Phlebotomine Vector Ecology in the Domestic Transmission of American Cutaneous Leishmaniasis in Chaparral, Colombia

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Abstract. Phlebotomine vector ecology was studied in the largest recorded outbreak of American cutaneous leishmaniasis in Colombia in 2004. In two rural townships that had experienced contrasting patterns of case incidence, this study evaluated phlebotomine species composition, seasonal abundance, nocturnal activity, blood source, prevalence of *Leishmania* infection, and species identification. CDC miniature light traps were used to trap the phlebotomines. Traps were set indoors, peridomestically, and in woodlands. Natural infection was determined in pools by polymerase chain reaction–Southern blot, and blood sources and species identification were determined by sequencing. Large differences were observed in population abundance between the two townships evaluated. *Lutzomyia longiflocosa* was the most abundant species (83.1%). Abundance was higher during months with lower precipitation. Nocturnal activity was associated with human domestic activity. Blood sources identified were mainly human (85%). A high prevalence of infection was found in *L. longiflocosa* indoors (2.7%) and the peridomestic setting (2.5%). *L. longiflocosa* was responsible for domestic transmission in Chaparral.

INTRODUCTION

Leishmaniasis is prevalent in 88 countries, affecting an estimated 12 million people. Approximately 2 million new cases are recorded each year, of which 500,000 are visceral leishmaniasis (VL) and 1,500,000 are cutaneous leishmaniasis (CL); however, these numbers are thought to be underestimates of the true disease burden.^{1,2} Because CL is not fatal, its control at individual and community levels has received little attention. Therefore, CL still requires additional study and support for the implementation of effective prevention and control strategies.²

CL was classified as a zoonosis in the New World, with the cycle of transmission occurring between phlebotomine vectors and wild reservoirs. Humans were considered an incidental host, infected only when entering the forest. In recent decades, the domestic environment has become a risk factor for the disease. In the 1980s, Brazil began to record CL cases in all age groups, suggesting transmission in domestic and peridomestic environments.³ One of the factors associated with the epidemiological change was the adaptation of some species of the subfamily Phlebotominae, vectors of *Leishmania* parasites, to altered ecosystems and other sources of blood meals, such as humans and domestic animals.^{3,4} In Colombia, increased human movement, mainly because of political instability, encouraged the geographical spread of *Leishmania* species.⁵ Simultaneously, *Leishmania* parasites have shown their ability to infect additional mammalian hosts^{6,7} and use additional phlebotomine species as vectors.^{8–10} These multiple factors have contributed to a significant increase in CL cases in Colombia in the past decade. During the 1990s, an average of 6,500 cases of CL was recorded each year; however, the past decade has recorded an average of approximately 11,000 cases per year, with peaks in 2005 and 2006 of about 20,000 cases per year.^{11–13}

The largest outbreak of CL reported in Colombia occurred in Chaparral County (municipio), Tolima Province (departa-

mento), which is located in the upper valley of the Magdalena River in the sub-Andean region of Colombia. A peak of 8,444 CL cases per 100,000 inhabitants was reported in 2004.¹⁴ The distribution of cases by age and gender was atypical, with children aged 15 years or less comprising 36.5% of the total of 2,835 cases and women comprising 35.5% of 1,811 adult cases (Hospital San Juan Bautista of Chaparral, unpublished data). These demographics suggested that transmission of the parasite had occurred in the household environment.^{10,14–16} Leishmaniasis was restricted to townships located between 1,000 and 2,000 m.¹⁷ The most frequently isolated parasite was *Leishmania* (*Viannia*) *guyanensis* (94.6% of 56 isolates), a species previously present in Colombia only in distant regions such as the Amazon and Orinoco River watersheds,^{18–20} the Caribbean coast, and localized Andean regions.^{9,10} In a preliminary survey, the high abundance of the phlebotomine, *Lutzomyia longiflocosa*, from the *verrucarum* group suggested that this species was the most likely vector. This species was previously reported as a potential vector because of its anthrophilic and endophagic behavior and its abundance in several other outbreaks of leishmaniasis in the sub-Andean region in the upper and middle Magdalena River valley.^{14,21–23} Other phlebotomine species collected in the preliminary survey in relatively low abundance (< 10%)—*L. columbiana*, *L. nunez-tovari*, and members of the *L. (Helcorcytomyia)* subgenus—were considered to have a limited role as vectors.^{14,21–23}

The current study characterized the composition and abundance of phlebotomine vector species in two townships (veredas) of Chaparral, Tolima, that had contrasting patterns of prevalence of infection during the outbreak. Additionally, vector ecology characteristics in terms of seasonality, nocturnal activity, blood source, and natural infection with *Le. (V.)* were observed to assess the risk of domestic transmission.

MATERIALS AND METHODS

Study area. The study was conducted in the central mountain range of the Colombian Andean region in Chaparral county. It was carried out in two townships with very different CL

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prevalence but similar geographic, environmental, and demographic conditions to identify differences in phlebotomine composition and abundance in relation to *Leishmania* parasite transmission. The townships, Agua Bonita ($3^{\circ}49'91''\text{N}$, $75^{\circ}33'57''\text{W}$) and Irco Dos Aguas ($3^{\circ}48'121''\text{N}$, $75^{\circ}37'80''\text{W}$), were selected for their contrasting patterns of incidence during the outbreak; in the former, the cumulative number of incident cases in 2004–2006 was 74% of the population, whereas in the latter, it was 1.3%. These two townships are located 16.2 km apart (in linear distance) but separated by the deep canyon of the Ambeima River, and they have similar altitudinal ranges: Agua Bonita is 986–2,378 m and Irco Dos Aguas is 932–2,153 m (Figure 1). Both are rural townships with population densities of 15.1 and 37.7 inhabitants/km², respectively. Coffee is the main crop in both areas.

Analysis of land coverage. Differences in land coverage between the study sites were evaluated using satellite images as described by Valderrama-Ardila and others.¹⁷ Land coverage categories were defined as (1) forest (mature forest, stratified old secondary forest, and mature planted forest); (2) paramo (natural grasslands of wet soils, usually at elevations above 3,200 m); (3) shrubs (early secondary growth

forest, including permanent cultivations such as coffee or fruit trees); (4) cultivation (annual cultivation such as corn); (5) grasslands (pastures below 3,000 m adjacent to cultivated areas); (6) water bodies (rivers, lakes, ponds, and reservoirs); (7) exposed soil (areas of degraded soil with almost no vegetation); and (8) urban areas (human settlements or development in urban or rural areas). Coverage percentages per township were calculated using supervised classification of LandSat images with ERDAS Imagine Software.

Phlebotomine abundance and seasonal variation (abundance substudy). *Initial cross-sectional study for house selection.* To select three houses per township with higher phlebotomine abundance, all houses from both townships were initially sampled. During these surveys, a Centers for Disease Control and Prevention (CDC) miniature light trap was located inside each house for one night (18:00–6:00 hours). Geopositioning and altitude were recorded. The percentage of positive houses and indoor phlebotomine abundance was calculated. Additional CDC traps were located at 10 m from the house to determine species and abundance in the peridomestic environment. Phlebotomine identification was carried out using the method by Young and Duncan.²⁴ The survey was carried out

FIGURE 1. Map of Chaparral, Tolima, Colombia, showing the townships (grey areas) affected by the CL outbreak and locations of Agua Bonita (high CL case prevalence) and Irco Dos Aguas (low CL prevalence).

in August and September of 2007, a low-precipitation period in which higher phlebotomine abundance was expected.

Phlebotomine sampling in selected houses. In each one of the three selected houses with high abundance of phlebotomine, five CDC miniature light traps were placed for 3 consecutive nights: one in an occupied bedroom (indoor), two outdoors at a distance of 10 m (peridomestic), and two in the nearest woodland (maximum distance of 90 m). All traps were placed 1.5 m above ground level; they were activated from 18:00 to 6:00 hours. Samples were collected each month from June 2007 to May 2008 except for October and March because of political instability in the area.

Habitat characterization in selected houses. The habitat in the 100-m radius around each house was characterized at point locations at 10-m intervals along eight transects centered on the house at 45° intervals using a trap-web pattern. Habitats were classified according to the following categories: hen house, pigsty, horse or cow stable, forest, shaded coffee plantation, unshaded coffee plantation, cultivation (short annual crops), shrubs, pasture, and others. Around each house, the percentages of points in each habitat category were used to characterize the habitat.

Rainfall patterns. The average monthly precipitation during the study period was used to analyze differences in phlebotomine relative abundance. Precipitation data were obtained from the nearest active weather station (Demonstration Farm of the Institute of Hydrology, Meteorology and Environmental Studies of Colombia Instituto de Hidrología, Meteorología y Estudios Ambientales [IDEAM]) located at 1,040 m (3°43'4.4"N and 75°29'44.3"W) and 10 km from Irco Dos Aguas and 12 km from Agua Bonita. The data from this station can be extrapolated to the sample sites in terms of precipitation patterns, because they are located under the same pluviometric regimens according to Bioclim data (www.worldclim.org). Temperature records, however, cannot be extrapolated because of the altitudinal range observed in the two townships.

Analysis of phlebotomine in selected houses. The abundance (density) of phlebotomines was expressed as the number of specimens of each species collected per trap/night. The data were analyzed by negative binomial regression because of the skewness and overdispersion relative to Poisson. The response variable was the number of phlebotomines captured in the 3 consecutive nights per month (collection performed monthly), and explanatory variables were compared in terms of mean density. Seasonal variation was graphed by comparing the monthly female phlebotomine collection with the average monthly precipitation.

Phlebotomine nocturnal activity (nocturnal substudy). In Agua Bonita, sampling to evaluate the nocturnal activity of phlebotomines was conducted in two of the previously selected houses for an additional period of 5 months (March to July 2009). Samples were taken monthly (every 4 weeks) to identify the behavior of the vectors during periods of low and high rainfall. Each sampling period was 4 nights in each of the houses. Two CDC miniature light traps were allocated to each house, one located in indoors and the second located peridomestically (10 m distant from the house). The traps were activated from 17:00 to 6:00 hours. The collection bag was changed each hour.

Phlebotomine capture rate was expressed as the number of specimens per species collected/trap per hour. Nocturnal activ-

ity was calculated based on the average of phlebotomine capture per hour in both houses per township. Statistical analyses to evaluate differences in phlebotomine captures per hour and per month were done using negative binomial regression.

Species identification. The phlebotomines collected were separated from other insects after immobilization of all insects with triethylamine (TEA; 04885-1; Fisher Scientific, Pittsburgh, PA). The immobilization was carried out by placing the collection bag of the CDC trap, including its insect contents, with a piece of cotton soaked with 1 mL TEA inside a plastic bag for 15 min. The phlebotomines were separated and placed in 70% alcohol until processed for identification and polymerase chain reaction (PCR). An initial identification was done with some specimens to associate the species with its external morphological features. This initial identification was made using the methods of Young and Duncan²⁴ and Galati²⁵ by clarifying the specimens in KOH and phenol. Because the phlebotomines were to be subjected to additional analysis by PCR, identification of all specimens was not possible using standard practice of clearing each specimen. Phlebotomine identification was, therefore, done by external morphological features. The separation of *L. longiflocosa* from other phlebotomine females was undertaken in several steps. First, the *L. verrucarum* group species were separated from the other Phlebotominae by the length of the palpomere, coloration of scutum, and size and indices of the insect wing.²⁶ Species within the *L. verrucarum* group were distinguished as follows. *L. longiflocosa* had the katepimeron dark at the base; the anterior coxae were darker than the median and posterior coxae. Some specimens also had darkened coloration for the katepisternum. *L. columbiana* exhibited a darkened coloration for the katepimeron, katepisternum, and the three pairs of coxae. *L. oresbia* differed by having the anepisternum, katepimeron, katepisternum, and the three pairs of coxae darkened. Because *L. nuneztovari* did not present a pigmentation pattern that differentiated it from the other species, the wing measurement δ was mainly used as a reference characteristic to distinguish this species. This measure was generally greater (0.31 mm [0.26–0.37]) than the measure of other *L. verrucarum* group species in the study area. In addition, the wing measurement index β (0.22 mm [0.2–0.26]) was less than δ . The *L. longiflocosa* and *L. columbiana* wing indices were also used to discriminate species by external morphology. The following parameters were used. *L. longiflocosa* β (0.24 mm [0.22–0.26]) was usually subequal to or greater than δ (0.22 mm [0.17–0.29]), and *L. columbiana* β (0.19 mm [0.15–0.23]) was usually subequal or smaller than δ (0.23 mm [0.16–0.32]). Identification by external morphological features was verified in a subsample (10%) that was cleared in KOH and phenol and observed microscopically using the Young and Duncan²⁴ and Galati²⁵ phlebotomine identification key.

Blood source analysis. Field-collected blood-fed phlebotomines were stored in separate vials with 70% alcohol. DNA was isolated using the DNeasy extraction kit (Qiagen) according to the manufacturer's recommendations and guidelines. Blood source identification was carried out using cytochrome b sequences as described.^{27,28} All DNA templates were tested with the primer pairs mammals C described by Molaei and others^{27,28} (CCATCCAACATCTCAGCATGATGAAA[f], GCCCTCAGAATGATATTTGTCCTCA[r], 395 bp) and a primer pair for avian (GCCAAATATCATTCTGAGGG GC[f], GGCGAATAGAAAATATCATTGTGG[r], 410 bp)

designed by us. All the amplifications using mammals C were sequenced, because this primer can amplify all types of blood sources, including birds (Molaei G, personal communication). Several amplicons from the avian primers designed by us were sequenced to verify the results against those results from the mammals C primers. The Taq PCR Master Mix Kit (Quiagen, Valencia, CA) was used to amplify all PCR using the selected primers. After the amplification was confirmed, AccuPrimePfx enzyme (Enzyme-High Fidelity PCR, Invitrogen Tech-Line SM Carlsbad, CA) was used to amplify the samples for sequencing. A total of 25 μ L reaction was prepared using 1 μ L template DNA, 2 μ L each primer (0.1–0.5 pmol/L), 2.5 μ L 10 $\times$ PCR buffer, 2 μ L MgCl₂ (15 mmol/L), 1 μ L desoxyribonucleic triphosphate [dNTP] (10 mmol/L), 0.25 μ L Taq DNA polymerase (1.25 U/reaction), and 14.25 μ L water. The PCRs were run in a PTC 200 (MJ Research). The PCR products were separated by electrophoresis on agarose gel 1.5% stained with ethidium bromide (0.5 mg/mL) and visualized on an ultraviolet transilluminator. Subsequently, the amplification products were purified using PCR Purification Kit QIAquick (Quiagen, Valencia, CA) and sequenced in reactions of cyclic sequencing (Yale University, New Haven, CT) using the sequencer 3730xl DNA analyzer (Applied Biosystems). The sequences were annotated using ChromasPro version 1.22 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) and identified by comparing the DNA sequence to GenBank databases (National Center for Biotechnology Information).

Leishmania infection. Parasite detection was carried out in the phlebotomine female specimens of *L. ongiflocosa* and *L. columbiana* collected in the seasonal substudy and all of the blood-fed specimens collected over the entire study. Female specimens (non-blood-fed) were pooled by species in vials of 20 or fewer, and blood-fed females were processed individually. The phlebotomine pools were grouped according to trap location, house number, month of capture, and site.

PCR. DNA from pooled or individual samples was extracted using the DNeasy extraction kit (Qiagen) in accordance with the manufacturer's instructions. DNA samples were amplified with the primers LV (5'-ATT TTT GAA CGG GGT TTC TG-3') and B1 (5'-GGG GTT GGT GTA ATA TAG TGG-3'), which specifically amplify a fragment of 700-bp mini circle kDNA of *Le. (V.)* species.^{29,30} The thermal profile was 95°C for 5 minutes, 35 cycles at 92°C for 1 minute, 66°C for 40 seconds, 72°C for 30 seconds, and a final extension at 72°C for 1 minute.

Electrophoresis and hybridization. PCR products were separated by electrophoresis on 1.5% agarose gels in 0.5 $\times$ TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) at 90 V for 45 min, and the bands were visualized and recorded with a Gel Doc 2000 (BioRad) gel documentation system. To prevent cross-contamination, separate bench areas for DNA extraction, PCR sample preparation, and amplification were used. To improve the sensitivity and specificity of PCR, a chemoluminescent Southern blot was performed.^{29,30} Briefly, agarose gels were processed in denaturation (1.5 M NaCl, 0.5 M NaOH) and neutralization (1 M Tris, pH 8.0, 1.5 M NaCl) buffer for 20 minutes each and blotted onto a nylon membrane (Hybond N+; Amersham Bioscience). The transfer was carried out by capillary action overnight using 10 $\times$ sodium chloride-sodium citrate (1.5 mol/L NaCl and 0.15 mol/L sodium citrate).

DNA was fixed to the membrane with ultraviolet light in a cross-linker (Spectronics) for 30 seconds in automatic mode. Membranes were hybridized at high stringency (65°C) with a 700-bp kDNA mini circle probe derived from *Le. (V.) guyanensis* that had been purified and cloned into PCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA) combined with the Alka-Phos Direct labeling and detection system with CDP-Star (Amersham-Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Membranes were then exposed for autoradiography for 1 hour at room temperature. Samples were considered positive when a band of the correct size (700 bp) was seen in the Southern blot, even if electrophoresis was negative. Two positive controls were used to verify that the tests were working. The first positive control was DNA extracted from a pool containing one experimentally infected *L. longipalpis* with *Le. (V.) panamensis* strain HOM/PA/71/LS94 and 19 females *L. longipalpis* without infection. The second control was DNA extracted from 1 $\times$ 10⁶ promastigotes of same strain of *Le. (V.) panamensis*. Two additional negative controls were used: (1) DNA of 20 *F.* *L. longiflocosa* females from an uninfected colony and (2) a PCR control (no DNA).

Analysis. Prevalence of infection in the phlebotomine pools was calculated assuming that at least one insect from each positive pool was infected, as described by Katholi and others.³¹ An R function for this calculation is given in Supplemental Table 1.³²

Identification of Leishmania species. Phlebotomines that were positive for *Leishmania* kDNA, reference strains (*Le. braziliensis* M2903, *Le. guyanensis* M4147, and *Le. panamensis* LS94), and two *Le. guyanensis* strains isolated from patients during the Chaparral epidemic were processed for species typing by direct sequencing of PCR products of the *Leishmania* 7SLRNA gene region as previously described.³³ PCR was carried out using primers LeishFW (5'-CATCCGTGACAGGATTCGAACC-3'), corresponding to a sequence approximately 200 bp upstream from the putative 7SL gene start sequence, and LeishRV (5'-CG TGGGGCTCAAGTGCGGACATG-3'), corresponding to sequence at position 36 bp upstream from the end of the putative 7SL gene sequence.³³ PCR products of approximately 430 bp were extracted from the gel and purified for the sequencing reaction using the QIAquick gel extraction kit (Qiagen, CA). Sequence analyses and single-nucleotide polymorphism (SNP) identification were performed using BioEdit v7.0.5 and Sequencher Demo version software. Similarity search was performed with BLAST. Multiple sequence alignment and editing were conducted using Bioedit (biological sequence alignment editor written for Windows 95/98/NT/2000/XP; <http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

RESULTS

Land coverage. Differences in land coverage between the townships were because of the historical use processes. Agua Bonita's main coverages were forest (43.2%) followed by cultivation (23.7%), shrubs (19.4%), and grasslands (13.7%), whereas Irco Dos Agua's main coverages consisted of shrubs (62.2%), cultivation (21.0%), grasslands (12.9%), and forest (3.9%). Here, cultivation includes corn, sugar cane, and

unshaded coffee, whereas shrubs includes shaded coffee plantation, thickets, small trees, and cacao trees. Land coverages had changed little since 1989.¹⁷ According to Holdridge Life Zones system, both townships are located in premontane very humid forest (bmh-PM; IGAC Holdridge Life Zone map with a scale of 1:500,000).³⁴

Phlebotomine abundance and seasonal variation (abundance substudy). *Initial cross-sectional study for house selection.* In Agua Bonita, 20 of 33 houses sampled were positive for phlebotomines (60.6%). Seven phlebotomine species were collected indoors. *L. longiflocosa* was the dominant species in dwellings (90% of 155 phlebotomines) and in the peridomestic area (43% of 68). This species infested the highest number of houses (41%); however, it tended to aggregate in seven houses, which contributed 76% (122/155 of the *L. longiflocosa* trapped). The more productive houses were located between 1,460 and 1,704 m.

In Irco Dos Aguas, 25% of the houses were positive for phlebotomines (8 of 32 houses). Only two species were identified in the 12 specimens collected: nine species were *L. longiflocosa* and three species were *L. (Helcocyrtomyia)* spp. Three positive houses located between 1,504 and 1,530 m were selected.

Species composition. Large differences in total number of phlebotomines captured in the selected three houses (abundance substudy) were observed between the two townships. The phlebotomines in Agua Bonita township constituted 99.3% of 10,001 phlebotomines captured (Table 1). In Agua Bonita, *L. longiflocosa* was the dominant species (83.1%) followed by *L. columbiana* (7.5%), *L. (Helcocyrtomyia)* spp. (4.7%), *L. trinidadensis* (2.6%), and *L. nuneztovari* (2%). In Irco Dos Aguas, 72 phlebotomines were collected, of which a large majority were either *L. longiflocosa* or *L. (Helcocyrtomyia)* spp.; these species occurred in similar numbers: *N* = 34 (47%) and 32 (44%), respectively.

Habitat characterization around selected houses. The main land use within 100 m radius of the three houses sampled in Agua Bonita was forest (40%) followed by unshaded coffee plantations (21%), shaded coffee plantations (15%), grasslands (11%), shrubs (4%), and annual crops (corn, sugar cane, and

beans; 9%). In Irco Dos Aguas, the main uses were unshaded coffee plantations (30%) and grasslands (28%) followed by shrubs (19%), annual crops (4%), forest (4%), and others (mainly cacao; 15%).

Seasonal variation. In Agua Bonita, the phlebotomine female abundance from the three selected houses (abundance substudy) varied significantly by month ($P < 0.001$). During the year, two periods of high precipitation (April–May and October–November) were followed by two periods with low precipitation (June–September and December–March) (Figure 2A and B). The phlebotomine abundance patterns were defined by the predominant species *L. longiflocosa*. This species showed a clear upward trend in the two periods of low rainfall (August–September and February) (Figure 2A). In Irco Dos Aguas, *L. longiflocosa* showed the same abundance pattern regardless of its low abundance (data not shown). Variations in capture rates were also observed among the collection habitats (Figure 2A). Increased collections were particularly evident during August and September in the peridomestic area and woodland and in February in the indoor and woodland areas (Figure 2A).

L. columbiana, the second most abundant species in Agua Bonita also showed an increase in abundance during the periods of low precipitation. This species was most frequently captured in peridomestic environment with the exception of July and May, when it was most abundant in woodland (Figure 2B). *L. (Helcocyrtomyia)* spp. showed higher abundance in the woodlands, with a slight increase between January and April (data not shown). *L. trinidadensis* populations were low throughout the year, but they were found mainly in woodland (data not shown). The low abundance of *L. nuneztovari* (*N* = 197) was insufficient to detect a seasonal trend (data not shown).

During the seasonal study, the cumulative precipitation at the local weather station (3,248 mm for 2007) was higher than the average of 2,853 mm estimated by Bioclim. This finding did not affect the bimodal precipitation pattern characteristic of the Andean region in Colombia. The increase in cumulative precipitation was a consequence of the La Niña effect that occurred from 2007 to 2008 (National Oceanic

TABLE 1

Density of phlebotomine species collected in the seasonal substudy indoors, peridomestically, and in woodland in the townships of Agua Bonita and Irco Dos Aguas

Species <i>Lutzomyia</i>	Indoors			Peridomestic			Woodlands			Total
	<i>N</i>	<i>L/t/n</i>	RR (CI)	<i>N</i>	<i>L/t/n</i>	RR (CI)	<i>N</i>	<i>L/t/n</i>	RR (CI)	<i>N</i>
Agua Bonita										
<i>longiflocosa</i>	480	5.3		3,891	22		3,881	23		8,252
<i>columbiana</i>	47	0.5	0.29 (0.10–0.84)	470	3	0.18 (0.12–0.28)	228	1.3	0.13 (0.08–0.21)	745
<i>(Helcocyrtomyia)</i>	15	0.2	0.15 (0.01–2.23)	50	0.3	0.04 (0.02–0.08)	400	2.02	0.07 (0.04–0.12)	465
<i>trinidadensis</i>	8	0.1	0.15 (0.01–2.23)	73	0.4	0.03 (0.01–0.07)	174	1.0	0.06 (0.03–0.11)	255
<i>nuneztovari</i>	46	0.5	0.26 (0.07–0.92)	67	0.4	0.06 (0.03–0.15)	84	0.5	0.06 (0.03–0.12)	197
<i>shannoni</i>				3	0.02	0.03 (0.00–0.23)	6	0.03	0.03 (0.00–0.23)	9
<i>lerayi</i>				3	0.02	0.02 (0.00–0.12)				3
<i>oresbia</i>							2	0.01	0.03 (0.00–0.23)	2
Subtotal	596	7		4,557	25		4,775	27		9,929
Irco dos Aguas										
<i>longiflocosa</i>	7	0.1		12	0.1		15	0.1		34
<i>(Helcocyrtomyia)</i>	6	0.1	0.80 (0.16–0.10)	6	0.03	0.75 (0.08–6.69)	20	0.1	1.33 (0.35–5.00)	32
<i>columbiana</i>	1	0.01	1.00 (0.06–15.96)	1	0.01	0.75 (0.08–6.69)	1	0.01	0.57 (0.07–4.62)	3
<i>trinidadensis</i>	1	0.01	1.00 (0.06–15.96)	1	0.01	0.80 (0.09–6.82)				2
<i>oresbia</i>							1	0.01	0.57 (0.07–4.62)	1
Subtotal	15	0.2		20	0.1		37	0.2		72
Total										10,001

CI = confidence interval; *L/t/n* = density of *Lutzomyia*/trap per night per place; *N* = total captures; RR = rate ratio vs. *L. longiflocosa*.

FIGURE 2. Densities of females/trap per night and monthly standard error. (A) *L. longiflocosa* and (B) *L. columbiana* captured indoors, peridomestically, and in woodland in Agua Bonita were related to total precipitation. Each left vertical axis has a break to facilitate visualization of low values.

and Atmospheric administration [NOOA] El Niño Southern Oscillation web page, www.cpc.ncep.noaa.gov/products/analysis_monitoring/ensostuff/ensoyears.shtml).

Phlebotomine nocturnal activity (nocturnal substudy). A total of 1,567 individuals (46.6% females and 53.4% males) belonging to eight species of the genus *Lutzomyia* and one specimen of genus *Warileya* were captured. A total of 188 phlebotomines were collected indoors, and 1,379 were captured peridomestically. *L. longiflocosa* was the main species collected indoors (70.0%) and peridomestically (76.0%) followed by *L. columbiana* (21.1% indoors and 0.2% peridomestic). The other species collected [*L. (Helcocyrtomyia)* spp., *L. nunez-tovari*, *L. trinidadensis*, *L. lerayi*, *L. shannoni*, *L. oresbia*, and *W. rotundipennis*] each represented less than 1% of the total captured indoors and peridomestic.

The activity of *L. longiflocosa* in the peridomestic environment peaked early, between 19:00 and 20:00 hours, before decreasing (Figure 3A). Indoor activity peaked between 23:00 and 24:00 hours and was low at the start and end of the

trapping times (Figure 3B). The nocturnal activity of *L. columbiana* in the peridomestic environment was maintained during most of the night, but a peak between 22:00 and 24:00 hours was observed indoors, which was also the case with *L. longiflocosa* (Figure 3A and B). Differences in phlebotomine abundance were observed among months ($P < 0.001$), showing an increase in phlebotomine abundance during the periods with lower precipitation (data not shown); however, the nocturnal activity pattern was maintained during the study period (March–July 2009).

Blood sources analysis. From 123 *Lutzomyia* collected with blood, 82 were amplified successfully with either of the primers tested, and in 70, the blood source was identified. Sixty of the samples (85%) contained human blood: 36 *L. longiflocosa*, 6 *L. (Helcocyrtomyia)* spp., 7 *L. (verrucarum)* spp., and 11 undetermined *Lutzomyia*. Nine contained avian blood (all *Gallus gallus*): 8 *L. longiflocosa* and 1 *L. (verrucarum)* spp. One blood meal from an undetermined *Lutzomyia* species contained blood from *Choloepus hoffmani*, the two-toed

FIGURE 3. Phlebotomine nocturnal activity of *L. longiflocosa* and *L. columbiana* in (A) peridomestic and (B) indoor environment.

FIGURE 4. *Leishmania* species identification by SNP typing of the 7SL RNA gene region in Colombia. 7SLRNA PCR (430 bp) products from *L. (V.)* reference strains (*L. V. brasiliensis* M2903, *L. V. guyanensis* M4147, and *L. V. panamensis* LS94), *L. guyanensis* strains from Chaparral patients (isolates 3), *Lutzomyia* infected by xenodiagnostic, and five *L. longiflocosa* samples (Blood21TOCHAB005, Blood66TOCHAB011, NonBlood16, NonBlood17, and NonBlood4) were sequenced, and species identification was determined by SNP analysis. Species-specific SNPs used for typing within the *L. (V.)* subgenus, which were previously reported by Stevenson and others,³³ are as follows: *brasiliensis* 80A, 91G, 98A, 124T, 161G, 164C, 167T, 341C *panamensis* 80T, 91A, 98G, 124A, 161A, 164A, 167A, 341C *guyanensis* 80T, 91A, 98A, 124A, 161A, 164A, 167T, and 341T.

sloth (Supplemental Table 2). Lack of taxonomic determination in sand flies was because blood-fed females are more difficult to identify; this difficulty is because, sometimes, the ingested blood is spread through the thorax.

Natural infection by *L. (V.)* in *L. longiflocosa*. In the seasonal substudy, female specimens from Agua Bonita—5,540 *L. longiflocosa* and 588 *L. columbiana*—were distributed into 309 and 78 pools, respectively. From Irco Dos Aguas, only 58 specimens of *L. longiflocosa* were analyzed in 19 pools. No infection with *L. (V.)* sp. was detected in *L. longiflocosa* from Irco Dos Aguas or in *L. columbiana* from Agua Bonita. In contrast, in *L. longiflocosa* samples from Agua Bonita, 66 of 309 pools were positive. Infected phlebotomines were observed in two of the sampled houses. All the sampled houses had a history of *Leishmania* cases (one to four cases per house) that had been treated previously; no active lesions

were observed during the sampling for phlebotomines. The highest prevalence of infection was found inside dwellings (2.7%) and in peridomestic environments (2.5%), whereas in the forest, the rates of infection were low (0.2%). Most of the infected phlebotomines were collected during the months with high population densities of *L. longiflocosa*, and higher prevalence was found indoors (Table 2).

Blood-fed phlebotomines were also tested for *Leishmania* infection; 8 of 123 samples were positive, and of these samples, 4 samples were human blood. The blood source of the other four blood meals was not identified (Supplemental Table 2).

Sequencing. Of the 29 kDNA-positive *L. longiflocosa* captured in Agua Bonita (21 from pools of non-blood-fed and 8 from blood-fed specimens), 6 samples were successfully sequenced (Figure 4). Of the sequenced kDNA samples, five samples were from *Le. (V.) guyanensis*, and

TABLE 2
Summary of infection with *L. (V.)* parasites using PCR–Southern blot in pools of *L. longiflocosa* by month and environmental setting

	2007						2008				Positive pools	Infection (%)
	June	July	Aug	Sept	Nov	Dec	Jan	Feb	Apr	May		
Indoors												
p(+)/tp	2/2	0/3	0/3	0/3	0/1	1/1	0/3	7/17	0/1	0/1	10/35	2.7
n	12	44	21	33	2	1	26	306	4	1	450	
Peridomestic												
p(+)/tp	1/6	2/6	10/57	17/28	0/2	0/3	6/10	16/19	1/2	0/2	52/135	2.5
n	97	101	1,125	537	23	45	172	380	17	3	2,500	
Woodland												
p(+)/tp	0/4	0/5	0/24	0/13	0/1	0/2	1/7	2/79	1/3	0/1	4/139	0.15
n	53	81	444	234	2	30	104	1,572	46	6	2,572	

p(+)/tp = positive pools/total pools.

one sample was identified only as *Le. (V.)* sp. because of an inability to get a complete sequence. The sequences from the *L. (V.) guyanensis* samples isolated (Blood21TOCHAB005, Blood66TOCHAB011, and NonBlood16) showed SNPs associated with *Le. (V.) guyanensis* isolated from a patient (isolate 3) from Chaparral. The other two sand fly isolates (NonBlood17 and NonBlood4) had identical sequences to the reference strain (M4147) of *Le. (V.) guyanensis*.

DISCUSSION

This study shows, for the first time, the natural infection of *L. longiflocosa* with parasites of the subgenus *L. (V.)*. This species was the most abundant phlebotomine species observed in the Chaparral County, Tolima Province, where the largest Colombian outbreak of cutaneous leishmaniasis was reported. The results showed that the most abundant species, *L. longiflocosa*, fed mainly on humans in the study sites and that infected phlebotomines were mainly found in the indoor and peridomestic environment. This finding indicated that *Leishmania* transmission was occurring in the domestic environment. Because *Le. (V.) guyanensis* was detected in the infected phlebotomines, the data confirmed that the *Leishmania* parasites continued to circulate in the post-epidemic period.

L. longiflocosa is endemic to Colombia and had been suggested as the principal vector of leishmaniasis in the sub-Andean region of the upper and middle Magdalena River valley.^{14,21–23} Similar conditions in terms of elevation, abundance, and domestic and peridomestic behavior of *L. longiflocosa* were observed in other foci located in the Provinces of Huila (Baraya, Tello and Neiva),²¹ Norte de Santander (Abrego),²² Tolima (Planadas),²³ and Tolima (Chaparral).¹⁴ The ability of this species to transmit *Leishmania* parasites was successfully evaluated under laboratory conditions with *Le. braziliensis*.³⁵

Clear differences in species composition and abundance of phlebotomines were noted. In Agua Bonita, abundance and species diversity were significantly greater than in Irco Dos Aguas. Although both townships have similar altitudinal ranges and human population densities, great differences were observed in land use. Land coverage, using remote sensing data, differs between the sites mainly in terms of forest and shrubs. Agua Bonita has nine times more forest area than Irco Dos Aguas (43.2% versus 3.9%) and one-third of the shrub coverage (19.4% versus 62.2%). Similar results were observed in the 100-m radius around the sampled houses: 39.8% was classed as forest in Agua Bonita compared with 4.2% in Irco Dos Aguas. Grassland and shrubs predominated in the latter. Similar proportions of coffee plantations around the houses were observed in both sites (36.4% in Agua Bonita and 29.5% in Irco Dos Aguas); however, in Agua Bonita, some were mixed with other trees to provide shade. Of the environments tested, forest had the highest phlebotomine capture rate. These observations suggested that the greater abundance of phlebotomines is associated with increased forest cover in the township of Agua Bonita. Previous spatial analysis of the region, using remote sensing, indicated that case incidence was associated with higher coverage with forest or shrubs (2.6% greater for each additional percent coverage).¹⁷ In turn, the differences found in phlebotomine abundance, especially of *L. longiflocosa*, may partially explain the difference in CL prevalence cases between the sites.

Periods of lower rainfall were inversely associated with abundance peaks of *L. longiflocosa* and *L. columbiana*. Additionally, higher prevalence of phlebotomine infection was found in the periods with higher vector abundance. These results suggest that transmission risk may be associated with human domestic areas and that risk would increase during the periods of highest abundance of phlebotomine activity, affecting persons without distinction of age or sex. This seasonal pattern is similar to the bimodal distribution of CL cases previously reported in the epidemic of Chaparral County,¹⁵ in which the peak of cases was observed in the months of October–December and April–June during the 2003–2006 outbreak (Hospital San Juan Bautista of Chaparral, unpublished data). The affected human populations were living in townships situated at the elevations inhabited by *L. longiflocosa* (1,000–2,200 m) and hence, were exposed to its biting during the dry periods when the densities were highest. Then, after an incubation period of 8–12 weeks, the leishmaniasis cases began to appear.³⁶ Risk of *Leishmania* transmission increased in the houses with higher phlebotomine abundance, which were located between 1,400 and 1,700 m, which has been observed in other studies.²¹

The nocturnal activity of *L. longiflocosa* showed clear hourly patterns. The major activity occurred early in the peridomestic environment (19:00–20:00 hours) and at midnight (23:00–24:00 hours) in the indoor environment, although activity was observed throughout the night in both environments (18:00–4:00 hours). These periods corresponded to when humans are predominantly in the domestic environment and suggest an adaptation of the vector to human behavior.

The PCR–Southern blot analysis of the 700-bp mini circle kDNA identified the presence of *Le. (V.)* infection in the phlebotomines. This technique increases the sensitivity because of the high number of copies of the mini circle kDNA present in the parasite, and it maintains specificity.³⁷ The sequence of the 7SLRNA in infected specimens was able to identify the species *Le. (V.) guyanensis* in six samples of *L. longiflocosa* (three samples had the same sequence observed in the isolate from a patient in Chaparral). These results also confirm previous reports of this *Leishmania* species circulating in this outbreak in humans and dogs.^{10,38} The presence of phlebotomine infected with *Le. (V.)* parasites in the post-epidemic period is surprising, because no active CL lesions were observed during these studies. However, *Leishmania* has been isolated from human lesions 9 years after their appearance and apparent resolution,³⁹ which has been confirmed by both molecular and biological methods in skin sampled from clinically unaffected areas in confirmed CL cases.^{40,41} Furthermore, individuals in endemic areas without clinical symptoms have been observed to harbor parasites.³⁰

The presence of *Le. (V.) guyanensis* in the area suggested that it has recently expanded its range using an anthropophilic phlebotomine vector very different from the vector in its natural enzootic foci, such as *L. umbratilis* and *L. antunesi*.⁴² In the Caribbean region of Colombia where *Le. (V.) guyanensis* has also been found, the vector has not been determined, and known vectors have not been observed in this region.⁹ The current results suggest that the parasite is exploiting multiple vector species in different foci.^{3,43,44}

The other potential vector species with known epidemiological importance in other transmission foci—*L. columbiana*, *L. (Helcocyrtomyia)* spp., and *L. nuneztovari*—are unlikely to have played a major role in transmission because of their low

abundance. However, this study was the first time that a natural infection of *Le. (V.)* was observed in *L. (Helcocyrtomyia)* spp. in Colombia. These species showed an annual population behavior similar to *L. longiflocosa* (i.e., an increase in occurrence in the months of lowest rainfall). This observation is consistent with the behavior of other phlebotomine species from the *Townsendi* series, with populations that are generally more abundant in dry periods.⁴⁵ The presence of several specimens of *L. oresbia*, *L. lerayi*, and *L. shannoni* reaffirmed the species diversity of *Lutzomyia* in Colombia, even at high altitudinal ranges (1,000–2,000 m).

In conclusion, *L. longiflocosa* is confirmed as the principal vector responsible for domestic transmission in Chaparral. Additionally, this study is the first report of natural infection in this species with parasites of the *Le. (V.)* as well as with the species *Le. (V.) guyanensis*. The presence of infections with *Le. (V.)* parasites in the phlebotomines documents the domestic presence of the parasite into the post-epidemic period. The phlebotomine infections and habitat distributions explain the high proportion of infections in women and children during the outbreak, again confirming domestic transmission in this focus. These conclusions will facilitate the design of prevention and control measures that focus on application at the beginning of the dry season in the sub-Andean regions of Colombia.

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