CANINE VISCERAL LEISHMANIASIS IN COLOMBIA: RELATIONSHIP BETWEEN CLINICAL AND PARASITOLOGIC STATUS AND INFECTIVITY FOR SAND FLY FLIES

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Abstract. We studied the reservoir competency of canines with distinct clinical presentations of Leishmania chagasi infection. The parasitologic status of asymptomatic and symptomatic dogs was determined by standard culture methods. Infectivity was assessed by multiple xenodiagnoses with Lutzomyia longipalpis, over a period of 2–11 months. Asymptomatic dogs were non-infective (0 of 5) while 2 of 7 oligosymptomatic dogs infected L. longipalpis, transmitting the parasites at low rates (range 0.9–5.2% of engorged flies). Polysymptomatic dogs transmitted L. chagasi more frequently (4 of 8 dogs) and reached higher infection rates (range 5.0–22.5% of engorged flies). The skin of the ear tended to be more infective to sand flies than that of the abdomen. Primerase chain reaction hybridization (PCR-H) was a sensitive method for detection of L. chagasi, yielding the highest positive rate in serum (16 of 17 dogs) with no distinction between clinical groups. No association between skin positivity by PCR-H and infectivity to sand flies was found. The infectivity of dogs from clinically comparable groups from Colombian and Mediterranean foci differed. This may be a reflection of varied nutritional conditions or vector competency of distinct sand fly species.

INTRODUCTION

Visceral leishmaniasis (VL) is a zoonotic disease of public health importance in which wild and domestic mammals act as reservoir hosts. Land exploitation has extended the natural cycle of Leishmania infantum (=Leishmania chagasi) to rural and periurban habitats where humans, dogs, and a few species of phlebotomine vectors are involved. Canines have been recognized as the main source of L. infantum in the peridomestic setting and therefore have been the target of control measures against the human disease. In recent years, epidemiologic studies have failed to conclusively demonstrate that elimination of dogs could have a long-lasting effect on the incidence of L. infantum infection in both dog and human populations. Therefore, understanding the circumstances in which parasites are transmitted from canines to sand flies is essential for the implementation of sound prophylactic and control measures.

Infectivity of dogs to sand flies has been investigated in the Mediterranean basin using specimens of the highly competent vectors Phlebotomus perniciosus and Phlebotomus ariasi while in South America most studies have been carried out in Brazil with the natural vector Lutzomyia longipalpis. Results obtained with L. infantum and its natural vectors in Europe differ from those described in earlier reports from South America. This discrepancy could be due to differences in parasite strains, nutritional status of dogs, and vector capacity of the species in different endemic foci. Also, it is possible that the diagnostic methods used to detect infected animals have not identified all the infective individuals. For these reasons, we carried out transmission studies in Colombian dogs at different clinical stages of natural or experimental L. chagasi infection. The parasitologic status was determined by standard culture methods and PCR-H. Infectivity was assessed by subjecting dogs to multiple xenodiagnoses over a period of several months using natural (Lutzomyia longipalpis) and experimental (Lutzomyia youngi) Leishmania vectors.

MATERIALS AND METHODS

Dogs. A group of twenty mongrel dogs composed of three puppies (< 5 months old) experimentally infected with L. chagasi and 17 adults collected in the VL-endemic area of Cundinamarca (4° 18’N, 74° 42’W), Colombia, were used in this study. Animals were maintained according to international and Colombian guidelines (Law 84/89) in a kennel located in a sand fly-free area near the city of Cali.

All dogs were serologically positive as determined by an enzyme-linked immunosorbent assay (ELISA) which used soluble Leishmania chagasi antigen (1 μg/well) and peroxidase-labeled goat anti-dog IgG (Kirkegaard and Perry Labs). Animals were considered infected when the optical density of the serum was > 3 standard deviations above that of healthy uninfected controls. The specificity and sensitivity of this method has shown to be 85–96% and 98%, respectively.

Diagnosis and clinical classification of L. chagasi infection. Leishmania chagasi infection was confirmed either by culture (whole blood or leukocyte fraction) and/or PCR-H of serum, skin samples (ear or abdomen), or popliteal lymph node aspirate as previously described. The primers used in PCR assays were designed to amplify the variable and conserved regions of the kinetoplast DNA minicircle of L. donovani. Skin samples were lysed with 100 μl of TE buffer (10 mM Tris, 1 mM EDTA) containing 50 μg of proteinase K for 2 hr at 37°C. Lymph node aspirates and serum (100 μl) were centrifuged at 10,000 × g for 5 min and lysed with 30 μg of proteinase K. DNA was extracted with a mixture of phenol-chloroform-isoamyl alcohol (Sigma, St. Louis, MO) and precipitated for 3 hr at −70°C with 1/10 volume sodium acetate (2.5 M), glycogen (20 μg) and 3 volumes of cold ethanol. The DNA pellet was centrifuged at 15,000 × g (30 min, 4°C), desiccated for 20 min with a vacuum pump and subsequently hydrated for 15 min at 50°C in 15 μl of water. Ten μl of diluted DNA (1:50 or 1:10 for skin or lymph node aspirate and serum, respectively) were used for the PCR reaction. A hot-start technique was used to avoid nonspecific amplification. The amplification product was transferred to a nylon membrane with a dot-blot apparatus (Schleicher and Schuell) and hybridized with a kDNA-specific biotin-labeled probe (BioPrime, GIBCO, BRL), which was detected according to manufacturer’s instructions (Photogene, GIBCO, BRL).
Xenodiagnosis of dogs infected with *Leishmania chagasi*

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<th>Clinical status and code no. of dogs</th>
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<th>Intensity of infection</th>
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* Asymptomatic at the time of the last xenodiagnosis, which was positive. LY = *Lutzomyia youngi*, LL = *Lutzomyia longipalpis*, nd = not done; na = not applicable; $+$ = 0–50 promastigotes, $+$+ = 50–100 promastigotes, +++ = 100–200 promastigotes, ++++ = >200 promastigotes.

Animals suffering visceral leishmaniasis commonly have one or more of the following signs: weight loss, enlarged lymph nodes, hepatosplenomegaly, keratoconjunctivitis, onychogryphosis, alopecia, dermatitis, skin ulcers, with bacterial secondary infection, anemia, and coagulopathies evidenced by bleeding especially hemoptysis. According to their clinical condition animals were divided into asymptomatic (n = 5), oligosymptomatic (one or two symptoms; n = 7), and polysymptomatic (> 2 symptoms; n = 8) as described by Mancianti and others.10

Experimental infection of three puppies was carried out either by the intradermal (n = 1) or intravenous route (n = 2) using promastigotes of *L. chagasi* from the stationary phase of growth (8 days of culture). This strain (MCAN/COL/98/CAT) was originally isolated from a polysymptomatic dog residing in the Cundinamarca focus. The puppy infected through the intradermal route (determined by PCR) remained asymptomatic while the other two puppies infected intravenously developed full-blown visceral leishmaniasis in approximately 3 months.

**Sand flies, Lutzomyia longipalpis**, the most important vector of visceral leishmaniasis in the Americas, was used for xenodiagnosis. Individuals were obtained from a colony established in 1994 at the National Institute of Health, Bogotá, Colombia. This colony originated from specimens collected in El Callejón, Cundinamarca and is currently maintained as a semi-closed population. *Lutzomyia youngi*, which was used as an experimental vector, was captured in an illuminated Shannon trap11 in the natural reserve of Mateguada (4° 35’N, 76° 12’W) in the municipality of Tulúa, Valle del Cauca where no transmission or promastigote infections in wild-caught individuals were detected during entomologic studies carried out between 1989 and 1999.

**Xenodiagnosis.** Sand flies (colonized and wild-caught) were maintained at 26°C and 80% relative humidity in 250 ml plastic containers covered with fine mesh. They were deprived of sugar 24 hr prior to xenodiagnosis. Dogs were anesthetized with xylacine (2 mg/kg; Rompun®, Bayer) and variable numbers of sand flies (30–60) were allowed to feed for 30 min on the skin of the abdomen or ears. Two dogs, one oligosymptomatic and one polysymptomatic, were used to compare the infectivity to sand flies according to the body site where insects obtained the blood meal. Unfed females were withdrawn from the cages where sand flies were maintained for 5–7 days on a sugar-water diet after they had taken their blood meal. Sand flies were examined for *Leishmania* promastigotes in the mid- and foregut by individual dissection and inspection of wet mounts at 400 ×. Dog infectivity was evaluated on the basis of percentage of infected flies and intensity of infection, determined by the approximate number of promastigotes in the sand fly gut (Table 1).

**RESULTS**

None of the five dogs considered asymptomatic at the beginning of the study was infective to *L. longipalpis* or *L. youngi* after multiple xenodiagnoses were performed at time intervals ranging from 1 to 8 months (Table 1). Two of seven dogs from the oligosymptomatic group were infective to sand flies. These dogs transmitted the parasite at low rates.
as indicated by the number of *L. longipalpis* (range 0.9–5.2%) and *L. youngi* (1.5%) infected after feeding (Table 1).

Polysymptomatic dogs transmitted *L. chagasi* to *L. longipalpis* more frequently (4 of 8) than the oligosymptomatic group, and infection rates of vectors also proved to be in a higher range (5.0–22.2%). In addition, sand flies that fed on polysymptomatic individuals tended to have larger numbers of promastigotes in the mid- and foregut, suggesting that animals at this stage of infection had a higher parasite burden in the skin than oligosymptomatic dogs (Table 1).

All dogs included in the study that were positive by culture or PCR-H were confirmed as infected either by culture or PCR-Hybridization (Table 2). The success rate in isolating *L. chagasi* by aspiration and culture from the popliteal lymph node was associated with the clinical condition of the animals. No parasites were cultured from lymph nodes of the 5 asymptomatic dogs while positive cultures were obtained from 4 (4 of 20). Although all the dogs that infected sand flies (n = 6) had positive lymph node cultures, a significant proportion of culture-positive individuals (5 of 11) were non-infective to sand flies (Tables 1 and 2). Experiments in which xenodiagnoses were carried out simultaneously on the ears and abdomen of an oligosymptomatic and a polysymptomatic dog showed that the skin of the ear tended to be more infective than that of the abdomen (Table 3).

PCR-H was a sensitive method to detect *L. chagasi* in samples obtained from different body sites of dogs at different stages of infection. PCR was less sensitive than PCR-H as shown by the higher numbers of positive individuals in all the clinical groups with the latter method (Table 2). Similar positive rates were found in serum (16 of 17, 94%), and lymph node (19 of 20, 95%) with no clear distinction between clinical groups. Lower sensitivity of PCR-H from the skin of the ear (70%) and abdomen (65%) was observed and no association was found between positivity at these sites and infectivity to sand flies (Tables 1 and 2).

Four individuals that were infective to the natural vector *L. longipalpis* were also exposed to *L. youngi* (an experi-
important reservoirs that not only transmit leishmanias to the sand fly vectors but also suffer overt disease. One of these species, the domestic dog, represents the most important reservoir of L. infantum in the peridomestic setting. Its epidemiologic role generally has been assessed by serology, a method that has not always correlated with the clinical status.

Several studies have established a strong association between therapy-related decreases in parasite burden, diminution of the infective potential of dogs, and improvement of the clinical condition. Our xenodiagnoses were performed on clinically characterized dogs free of intestinal parasites maintained under standard nutritional regimes. Thus, variables that may influence infection evolution and infectivity to vectors were controlled for.

The data derived from study of these dogs are similar to previous observations in Brazil by Sherlock, who reported that asymptomatic dogs are modest sources of L. chagasi for the vectors, and that individuals at an advanced stage of disease become more infective to sand flies. Unfortunately, since no clinical description of the dogs actually used in these experiments was presented, the association of positive xenodiagnosis and disease status remains unclear.

The molecular method used in our study to detect L. chagasi, which was PCR-H of samples from different body sites, failed to provide a reliable predictor of dog infectivity to vectors. It is possible that skin biopsies included areas of amastigote-laden tissue at depths beyond the reach of the sand fly faciscle (piercing-sucking mouthparts) during feeding.

The fact that several dogs positive by lymph node culture were not infective to sand flies (5 of 11) suggests that parasite dissemination follows a sequential, compartmentalized pattern in which lymphoreticular organs reach higher burdens at earlier stages of infection than the skin. Since L. chagasi was isolated from the blood of only one polysymptomatic and one oligosymptomatic dog, we believe that L. chagasi is circulating in low numbers, below the threshold of detection by standard culture methods. This is supported by the fact that PCR-H detected L. chagasi in sera from 16 of 17 animals tested regardless of the clinical status. We cannot rule out the possibility that molecular techniques could also detect parasite DNA from non-viable or disrupted organisms as has been suggested for Toxoplasma gondii, Mycoplasma gallisepticum, and Chlamydia trachomatis.

Poor nutrition and to some extent stress have been considered important factors negatively influencing the clinical status of hosts infected with Leishmania. In general, the dogs included in the present study were undernourished and infected with ecto- and endoparasites when they were first identified. Their health in the kennel improved following deworming and the establishment of an adequate nutritional regime. Therefore, with the exception of 3 polysymptomatic dogs that died of VL, some animals categorized as polysymptomatic became oligosymptomatic (2 of 8), and one of seven oligosymptomatics reverted to asymptomatic at the end of the study. A polysymptomatic dog that initially failed to infect sand flies became increasingly more infective, concomitantly with the appearance of additional VL signs and a marked deterioration of the clinical condition.

It should be noted that one of the dogs initially included as polysymptomatic improved clinically but not parasitologically because 3 months after clinical recovery it remained culture positive (lymph nodes and blood) and infective to sand flies. This observation suggests differences in the parasitologic status and infectivity to vectors of asymptomatic dogs at the beginning of the infection (i.e., low parasite burden) compared to those that acquire this status after remission from overt disease (i.e., high parasite burden).

Available data suggest that Phlebotomus perniciosus may be a more competent vector than L. longipalpis. Molina and others, using naturally infected dogs from an endemic area in Spain, showed that a high proportion of asymptomatic dogs transmitted L. infantum to P. perniciosus. This study also indicated that there was no difference in the infective potential of the distinct clinical groups of dogs. The infection rate of P. perniciosus reported by different authors usually ranged between 32–92% while that of L. longipalpis from different studies, including ours, ranged from 13% to 29%. Two alternative hypotheses could be postulated: 1) the threshold for infecting P. perniciosus is lower than that necessary to infect L. longipalpis, and for this reason asymptomatic dogs with lower parasite burdens in the skin are still capable of transmitting L. infantum to the former vector species; or 2) dogs in Europe, because of better nutrition, remain asymptomatic despite having higher parasite burdens than the undernourished, Latin American dogs, and consequently are more infective to P. perniciosus.

In some of our experiments we included Lutzomyia youngi, a putative vector of Leishmania Viannia braziliensis, which is distributed in Costa Rica, Venezuela, and Colombia. We were able to infect this sand fly species on polysymptomatic and oligosymptomatic dogs. Although infection rates were lower than those observed for L. longipalpis (and dog infection by sand fly bite was not attempted), these observations underscore the need to evaluate the risk of introducing infected dogs in areas where sand flies different from the natural vectors (L. longipalpis, Lutzomyia evansi) are present. As opposed to the European foci, certain regions in the Neotropics have intense sand fly biting activity all year round, which could compensate for the low vector capacity. Moreover, potential wild reservoirs such as Didelphis marsupialis and Proechimys spp., which are widely distributed in these areas, and represent links between the peridomestic and sylvatic habitats, could become infected, contributing to the establishment of new foci.

Deane and Grimaldi reported that cutaneous parasitism in canines is frequent, and our results suggest an uneven distribution of Leishmania in the skin of different body sites. In our dogs, the skin from the ears was a better source of
Leishmania for the vectors than the abdominal skin. The ears of dogs living in rural areas are frequent targets of hematophagous arthropods (black flies, gnats, mosquitoes, ticks, sand flies), and although the saliva of many Diptera and Acarina may contain antihistamine substances, the fact is that inflammatory lesions in this body site are common, probably due to pruritus and subsequent scratching. The presence of inflammatory cells, including those parasitized by Leishmania, suggested previously as playing a role in dissemination, could account for the local maintenance of parasite burdens above the transmission threshold, as opposed to the skin of the abdomen, where arthropod feeding (and scratching) is less frequent. Also, it has been postulated that the cooler sites of the body, such as the ears, could propagate an increased localization and/or reproduction of leishmanias as compared to warmer areas, thus favoring transmission. Studies in humans have shown that the dermal capillary density varies according to the body site, and that the head-neck region is significantly more irrigated than the abdomen. Therefore (extrapolating these findings to canines) one would expect that a richer capillary bed, mainly of the papillary dermis, would facilitate the arrival or permeation of a large number of infected cells into the blood pool produced by sand fly teeth during feeding. The fact that infectivity of our polysymptomatic dogs could be demonstrated by xenodiagnoses carried out on the abdomen (data not shown), concur with the inference that animals at this clinical stage had higher parasite burdens than oligosymptomatic dogs.

Regarding immunoprophylaxis, and assuming that L. longipalpis has a lower vector capacity than P. perniciosus, vaccination strategies that would prevent dogs from developing high parasite burdens, could reduce transmission more effectively in Latin America than in the Mediterranean basin.

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