CROSS-SECTIONAL SEROSURVEY OF FELINE LEISHMANIASIS IN ECOREGIONS AROUND THE NORTHWESTERN MEDITERRANEAN

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Abstract. A cross-sectional serosurvey using *Leishmania infantum* ELISA was performed on 445 cats living in ecoregions around the Northwestern Mediterranean basin; 58 cats from an area of the US where leishmaniasis is not endemic were used as negative controls. ELISA results were further confirmed in 69 cats by Western blot (WB). Finally, 76 of them were also tested for FeLV and FIV. Seroprevalence by ELISA-prot A was 6.29%, and that by ELISA-IgG was 5.25%. Positive cat sera recognized patterns of polypeptides in WB, including *L. infantum*-specific antigenic fractions. There was no association with retroviruses. *Leishmania*-specific antibodies are prevalent in cats living in ecoregions around the Northwestern Mediterranean basin; thus, leishmaniasis must be included in the differential diagnosis of diseases in cats living in these ecoregions. Their role as peridomestic reservoirs for *L. infantum* needs further characterization, but it could be hypothesized that the cat is a secondary reservoir host, rather than an accidental one.

INTRODUCTION

Leishmaniases are endemic in 88 countries on 4 continents. In terms of global disease load, the leishmaniases are the third most important vector-borne disease, after malaria and lymphatic filariasis, causing 2.4 million disability-adjusted life years lost and 59,000 deaths in 2001.[1] The causative agents are parasitic protozoan of the genus *Leishmania*. Over 20 species and subspecies of *Leishmania* infect human beings, and sand flies from the genus *Phlebotomus* spp. and *Lutzomyia* spp. are the proven vectors of human visceral and cutaneous and also of canine (CaL) leishmaniases. *Leishmania* can cause a broad spectrum of clinical outcomes, ranging from self-healing skin ulcers, to severe, life-threatening disease. Some leishmaniases are a widespread serious zoonotic disease with a great impact on public health. Understanding how the disease behaves in domestic and wild animals is therefore of great value both in human and veterinary medicine.

Dog is the main peridomestic reservoir for zoonotic leishmaniasis caused by *Leishmania infantum* (= *L. chagasi*) in the Pelearctic and in the Neotropic ecozones. The prevalence of infection in dogs living in the ecoregions around the Northwestern Mediterranean basin reaches a remarkable 67% in areas where it is highly endemic,[2] much higher than previously published results. Because of the feeding habits of sand flies and marked lack of host preference,[3] other vertebrate species are potential reservoir hosts. In fact, strains of *L. infantum* have been isolated and identified in mammals other than humans and dogs living in Western Palearctic, such as foxes,[4,5] rats,[6] horses,[7] and cats.[8]

Leishmaniasis in cats was first described in 1912 in Algeria in a sample of bone marrow from a 4-month old kitten[9] living as a pet in the same house where a dog suffered from CaL and a child was affected by visceral leishmaniasis. Since then, asymptomatic infection or clinical disease in domestic cats caused by *L. infantum* has been reported in ecoregions around the Mediterranean basin sporadically. Cutaneous forms consisting of ulcerocrusted dermatitis, nodular dermatitis, alopecia, and scaling are those most frequently described in feline leishmaniasis,[10–14] and visceral forms with liver, spleen, lymph nodes, and kidney involvement have been less commonly described.[11,15–18] Coinfection with immunosuppressive viruses, both feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV), has been recently confirmed.[12] Experimental infection with *L. infantum* has also been achieved with cats showing serum antibody titers but without clinical symptoms.[19]

Little information is available about epidemiology of feline leishmaniasis caused by *L. infantum* in the Southwestern Palearctic.[20] Over the past years, 8 epidemiologic studies have been carried out in these ecoregions.[12,21–27] with seroprevalences ranging from 62% to 0.9%. In Spain, 115 of 117 cats screened using enzyme-linked immunosorbent assay (ELISA) were negative and 2 were uncertain for *L. infantum* antibody detection.[26]

The aim of the present study was to characterize the epidemiology of feline leishmaniasis in ecoregions around the Northwestern Mediterranean basin by means of a broad cross-sectional serosurvey, to gain an understanding of the role of the cat in this zoonosis.

MATERIAL AND METHODS

Study subjects. Sera collected from 445 cats from the Northeastern Iberian Peninsula and Balearic Islands were studied. Cats were from three different areas: Barcelona (*N* = 390), Tarragona (*N* = 12), and the island of Mallorca (*N* = 43). Clinical veterinarians collected all samples. Samples from Barcelona had two different origins: 255 stray cats from the animal pound of Barcelona and 135 cats examined at the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona.

Complete history was not available for all cats. One hundred and one were male and 76 cats were female. Ninety-three were clinically healthy and 63 presented clinical signs for a variety of diseases. The mean ± SD age for 134 cats was 4.1 ± 4.1 years, ranging from 3.6 months to 17 years. Breed

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was known for 168 cats with 152 European, 9 Persian, and 7 Siamese. No other information was available.

Sera collected from 58 cats seen for medical reasons at the North Carolina State University Veterinary Teaching Hospital, Raleigh, NC, where leishmaniasis is not endemic, were used as negative controls to establish the cut-off for serological techniques.

ELISA. To increase the robustness of the serosurvey results, 2 different ELISAs were carried out. ELISA-protein A (prot A) was performed on all cat samples, and ELISA-Immunoglobulin (Ig) G was performed on a subset of 305 samples.

An ELISA previously described for dog sera was adapted to cat sera.[28] Briefly, microtiter plates were coated with 0.1 mL of *L. infantum* (MHOM/FR/78/LEM-75 zymodeme MON-1) antigen (20 μg mL^{-1} in 0.1 M carbonate-bicarbonate, pH 9.6) and incubated overnight at 4°C. One hundred microliters per well of cat sera, diluted 1:200 in PBS-0.05% Tween 20 (PBST)-1% dried skimmed milk (PBST-M), was incubated for 1 h at 37°C. After 3 washes with PBST and 1 wash with PBS, 100 μL per well of protein A (prot A) (0.2 μg mL^{-1} dilution in PBST-M buffer; Sigma, St. Louis, MO) or anti-cat IgG (1:5000 in PBST-M; Cappel, Durham, NC), both conjugated to horseradish peroxidase (HRPO), was added. These conjugates were incubated for 1 h at 37°C, and then the plates were rewashed. The substrate solution (*ortho*-phenylenediamine, 0.4 mg mL^{-1}; Sigma) plus H$_2$O$_2$ (0.4 μL mL^{-1}) in 0.1 M phosphate/citrate buffer, pH 5.0, was added at 200 μL per well and developed for 20 min at 24°C. The reaction was stopped with 50 μL of 3 M H$_2$SO$_4$. Absorbance values were read at 492 nm in an automatic micro-ELISA reader (Anthos 2001, Anthos Labtec Instruments, GmbH, Eugendorf, Austria). The reaction was quantified as ELISA units (EU) related to a positive cat sera used as a calibrator and arbitrarily set at 100 EU. This cat has confirmed leishmaniasis by bone marrow cytology, inmunohistochemistry staining of ocular tissues, and bone marrow polymerase chain reaction.[18] All determinations included the calibrator serum as a positive control and serum of a cat from an area where leishmaniasis is not endemic as a negative control.

The cut-off was established at 44 EU for prot A and at 53 EU for IgG (mean + 4 standard deviations of sera of 58 cats from an area where leishmaniasis is not endemic). Negative results were established at 29 EU for prot A and at 36 EU for IgG (mean + 2 standard deviations of sera of 58 cats from an area where leishmaniasis is not endemic). Uncertain results were established between positive and negative results for both conjugates.

Immunoblot analysis. To confirm ELISA results, 25 positive cat sera by ELISA and 44 negative cat sera from an area where leishmaniasis is not endemic were assessed by Western blot (WB). WB was performed as described elsewhere.[29] Antigen (3 × 10^8 promastigotes mL^{-1}) in sample buffer (0.5 M Tris-HCl, pH 6.8, 0.01 M EDTA, 5% sodium dodecylsulfate (SDS), 5% 2-mercaptoethanol, 0.0125% bromophenol blue) was run in 0.1% SDS-13% polyacrylamide gels. Immunoblot was carried out with sera at 1:50 dilution in 20 mM Tris, 0.13 mM NaCl, pH 7.6, containing 0.05% Tween 20 (TST) and 1% dry skimmed milk. Prot A-HRPO (Sigma) at 1:1000 dilution was used as secondary detection probe. Color was developed with 4-chloro-1-naphthol (Sigma) and H$_2$O$_2$, and the reaction was stopped with tap water. The relative molecular weights of

the bands detected were calculated with Quantity One® software (version 4.1.1, Bio-Rad, Segrate, Italy) related to the biotinylated SDS-PAGE standard (Low Range, Bio-Rad Laboratories, Hercules, CA).

Detection of FeLV antigen and FIV antibody. To evaluate the impact of immunosuppressive retroviruses, 76 of them were tested for FeLV antigen and for FIV antibody. Detection of FeLV antigen (p27) and FIV antibody was performed with a commercial assay kit (SNAP® FIV Antibody/FeLV Antigen Combo Test; IDEXX Laboratories, Westbrook, ME).

Statistical analysis. Chi square was used to test for associations, and McNemar's test to look at the agreement of the tests. Differences were considered significant if the P value was < 0.05. The program WinEpinfo 5.0 was used to test for agreement between tests.

RESULTS

ELISA. The seroprevalence by ELISA-prot A was 6.29%, and the seroprevalence by ELISA-IgG was 5.25%. There were also 13.03% and 6.56% of uncertain results for ELISA-prot A and ELISA-IgG, respectively. Levels of *Leishmania*-specific antibodies using prot A in positive cat sera ranged from 44.3 EU to 296.3 EU, with a mean of 79.5 EU (95% confidence interval [CI]: 59.1–99.9), and uncertain levels of *Leishmania*-specific antibodies ranged from 28.6 to 42.9 EU, with a mean of 34.4 EU (95% CI: 33.3–35.5). Levels of *Leishmania*-specific IgG antibodies in positive cat sera ranged from 53.5 to 213.7 EU, with a mean of 88.0 EU (95% CI: 67.4–108.6), and uncertain levels of anti-*Leishmania* IgG antibodies ranged from 36.3 to 52.0 EU, with a mean of 43.5 EU (95% CI: 41.4–45.6). There was an overall good agreement between tests (McNemar's test, P = 0.7389). There was not a statistical association between geographical origin, clinical status, age, or sex and positive results for *L. infantum* ELISA.

Immunoblot analysis. ELISA-positive cat sera, including the calibrator cat, recognized variable patterns of polypeptides with molecular masses ranging from 14 through 69 kDa, which included *L. infantum* specific antigen fractions (Figure 1). The highest sensitivity was found for bands of 69 (this value includes fractions of this and higher masses), 54, 34, 28, and 16 kDa (Table 1). Specificity of the bands was determined with those sera from areas where leishmaniasis is not endemic. Sera samples from areas where leishmaniasis is not endemic reacted with 1–5 polypeptides of *L. infantum* antigen, with the most frequent band being detected at 69 kDa (Table 1). No sera from cats living in areas where leishmaniasis is not endemic reacted with polypeptides of low molecular mass (< 26 kDa) that are considered to be the most specific in diagnosis of human and canine leishmaniasis (Figure 1).[29,30]

Detection of FeLV antigen and FIV antibodies and relationship with *Leishmania* antibodies. FeLV antigens were detected in 7.8% of the cats (6 out of 76), and FIV-specific antibodies were found in 6.5% of the cats (5 out of 76). Both retroviruses were detected in 1.3% of the cats (1 out of 76). Only one cat that was positive to FeLV antigen was also positive to *L. infantum* serology, both by ELISA-prot A and ELISA-IgG. No cat presented FIV- and *L. infantum*-specific antibodies. Therefore, no association was found between FeLV antigens or FIV-specific antibodies and *L. infantum*-specific antibodies.
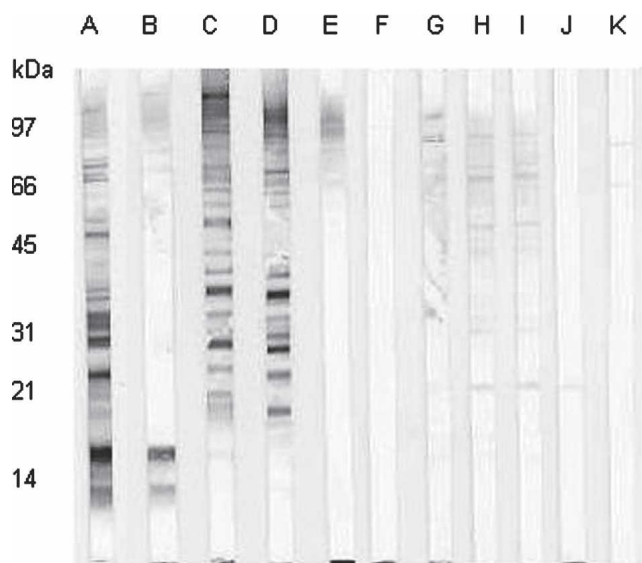
FIGURE 1. PAGE of ELISA-positive cat sera.

TABLE 1
Recognition of *L. infantum* antigens by sera of cats

Band (kDa)	Total (N = 69)		Endemic (N = 25)		Non-endemic (N = 44)	
	n	%	n	%	n	%
69	31	44.9	16	64.0	15	34.1
67	6	8.7	1	4.0	4	9.1
66	6	8.7	2	8.0	4	9.1
64	8	11.6	5	20.0	3	6.8
63	6	8.7	4	16.0	2	4.5
62	2	2.9	0	0.0	2	4.5
60	4	5.8	0	0.0	4	9.1
59	2	2.9	1	4.0	1	2.3
56	14	20.3	6	24.0	8	18.2
54	13	18.8	10	40.0	3	6.8
52	9	13.0	3	12.0	6	13.6
50	14	20.3	6	24.0	8	18.2
48	9	13.0	6	24.0	3	6.8
46	5	7.2	3	12.0	2	4.5
45	2	2.9	1	4.0	1	2.3
43	4	5.8	3	12.0	1	2.3
42	4	5.8	2	8.0	2	4.5
40	3	4.3	1	4.0	2	4.5
39	1	1.4	1	4.0	0	0.0
36	5	7.2	3	12.0	2	4.5
34	9	13.0	8	32.0	1	2.3
33	6	8.7	4	16.0	2	4.5
32	4	5.8	4	16.0	0	0.0
31	1	1.4	1	4.0	0	0.0
30	8	11.6	5	20.0	3	6.8
28	7	10.1	7	28.0	0	0.0
26	3	4.3	2	8.0	1	2.3
24	6	8.7	6	24.0	0	0.0
22	2	2.9	2	8.0	0	0.0
21	4	5.8	4	16.0	0	0.0
20	3	4.3	3	12.0	0	0.0
18	5	7.2	5	20.0	0	0.0
16	8	11.6	8	32.0	0	0.0
14	4	5.8	4	16.0	0	0.0
Mean number of bands	3.17		5.52		1.84	

DISCUSSION

The main peridomestic reservoir for *L. infantum* in the ecoregions around the Mediterranean basin is the dog, with a seroprevalence ranging from 2% to 30%. In endemic focuses of leishmaniasis in the Northeastern Iberian Peninsula, the seroprevalence of CaL approaches 10%,[31] and in the island of Mallorca it reaches 30%.[2] In contrast with the large studies performed in dogs, little information is available about the role of other mammals in the life cycle of *L. infantum*. Sporadic cases of feline leishmaniasis have been reported in the Southwestern Palearctic.[8,11,12,17] The present study reports seroprevalences for cats living in ecoregions around the Northwestern Mediterranean basin of 6.29% and 5.25% when using ELISA-prot A and ELISA-IgG, respectively. Therefore, the result shows that *L. infantum*-specific antibodies are prevalent in cats from these ecoregions. The ELISA conditions required a 4-fold increase in serum and 3-fold increase in conjugate concentrations as compared with the ELISA routinely used in our laboratory to detect canine antibodies to *L. infantum*. Thus, the actual antibody levels detected in cats are putatively much lower than those observed in dogs. This finding is in agreement with those few clinical feline leishmaniasis cases reported in areas where the disease is endemic.

Data on seroprevalence for feline leishmaniasis are very disparate. In Italy, using indirect fluorescent antibody test, seroprevalence in Liguria and Tuscany was 0.9%,[12] but in Sicily it was 62%.[23] However, the cat populations studied were different: the first study was performed on clinically healthy cats, and the second was performed on FIV-positive cats. These different results obtained in the same biogeographical area could be related to the ELISA conditions used. The higher dilutions of sera and conjugate used in the former study, similar to those used for CaL, possibly prevented the detection of the low rate of antibodies present in feline infections.

WB has shown high sensitivity and specificity for the diagnosis of leishmaniasis in humans and dogs. The pattern of bands observed in ELISA-positive cats is in concordance with that observed in human visceral leishmaniasis and in CaL, with no sera collected from cats living in areas where leishmaniasis is not endemic reacting with low molecular weight polypeptides, which are considered the most specific,[29,30] thus confirming ELISA results. The significance of bands of high molecular mass, in particular that of 69 kDa, is not clear as it appears both in cats from areas where leishmaniasis is endemic and also in those from areas where it is not endemic; however, this same finding has been described for dogs and humans.[29] One possible explanation could be the presence of cross-reactive antibodies to heat shock protein 70 family in the sera of some cats living in areas where leishmaniasis is not endemic, the occurrence of which has been described in a wide variety of medical problems.

The rate of infection among dogs living in an area of leishmaniasis endemicity is considerably higher than previously described in seroepidemiological studies,[32] as serology is often not sensitive enough to detect every infection. Other approaches used to determine the rate of infection, such as molecular techniques[33] or cellular immunity tests,[34,35] resulted in higher rates of prevalence than conventional serological studies. In this manuscript, we studied *Leishmania* exposure in cats only by means of serology, so it is possible that we have underestimated the actual rate of infection. Therefore, further broad surveys using other techniques, such as polymer-

ase chain reaction and cellular immunity tests, should be performed in cats to better estimate *Leishmania* infection and/or exposure.

Whether the low prevalence of infection or disease in cats from areas where leishmaniasis is endemic may be due to underreporting or to the fact that cats have a high degree of natural resistance to *L. infantum* is unknown. On the one hand, the most common manifestation of feline leishmaniasis is not the severe visceral form but the cutaneous one, with cats showing low levels of specific antibodies. On the other hand, naturally infected cats do not recover without specific antileishmanial therapy;[18] furthermore, co-infection with immunosuppressive retroviruses leads to parasite dissemination and visceralization. These findings are in concert with the situation that occurs in humans,[36] and also in other mammals such as horses,[37] or in the subgroup of resistant dogs living in areas where leishmaniasis is endemic.[35] Thus, our hypothesis is that the immune response in cats, mainly cellular immunity, is effective enough to control the infection and confer a certain degree of natural resistance, if there are not immunosuppressive events.

Viral, bacterial, rickettsial, fungal, and protozoan opportunistic infections have been associated with retroviral infections in cats because of the immunosuppressive stage that both FeLV and FIV can induce. The prevalence of infection for FeLV and FIV in the present study was comparable to that observed in other studies in the same ecozone, but no association between these retroviruses and *Leishmania*-specific antibodies was found. Because the infection rate was so low, and the numbers of positive FeLV and FIV cats were so small, any association between leishmaniasis and FeLV or FIV—if it exists—will be very difficult to find. In the study on FIV-positive cats in Sicily, associated seroreactivity to *Leishmania* was much higher (62%) than our results,[23] suggesting that in areas where the disease is endemic there may be retroviral infections in the origin of opportunistic infections like leishmaniasis, as is the case for HIV-infected human patients.[36] However, future studies should investigate in greater depth the possible association between *Leishmania*, FeLV, and FIV infections.

Transmission of feline leishmaniasis is presumed to be sand fly-related. Trophotaxis in sand flies depends on the species involved, and sand flies can also behave with a marked lack of host preference.[38] *Lutzomyia* spp. sand flies, vectors of leishmaniasis in the Neotropic ecozone, have been reported feeding on cats in Peru.[39] Studies performed in the Iberian Peninsula on host-feeding patterns of *Phlebotomus perniciosus,* one of the main vectors of *L. infantum* in the Palearctic ecoregions of the Mediterranean biome, identified cat blood meals in all locations studied.[40] Consequently, the possibility of sand flies feeding on domestic cats is real. However, to resolve the issue of transmission it should be studied by means such as xenodiagnosis, evaluating the infectiousness of cats to phlebotomine vectors feeding on them.

The present study is by far the largest seroepidemiological survey on feline leishmaniasis to date. On the basis of the results of this and other studies,[10–18] leishmaniasis must be included in the differential diagnosis of dermatoses or systemic disease in cats living in biogeographical areas of leishmaniasis endemicity. From an epidemiologic point of view, environmental changes, growing demographics, and human and animal mobility are contributing factors to the modification of the biogeographical distribution and also to the incorporation of new hosts. These changing features confirm leishmaniases as reemerging zoonoses.[41] In this setting, the role of the cat as a peridomestic reservoir for *L. infantum* remains controversial and warrants further research, but, according to the reported prevalence and other evidence,[39,40] it could be hypothesized that cats are a secondary reservoir host rather than simply an incidental one, as has been suggested.[42]

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