

Prevalence of *Leishmania infantum* Infection in Dogs Living in an Area of Canine Leishmaniasis Endemicity Using PCR on Several Tissues and Serology

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We studied and compared the prevalence of *Leishmania* infection and the seroprevalence and the prevalence of canine leishmaniasis in an area where canine leishmaniasis is endemic. One hundred dogs living on the island of Mallorca (Spain) were studied. In this study, we clinically examined each dog for the presence of symptoms compatible with leishmaniasis, determined the titer of anti-*Leishmania* antibodies, and investigated the presence of *Leishmania* DNA by PCR in skin, conjunctiva, and bone marrow samples of each dog. The prevalence of the disease and the seroprevalence were 13 and 26%, respectively. In 63% of the dogs, *Leishmania* DNA could be detected by PCR in at least one of the tissues studied. The results of positive PCR in the bone marrow, the conjunctiva, and the skin were 17.8, 32, and 51%, respectively. The prevalence of the infection, 67%, was calculated using all animals that were seropositive and/or positive by PCR with any tissue. The results showed that the majority of dogs living in an area where canine leishmaniasis is endemic are infected by *Leishmania* and that the prevalence of infection is much greater than the prevalence of overt *Leishmania*-related disease.

Canine leishmaniasis is a severe systemic disease of dogs caused by the protozoan parasite *Leishmania infantum*. Clinical manifestations of the disease include nonpruritic skin lesions, such as exfoliative dermatitis and ulcerations, local or generalized lymphadenopathy, loss of weight, poor appetite, ocular lesions, epistaxis, lameness, renal failure, and diarrhea (5, 9, 14, 25). Leishmaniasis is a zoonotic disease for which dogs are considered the chief reservoir of the parasite. The disease is endemic in the Mediterranean basin, where seroprevalence ranges between 10 and 37% (10, 24). There are, however, several studies suggesting that the rate of infection is higher than the figures found by serological investigations. A survey performed using the PCR and immunoblotting techniques found that most dogs living in southern France had been exposed to *Leishmania* (3). These results agree with another study that found a rate of *Leishmania* infection of 65% for dogs living in Portugal by using serology and cell-mediated tests (4).

The percentage of infected dogs living in an area where canine leishmaniasis is endemic has major public health implications. It was demonstrated that infected, but asymptomatic, dogs were sources of the parasite for phlebotomine vector sandflies and as a consequence play an active role in the transmission of *Leishmania* (15).

The present study was designed to investigate and compare the prevalence of *Leishmania* infection, the seroprevalence and the prevalence of the disease in a canine population living

in an area where canine leishmaniasis is endemic. One hundred dogs living on the island of Mallorca (Spain) were included in this study. Veterinarians clinically examined all dogs, and the titer of anti-*Leishmania* antibodies was determined. The presence of *Leishmania* DNA in each dog was investigated by PCR with three tissues: skin, eye conjunctiva, and bone marrow.

MATERIALS AND METHODS

Animals. The study was carried out on the Island of Mallorca, an area of canine leishmaniasis endemicity. The subjects of the study were 100 dogs from different breeds and ages, which had to be euthanized in the Animal Pound of Palma de Mallorca for reasons related to city sanitation policy.

Sampling. Prior to sampling and euthanasia, all dogs were examined to detect clinical symptoms compatible with canine leishmaniasis. The dogs were then premedicated with acepromazine maleate and anesthetized intravenously with sodium thiopental.

Blood was collected by cephalic or jugular venepuncture, and the serum samples for detecting and quantifying specific antibodies to *Leishmania* were stored at -80°C . Three types of tissues for PCR were sampled: bone marrow, skin, and eye conjunctiva. Bone marrow aspirates were obtained from the costochondral junctions by using a 22 gauge needle. Cutaneous samples were collected from the upper part of the muzzle by punch biopsy with a diameter of 5 mm and with each biopsy weighing approximately 30 mg. Conjunctiva samples were obtained using scissors, with each biopsy weighing approximately 30 mg. Samples were stored at -20°C before DNA extraction. After sampling was completed, dogs were euthanized using an overdose of parenteral barbiturates.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed as previously described (20). Briefly, microtiter plates were coated with a $20\text{-}\mu\text{g ml}^{-1}$ concentration of *L. infantum* antigen in 0.1 ml of coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) and incubated overnight at 4°C . One hundred microliters of dog sera per well was diluted 1:400 in phosphate-buffered saline (PBS)–0.05% Tween 20 (PBST)–1% dried skim milk and was incubated for 1 h at 37°C . After washing three times with PBST and once with PBS, 100 μl of anti-dog immunoglobulin G (IgG) (1:20,000) conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, Tex.) was added. This conjugate was incubated for 1 h at 37°C , and then the plates were rewashed. The substrate

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TABLE 1. Clinical status, serology, and PCR results, including the three tissues sampled for each dog, in a canine population living in an area of Mallorca where leishmaniasis is endemic

Group no.	Serology ELISA result	Presence of <i>Leishmania</i> DNA			Clinical status		
		Bone marrow	Conjunctiva	Skin	No. of symptomatic dogs	No. of asymptomatic dogs	Total no. of dogs
1	+	+	+	+	9	4	13
2	+	-	-	+	2	6	8
3	+	-	-	-	0	4	4
4	+	+	-	-	0	1	1
5	-	-	+	+	0	10	10
6	-	-	-	+	2	17	19
7	-	-	+	-	0	9	9
8	-	+	-	+	0	1	1
9	-	+	-	-	0	2	2
10	-	-	-	-	0	33	33

solution (ortho-phenylene-diamine, 0.4 mg/ml) (Sigma) and H₂O₂ (0.4 µl/ml) in 0.1 M phosphate-citrate buffer (pH 5.0) was added at 200 µl/well and developed for 20 min at 24°C. The reaction was stopped with 50 µl of 3 M H₂SO₄. Absorbances were read at 490 nm in an automatic microELISA reader (EL 312e microplate; Bio-tek Instruments).

Sera from 28 dogs not infected with *L. infantum* that were living in a region where it is endemic were tested to set up a cutoff for IgG-specific ELISA determinations. The cutoff absorbance was established as the mean plus 3 standard deviations, resulting in 0.236 for IgG (mean, 0.099; standard deviation, 0.0456). All determinations included serum from a sick dog with a confirmed infection as a positive control and serum from a healthy dog as a negative control.

DNA isolation. (i) Bone marrow. Samples of DNA were prepared as previously described (22). Briefly, 115-µl bone marrow samples were washed and centrifuged three times in Tris-EDTA buffer (pH 8.0), and the leukocyte pellet was incubated in 0.1 ml of lysis buffer (50 mM potassium chloride, 10 mM Tris-HCl [pH 8.4], 0.5% Tween 20, and 100 µg of proteinase K/ml) at 56°C overnight. Proteinase K was inactivated by incubating the samples at 95°C for 10 min before using them in the PCR.

(ii) Conjunctiva and skin tissue. Conjunctiva and skin biopsies were digested overnight in the presence of sodium dodecyl sulfate and proteinase K at final concentrations of 2% and 0.2 mg/ml, respectively, in 1 ml of TE buffer (50 mM Tris [pH 8.0], 20 mM EDTA). Afterwards, DNA was isolated by double phenol-chloroform extraction (23).

PCR. *Leishmania*-specific oligonucleotide primers SP176 (5'-TCTTGGCGGG GAGGGGGTG-3') and SP177 (5'-TTGACCCCAACCACATTTTA-3') were used to amplify a 120-base-pair fragment of *Leishmania* kinetoplast DNA minicircles (21). PCR was conducted in a 50-µl final reaction mixture containing PCR buffer, 1.5 mM MgCl₂, 0.1 mM concentrations of each deoxynucleoside triphosphate, 0.3 µM concentrations of each primer, 3 µl of supernatants of digested tissue, and 1.25 U of *Taq* polymerase (Ecogen). Reactions were carried out in an automatic thermocycler (Perkin-Elmer) with a thermal cycling profile of 94°C for 3 min, 35 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and finally 72°C for 5 min, and at that point the thermocycler maintained a constant temperature of 4°C. Positive controls containing 10 ng of genomic of *Leishmania* DNA and a negative control without template DNA were included. Amplified fragments were analyzed by electrophoresis in a 2.5% agarose gel containing ethidium bromide (0.5 µg/ml) at 100 V for 1 h. A φX174 HaeIII (DNA MWM V; Boehringer Mannheim) was used as a molecular weight marker.

To ensure that negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation, or PCR inhibition, sample DNA was also amplified for β-actin by using a forward primer (5'-ACC TGGAGTTCGAGGYTCGGA-3') and a reverse primer (5'-AAGTAACCTG GTTGGTGAAGCAG-3') (12). When samples did not yield amplification products, they were extracted again until amplification products were obtained.

RESULTS

Thirteen animals presented one or more clinical signs of canine leishmaniasis while 87 dogs were asymptomatic. Twenty-six of the dogs were seropositive. Eleven of them also

showed clinical signs while 15 were clinically healthy dogs. Two dogs with clinical signs of leishmaniasis were seronegative.

The presence of parasite DNA was detected in 63 dogs for at least one of the three tissues investigated. The results of the PCRs with the different tissues were as follows: 17 out of 95 dogs (17.8%) had positive bone marrow, 32 out of 100 dogs (32%) had positive conjunctiva, and 51 out of 100 dogs (51%) had positive skin. *Leishmania* DNA was detected in all animals presenting clinical signs.

Results for clinical status, serology, and PCR, including the three tissues sampled for each dog, are shown in Table 1.

The prevalence of the infection, 67%, was calculated by adding all animals that were seropositive and/or positive for PCR with any tissue.

DISCUSSION

According to our results, the prevalence of canine leishmaniasis in Mallorca and the seroprevalence are 13 and 26%, respectively. These results are in agreement with those obtained by various authors throughout the Mediterranean basin (6, 24, 27). For canine leishmaniasis, serology is considered to be a sensitive and useful technique and is well correlated with clinical signs. However, the meaning of asymptomatic but seropositive dogs (15 out of 26) is difficult to explain without a follow-up study. Undoubtedly, this condition indicates previous contact with the parasite, but we do not know whether these dogs are immune resistant animals or whether they will subsequently develop the disease (4).

The prevalence of infection that we found in Mallorca (67%) is very high, although lower than the 80% found in France using a smaller number of dogs (3). Thus, our study and others confirm that the prevalence of *Leishmania* infection has been underestimated (3, 4). These data are useful for epidemiological studies and would provide a better estimation of the level of transmission of the parasite.

The frequency of DNA parasite detection was different in different tissues. The low percentage of positive bone marrow PCR (17%) suggests that a hematogenous dissemination to the bone marrow takes place only in part of the animals. Consequently, the detection of *Leishmania* DNA in bone marrow by using PCR is not an adequate method to detect *Leishmania* infection in dogs. Our results, therefore, disagree with those of

other authors who found bone marrow PCR a better diagnostic method than serology (1). On the other hand, half of the dogs studied were positive to parasite DNA detection in the skin. This result indicates that skin is the major tissue reserve of parasites in dogs and that PCR in skin biopsy is a sensitive method to detect infection. This finding concerns the biology of the parasite because the skin is the most accessible tissue for the vector. Furthermore, cutaneous samples were collected from the upper part of the muzzle where most sandflies take their blood meal (13).

In the compartmental mathematical model of canine leishmaniasis, it was assumed that asymptomatic dogs were not infectious for sandflies (8, 11). However, other authors showed that infectivity of dogs presenting *Leishmania* infection is not exclusively linked to the symptomatic stage of the disease: they found that three out of five asymptomatic but seropositive dogs transmitted the parasite to the sandfly vectors (15). Our results support the idea that asymptomatic dogs must be considered infectious for sandflies. We showed that 54% of healthy dogs living in an area where *Leishmania* is endemic must be considered asymptomatic carriers of *Leishmania*. Further studies are needed to ascertain the potential of asymptomatic dogs to transmit *Leishmania* to vector sandflies.

Studies of the immune response against the *Leishmania* parasite in the dog have revealed that T lymphocytes and the cytokines they produce play a crucial role in determining whether an infection with this intracellular pathogen results in either protective immunity or progressive disease (17, 18, 19). In the population of our study, it would have been interesting to study the cellular immune response using a field tool such as leishmanin skin test. This was not, however, possible for ethical reasons, as we would have needed to previously manipulate animals not destined for research. However, it has been reported that 40% of the asymptomatic dogs living in Portugal (4) and that 48% of the asymptomatic dogs living in Mallorca (26) had demonstrable parasite-specific cellular immunity. We found that 37% of asymptomatic dogs were PCR positive in the skin and/or conjunctiva and were seronegative. Probably, dogs with suitable cellular immunity could control the spread of *Leishmania* parasite and live in equilibrium with the parasite in the skin and mucosal regions. This fact may explain the high rate of infection due to the whole spectrum of immune response to *Leishmania* in the canine population (4). In human beings, a strong association between the decrease in the number of CD4⁺ T lymphocytes and the increase in the infectivity of people coinfecting with *L. infantum* and human immunodeficiency virus to the sandflies has been reported recently (16).

The high prevalence of *Leishmania* infection in regions where leishmaniasis is endemic has to be taken into account in any campaign aimed at controlling canine leishmaniasis. In fact, some authors have demonstrated that removing seropositive dogs is an insufficient method to eradicate canine leishmaniasis (2, 7).

Vaccines against *Leishmania* are a goal for the scientific community working on human and canine leishmaniasis. Potential vaccines could act by either destroying the parasite and/or preventing disease pathology. While the latter type of vaccine would prevent severe disease and morbidity among immunized dogs, the parasite transmission cycle would remain intact. This is especially important because of the high preva-

lence of *Leishmania* infection that we have shown, suggesting that asymptomatic dogs act as reservoirs for parasite transmission to sandflies. Thus, potential canine vaccines must induce sterile immunity, eliminating amastigotes that reside in apparently healthy skin. This type of vaccine would both prevent canine leishmaniasis and result in decreased human disease.

In conclusion, this study demonstrates that the prevalence of *Leishmania* infection in an area of endemicity is higher than assumed and that the main tissue reserve of the parasite in dogs is the skin. This information is essential for designing and implementing appropriate control measures and must be addressed when evaluating the efficacy of any vaccine.

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