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Leishmania (*Viannia*) Infection in the Domestic Dog in Chaparral, Colombia

Julián Santaella, Clara B. Ocampo,* Nancy G. Saravia, Fabián Méndez, Rafael Góngora, Maria Adelaida Gomez, Leonard E. Munstermann, and Rupert J. Quinnell

Centro Internacional de Entrenamiento e Investigaciones Médicas, Cali, Colombia; Escuela de Salud Pública, Universidad del Valle, Cali, Colombia; Department of Public Health, Yale University, New Haven, Connecticut; Institute of Integrative and Comparative Biology, University of Leeds, Leeds, United Kingdom

Abstract. Peridomestic transmission of American cutaneous leishmaniasis is increasingly reported and dogs may be a reservoir of *Leishmania* (*Viannia*) in this setting. We investigated the prevalence of infection in dogs in Chaparral County, Colombia, the focus of an epidemic of human cutaneous leishmaniasis caused by *Leishmania* (*Viannia*) *guyanensis*. Two (0.72%) of 279 dogs had lesions typical of cutaneous leishmaniasis that were biopsy positive by kinetoplast DNA polymerase chain reaction–Southern blotting. Seroprevalence was 2.2% (6 of 279) by enzyme-linked immunosorbent assay. Buffy coat and ear skin biopsy specimens were positive by polymerase chain reaction–Southern blotting in 7.3% (10 of 137) and 11.4% (12 of 105) of dogs, respectively. Overall 20% of dogs (21 of 105) showed positive results for one or more tests. Amplification and sequencing of the *Leishmania* 7SL RNA gene identified *L. guyanensis* in one dog and *L. braziliensis* in two dogs. No association was identified between the risk factors evaluated and canine infection. Dogs may contribute to transmission but their role in this focus appears to be limited.

INTRODUCTION

New World cutaneous leishmaniasis (CL) is a group of diseases mainly caused by *Leishmania* (*Viannia*) spp. and characterized by a spectrum of clinical manifestations in humans, ranging from localized dermal ulcers to mucocutaneous lesions.[1] Although death from CL is rare, cutaneous lesions may generate psychological, social, and economic problems in affected populations. Moreover, chronic manifestations such as mucocutaneous disease are often refractory to chemotherapy and represent an important public health concern. The number of reported cases of leishmaniasis has increased in Colombia from an average of 6,500 new cases per year in the 1990s to approximately 20,000 cases reported in 2005 and 2006, of which 99% were CL.[2]

Human CL in the Americas is considered a zoonotic disease, with human infection resulting from transmission from sylvatic mammalian reservoirs by bites of the sand fly vector (*Lutzomyia* spp.). Cutaneous leishmaniasis in Colombia is usually associated with occupational contact with forest areas by adult males; few infections occur in young children.[3] The largest epidemic of CL recorded in Colombia occurred in Chaparral County, Department of Tolima during 2003–2004, when 2,346 cases were recorded in an area where few cases had previously been reported (2–3 cases per year).[3] Since 2005, the number of new cases has decreased in the area, but transmission has continued at a lower rate. Notably, during 2003–2006, 8.5% of cases occurred in children 0–4 years of age, consistent with domestic transmission. *Leishmania* (*Viannia*) *guyanensis* was the principal etiologic agent of this epidemic, and was identified in 94.6% (53 of 56) of human samples.[4] Interestingly, *L. guyanensis* had not been previously reported in the Andean region of Colombia, and the epidemic may have resulted from introduction of the parasite into an immunologically naïve population as a result of human migrations from the Amazon region.

The mammalian reservoir(s) involved in the Chaparral County epidemic are unknown. Evidence for domestic transmission suggests involvement of one or more peridomestic reservoirs. The domestic dog (*Canis familiaris*) is well known as a zoonotic reservoir for visceral leishmaniasis caused by *Leishmania infantum*, and there is increasing evidence for its role as reservoir for *Leishmania* (*Viannia*) species. Several studies in South America have shown that dogs can be experimentally infected with *L.* (*Viannia*) species and that a high proportion of the canine population is naturally infected.[5–9] However, dog infections have not been characterized in areas where *Leishmania* (*Viannia*) *guyanensis* is transmitted.

The overall objective of the current study was to examine the role of dogs as a potential reservoir host in the Chaparral County focus. Specific aims of the study were to 1) determine the prevalence of *Leishmania* (*Viannia*) infection in dogs and identify the *Leishmania* species involved, 2) compare different diagnostic methods, and 3) explore possible risk factors for canine infection.

MATERIALS AND METHODS

Study area. Chaparral County is located in the Department of Tolima (3°43′N, 75°30′W) in the western chain of the Colombian Andes Mountains. Chaparral County consists of small rural townships (veredas) located 300–3,700 meters above sea level. The townships in which transmission was recorded are located 1,000–2,000 meters above sea level in the Ambeima and Hermosas Canyons. At these elevations, hillsides are largely cleared for farming, but patches of natural forest still remain.[10]

Sample collection. Samples were collected during July–October 2007 from 279 dogs in the 13 townships with the highest cumulative incidence (30–99%) of human cases of CL during 2003–2006. Sampling was conducted in conjunction with a rabies vaccination program implemented by the Tolima Secretary of Health. Dogs were gathered at a central location in each township, and owner permission was obtained by formal informed consent before each dog was sampled. This study was performed in accordance with national guidelines and international standards for humane care and use of laboratory animals, and reviewed and approved by the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) institutional review committee for

*Address correspondence to Clara B. Ocampo, Centro Internacional de Entrenamiento e Investigaciones Medicas, Carrera 125 No. 19–225, Cali, Colombia. E-mail: claraocampo@cideim.org.co

research in animals, Federal Wide Assurance no. A5643-01, U.S. Department of Health and Human Services, and the committee for the ethical evaluation of experimental animal research of the Universidad del Valle Faculty of Health, Cali, Colombia.

Each dog was examined for leishmaniasis-like lesions or scars. If lesions were observed, the dog was sedated by intramuscular administration of xylazine (0.5–1 mg/kg) and acepromazine (0.5–1 mg/kg). Lesion biopsy specimens and scrapings from the lesion edge were taken by using a sterile scalpel. Biopsy specimens were placed in 1.5-mL sterile polypropylene tubes containing 100% ethanol and kept at 4°C for up to 10 hours and –20°C for long-term storage. Lesion scrapings were smeared onto a microscope slide and held at ambient temperature. Slides were stained with Giemsa and microscopically examined for parasites. Lesion aspirates were obtained from the edges of lesions using a 1-mL tuberculin syringe containing 0.1 mL of buffered saline solution containing penicillin and streptomycin. Aspirates were inoculated into tubes containing Senekjie's diphasic culture medium (4 tubes per lesion). Tubes were transported to the laboratory at ambient temperature and then incubated at 27°C for 4 weeks and microscopically inspected on a weekly basis for parasite growth.

A blood sample (4 mL) was obtained from all dogs by venipuncture of the cephalic vein and placed in sterile, EDTA-coated tubes. Tubes were kept at 4°C until processing. Blood was centrifuged at 2,800 rpm for 20 minutes, and the buffy coat and serum were collected and stored at –20°C for up to 4 days. Samples were then transported to Cali, Colombia and stored at –70°C.

Biopsy specimens of normal ear skin (0.2–0.5 cm^2) were taken from each dog (n = 263) after consent of the owner was provided by using a sterile scalpel blade and after administration of local anesthesia (0.1 mL of lidocaine). Biopsy specimens were stored in 100% ethanol as described above.

Negative and positive controls. Twenty-five dogs from an urban area of Cali to which CL was not endemic served as negative controls. The positive control serum consisted of a high-titer serum sample from a dog with an active cutaneous lesion from Villavicencio, Department of Meta, Colombia. The causative species of the positive control was identified as *Leishmania* (*Viannia*) *panamensis* by using monoclonal antibodies and isoenzyme electrophoresis.[11,12]

Serologic analysis. IgG against *Leishmania* were quantified by using an enzyme-linked immunosorbent assay (ELISA). Soluble antigen was obtained from *L. panamensis* (strain MHOM/COL/1986/1166; CIDEIM) cultured in Senekjie's medium. Parasites were washed in sterile phosphate-buffered saline (PBS), lysed by using a freeze–thaw cycle at –45°C for 10 minutes, and centrifuged at 12,000 rpm for 20 minutes at 4°C. Soluble protein concentration was quantified by using the Bradford method. Antigen was diluted to a concentration of 50 µg/mL in coating buffer (0.015 M $NaCO_3$, 0.035M $NaHCO_3$, pH 9.6), and applied (50 µL/well) to alternate rows of a 96-well plate (Immunolon I; Dynatech Laboratories, Chantilly, VA). Plates were incubated overnight at 4°C, washed three times with washing buffer (PBS, 0.1% Tween 20, pH 7.4), blocked with 200 µL of blocking buffer (coating buffer, 10% fetal bovine serum), and incubated at 37°C for 1 hour. After three washings, dog serum samples (50 µL/well) were added at dilutions of 1:200, 1:400, and 1:800 in incubation buffer (PBS, 10% fetal bovine serum). Each dilution of serum was added to an antigen-coated and non–antigen-coated well to control for nonspecific binding.

Plates were incubated at 37°C for 2 hours and washed three times with washing buffer. Horseradish peroxidase–conjugated rabbit anti-dog IgG (Sigma-Aldrich Laboratories, St. Louis, MO), diluted 1:1,000 in incubation buffer (100 µL/well) was then added and incubated at 37°C for 1 hour. Plates were washed three times and developed at room temperature for 1 hour with 100 µL/well of a 1:1 mixture of ABTS substrate (2,2′ azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]) and peroxidase solutions (ABTS microwell peroxidase substrate system; Kirkegaard and Perry Laboratories, Gaithersburg, MD). Absorbance was read at 405 nm. Because *Trypanosoma* transmission has not been reported in the area of the study, *Trypanosoma*-specific ELISAs were not performed.

Absorbance (optical density) values were expressed as arbitrary units of antibody/milliliter relative to a positive control serum as described.[13] On each plate, the positive control serum was titrated two-fold at dilutions from 1:50 to 1:409,600. This serum was assigned an arbitrary number of antibody units, 25,600/mL, defined as the reciprocal of the highest dilution at which absorbance was greater than 3 SD above background values (wells without serum). Absorbance values were calculated as observed absorbance in the antigen-coated well minus the absorbance from the non-antigen well. A standard line was fitted to the positive control serum absorbance values by using a log-logit transformation over the range of dilutions 1:50–1:25,600.[13] The slope and intercept of the line was calculated by linear regression, and the absorbance of each test serum was expressed as the geometric mean antibody units/milliliter by using the standard line. A sample was considered positive when its log-antibody titer was greater than 3 SD above the mean of the log-antibody titer of the 25 negative control serum samples. This threshold was set at 3.02 log units/mL (1,050 units/mL).

Polymerase chain reaction–Southern blot. DNA from buffy coat, ear skin, and lesion samples was extracted by using the DNeasy extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A kinetoplast DNA (kDNA) PCR was conducted as described by Vergel and others[14] according to modifications reported by Figueroa and others.[15] The primers LV (5′-ATT TTT GAA CGG GGT TTC TG-3′) and B1 (5′-GGG GTT GGT GTA ATA TAG TGG-3′), amplify a 700-basepair fragment of the kDNA minicircle of *L.* (*Viannia*) species.[14] Specificity of these primers was tested by using control DNA from dog, *L. chagasi*, *Trypanosoma cruzi*, and *T. rangeli*. No amplification of dog or trypanosome DNA was observed. Amplification of *L. chagasi* DNA was weak and produced a multiple band pattern, but not the 700-basepair band characteristic of *L.* (*Viannia*). The PCR thermal profile was 35 cycles at 95°C for 5 minutes, 92°C for 1 minute, 66°C for 40 seconds, and 72°C for 30 seconds. The PCR products were analyzed by electrophoresis on 1.3% agarose gels in 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0). Separate areas for DNA extraction, PCR sample preparation, and amplification were used to avoid cross-contamination.

A chemiluminescent Southern blot was performed to improve the sensitivity and specificity of PCR as described elsewhere.[14,16] 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) buffers for 20 minutes each, and blotted

onto a nylon membrane (Hybond N+; Amersham Bioscience, Piscataway, NJ). Membranes were hybridized at high stringency (65°C) with a probe derived from *L. panamensis* kDNA (GenBank accession no. HQ878382) and the Alka-Phos Direct Labeling and Detection System with CDP-Star (Amersham-Pharmacia Biotech, Little Chalfont, United Kingdom) 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 basepairs) was seen in the Southern blot, even if a negative result was obtained after agarose gel electrophoresis of PCR products. Seven blood samples of dogs from Cali were used as negative controls for the PCR–Southern blot.

Identification of *Leishmania* species. Buffy coat samples that were positive for *Leishmania* kDNA, reference strains (*L. braziliensis* M2903, *L. guyanensis* M4147, and *L. panamensis* LS94), and two *L. guyanensis* strains isolated from patients during the Chaparral County epidemic[4] were processed for species typing by direct sequencing of PCR products of the *Leishmania* 7SL RNA gene region as described.[17] The PCR was carried out by using primers LeishFW (5′-CATCCGTGACAGGATTCGAACC-3′), corresponding to a sequence of approximately 200 basepairs upstream from the putative 7SL gene start sequence, and LeishRV (5′-CGTGGGGCTCAAGTGCGGACATG-3′), corresponding to sequence at a position 36 basepairs upstream from the end of the putative 7SL gene sequence.[17] The PCR product of approximately 430 basepairs was extracted from the gel and purified for the sequencing reaction by using the QIAquick Gel Extraction Kit (Qiagen). Sequence analyses and single nucleotide polymorphism (SNP) identification were performed by using BioEdit v7.0.5 and Sequencher Demo version software. Similarity search was performed by using BLAST. Multiple sequence alignment and editing were conducted by using ClustalW2 and CLC DNA Workbench 5.5 limited mode.

Risk factors for canine infection. Information on potential risk factors for infection was obtained by using a standard questionnaire given to dog owners at the time of sampling. Individual households were not visited because of armed conflict in the study area. Canine infection was assessed as a positive ELISA or PCR–Southern blot result for an individual dog. Information was obtained for the following risk factors for canine infection: age and sex, fur length (≥3 cm and <3 cm), condition (malnourished or well nourished), and whether the dog was used for hunting. Information was also obtained for household risk factors for canine infection, including presence of one or more past human CL cases in the household, number of dogs and other domestic animals in the household area (swine, fowl, bovines, equines, and ovines), approximate distance from the house to the nearest forest patch, and reported presence of sand flies (*Lutzomyia* spp.) in the house.

Statistical analysis. Differences in the proportion of dogs with positive results for each diagnostic test were compared by using McNemar's test with exact probabilities. Agreement between diagnostic tests was quantified by using the kappa statistic. Individual and household risk factors for canine infection were analyzed by exact logistic regression because the number of *Leishmania*-positive dogs was low. Exact confidence intervals (CIs) and significance were calculated by using mid-*P* values. All analysis was performed by using Stata version 11.0 (Stata Corp LP, College Station, TX).

RESULTS

***Leishmania* infection in domestic dogs.** A total of 279 dogs were examined, of which 73.4% (204 of 279) were male and 40.1% (113 of 279) were ≥ 3 years of age. Dogs ≥ 3 years of age would have been alive in the leishmaniasis-endemic area at the time of peak incidence and transmission to humans. Of the 74 females, 6 (8.1%) were pregnant and 7 (9.5%) were lactating. Most dogs were of mixed breed (77.7%), were free to roam (99%) and slept outside the house (97%). Ninety seven (38.2%) dogs were used for hunting activities.

The 279 dogs belonged to 197 owners, of whom 60.7% (119 of 197) reported previous CL in at least one person in their household. All the dogs were examined clinically and serologically; 137 dogs were tested by PCR-Southern blot for buffy coat. Of these dogs, 105 were tested by PCR–Southern blot with ear skin biopsy specimens.

Two dogs (0.7%) had active CL-like lesions; each had two or three small ulcerated lesions in the scrotum area. Lesions were covered with fibrous tissue, but bled easily when cleaned with a sterile dressing. The owners of both dogs reported that lesions had been present for at least two years. Cultures from lesion aspirates and slides of lesion scrapings were both negative when examined microscopically. However, lesion biopsy specimens from both dogs were positive for *Leishmania* (*Viannia*) spp. parasites by kDNA PCR–Southern blot. Both dogs were serologically positive by ELISA, and ear skin biopsy specimens from these dogs were positive by PCR–Southern blot, although the result of a PCR–Southern blot for buffy coat samples was negative. No CL scars were seen in any of the dogs studied.

Serologic testing showed 6 (2.2%) of 279 dogs had positive ELISA results (including the two clinical cases above) (Table 1). Titers of seropositive dogs were relatively low (geometric mean = 2,902 units/mL, range = 1,218–6,616 units/mL) compared with the positive control serum (25,600 units/mL). The proportions of dogs with positive PCR–Southern blot results

TABLE 1

Percentage of dogs from Chaparral, Colombia positive for *Leishmania* (*Viannia*) infection by ELISA and PCR–Southern blot on buffy coat and ear skin biopsy specimens*

Dogs	% Positive (no. tested/total)			
	ELISA	PCR–Southern blot on buffy coat	PCR–Southern blot on skin biopsy specimen	Total positive by any test
All tested dogs	2.2% (6/279)	7.3% (10/137)	11.4% (12/105)	–
95% CI	(0.8–4.6%)	(3.6–13.0%)	(6.0–19.1%)	
Dogs tested by all three methods	5.7% (6/105)	7.6% (8/105)	11.4% (12/105)	20.0% (21/105)
95% CI	(2.1–12.0%)	(3.3–14.5%)	(6.0–19.1%)	(12.8–28.9%)

* ELISA = enzyme-limked immunosorbent assay; PCR = polymerase chain reaction; CI = confidence interval.

were higher: 7.3% (10 of 137) of buffy coat samples and 11.4% (12 of 105) of ear skin biopsy specimens. None of the tested samples from the negative control dogs were positive by PCR–Southern blot.

The combined prevalence of infection in those dogs evaluated by using the three diagnostic tests was 20.0% (21 of 105) (Table 1). There were no significant differences in sensitivity between the three tests ($P > 0.15$ for each pairwise comparison). Agreement between ELISA and skin biopsy PCR–Southern blot results was weakly significant (kappa = 0.27, 95% CI = 0–0.66, $P = 0.0011$); three of six dogs with positive ELISA results had positive skin biopsy results. Agreement was not significant between PCR–Southern blot for buffy coat and either ELISA (kappa = –0.07; $P = 0.76$) or PCR–Southern blot on skin biopsy specimens (kappa = 0.12, 95% CI = 0–0.52, $P = 0.11$).

Sequencing. Of the 10 kDNA-positive buffy coat samples, 9 were processed for *Leishmania* species identification by 7SL RNA gene sequencing. From these samples, three amplification products were successfully sequenced. All sequences were clearly identified as *L.* (*Viannia*) spp. Sequence from one dog (dog 8) was identified as *L. guyanensis* on the basis of species-specific SNP typing.[17] The full sequence was almost identical to that of the reference strain of *L. guyanensis*, differing only at position 141 (G→A), which has not been described as a species-specific SNP (Figure 1). This sequence differed at two positions (G77C, A141G) from the two human *L. guyanensis* isolates (isolates 3 and 9). Sequence from dog 5 was identical to that of the *L. braziliensis* reference strain. Sequence from dog 4 was closest to *L. braziliensis* on the basis of species-specific SNPs, though it differed at 4 positions (G77C, C87T, C124T, C127T).

Risk factors for *Leishmania* infection in dogs. Characteristics of the dog population are shown in Table 2. The prevalence of canine infection measured by ELISA or PCR–Southern blot generally increased with age, although the increase was not significant (Table 2). Prevalence by each test was also higher in households where the owners reported the presence of sand flies in the house, approaching significance for the ELISA ($P = 0.054$). No correlation was seen between dog infection and previous human infection in the household (Table 2). Prevalence (by each diagnostic test) did not differ significantly between townships ($P > 0.13$, by exact test). No significant associations were observed when positivity by either ELISA or PCR–Southern blot was used as an outcome variable.

DISCUSSION

Natural infection of the domestic dog has been reported in countries in Latin America with the following species of *Leishmania* (*Viannia*): *L. braziliensis*, *L. peruviana*, *L. panamensis*, and *L. colombiensis*.[7] In Colombia, canine infection with *L. braziliensis* and *L. panamensis* has been reported,[5,18] but without prevalence of infection or disease data. In the current study, canine infection was documented in an area where the predominant species transmitted to humans was *L. guyanensis*. Sequencing of the 7SL RNA gene region enabled identification of *L. guyanensis* and *L. braziliensis* in one and two

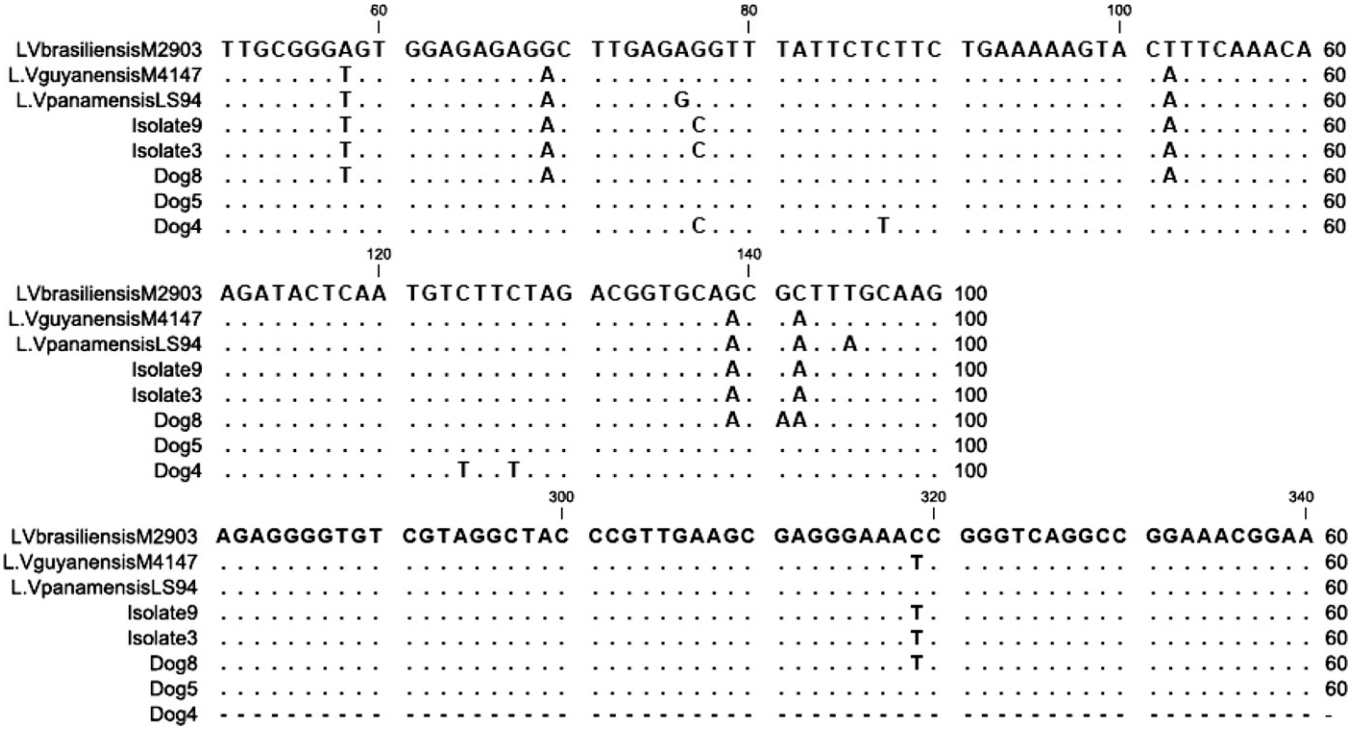

FIGURE 1. *Leishmania* species identification by single nucleotide polymorphism typing of the 7SL RNA gene region, Colombia. 7SLRNA PCR (~430 bp) products from *L.* (*Viannia*) reference strains (*L. braziliensis* M2903, *L. guyanensis* M4147 and *L. panamensis* LS94), *L. guyanensis* strains from Chaparral patients (isolates 3 and 9) and dog buffy coat samples (dogs 4, 5 and 8) were sequenced and species identification was determined by SNP analysis. Species-specific SNPs used for typing within the *L.* (*Viannia*) subgenus, as previously reported by Stevenson,[17] are as follows:
L. braziliensis 58A, 69G, 76A, 102T, 139G, 142C, 145T, 319C
L. panamensis 58T, 69A, 76G, 102A, 139A, 142A, 145A, 319C
L. guyanensis 58T, 69A, 76A, 102A, 139A, 142A, 145T, 319T

TABLE 2
Association between individual and household risk factors and *Leishmania* (*Viannia*) infection in dogs from Chaparral, Colombia*

Risk factor	% Positive (no. tested/total)		
	ELISA (6/279)	Buffy coat PCR–Southern blot (10/137)	Ear skin PCR–Southern blot (12/105)
Age of dog, years			
0–3	0.5% (1/199)	6.7% (6/90)	10.8% (7/65)
4–10	6.3% (5/80)	8.5% (4/47)	12.5% (5/40)
OR (per year) (95% CI)	1.30 (0.95–1.74)†	1.17 (0.90–1.50)	1.03 (0.77–1.34)
Sex of dog			
M	2.5% (5/204)	7.4% (8/108)	11.3% (9/80)
F	1.4% (1/74)	6.9% (2/29)	12.0% (3/25)
OR (95% CI)	0.55 (0.02–4.03)	0.93 (0.13–4.29)	1.07 (0.22–4.19)
Fur length, cm			
Short (< 3)	2.6% (5/192)	4.2% (4/96)	12.2% (9/74)
Long (≥ 3)	1.4% (1/71)	15.2% (5/33)	12.5% (3/24)
OR (95% CI)	0.54 (0.02–3.95)	4.05 (0.96–18.0)†	1.03 (0.21–4.04)
Dog condition			
Well nourished	2.8% (4/152)	6.3% (5/80)	15.0% (9/60)
Malnourished	1.6% (2/127)	7.7% (4/52)	7.3% (3/41)
OR (95% CI)	0.55 (0.07–3.17)	1.25 (0.29–5.16)	0.45 (0.09–1.72)
Reported presence of sand flies in the house			
No	0.0% (0/106)	4.7% (2/43)	5.9% (2/34)
Yes	3.5% (6/172)	8.6% (8/93)	14.3% (10/70)
OR (95% CI)	5.16 (0.97–∞)†	1.92 (0.42–13.8)	2.65 (0.60–18.7)
Previous human CL case in the house			
No	0.9% (1/110)	8.0% (4/50)	11.8% (4/34)
Yes	3.0% (5/169)	6.9% (6/87)	11.3% (8/71)
OR (95% CI)	3.31 (0.45–79.7)	0.85 (0.22–3.59)	0.95 (0.27–3.88)
Distance from house to forest, meters			
Far (≥ 200)	3.0% (4/132)	6.2% (4/65)	14.6% (7/48)
Near (< 200)	1.4% (2/147)	8.3% (6/72)	8.8% (5/57)
OR (95% CI)	0.44 (0.06–2.54)	1.38 (0.36–5.80)	0.57 (0.15–1.96)
No. dogs in household			
OR (per dog) (95% CI)	1.19 (0.48–2.84)	0.92 (0.40–2.01)	0.71 (0.31–1.49)
No. other domestic animals			
OR (per 10 × change) (95% CI)	1.02 (0.302.72)	0.35 (0.08–1.01)†	0.83 (0.39–1.62)

* ELISA = enzyme-limked immunosorbent assay; PCR = polymerase chain reaction; OR = odds ratio; CI = confidence interval; CL = cutaneous leishmaniasis.
† $0.05 < P < 0.10$.

dogs, respectively, although parasite species was not identified in most infected dogs. *Leishmania guyanensis* infection has not been previously confirmed in the domestic dog, although infection with *L.guyanensis/L.braziliensis* hybrids has been reported in Venezuela.[19]

The overall prevalence of canine infection in the study area was 20%, although the prevalence using any single diagnostic test was lower. Using serologic analysis alone, a prevalence of only 2.2% was observed. Prevalence of *Leishmania* (*Viannia*) infection in dogs elsewhere in South America varies from 0% to 63%, depending on the study site and diagnostic test.[7] Although the overall prevalence of infection in Chaparral County was comparable with that reported in other studies, the prevalence of CL-like lesions in dogs (0.7%) was low.[7] Both lesion-positive dogs had lesions for more than two years; low parasite numbers would be expected in such chronic lesions.[20] This finding may explain the failure to detect parasites in lesions by culture or microscopy, although method issues associated with working in a remote field site cannot be excluded.

Few previous studies have compared the sensitivity of several diagnostic tests for canine infection. In this study, PCR–Southern blot of ear skin samples was the most sensitive method. The level of agreement between the results for buffy coat and ear skin was weak, suggesting that parasite loads in both sites were low and resulted in a low probability of detection by any test. These results indicated that surveys that rely on serologic analysis may underestimate the prevalence of infection, and that multiple PCRs may be needed for an accurate assessment of prevalence. The relatively high sensitivity of PCR using buffy coat was unexpected. Our findings differ from those for *L. braziliensis/L. peruviana* dog infection in Peru, where ELISA was more sensitive than PCR of blood (20% seroprevalence compared with 9% PCR positivity).[9] These differences indicate either that antibody responses in the Chaparral County study area were short-lived, that not all infected dogs seroconverted, or that unspecified method dissimilarities may be responsible.

There was a low prevalence of symptomatic and seropositive dogs compared with PCR-positive dogs; the proportion of symptomatic infection was only 8.7% (2 of 21 infected dogs). In contrast, the proportion of symptomatic infection with *L. braziliensis/L. peruviana* in Peru was 15%[9] and in Argentina, 72% of seropositive dogs had lesions.[20] These comparisons suggest that the species or strains of parasite circulating in dogs in Chaparral County were less pathogenic. Sampling bias may also have affected the prevalence estimates because the study relied on passive sampling alone (because visits to households were not possible). Questionnaire data showed that dog owners brought 70% of their dogs to the vaccination site, but the number of dog owners not present was unknown. It is possible that sick dogs were less likely to be brought for vaccination, and therefore the prevalence of symptomatic infection may have been underestimated. The sampled dogs had a strongly biased sex ratio (73% male). This finding may indicate a sampling bias, although the lack of an association between prevalence and gender suggests that this

was not a critical bias. Moreover, the sex ratio is likely to have been male-biased because male dogs are preferred as working dogs in this area.

Sequencing results suggest that *L. guyanensis* and *L. braziliensis* are circulating in the dog population in this area. Unfortunately the low sequencing success, probably caused by low parasite numbers in asymptomatic infection, does not enable an estimate of the relative prevalence of each species. This finding complicates the interpretation of the prevalence data because it is likely that the sensitivity of different diagnostic tests may vary with species. The presence of *L. braziliensis* has not been reported in the area. Of 56 human isolates, 53 were identified as *L. guyanensis* and 3 as *L. panamensis*.[4] In the present study, the two dogs with confirmed *L. braziliensis* infections were born in the study area, suggesting autochthonous transmission. However, this is an area of high human migrations, and it is possible that *L. braziliensis* is a recent introduction. Species-specific SNPs enabled typing of *Leishmania* species in these samples. However, sequence from one dog differed at four positions from the reference strain of *L. braziliensis*, and the *L. guyanensis* canine sequence differed from the reference strain at one position and the patient strains of *L guyanensis* at 2 positions. Although these variations did not occur within the reported species-specific SNPs, the significance of this sequence variation is unknown because intraspecific sequence variation at this locus has not been examined.

Risk factor analysis did not show significant risk factors for *Leishmania* infection in dogs. The lack of significant associations at $P < 0.05$ may reflect the low prevalence found with each test, which resulted in lowered power of the analyses. Furthermore, household risk factors were obtained by report of the householder rather than by direct observation and may have introduced reporting errors. The prevalence of infection was generally higher in older dogs, but differences were low and not significant. The lack of a strong association between infection status and dog age can be attributed to a high recovery rate from detectable infection, as has been observed in Peru where the recovery rate was 0.5/year.[21] The association, although weak, between householder-reported presence of sand flies in the house and seroprevalence, coupled with the lack of an association between hunting and infection, suggests that dogs acquired infection in the peridomestic environment. This finding is consistent with the hypothesis that human transmission in this area is peridomestic, and the putative vector *Lutzomyia longiflocosa* is known to occur inside houses in the study area.[22] We were unable to detect an association between canine and human infection in the household. However, dogs were sampled several years after the peak of human transmission. Such associations have previously been reported in Argentina and Peru.[20,23]

Identification of *L. guyanensis* in an asymptomatic dog suggests that dogs could serve as a reservoir of infection in this area. This dog lived in a household with a history of human cases which at the time of sampling had one person with an active lesion. Interpretation of the epidemiologic data is complicated by the fact that dogs were sampled several years after the peak incidence in humans. However, the much higher prevalence of symptomatic infection in humans in the study site (up to 99%) compared with dogs, and the presence of *L. braziliensis* in dogs but not humans, suggests that dogs do not play a major role in transmission to humans in this focus. The presence of infected dogs does not prove that dogs can transmit infection to the vector. Infectiousness of dogs infected with *L. infantum* is positively correlated with symptomatic infection and high levels of antibodies against *Leishmania*.[24] If the same is true for *Leishmania* (*Viannia*) infections, dogs in the Chaparral County study area may not be highly infectious, given the low serologic and clinical prevalence. However, the presence of parasite DNA in normal ear skin and blood of asymptomatic dogs supports the availability of parasites for sand fly uptake. These results confirm that dissemination of parasites to extra-lesional sites occurs in infected dogs, as has been shown for *L. guyanensis* in *Choloepus* sloths (the sylvatic host),[25] and for *Leishmania* (*Viannia*) in dogs in Peru.[9]

Confirmation of the ability of infected dogs to transmit infection requires xenodiagnostic studies. Previous studies in which sand flies were fed on lesions of dogs with symptomatic *L. braziliensis* infection have produced contrasting results: xenodiagnosis perfomed on two Colombian dogs using 3 different local vectors of cutaneous leishmaniasis and colonized *Lutzomyia longipalpis* failed to infect any of the sandflies,[5] while *Lutzomyia whitmani* were infected after feeding on 3 of 9 Brazilian dogs.[26] Also, less than 1% of *Lutzomyia youngi* was found to be infected after being fed on two dogs that had lesions produced by *Leishmania* (*Viannia*) parasites.[27] These studies, although small, suggest that symptomatic dogs are not highly infectious. Further xenodiagnostic studies are necessary to determine whether asymptomatic dogs are able to infect sand flies.

In summary, these results provide evidence of canine infection with *L. guyanensis* and *L. braziliensis* in an area where human *L. guyanensis* transmission occurs. Further studies, particularly xenodiagnosis, will be essential to determine whether dogs can be a reservoir for human infection in this epidemiologic setting.

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Authors' addresses: Julián Santaella, Clara B. Ocampo, Nancy G. Saravia, Rafael Góngora, and Maria Adelaida Gomez, Centro Internacional de Entrenamiento e Investigaciones Medicas, Cali, Colombia, E-mails: julian.santaella@gmail.com, claraocampo@cideim.org.co, saravian@cideim.org.com, rafael_gongora@cideim.org.co, and maria.adelaida.gomez@gmail.com. Fabián Méndez, Escuela de Salud Publica, Universidad del Valle, Cali, Colombia. E-mail: famendez@grupogesp.org. Leonard E. Munstermann, School of Medicine, Yale University, New Haven, CT, E-mail: leonard.munstermann@yale.edu. Rupert J. Quinnell, Institute of Integrative and Comparative Biology,

University of Leeds, United Kingdom, E-mail: r.j.quinnell@leeds.ac.uk.

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