of American cutaneous leishmaniasis in Chaparral, Colombia. Am J Trop Med Hyg. 2011 Nov [cited 2013 Mar 7]; 85(5):847–56. https://doi.org/10.4269/ajtmh.2011.10-0560 PMID: 22049038

9. Akhoundi M, Kuhlis K, Canning A, Votýpka J, Marty P, Delaunay P, et al. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sand flies. PLoS Negl Trop Dis. 2016; 10(3):1–40.

10. Cotton JA. The Expanding World of Human Leishmaniasis. Trends Parasitol. 2017; 33(5):341–4. https://doi.org/10.1016/j.pt.2017.02.002 PMID: 28319012

11. Alexander B, Lopes de Carvalho R, McCallum H, Pereira MH. Role of the domestic chicken (Gallus gallus) in the epidemiology of urban visceral leishmaniasis in Brazil. Emerg Infect Dis. 2002; 8(12):1480–5. https://doi.org/10.3201/eid0812.010485 PMID: 12498667

12. Ashford R. Leishmaniasis reservoirs and their significance in control. Clin Dermatol. 1996; 14:523–32. PMID: 8889331

13. Rotureau B. Ecology of the Leishmania species in the Guianan ecoregion complex. Am J Trop Med Hyg. 2006; 74(1):81–96. PMID: 16407350

14. Travi B, Arteaga L, Leon A, Adler G. Susceptibility of Spiny Rats (Proechimys semispinosus) to Leishmania (Viannia) panamensis and Leishmania (Leishmania) chagasi. 2002; 97(September):887–92. PMID: 12386716

15. Abbasi I, Cunio R, Warnburg A. Identification of Blood Meals Imbibed by Phlebotomine Sand Flies Using Cytochrome b PCR and Reverse Line Blotting. Vector-Borne Zoonotic Dis. 2009; 9(1):79–86. https://doi.org/10.1089/vbz.2008.0064 PMID: 18973440

16. Sant’Anna M, Jones N, Hindley A, Mendes-Sousa R, Dillon R, Cavalcante R, Alexander B, Bates P. Blood meal identification and parasite detection in laboratory-fed and field-captured Lutzomyia longipalpis by PCR using FTA databasing paper. Acta Trop. 2008; 107:230–37. https://doi.org/10.1016/j.actatropica.2008.06.003 PMID: 18606150

17. Bejarano EE, Uribe S, Rojas W, Vélez ID. Phlebotomine sand flies (Diptera: Psychodidae) associated with the appearance of urban leishmaniasis in the City of Sincelejo, Colombia. Mem Inst Oswaldo Cruz. 2002; 97:645–7. PMID: 12219128

18. Cortés LA, Fernández JJ. Especies de Lutzomyia en un foco urbano de leishmaniasis visceral y cutánea en El Carmen de Bolívar, Bolívar, Colombia. Biomedica. 2008;433–40.

19. Patiño-londoño SY, Salazar LM, Acero CT, Velez Bernal ID. Aspectos socioepidemiológicos y culturales de la leishmaniasis cutánea: concepciones, actitudes y prácticas en las poblaciones de Tierralta y Valencia, (Córdoba, Colombia). Salud Colect. 2017; 13(1):123–38. https://doi.org/10.18294/sc.2017.1079

20. Ramirez JD, Hermández C, León CM, Ayala MS, Flórez C, González C, et al. Taxonomy, diversity, temporal and geographical distribution of Cutaneous Leishmaniasis in Colombia: A retrospective study. Sci Rep. 2016; 6.

21. Montoya-Lerma J, Ferro C. Flebótomos (Diptera: Psychodidae) de Colombia. Santafé de Bogotá: Centro Editorial Javeriano; 1999.

22. Contreras MA. Lutzomyia spp. (Diptera: Psychodidae) en zonas cafeteras de la región andina colombiana: taxonomía e importancia médica. M.Sc Thesis, Universidad Nacional de Colombia. 2013. Available from: http://www.bdigital.unal.edu.co/9510/1/64703281.2013.pdf

23. Vivero RJ, Quintero LS, Peña HC, Alvar-Beltrán J, Tovar C, Atencia CM, et al. Composition and distribution of medically important phlebotomines (Diptera: Psychodidae) in the municipalities of Tierralta and Valencia (córdoba, Colombia). J Vector Borne Dis. 2017; 54(1):87–95. PMID: 28352051

24. González C, Cabrera OL, Munstermann LE, Ferro C. Distribución de los vectores de Leishmania infantum (Kinetoplastida: Trypanosomatidae) en Colombia. Biomédica. 2006; 26(24):64–72.

25. Bejarano EE, Sierra D, Vélez ID. Novedades en la distribución geográfica del grupo verrucarum (Diptera: Psychodidae) en Colombia. Biomédica. 2003; 23:341–50.

26. Young D, Duncan M. Guide to identification and geographic distribution of Lutzomyia and sand flies in Mexico, West Indies, Central and South America (Diptera: Psychodidae). Mem Am Entomol, 1994

27. Galati EAB. Apostila de Bioecologia e Identificação de Phlebotominae (Diptera, Psychodidae)—Departamento de Epidemiologia, Faculdade de Saúde Pública da USP, São Paulo, Brasil, 2005.
28. Hernández C, Alvarez C, González C, Ayala MS, León CM, Ramírez JD. Identification of Six New World Leishmania species through the implementation of a High-Resolution Melting (HRM) genotyping assay. Parasit Vectors. 2014; 7(501):1–7.

29. El Tai N, Osman O, El Fari M, Presbier W, Schönnian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. T Roy Soc Trop Med H. 2000; 94(5):575–579.

30. Molaei G, Andreatis TG, Armstrong PM, Diuk-Wasser M. Host-feeding patterns of potential mosquito vectors in Connecticut, U.S.A.: molecular analysis of bloodmeals from 23 species of Aedes, Anopheles, Culex, Coquillettidia, Pseudophora, and Uranotaenia. J Med Entomol. 2008; 45(6):1143–51. PMID: 19058640

31. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994; 3(5):294–9. PMID: 7881515

32. Romero RL, Lastre M, Pérez A, Bejarano E. DNA barcoding to identify species of phlebotomine sand fly (Diptera: Psychodidae) in the mixed leishmaniasis focus of the Colombian Caribbean. Acta Trop. 2016; 159:125–31. https://doi.org/10.1016/j.actatropica.2016.03.017 PMID: 26992298

33. Contreras MA, Vivero RJ, Velez ID, Porter CH, Uribe S. DNA Barcoding for the identification of sand fly species (Diptera, Psychodidae, Phlebotominae) in Colombia. PLoS Negl Trop Dis. 2014; 9(1), e85496.

34. Azpurua J, De La Cruz D, Valderama A, Windsor D. Lutzomyia sand fly diversity and rates of infection by Woltbachia and an exotic Leishmania species on barro Colorado Island, Panama. PLoS Negl Trop Dis. 2010; 4(3), E627. https://doi.org/10.1371/journal.pntd.0000627 PMID: 20231892

35. Cochero S, Anaya Y, Díaz Y, Paternina M, Luna A, Paternina L, et al. Infección natural de Lutzomyia cayennensis cayennensis con parasitos tripanosomatídeos (Kinetoplastida: Trypanosomatidae) en Los Montes de María, Colombia. Rev Cubana Med Trop. 2007; 59(1):35–9. PMID: 23427416

36. Travi BL, Adler GH, Lozano M, Cadena H, Montoya-Lerma J. Impact of habitat degradation on phlebotomine (Diptera: Psychodidae) of tropical dry forests in Northern Colombia. J Med Entomol. 2002; 39(3):451–6. PMID: 12061439

37. Ferro C, Morrison AC, Torres M, Pardo R, Wilson ML, Tesh RB. Age structure, blood-feeding behavior and Leishmania chagasi infection in Lutzomyia longipalpis (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. J Med Entomol. 1995; 32(5):618–629. PMID: 7473616

38. Le Pape P. Écophi diologie de la leishmaniose à Leishmania infantum = L. chagasi dans la plaine des caraïbes (Colombie): corrélation vecteur et réservoir canin. PhD thesis. Université de Montpellier. 1992. Available from: http://www.theses.fr/1992MON1T005

39. Christensen HA, Fairchild GB, Herrera A, Johnson CM, Young DG, Vasquez AN. The ecology of cutaneous leishmaniasis in the Republic of Panama. J Med Entomol. 1983; 20: 463–484 PMID: 6358496

40. Feliciangeli MD, Rabinovich J. Abundance of Lutzomyia ovallesi but not Lu. gomezi (Diptera: Psychodidae) correlated with cutaneous leishmaniasis incidence in north-central Venezuela. Med Vet Entomol 1998; 12: 121–131. PMID: 9622364

41. Rodríguez N, Aguilar CM, Barrios MA, Barker DC. Detection of Leishmania braziliensis in naturally infected in-dividual sand flies by the polymerase chain reaction. Trans R Soc Trop Med Hyg 1999; 93: 47–49.

42. Santamaría E, Ponce N, Zipa Y, Ferro C. Presencia en el peridomicilio de vectores infectados con Leishmania (Vianna) panamensis en dos focos endémicos en el occidente de Boyacá, piedemonte del valle del Magdalena medio, Colombia. biomedica. 2006; 26:82–94.

43. Barreto M, Burbano ME, Barreto P. Lutzomyia Sand Flies (Diptera: Psychodidae) from Middle and Lower Putumayo Department, Colombia, with New Records to the Country. Mem Inst Oswaldo Cruz. 2000; 95(Feb ruary):633–9. PMID: 10998213

44. Duque P, Velez ID, Morales M, Sierra D. Sand flies fauna involved in the transmission of cutaneous leishmaniasis in Afro-Colombian and Amerindian communities of Choco, Pacific Coast of Colombia. Neotropical Entomology. 2004; 33 (2): 255–264.

45. Sandoval CM, Angulo VM, Gutierrez R, Muñoz G, Ferro C. Espèces de Lutzomyia (Diptera: Psychodidae) possibles vectores de leishmaniasis en la ciudad de Bucaramanga, Santander, Colombia. biomedica. 1998; 18(2):161–8.

46. Bejarano EE, Uribe S, Rojas W, Vélez ID. Phlebotomine sand flies (Diptera: Psychodidae) associated with the appearance of urban leishmaniasis in the City of Sincelejo, Colombia. Mem Inst Oswaldo Cruz. 2002; 97:645–7. PMID: 12219128

47. Montoya-Lerma J, Cadena H, Oviedo M, Ready P, Barazarte R, Travi B, et al. Comparative vectorial efficiency of Lutzomyia evansi and Lu. longipalpis for transmitting Leishmania chagasi. Acta Tropica. 2003; 85: 19–29 PMID: 12505180
48. Montoya-Lerma J, Lane RP. Factors affecting host preference of *Lutzomyia evansi* (Diptera: Psychodidae), a vector of visceral leishmaniasis in Colombia. Bull Entomol Res. 1996; 86(1):43.

49. Travi BL, Jaramillo C, Montoya J, Segura I, Zea A, Goncalves A, et al. Didelphis marsupialis, an important reservoir of *Trypanosoma* (schizotrypanum) *cruzi* and *Leishmania (Leishmania) chagasi* in Colombia. Am J Trop Med Hyg. 1994; 50(5):557–65. PMID: 8203703

50. Vergel C, Palacios R, Cadena H, Posso CJ, Valderrama L, Perez M, et al. Evidence for *Leishmania (Viannia)* Parasites in the Skin and Blood of Patients Before and After Treatment. J Infect Dis. 2006; (3):503–11.

51. Paternina LE, Verbel-Vergara D, Romero-Ricardo L, Pérez-Doria A, Paternina-Gómez M, Martínez L, et al. Evidence for anthropophily in five species of phlebotomine sand flies (Diptera: Psychodidae) from northern Colombia, revealed by molecular identification of bloodmeals. Acta Trop. 2016; 153:86–92. https://doi.org/10.1016/j.actatropica.2015.10.005 PMID: 26464046

52. Feliciangeli M. D. Natural breeding places of phlebotomine sand flies. Medical and veterinary entomology. 2004; 18(1), 71–80. PMID: 15009450

53. Vieira VP, Ferreira AL, dos Santos CB, Leite GR, Ferreira GE, Falqueto A. Peridomestic breeding sites of Phlebotomine sand flies (Diptera: Psychodidae) in an endemic area of American cutaneous leishmaniasis in Southeastern Brazil. The American journal of tropical medicine and hygiene. 2012; 87 (6):1089–1093. https://doi.org/10.4269/ajtmh.2012.12-0470 PMID: 23091196

54. Vivero RJ, Torres-Gutierrez C, Bejarano EE, Peña HC, Estrada LG, Florez F et al. Study on natural breeding sites of sand flies (Diptera: Phlebotominae) in areas of *Leishmania* transmission in Colombia. Parasites & vectors. 2015; 8(1):116.

55. Schmidt KA, Ostfeld RS. Biodiversity and the dilution effect in disease ecology. Ecology. 2001; 82 (3):609–19.

56. Morrison AC, Ferro C, Tesh RB. Host preferences of the sand fly *Lutzomyia longipalpis* at an endemic focus of American visceral leishmaniasis in Colombia. Am J Trop Med Hyg. 1993; 49:68–75. PMID: 8352394