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Phlebotomine (Diptera: Psychodidae) species and their blood meal sources in a new leishmaniasis focus in Los Montes de María, Bolivar, Northern Colombia

Especies de flebotomíneos y sus fuentes de ingesta sanguínea en un nuevo foco de leishmaniasis en Los Montes de María, Bolívar, Norte de Colombia

Phlebotomine and its blood meals, northern Colombia

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Marlon Mauricio Ardila and Alveiro Pérez-Doria: conceptualized and planned the study.

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Yeisson Cera-Vallejo, Marlon Mauricio Ardila and Alveiro Pérez-Doria: conducted specimen collection, dissections, and taxonomical identification.

Yeisson Cera-Vallejo, Lina Martínez and Alveiro Pérez-Doria: performed molecular biology assays.

Yeisson Cera-Vallejo and Marlon Mauricio Ardila: drafted the initial english version of the manuscript, and all authors subsequently contributed to revisions.

Introduction. In 2018, El Alférez Village in Los Montes de María (Bolívar Department-Northern Colombia), macro-focus of leishmaniasis, recorded its first case, highlighting changes in the distribution and eco-epidemiology of the disease, although interactions between vectors and local fauna remain unknown.

Objetive. To evaluate the diversity of sandflies and their blood meal sources in the community of El Alférez, El Carmen de Bolívar (Department of Bolívar-Colombia).

Materials and methods. Sandflies were collected in 2018 using LED-based light traps in domestic/peridomestic/sylvatic ecotopes and identified at the species level. Multiplex PCR targeting the mitochondrial Cytb gene was used to analyse blood from the digestive tract.

Results. *Lutzomyia evansi* was the most abundant species (71.85%-n=485/675), followed by *Lutzomyia panamensis, Lutzomyia gomezi, Lutzomyia trinidadensis, Lutzomyia dubitans*, *Lutzomyia abonnenci* and *Lutzomyia aclydifera*. 25.00% (n=25/100) of the species analyzed showed bloodmeals from *Canis familiaris* (36.00%-n=9/25), *Ovis aries* (36.00%-n=9:/25), *Bos taurus* (24.00%-n=6:/25)*, Sus scrofa* (20.00%-n=5:/25) and *Homo sapiens* (8.00%-n=2/25). *Lu. evansi* was the species with the highest feeding frequency (68.00%-n=17/25), predominantly on a single species (44.00%-n=11/25) or a combination of different species (24.00%-n=6/25).

Conclusion. Results indicate eclectic feeding behaviour in *Lu. evansi*, implying potential reservoir hosts for *Leishmania* spp. and increasing transmission risk. This study is a first step towards understanding the diversity of mammalian blood sources used by the sandflies community. This is crucial for vector identification and the formulation of effective control measures.

Keywords: *Lutzomyia evansi*; *Lutzomyia panamensis; Lutzomyia gomezi;* multiplex polymerase chain reaction; citocromos B.

Introducción. En 2018, la Vereda El Alférez en Los Montes de María (Departamento de Bolívar-Norte de Colombia), macrofoco de leishmaniasis, reportó su primer caso, destacando cambios en la distribución y ecoepidemiología de la enfermedad, aunque las interacciones entre vectores y fauna local son desconocidas.

Objetivo. Evaluar la diversidad de flebotomíneos y su fuente de alimentación sanguínea en la comunidad de El Alférez, El Carmen de Bolívar (Departamento de Bolívar-Colombia).

Materiales y métodos. Se recolectaron los flebotomíneos en 2018 utilizando trampas de luz LED en ecótopos domésticos/peridomésticos/silvestres y se identificaron a nivel de especie. Se utilizó una PCR múltiple dirigida al gen mitocondrial Cytb para analizar la sangre del tracto digestivo.

Resultados. *Lutzomyia evansi* fue la especie más abundante (71,85%-n=485/675), seguida por *Lutzomyia panamensis, Lutzomyia gomezi, Lutzomyia trinidadensis, Lutzomyia dubitans, Lutzomyia abonnenci* y *Lutzomyia aclydifera*. El 25,00% (n=25/100) de las especies analizadas mostraron fuentes de ingesta sanguínea de *Canis familiaris* (36,00%-n=9/25), *Ovis aries* (36,00%-n=9/25), *Bos taurus* (24,00%-n=6/25), *Sus scrofa* (20,00%-n=5/25) y *Homo sapiens* (8,00%-n=2/25). *Lu. evansi* mostró ser la especie con la mayor frecuencia de alimentación (68,00%-n=17/25), predominantemente de una sola especie (44,00%-n=11/25) o de una combinación de diferentes especies (24,00%-n=6/25).

Conclusiones. Los hallazgos indican un comportamiento alimenticio ecléctico en *Lu. evansi,* implicando potenciales reservorios para *Leishmania* spp. y elevando el riesgo de transmisión. Este estudio es un primer paso para comprender la diversidad de fuentes sanguíneas de los mamíferos utilizadas por los flebotomíneos. Esto es crucial para identificación del vector y la formulación de medidas de control eficaces.

Palabras clave: *Lutzomyia evansi*; *Lutzomyia panamensis; Lutzomyia gomezi;* reacción en cadena de la polimerasa multiplex; citocromos B.

Phlebotomine sandlflies (Diptera, Psychodidae: Phlebotominae) are vectors of *Leishmania* spp. (Euglenozoa, Kinetoplastea, Trypanosomatidae) and other trypanosomatids, bacteria and arboviruses (1).

Historically, Los Montes de María (Bolívar Department) has been the area with the highest incidence of visceral leishmaniasis (VL) and some cases of cutaneous leishmaniasis (CL) in Colombia. A total of 1,781 cases of CL and 54 cases of VL were reported in this department between 2014 and 2019 (2). The wide temperature range (21°C-33°C), 90% relative humidity, abundant leaf litter, high rurality, poverty and a diverse peridomestic (PD) mastofauna would explain the high presence of phlebotomines and 33.4% of cases of VL/100,000 in El Carmen de Bolívar (Tropical Dry Forest-TDF) (3).

Numerous studies have been carried out in Los Montes de María focusing on the identification of potential reservoirs of *Leishmania* and others trypanosomatids (4-11), as well as on the diversity of phlebotomine fauna and the detection of natural infections with *Leishmania* spp. or other trypanosomatids (6,9,12-28). In parallel, the sources of blood intakes of phlebotomine sandflies have been examined (27,29-31).

Bolívar Department presents 20 phlebotomine sandfly species (23,32), among which the following stand out *Lutzomyia evansi* (Núñez-Tovar, 1924), incriminated as a vector of *Leishmania chagasi* (Cunha & Chagas, 1937) and *Le. braziliensis* (Vianna, 1911), etiologic agents of VL and CL, respectively; *Lu. gomezi* (Nitzulescu, 1931) vector of *L. braziliensis* and *L. panamensis* (David & Craft, 2009); and *Lu. panamensis* (Shannon, 1926), vector of *L. panamensis*, etiological agents of CL (27,33).

Several studies have been carried out on the phlebotomine sandfly fauna of the department. A total of 19,649 phlebotomine have been identified, of which *Lu. evansi*

stands out due to its greater abundance (4,7,14,20,21,28; Hernández-Bolívar et al. in preparation; Herrera et al. preparation).

However, one study from the region, reports that females of *Lu. evansi* fed on *Homo sapiens* (16.73%-n=82/490), *Capra hircus (*16.32%-n=80/490), *Sus scrofa* (12.45%-n=61/490)*, Bos indicus* (11.63%-n=57/490) and *Canis familiaris* (9.79%-n=48/490) (30). In other areas of Los Montes de María, polimerase chain reaction (PCR) has also been used as a highly sensitive technique to identify groups of mammals that have served as blood sources for *Lutzomyia* (29,30). The study of phlebotomine fauna involved in the transmission of *Leishmania* spp. and its blood sources, in El Carmen de Bolívar, could get better knowledge of the possible transmission net, in domestic (D) and PD ecotopes, in an area of high endemicity for VL and some cases of CL in Colombia. Therefore, the present study would evaluate the diversity of sandflies associated with the village of El Alférez (municipality of El Carmen de Bolívar- Bolívar Department) in northern Colombia and would constitute a molecular approach for the identification of blood sources found in the digestive tract of sandflies.

Materials and methods

The study was carried out in El Alférez, a rural area in of El Carmen de Bolívar Municipality (09°45'38''N; 075°10'19.1''W) in the Bolivar Department, northern Colombia, endemic for both VL and CL. This region is a TDF with bimodal rainfall (May-June with 68 mm^3 and September-November with 104 mm^3). The landscape includes patches of fruit trees such as cocoa, avocado, and timber trees (23). Phlebotomines were collected in four sampling events (January-November 2018) using LED-based light traps strategically placed in the D, PD and Sylvatic (S) ecotopes, following the signing of informed consent by the head of the household.

The S ecotope was defined as an area more than 100 m in diameter from the dwelling. Trapping was carried out on three consecutive nights from 18:00 to 06:00, resulting in a total trapping effort of 1,728 trapping hours.

Females with evidence of blood remnants in the abdomen were carefully preserved in Eppendorf® microtubes. The last three abdominal segments of each female were isolated, cleared in lactophenol (1:1) and carefully examined (400X). Taxonomic keys and reference images were used to analyse morphometric and anatomical characters (34). 100 fattened females were randomly selected for DNA extraction. This was done using the salting out method described in previous work (35). A multiplex conventional PCR assay chosen as a selective and efficient method to identify mixed blood sources in phlebotomines associated with human dwellings, minimising problems related to the universality of primers that could amplify the genome of insects or multiple vertebrate species.

In details, the extracted DNA was quantified and utilized for a multiplex PCR assay designed in function to amplify fragments of the mitochondrial cytochrome B (CytB) gene. The targeted species-specific amplicons included *Ovis aries* (132 bp), *Homo sapiens* (334 bp), *Sus scrofa* (453 bp), *Bos taurus* (561 bp) and *C. familiaris* (680 bp) and employing primers described in other studies (36).

The final PCR mixture was performed in a final volume of 12 µL with 4 µL of target DNA, 10 µL of reaction mixture containing GoTaq® Green Master Mix 2X (Promega, Madison, USA), primer mix 10 µM and ultrapure water. The melting temperature was estimated based on the average of the temperatures of each primer and subtracted 5 degrees, resulting in temperature 58°C, the rest of the profile was programmed according to the manufacturer's specifications. PCR products were analyzed using 2 % agarose gel electrophoretic run, in TBE buffer (Tris-Borate-EDTA) 1X

supplemented with SYBR Green, the amplicons size was estimated by comparison with a molecular marker DNA Ladder (Thermo Fisher Scientific, CA, 100-1,500 bp). All specimens were collected under the "Permiso Marco de Recolección de Especímenes de Especies Silvestres de la Diversidad Biológica con Fines de Investigación Científica No Comercial" Resolution 0391 of April 11, 2016 expedited by the "Autoridad Nacional de Licencias Ambientales (ANLA)" granted to the "Universidad Sucre". All animal experiments were conducted in accordance with the guidelines outlined in Resolution 008430 of 1993 and 2378 of 2008, issued by the Ministry of Health and Social Protection of Colombia, which establishes scientific, technical, and administrative norms for research in the health field. The research project has been approved by the Bioethics Committee of the University of Sucre in its ordinary meeting no. 07 of the year 2019. The committee functions in accordance with Resolution No. 28 of 2005, resolution No. 100 of 2009 of the Academic Council and resolution No. 04 of 2010 of the Superior Council of the University of Sucre.

Results

A total of 675 specimens of *Lutzomyia* genus were collected. Seven species were identified: *Lu. evansi* 71.85% (485/675), *Lu. gomezi*, 15.55% (105/675), *Lu. panamensis* 4.74% (32/675), *Lu. aclydifera* (Fairchild & Hertig, 1952) and *Lu. trinidadensis* (Newstead, 1922) with 0.30% each (2/675), *Lu. dubitans* (Sherlock, 1962) and *Lu. abonnenci* (Floch & Chassignet, 1947) with 0.14% each (1/675); 47 individuals, 6.96% (47/675) were not identified.

The highest abundance and richness were recorded in the PD with 44.00% (297/675) and the S with 85.71% (6/7) of the identified species, respectively. The lowest abundance and richness were recorded in ecotope D (table 1).

The amplification of the Cytb gene from the genetic material contained in the digestive tract of the phlebotomine showed amplicons for a single and multiple blood meal in 25.0% of the specimens (25/100) (figure 1).

Canis familiaris, *H. sapiens*, *B. taurus*, *S. scrofa* and *O. aries* appeared as blood sources; the blood of *C. familiaris* was predominant in the intestinal contents of the studied phlebotomines. Specifically, it was present in 66.0% (6/9) of the mixed blood meals and in 18.7% (3/16) of the single blood meals.

Lutzomyia evansi (17 individuals fed) was the species with the most generalist feeding habits, single and mixed blood meals were observed, followed by *Lu. gomezi* (six individuals fed) with five single intakes and one mixed bloodmeals, while that *Lu. panamensis* had only two mixed intakes (table 2).

Discussion

The abundance and species diversity of the sandflies collected in El Alférez, Los Montes de María, can be explained by the fact that this region is a TDF corridor with deciduous trees, abundant organic matter from decomposing litter, alkaline soils, and little human intervention, which is a suitable habitat for phlebotomines (26,37). In addition, there are abundant domestic and synanthropic fauna in the region that could sustain to the *Lutzomyia* spp. life cycle (38).

In Colombia, *Lutzomyia* has a wide distribution throughout the country and 20 species have been recorded in the Bolívar Department (23,32), of which seven (35.0%) of these species were found in this research.

The composition and abundance of phlebotomine sandflies identified in this study show patterns similar to previous research in the Colombian Caribbean, with *Lu. evansi* dominating, followed by *Lu. gomezi* and *Lu. panamensis* (6,9,12-16,18,19,21-23,26,27,39,40). Specifically, the high abundance of *Lu. evansi* aligns with the

findings from previous studies in the northern regions of Colombia, including the departments of Atlántico (39,40), Córdoba (12–14,26,27), Sucre (15,18,19,21,26), and Bolívar (6,16,22,23), with abundances of 74.92%, 86.38%, 90.80%, and 64.66%, respectively.

The significant presence of *Lu. evansi* in these study areas suggests a remarkable adaptability of the species to highly anthropized environments. Such adaptability could be attributed to the eclecticism of the diet of *Lu. evansi* (26). In addition, the loss and fragmentation of the original habitat due to agricultural activities, a characteristic of the TDF, may be associated with this species (39).

The results show that *Lu. evansi* uses both *B. taurus* and *C. familiaris* as a blood source, like other records in the country, particularly in Los Montes de María (Sucre Department) (29). This behaviour correlates closely with the intense livestock activity in the Colombian Caribbean, indicating that, *Lu. evansi* would have more opportunities to use *B. taurus* as a highly available blood source (29).

Although the role of cattle in the epidemiological cycle of leishmaniasis in Colombia has traditionally been underestimated, studies in Brazil have shown that *B. taurus* can be infected with *Le. infantum* (41). This trend suggests a possible link between livestock practices and leishmaniasis transmission. It highlights the need to further investigate the role of these domestic animals in disease dynamics.

The evidence of pigs (*S. scrofa*) as a blood source by *Lu. evansi* is compared with other authors who have also identified this mammal as a nourishment font (29). The zooanthropophilic habits of *Lu. evansi*, and its greater abundance in D and PD where pig farms and farmers are usually found, constitute a risk scenario for *Leishmania* spp. transmissions.

Other specimens of *Lu. evansi* have presented blood intakes from *O. aries*-sheep, a mammal with no clear role as a host for *Leishmania* spp. but which is very common in the PD. In addition, phlebotomines lay their eggs in soils rich in organic matter, mainly due to pig, sheep, and cattle farming, which provides nutrients for larval instars of *Lutzomyia* spp. (38).

A single specimen of *Lu. evansi* showed DNA traces of *H. sapiens* blood in its intestinal content, in contrast with the high frequency of this source in exemplars of the urban area of Sucre Department and in anthropophilic habits described in rural areas of Los Montes de María (12,27,28,30).

The multiple intakes and the probable eclecticism of *Lu. evansi*, in terms of the blood source (as evidenced in the present work) would ensure the transmission of *Leishmania* to mammals restricted to D, PD and S, including human. However, the low abundance of this species fed with *H. sapiens* may be a limitation since zoonotic scenario, requiring further studies.

Lu. gomezi, the second most abundant species recorded in this study, turned out to be an epidemiologically important species in Colombia, as it is distributed in 28/32 departments (32) and has been reported to be infected with *L. panamensis* and *L. braziliensis,* agents of CL (27).

It is important to emphasise that although the role of *O. aries* in the transmission cycle of *Leishmania* is unknown, it cannot be excluded as a potential reservoir, as five individuals presented DNA traces for *O. aries.*

Regarding *Lu. panamensis*, which showed a low frequency in PD and S, it is known for its importance in medical entomology, as it has a distribution in 14 Latin American countries and has been found in 22 departments in Colombia (32). This species,

considered highly anthropophilic, is the primary vectors of *Le. panamensis*, etiological agent of CL in Colombia (27).

The finding of a source of blood ingestion of *Lu. panamensis* would probably evidence an eventual anthropophilic habit, which was already referred in northern Colombia (Sucre and Córdoba) (27,29).

In the present research, negative results for sources of intake were obtained in 75.00% (n=75/100) of the individual. Is highly probably that the other individuals evaluated did not reveal bloodmeals, by prolonged fasting, maybe because the females were fertilized but not ready to oviposite or its feed from other wildlife vertebrates no evaluated in this study.

Lutzomyia trinidadensis, a species with a wide distribution in the Neotropic (in 15 Latin American countries) and in 20 departments of Colombia (32), was found in low proportion in this sampling, perhaps because it was collected during the rainy season characterized by a low abundance of the species.

In neighbouring Venezuela, Bonfante-Garrido *et al.* (42) found in this species, promastigotes which, when inoculated into hamsters, showed tissue invasion by amastigotes morphologically compatible with *L. venezuelensis*. This suggests that, *Lu. trinidadensis* is a potential vector of *Leishmania* in Venezuela and highlights the need to monitor its populations in Colombia.

The presence of *Lu. abonnenci* is important because of its widespread distribution in Bolivia, Brazil, Ecuador, French Guiana, Panama, Peru, Suriname, Venezuela, and Colombia, in the latter with a low frequency in 11 departments including Bolivar (32) and no vector role in *Leishmania* spp. transmission.

Lutzomyia dubitans, the other species found, has been reported in Brazil, Costa Rica, Panama, Trinidad and Tobago, and is distributed in 16 departments of Colombia (32), its role in the transmission of *Leishmania* spp. is uncertain. *Lutzomyia aclydifera* of the *dreisbachi* group is a sparsely distributed species in Colombia, with records from Antioquia, Chocó, Valle del Cauca and, more recently, Bolívar Department (23,32). As with *L. dubitans*, its role as a vector for *Leishmania* spp. is unknown.

Although it was not possible to identify all mammalian species as the main blood source, the technique allowed the detection of the most abundant domestic species, as potential nourishment source, in areas close to human dwellings (27,29-31). Technology such as sequencing would provide greater accuracy in identifying the blood source species. However, it would also prevent the identification of mixed blood sources without cloning, in addition to being able to observe sequences corresponding to the insect (30).

Finally, the application of multiplex PCR for the detection of DNA from domestic vertebrates allowed us to identify the blood meals of three species with high epidemiological value, *Lu. evansi, Lu. gomezi and Lu. panamensis*, which are fed by *B. taurus, H. sapiens, C. familiaris, O. aries* and *S. scrofa* (D and PD fauna), indicating the first approach to mammals that serve as blood source and act as possible hosts/reservoirs of *Leishmania* spp. for El Carmen de Bolívar. The presence of this phlebotomine-mammalian binomial in D and PD would represent a risk.

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Conflicts of interest

None declared.

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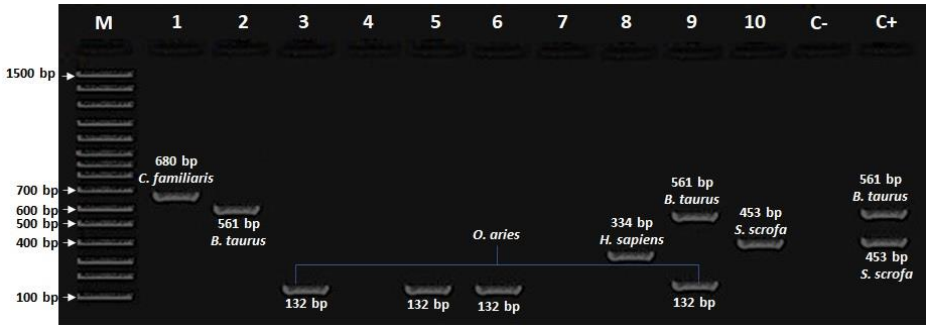

Figure 1. Agarose gel electrophoresis (2%-80V/70 min) of amplicons mitochondrial cytochrome B gene isolated from intestinal content of sandflies from the Vereda El Alférez, rural area of El Carmen de Bolívar (Bolívar), northern Colombia. **M**: Molecular marker (100-1,500 pb); **Lane 1:** DNA of *Lutzomyia evansi* (amplicon for *Canis familiaris*); **Lane 2:** DNA of *Lutzomyia evansi* (amplicon for *Bos taurus*); **Lane 3, 5, 6:** DNA of *Lutzomyia gomezi* (amplicons for *Ovis aries*); **Lane 4, 7:** DNA of *Lu. evansi* (did not present amplicon)**; Lane 8:** DNA of *Lu. evansi* (amplicon for *Homo sapiens*); **Lane 9:** DNA of *Lu. gomezi* (amplicons for *B. taurus/O. aries*); **Lane 10:** DNA of *Lu. evansi* (amplicon for *Sus scrofa*); **C-:** no template control (NTC); **C+:** Positive control (mixture of genetic material from *B. taurus* and *S. scrofa)*.

Table 1. Diversity and abundance of phlebotomines collected in the Village of El Alférez, El Carmen de Bolívar (Bolívar), northern Colombia.

Species	D	PD	S	Total (%)
**Lu. evansi*	112	216	157	485 (71.85%)
**Lu. gomezi*	31	50	24	105 (15.55%)
**Lu. panamensis*	1	14	17	32 (4.74%)
Lu. trinidadensis	0	1	1	2 (0.30%)
Lu. dubitans	0	1	0	1 (0.15%)
Lu. aclydifera	0	0	2	2 (0.30%)
Lu. abonnenci	0	0	1	1 (0.15%)
Lutzomyia spp.	17	15	15	47 (6.96%)
Total	161	297	217	**675 (100%)**

D: Domestic; PD: Peridomestic; S: Sylvatic. *Species of epidemiological importance in *Leishmania* transmisión.

Table 2. Mixed and unique blood meals for medically important phlebotomine species collected in the Village of El Alférez, El Carmen de Bolívar (Bolívar), northern Colombia.

Species	Mixed bloodmeals	No.	Single bloodmeals	No.
Lu. evansi	*O. aries/S. scrofa/C. familiaris*	1	*C. familiaris*	3
	O. aries/C. familiaris	2	*H. sapiens*	1
	C. familiaris/B. taurus	2	*O. aries*	5
	O. aries/S. scrofa/B. taurus	1	*B. taurus*	1
			S. scrofa	1
Lu. gomezi	*S. scrofa /B. taurus*	1	*O. aries*	5
Lu. panamensis	*S. scrofa/H. sapiens*	1	-	-
	B. taurus/C. familiaris	1	-	-
Total		**9**		**16**