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Visceral Leishmaniasis in a New York Foxhound Kennel

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Although endemic throughout much of the world, autochthonous visceral leishmaniasis has been reported on only 3 previous occasions in North America. After diagnosis of visceral leishmaniasis in 4 foxhounds from a kennel in Dutchess County, New York (index kennel), serum and ethylenediamine-tetraacetic acid (EDTA)-anticoagulated blood were collected from the remaining 108 American or cross-bred foxhounds in the index kennel and from 30 Beagles and Basset Hounds that were periodically housed in the index kennel. Samples were analyzed for antibodies to or DNA of tickborne disease pathogens and *Leishmania* spp. Most dogs had antibodies to *Rickettsia* spp., *Ehrlichia* spp., *Babesia* spp., or some combination of these pathogens but not to *Bartonella vinsonii* (*berkhoffi*). However, DNA of rickettsial, ehrlichial, or babesial agents was detected in only 9 dogs. Visceral leishmaniasis was diagnosed in 46 of 112 (41%) foxhounds from the index kennel but was not diagnosed in any of the Beagles and Basset Hounds. A positive *Leishmania* status was defined by 1 or more of the following criteria: a *Leishmania* antibody titer $\geq 1:64$, positive *Leishmania* polymerase chain reaction (PCR), positive *Leishmania* culture, or identification of *Leishmania* amastigotes by cytology or histopathology. The species and zymodeme of *Leishmania* that infected the foxhounds was determined to be *Leishmania infantum* MON-1 by isoenzyme electrophoresis. Foxhounds that were >18 months of age or that had traveled to the southeastern United States were more likely to be diagnosed with visceral leishmaniasis. Transmission of *Leishmania* spp. in kennel outbreaks may involve exposure to an insect vector, direct transmission, or vertical transmission.

Key words: Canine; Dogs; *Leishmania;* Sand fly.

Whereas human and canine visceral leishmaniasis are well established in parts of Asia, Africa, southern Europe, and Central and South America, visceral leishmaniasis has been reported infrequently in humans or domestic animals in the United States and Canada. Most cases of canine visceral leishmaniasis in the United States and Canada have been associated with a history of international travel.[1–4] However, 3 cases of canine visceral leishmaniasis have been reported in which no history of foreign travel was identified, including a pet dog from Texas, an English foxhound maintained in a closed research colony at The Ohio State University, and an American foxhound from a kennel in Oklahoma.[5–7] In these reports, infected dogs had not traveled outside of the United States, and neither a source of infection nor an insect vector was ever identified.

Visceral leishmaniasis is a zoonotic protozoal disease caused by various *Leishmania* species. The organisms are thought to be spread almost exclusively by an insect vector, the sand fly. The species of *Leishmania* is often predictive of the form of disease in both humans and domestic animals. Forms of leishmaniasis include visceral, cutaneous (including diffuse cutaneous), and mucocutaneous. The annual global incidence of leishmaniasis in humans is about 2 million cases (1.5 million cases of the cutaneous form and 500,000 cases of the visceral form).[8] The annual incidence in dogs, which serve as the reservoir host for human visceral leishmaniasis in many parts of the world, is unknown.

In this study, we describe an outbreak investigation involving a privately owned foxhound kennel in New York (index kennel) in which many of the dogs were exposed to or infected with *Leishmania* spp. Initial contact with the index kennel evolved from a telephone consultation related to serological evidence of coinfection with multiple tickborne organisms in the sick foxhounds. A veterinarian (EK) practicing in southern New York contacted one of the authors (EBB) at North Carolina State University College of Veterinary Medicine (NCSU-CVM) for assistance when appropriate treatment for several suspected tickborne pathogens failed to resolve the disease. Before the investigation, at least 7 foxhounds had died or been euthanized because of severe debilitation, and approximately 30 foxhounds in the active hunting pack were unable to perform as expected because of exercise intolerance and poor condition. Five foxhounds were sent to NCSU-CVM for diagnostic evaluation, 4 of which were confirmed to have visceral leishmaniasis. With the assistance of the Centers for Disease Control (CDC), Walter Reed Army Institute of Research (WRAIR), and the Istituto Superiore di Sanita, an investigation was initiated to determine the extent of infection, the infecting species of *Leishmania,* and whether transmission was occurring within the index kennel.

Materials and Methods

Canine Selection

The index kennel was located in Dutchess County, New York. Foxhounds have been raised on this property since 1928. When the initial dogs were identified, the index kennel contained approximately 120

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American or crossbred foxhounds ranging in age from neonates to 11-year-old adults. In general, the members of the active hunting pack consisted of adult dogs (≥18 months of age) used to hunt gray and red foxes in Dutchess County (95% of the year) and in the southeastern United States (5% of the year). Younger foxhounds (<18 months of age) were still in training and had not left Dutchess County. Once the initial dogs were diagnosed, it was hypothesized that the vague clinical signs of illness and deaths experienced in the index kennel were related to visceral leishmaniasis rather than to tickborne diseases. Subsequently, some foxhounds were determined to be infected on the basis of clinical presentation, as assessed by the attending veterinarian and kennel manager, and on the basis of indirect immunofluorescent antibody (IFA) titers to *Leishmania* antigens.

To facilitate reporting of the data generated during the investigation, foxhounds were divided into groups. Group I (n = 4) consisted of foxhounds selected by the kennel manager for evaluation at NCSU-CVM and in which visceral leishmaniasis was confirmed by serologic and molecular testing and evaluation of tissues obtained at postmortem examination. Group II (n = 101) included the majority of the kennel population: 70 adult foxhounds actively used for hunting and 31 young foxhounds still in training. Group IIa (n = 19) consisted of a subpopulation of group II foxhounds for which there was strong serological evidence of *Leishmania* spp. infection (reciprocal titers ≥256) and for which hematological and serum biochemical results were obtained during the initial stage of the outbreak investigation. Group IIb (n = 12) represented a subpopulation of clinically healthy, seronegative and PCR-negative group II foxhounds for which hematological and serum biochemical results were obtained concurrently with those for group IIa. Group IIc (n = 70) was comprised of the remaining group II foxhounds which did not fit the inclusion criteria for groups IIa or IIb. Group III (n = 7) was studied retrospectively and consisted of foxhounds infected with *Leishmania* that died or were euthanized prior to the investigation. Group IV (n = 30) consisted of a mixed population of healthy Beagle and Basset hounds that had been maintained in the index kennel for 2–3 months biennially for a 5 year period prior to the investigation. This period included the year of the investigation. Although no detailed information regarding their travel history was available, the Beagle and Basset hounds often traveled with the foxhounds of the index kennel to the southeastern United States. For identification purposes in the paper, foxhounds were designated by numbers and Beagle and Basset hounds were designated by letters.

Diagnostic Evaluation

The protocol for evaluation of group I foxhounds consisted of a history, physical examination, CBC, serum chemistry profile, and urinalysis. Foxhound 1 in group I underwent additional diagnostic testing, including urine culture, urine protein : creatinine (UPC) ratio, serum protein electrophoresis, lymph node and bone marrow aspirates, synovial fluid cytology from the carpal and tarsal joints, and culture of tarsal joint fluid for *Mycoplasma* spp. and *Leishmania* spp. promastigotes. All group I foxhounds were euthanized and examined postmortem. For groups IIa and IIb, CBCs and serum biochemical profiles were submitted to a commercial diagnostic laboratory.[a] In addition, specific gravity (SG) and dipstick analyses were determined from voided urine samples on 12 of 19 group IIa foxhounds.

Treatment

A variety of treatments for visceral leishmaniasis, including a botanical supplement[b] recommended by a local homeopathic veterinarian and meglumine antimoniate,[c] diaminazine aceturate,[d] and allopurinol recommended by NCSU-CVM, were employed by the attending veterinarian. The following dogs received 1 or more of these therapies between April 2000 and August 2000: foxhounds 1–3, 6–9, 11, 13, 15–16, and 19 in group IIa, foxhounds 8 and 11 in group IIb, and foxhounds 14, 25, and 59 in group IIc. Since September 2000, all treated foxhounds have been maintained with allopurinol alone.

Serology and PCR

Serum and ethylenediamine-tetraacetic acid (EDTA)-anticoagulated blood were collected from all foxhounds (groups I, II, and III) at an initial sampling date (October 1999–February 2000) and from group II foxhounds in April 2000, July 2000, and September 2000. All samples collected before February 2000 were obtained for the purpose of serological testing for tickborne disease and before identification of visceral leishmaniasis in the index kennel. These samples were obtained from a reference laboratory for retrospective testing for visceral leishmaniasis. Serum and EDTA-anticoagulated blood were collected from group IV dogs in April 2000. Serum samples were used for IFA testing for antibodies to *Ehrlichia canis, Ehrlichia equi,* and *Rickettsia rickettsii* by NCSU-CVM and for *Leishmania* spp. by the CDC, and EDTA-anticoagulated blood was used for PCR detection of the DNA of *Babesia* spp., *Ehrlichia* genus and species, *Rickettsia* spp., and *Leishmania* spp. by NCSU-CVM.

Immunofluorescent Antibody Testing

NCSU-CVM. An IFA test was used to detect antibodies to *E canis* (Florida), *E equi* (New York, human origin), *R rickettsii* (Domino, canine origin), *Bartonella vinsonii* (*berkhoffi)* (North Carolina, canine origin), *Babesia canis,* and *Babesia gibsoni* (North Carolina, canine origin) by means of previously described techniques.[9,10] Briefly, serial 2-fold dilutions of sera from dogs were reacted with fluorescein isothiocyanate anti-canine immunoglobulin G conjugate.[e] End-point titers were determined as the last dilution at which brightly stained organisms could be detected on a fluorescence microscope with exciter and barrier filters. Titers ≥1 : 64 were considered indicative of exposure to or early infection with *E canis, E equi, B vinsonii, B canis,* and *B gibsoni.* Titers ≥1 : 16 were considered indicative of exposure to or early infection with *R rickettsii.* All groups were tested for antibodies to *E canis, E equi,* and *R rickettsii.* Groups I, IIa, IIb, and III were tested for antibodies to *B vinsonii* (*berkhoffi*), *B canis,* and *B gibsoni.*

CDC. IFA testing with cultured promastigotes of *Leishmania donovani* was performed by the CDC as described.[11,12] Serum samples were screened at 1 : 16 and 1 : 32 dilutions. All 1 : 32 titers were repeated and titered to end point with serial dilutions. A titer of ≥1 : 64 to *Leishmania* antigens was considered indicative of previous exposure to or infection with *Leishmania* spp. Seroconversion was defined as a 4-fold rise in titer.

PCR

Total DNA was extracted from 200 μL EDTA-anticoagulated blood according to the manufacturer's instructions[f] from groups I, II, III, and IV for *Ehrlichia, Rickettsia, Babesia,* and *Leishmania* spp. amplification. Positive and negative controls were performed in conjunction with each assay.

Babesia *Amplification.* Amplification of babesial DNA was performed as previously described.[9]

Ehrlichia *Genus Amplification.* A 1-tube nested PCR was performed by means of outer primers EHR-OUT1 and EHR-OUT2 and inner primers GE2f and EHRL3-IP2 to amplify a 122-bp product as previously described.[10]

Ehrlichia *Species Amplification.* For all samples in which an *Ehrlichia* genus primer was obtained, a 1-tube nested PCR amplification for the detection of *E canis, Ehrlichia chaffeensis, Ehrlichia ewingii, E equi,* and *Ehrlichia platys* was performed without modification.[9,10]

Rickettsia *Amplification.* As previously described, a 1-tube nested PCR analysis that can detect both spotted fever and typhus group *Rickettsia* spp. was used.[13]

Leishmania *Amplification.* The primers R221 5′-GGTTCCTTTCCTGATTTACG-3′ and R332 5′-GGCCGGTAA-AGGCCGAATAG-3′ have been previously reported, and they were used to amplify an ≅600-bp fragment of the 18S rRNA gene.[14] The reaction conditions were as follows: The PCR reaction master mix

contained 200 μM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.9 mM MgCl, 50 pmol each of the forward and reverse primers, and 1.25 U of *Taq* polymerase.[g] Forty-seven microliters of the master mix was aliquoted into each reaction, and 3 μL of template DNA was added to each tube. Amplification conditions were 95°C for 5 minutes once, then 95°C for 45 seconds, 58°C for 45 seconds, and 72°C for 45 seconds for 50 cycles, followed by an extension at 72°C for 5 minutes. Positive and negative controls consisted of leishmanial DNA and water, respectively. The PCR products were electrophoresed in a 1% agarose gel containing 0.2 mg/mL ethidium bromide and were visualized under ultraviolet light. To confirm the target sequence, the PCR product from foxhound 1, group I was cloned into a plasmid, and the DNA sequence was determined from 3 separate clones in both directions with a Li-Cor model 4200LX DNA sequencer.[h] The DNA sequence was compared to known sequences with the BLAST program at the National Center for Biotechnology Information.

Level of Agreement and Risk Analysis

By means of the kappa statistic, the level of agreement between IFA and PCR tests for visceral leishmaniasis was determined for the January/February 2000 sampling period (ie, before any therapeutic intervention for visceral leishmaniasis). The level of agreement was not determined for any other sampling period because subsequent treatment of some of the infected foxhounds may have negatively influenced PCR results. The relative sensitivity of the PCR test was calculated with *Leishmania* culture-positive foxhounds as the standard. Odds ratios were calculated to determine whether or not an association existed between age or travel and the presence of antibodies against *E equi* or *R rickettsii* and positive *Leishmania* status. The number of foxhounds positive for antibodies to *E canis* was low (n = 6), and Fisher's exact test was performed to determine if a correlation between *E canis* seroreactivity and positive *Leishmania* status existed. A positive *Leishmania* status was defined by 1 or more of the following criteria: *Leishmania* antibody titer ≥1 : 64, positive *Leishmania* PCR, positive *Leishmania* culture, or identification of *Leishmania* amastigotes by cytology or histopathology. The variables, age and travel, could not be separated from each other because only adult foxhounds (age >18 months) had a history of travel.

Histopathology

Postmortem examinations were performed by one of the authors (LT) on all 4 group I foxhounds at NCSU-CVM. After partial postmortem examination by the attending veterinarian (EK), 3 foxhounds (dogs 1–3 in group III) were preserved by freezing until delivery to NCSU-CVM. Despite the intervening time interval and freezing artifact, these animals were in adequate condition for full postmortem examination. Tissues from 4 additional foxhounds (dogs 4–6 in group III) examined postmortem by the attending veterinarian and the uterus and placenta of foxhound 13 (group IIa) collected after Cesarean section and ovariohysterectomy for dystocia also were examined by histopathology at NCSU-CVM. All of the puppies from the litter of foxhound 13 (group IIa) died spontaneously within 24–48 hours after birth and were submitted for postmortem examination at NCSU-CVM. All tissues were fixed in 10% formalin, trimmed, and processed routinely. Then, 5-μ-thick tissue sections were stained with hematoxylin and eosin for microscopic examination.

Culture of Promastigotes

NCSU-CVM. One milliliter of aseptically collected tarsal synovial fluid from foxhound 1 (group I) was diluted with 4 mL of RPMI 1640 containing 10% fetal bovine serum (FBS). One milliliter of this mixture then was added to 25 cm^2 of 030 canine cells (monocytoid). When the cells were observed 24 hours after culture initiation, almost all had detached from the plastic flask, potentially in reaction to inflammatory cytokines from the synovial fluid. The cells were washed twice in fresh medium and returned to the flask, where reattachment occurred. The cultures were incubated at 35°C under an atmosphere of 5% CO_2/air, and the medium was replaced 1–2 times weekly.

Fig 1. Foxhound 1, group 1. Facial, aural, and periocular dermatitis is a manifestation of dermatologic disease caused by visceral leishmaniasis.

On day 5 after culture initiation, nonadherent cells were transferred either to Novy-Nicoll-McNeal (NNN) medium-based cultures with an overlay of M199/10% FBS and incubated at 23–25°C or to liquid cultures (M199/30% FBS) in 15-mL screw-capped glass culture tubes. Both NNN and liquid cultures were vigorously agitated daily to provide a suitable aerobic environment. Promastigotes were observed 10 days after inoculation of NNN cultures, which were passaged every 3 weeks by transferring 0.1 mL of supernatant to new cultures. After 5 passages, growth was never vigorous (1–10 promastigotes per 50 μL), and NNN cultures were discontinued. Promastigotes were observed in liquid cultures 23 days postinfection, and, in contrast to NNN cultures, vigorous growth (several thousand organisms/mL) was observed by day 39. These cultures were passaged at intervals of 1–3 weeks in upright 25-cm^2 Corning tissue culture flasks containing 10–20 mL of medium.

WRAIR. A fine needle aspirate of a popliteal lymph node from subclinical foxhounds (n = 8) or 0.1 g of surgically removed popliteal lymph node from clinical foxhounds (n = 4) was added to a 25-cm^2 culture flask containing 12 mL MM1 medium with the addition of 200 U penicillin and 0.2 mg streptomycin (hereafter referred to as ''transport medium'').[15] Cultures were monitored daily for the 1st 10 days postinoculation for parasitic, bacterial, or fungal growth with an inverted microscope and without opening the culture flask. If the culture remained negative for leishmanial growth at 10 days, the medium was exchanged aseptically in a laminar flow hood after centrifugal spin at 700 g for 10 minutes at 4°C. A majority of the resultant supernatant was decanted to approximately 4 mL of fluid above the pellet. Fresh transport medium was added to the pellet. Observation for parasite growth continued for 50 days with centrifugal culture medium replacement at 10-day intervals until a culture was declared negative for leishmanial growth. Cultures growing *Leishmania* spp. and showing no visual bacterial contamination were transferred to medium that contained decreasing antibiotic concentrations over time until they were growing antibiotic free. Once rapidly growing monoxenic and antibiotic-free cultures were achieved, they were cryopreserved in MM1 medium plus 15% glycerol after a controlled rate freeze. At the time of cryopreservation, the cultures were subdivided, and half were sent for isoenzyme characterization. The remaining half of them were retained as stock.

Isoenzyme Characterization

Aliquots of 13 cultures from WRAIR were submitted to one of the authors (MG), and isoenzyme characterization was performed as previously described.[16]

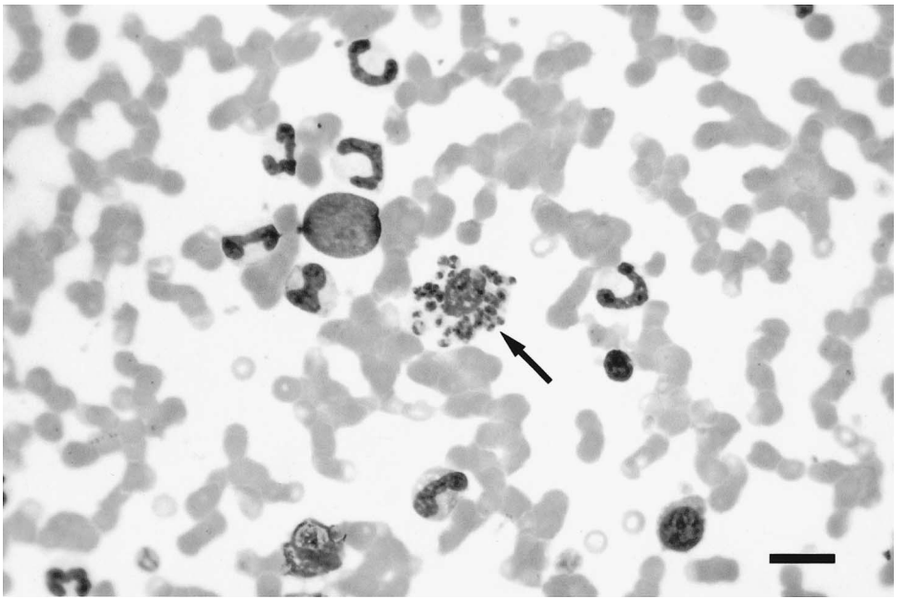

Fig 2. Synovial fluid cytology from foxhound 1, group 1. Multiple *Leishmania infantum* amastigotes are present in macrophages. Wright-Giemsa stain. 330×. Bar = 10 μm.

Results

Diagnostic Evaluation

On physical examination, group I foxhounds (n = 4) received poor body condition scores of 2–3 out of 9, with 5 defined as normal body weight. All 4 foxhounds had facial, aural, and periocular alopecia (Fig 1) and generalized lymphadenopathy, and 2 foxhounds had carpal or tarsal joint effusion or both. Foxhound 1 had a grade II/VI systolic murmur that was loudest over the mitral valve area and raised, firmly attached, subcutaneous masses on the right and left lateral thorax and over the dorsum at approximately T11–T12. Diagnosis of leishmaniasis in the initial dog (foxhound 1 in group I) was made by identification of amastigotes consistent with *Leishmania* spp. within macrophages in synovial fluid from both tarsal joints (Fig 2). Mononuclear inflammation was observed in synovial fluid from the right carpal joint, but no amastigotes were seen. Cytology of a popliteal lymph node aspirate was compatible with lymphoid hyperplasia. Serum protein electrophoresis confirmed a polyclonal gammopathy (albumin = 1.8 g/dL [normal = 2.7–4.4], gamma globulins = 9.14 g/dL [normal = 0.4–1.0]). Bone marrow aspirate cytology identified a hyperplastic marrow with a left shift, marked monocytosis, lymphocytosis, plasmacytosis, megakaryocytosis, and moderate erythroid hypoplasia. Numerous amastigotes consistent with *Leishmania* spp., both free and within macrophages, were seen in the marrow. *Leishmania* spp. were successfully cultured from synovial fluid. *Mycoplasma* spp. were not cultured from synovial fluid. Foxhound 3 had a raised, ulcerated dermal mass on the left perineum and a focal area of hyperreflectivity adjacent to the right optic disk. Fundic examinations were normal in the other group I foxhounds. In general, physical examinations performed by the attending veterinarian on group II and III foxhounds were similar to findings in group I foxhounds, but details of the examinations were not recorded. Poor body condition, focal or generalized dermatitis, and lymphadenopathy were common findings in group II and III foxhounds.

Ten of 23 (43%) group I and IIa foxhounds were anemic (Table 1). Nine (90%) were mildly anemic (hematocrit [HCT] = 26–36%; normal range = 37–55%), and 1 dog was severely anemic (HCT = 19%). Based on reticulocyte counts, the anemia in all group I foxhounds was nonregenerative. The type of anemia could not be definitively characterized as regenerative or nonregenerative in group IIa foxhounds because reticulocyte counts were not performed. However, red blood cell indices (mean corpuscular volume [MCV] and mean corpuscular hemoglobin concentration, [MCHC]) and morphology were within reference range in all group IIa foxhounds, suggesting lack of regeneration. No consistent pattern was observed among the total white blood cell or differential cell counts in groups I, IIa, and IIb, except for lymphocytosis in 8 of 19 (42%) group IIa foxhounds and in 4 of 12 (33%) group IIb foxhounds. Despite severe debilitation, group I foxhounds had lymphocyte counts within reference range. Moderate numbers of reactive lymphocytes and occasional immature monocytes were reported on blood smear examinations of all 4 dogs in group I. Serum biochemical abnormalities included hyperproteinemia (21 of 23, 91%) characterized by hyperglobulinemia (13 of 23, 56%), hypoalbuminemia (6 of 23, 26%), mild to moderate hyperamylasemia (18 of 23, 78%), and azotemia (11 of 23, 48%) (Table 1). Hyperamylasemia was not accompanied by azotemia in 6 of 23 (26%) foxhounds (Table 1). All group I foxhounds were capable of concentrating their urine to an SG >1.020. Semiquantitative estimation of proteinuria (urine dipstick) was 4+ in all group I foxhounds. The UPC ratio was abnormal (UPC ratio = 5; normal = <1) in foxhound 1 in group I. Of the 12 foxhounds in group IIa for which urine SG and dipstick results were available, all but 2 had urine that was concen-

Table 1. Hematologic and biochemical laboratory findings in 23 *Leishmania*-infected foxhounds (groups I and IIa) and 12 healthy foxhounds (group IIb).

	% HCT	Lymphocytes $\times 10^3$	Platelets $\times 10^3$	Albumin (g/dL)	Globulin (g/dL)	BUN (mg/dL)	Cr (mg/dL)	Amylase (IU/L)
NCSU reference range	32.7–57.7	1.0–5.0	181–350	2.8–3.8	2.7–3.0	6–23	0.9–1.5	183–530
Group I (n = 4)								
Range	28.9–33.3	1.9–3.2	253–362	1.5–2.3	4.1–10.4	12–108	1.1–3.5	738–1,181
Mean	31.3	2.3	314	1.8	7.1	43	1.9	858
New York reference range	37–55	1.5–7.0	150–700	2.3–3.2	2.2–4.8	10–28	0.4–1.8	200–1,000
Group IIa (n = 19)								
Range	19–50	2.0–15.0	150–383	2.2–3.6	3.9–8.7	13–131	1.0–7.1	509–1,742
Mean	37.0	6.2	215	2.7	5.0	38	2.2	1,229
Group IIb (n = 12)								
Range	42–56	2.6–14.0	150–301	2.8–3.9	3.7–4.5	12–22	1.0–1.6	716–2,280
Mean	50.1	6.5	194.8	3.3	4.0	15.6	1.2	939.5

BUN, blood urea nitrogen; Cr, creatinine; HCT, hematocrit; NCSU, North Carolina State University.

trated. Foxhound 1 in group IIa was mildly azotemic (blood urea nitrogen [BUN] = 30 mg/dL [normal = 10–28], and creatinine = 1.9 mg/dL [normal = 0.4–1.8]) and repeatedly hyposthenuric (SG = 1.001). Foxhound 10 in group IIa was azotemic (BUN = 53 mg/dL [normal = 10–28], and creatinine = 2.6 mg/dL [normal = 0.4–1.8]) and had a urine SG of 1.015. Based on urine dipstick evaluation, moderate to severe proteinuria (≥2+) was present in 9 of 12 (75%) group IIa foxhounds. None of the group IIb foxhounds was anemic, hyperglobulinemic, or azotemic at the time they were selected for comparison to the group IIa foxhounds.

Treatment

Treatment start dates, initial treatment, and treatment changes were determined primarily by the attending veterinarian (EK) in consultation with NCSU-CVM and a local homeopathic veterinarian. Toxicity or apparent ineffectiveness limited the use of some treatments and required change to other treatments over a relatively short period of time. It is difficult or impossible to draw conclusions about success or failure of treatments because of rapid treatment changes.

Serology

NCSU-CVM. Serological evidence of exposure (reciprocal titers ≥64) to *Ehrlichia* spp. pathogens was found in groups IIa (4 of 19, 21%), IIb (7 of 12, 58%), IIc (16 of 70, 23%), III (3 of 7, 43%), and IV (4 of 30, 13%). Reciprocal titers against the antigens for *E canis* and *E equi* ranged from 64 to 1,024. Of the foxhounds that were seroreactive to *Ehrlichia* spp., more sera (27 of 34, 79%) were reactive only against *E equi* antigens compared to reactivity to *E canis* antigens only (4 of 34, 12%). A few sera (3 of 34, 9%) were reactive against both antigens. Serological evidence of exposure to *Rickettsia* spp., with reciprocal titers ranging from 64 to 512, was found in 72 of 112 (64%) foxhounds representing all 4 groups (43–100%). Only 2 foxhounds (dog 3 in group I and dog 14 in group IIa) were seroreactive to *B vinsonii* (*berkhoffi)* antigens, with reciprocal titers of 64 and 32, respectively. Serological evidence of exposure to *B canis*, with reciprocal titers ranging from 64 to 512, was found in groups I (2 of 4, 50%), IIa (6 of 19, 32%), and III (3 of 7, 43%). All foxhounds in the groups tested (n = 42) were seronegative for *B gibsoni* antibodies.

CDC. When 1st evaluated, strong seroreactivity to *Leishmania* antigens (titers ≥1 : 256) was detected in all group I, IIa, and III foxhounds (n = 30). Group IIb foxhounds (n = 12) were not seroreactive to *Leishmania* spp. antigens. In group IIc (n = 70), 67 foxhounds were not seroreactive or had reciprocal titers <64, and 3 had reciprocal titers of 64.

When reevaluated in April, July, and September 2000, all surviving group IIa foxhounds still were strongly seroreactive. In July 2000, foxhounds 8 and 11 (group IIb) seroconverted. Seroconversion occurred in foxhounds 21 and 24 (group IIc) in April 2000, in foxhounds 14 and 22 (group IIc) in July 2000, and in foxhounds 25, 27, 59, and 36 (group IIc) in September 2000.

PCR

Ehrlichia spp. DNA was amplified from the blood of 6 of 111 dogs (1 of 4 in group I, 1 of 70 in group IIc, 1 of 7 in group III, and 3 of 30 in group IV). DNA from *E platys* was amplified from foxhound 4, group III. DNA from *E equi* and *E platys* was detected in Basset Hound B in group IV. DNA of the genus *Ehrlichia*, identified in the remaining 5 dogs (dog 2 in group I, dog 6 in group IIc, and Basset Hounds A, C, and D in group IV), could not be further characterized as to species.

Rickettsia spp. DNA was amplified from the blood of 2 dogs (dog 4 in group I and dog 11 in group IIb). Dog 4 (group I) had recently received a transfusion from PCR-positive dog 11 (group IIb). Reciprocal titers 2 days and 1 month after transfusion were 32 and 64, respectively, after which dog 4 (group I) was euthanized.

One foxhound (dog 9 in group IIa) had *B canis* antibodies (titer 1 : 256) and a PCR product using primers for the genus *Babesia.*

In January/February 2000, leishmanial DNA was detected by PCR in EDTA-anticoagulated peripheral blood in 4

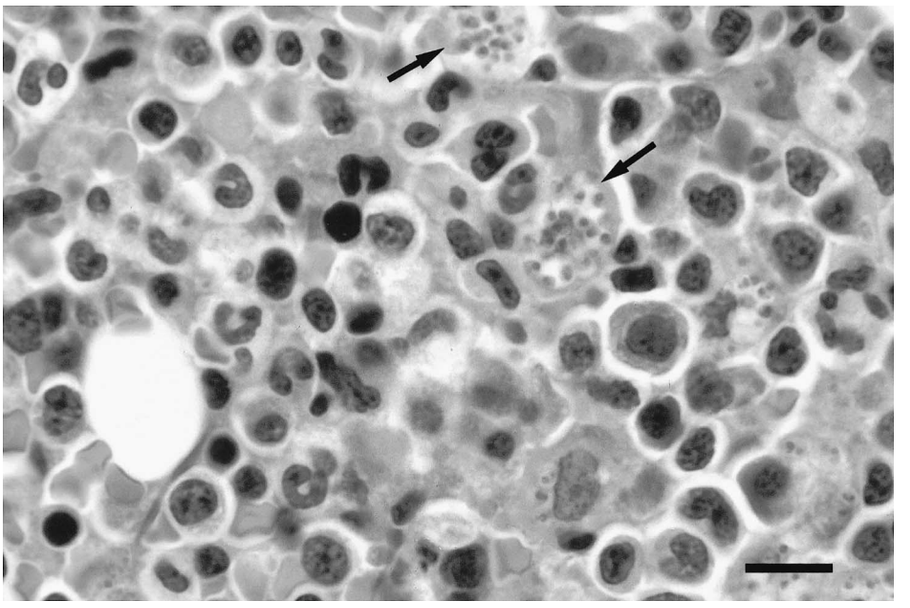

Fig 3. Section of spleen from foxhound 1, group 1. Numerous *Leishmania infantum* amastigotes are seen in macrophages. Hematoxylin and eosin. 500×. Bar = 10 μm.

of 4 (100%) group I foxhounds, 17 of 19 (89%) group IIa foxhounds, and 6 of 7 (86%) group III foxhounds. DNA was not detected by PCR in groups IIb and IIc. The DNA sequence of the PCR product from dog 1 in group I was found to have ≥99% sequence similarity to known leishmanial 18S rRNA gene sequences. Unfortunately, this region of the 18S rRNA gene cannot be used for species differentiation.

In April 2000, dogs 8 and 11 (group IIb) and dogs 24, 46, 64, and 67 (group IIc) were newly PCR positive for leishmanial DNA. In July 2000, dog 22 (group IIc) became *Leishmania* PCR positive, and in September 2000, 2 foxhounds (dogs 36 and 53 in group IIc) were newly identified as *Leishmania* PCR positive.

Level of Agreement and Risk Analysis

Based on the kappa statistic, substantial agreement existed between *Leishmania* IFA and PCR tests during the January/February 2000 sampling period. The few test disagreements occurred as a result of samples that were IFA positive but PCR negative. With the 13 *Leishmania* culture-positive dogs as a standard, the relative sensitivity of the PCR test was 85% (11 of 13). Despite reciprocal titers of 512, dogs 7 and 12 were PCR negative at the January/February 2000 sampling period. No association was observed between positive *E equi* or *R rickettsii* status and positive *Leishmania* status. A significant correlation was found between positive *Leishmania* status and *E canis* seroreactivity ($P = .03$). Foxhounds with positive *Leishmania* status were 5 times (OR = 5.4, CI = 1.78–19.51) more likely to have traveled to the southeastern United States or to be >18 months of age.

Histopathology

Postmortem examinations were performed on 7 foxhounds (dogs 1–4 in group I and dogs 1, 2, and 3 in group III). The majority (6 of 7, 86%) had generalized lymphadenopathy, hepatomegaly, splenomegaly, emaciation, and facial, aural, and periocular alopecia. Several foxhounds (dogs 2, 3, and 4 in group I) had grossly abnormal kidneys, characterized by a mottled yellow and tan capsular surface, relatively pale tan cortices, and yellow and tan streaks on cut surface. These dogs also had enlarged parathyroid glands. Foxhound 1 in group I had small amounts of brown synovial fluid in the carpal, tarsal, and stifle joints and numerous subcutaneous nodules (1–5 cm in diameter). Foxhound 2 in group I had brown cloudy synovial fluid in the right carpus. Other lesions consisted of prostatic enlargement (2 older males), cecal trichuriasis (2), and enteric nematodiasis (3). Foxhound 3 in group I had a multinodular yellow firm mass, which effaced the majority of the left kidney, several firm yellow intramural plaques in the right and left atria (1 cm in diameter), a thyroid mass, a firm nodular ulcerated mass in the left perineum (5 by 2 by 3 cm), and a markedly enlarged left external iliac lymph node (7 by 3 by 2 cm).

Histology was performed on the 7 foxhounds described above and on formalin-fixed tissues from 4 additional foxhounds. Microscopic lesions included lymphocytic, plasmacytic, or histiocytic inflammation and fibrosis, ranging in severity from mild to severe in the liver, kidney, conjunctiva, skin, joint capsules, subcutaneous nodules, spleen, lymph nodes, lung, pancreas, heart, skeletal muscle, sclera, ocular fundus, prostate, adipose tissue, esophagus, spinal cord, bone marrow, bladder, stomach, colon, or small intestine. In the lymph nodes, lymphoid follicles were not prominent, and a mild to marked sinus histiocytosis was present. Macrophages had intracytoplasmic red blood cells, hemosiderin, or ceroid, and large numbers of plasma cells were evident in medullary cords. As shown in the spleen of foxhound 1 (group I) (Fig 3), numerous protozoal organisms were present. These organisms were oval shaped,

2 by 5 μ in diameter, and had small nuclei and kinetoplasts (morphologically consistent with *Leishmania* spp.). Organisms were identified in macrophages in 7 of 11 foxhounds (dogs 1–4 in group I and dogs 2, 5, and 7 in group III). Leishmanial amastigotes were identified in kidney, spleen, bone marrow, lymph nodes, subcutaneous nodules, liver, skin, joint capsule, conjunctiva, bladder, stomach, adrenal cortical adenoma, and esophagus. In addition to renal interstitial inflammation, all of the foxhounds had mild to severe membranous glomerulopathy with thickening of the basement membranes of glomerular capillaries, Bowman's capsules, and tubules, with or without proteinaceous fluid within renal tubules. Foxhounds with gross renal lesions tended to have more advanced histologic lesions. Parathyroid hyperplasia was evident in these foxhounds. Protozoa accompanied by necrotizing vasculitis were identified in the bladder of foxhound 1 (group III). One foxhound with only formalin-fixed tissues available (6, group III) had an adrenal cortical adenoma. Two foxhounds with prostatic enlargement grossly (dogs 2 and 3 in group I) had prostatic hyperplasia, and 1 had a testicular seminoma. One of these dogs also had evidence of testicular degeneration, as did 1 other male. Foxhound 3 in group 1 also had a renal carcinoma, thyroid carcinoma, and well-differentiated hepatoid gland tumor with metastasis to the external iliac lymph node. The atrial plaques described grossly in this foxhound consisted of inflammatory cells similar to those found in other organs and an immature round cell population.

Protozoal organisms were not detected in the uterus, placenta, or 24- to 48-hour-old puppies of foxhound 13 (group IIa). Colonies of coccobacilli with accompanying foci of necrosis were evident in the lungs and liver of 1 puppy, suggesting septicemia as a possible cause of death.

Culture of Promastigotes

NCSU. Promastigotes of *Leishmania* spp. were cultured successfully from the synovial fluid of foxhound 1 (group I) by the methods described.

WRAIR. Fourteen cultures were collected from 12 foxhounds (dogs 1–3, 5, 7, 10–12, 15, 16, 18, 19) from group IIa by the methods described. In addition to a popliteal lymph node sample, a culture was obtained from a tail lesion in foxhound 3 and from a tongue lesion in foxhound 12. One culture (tongue lesion culture from foxhound 12) was discarded because of uncontrollable bacterial contamination. Foxhounds 2, 3, 11, and 12 were considered clinical for visceral leishmaniasis, whereas foxhounds 1, 5, 7, 10, 15, 16, 18, and 19 were considered subclinical for visceral leishmaniasis. Promastigotes of *Leishmania* spp. were cultured successfully from all 12 foxhounds.

Isoenzyme Characterization

Isoenzyme characterization of all 13 *Leishmania* spp. isolates from group IIa was consistent with the zymodeme *Leishmania infantum* MON-1. The cultures (popliteal lymph node and tail lesion) derived from foxhound 3, group IIb contained 2 zymodemes, *L infantum* MON-1 and MON-2.

Discussion

At the time the initial cases for this study were recognized, 29% (33 of 112) of the foxhounds in the index kennel were seroreactive (titers $\geq$1 : 64) to *Leishmania* antigens. This result suggested a high rate of exposure or infection with *Leishmania* spp., particularly because serologic testing often underestimates the true prevalence of infection.[17] Based on PCR amplification of leishmanial DNA or observation of amastigotes in tissues, 78% (26 of 33) of the seroreactive foxhounds were confirmed to be infected with *L infantum.* Two of the 3 previously published reports of canine visceral leishmaniasis in Ohio and Oklahoma involved foxhounds (1 English [Ohio], 1 American [Oklahoma], both maintained in kennel populations).[5,7] Two additional outbreaks of visceral leishmaniasis in foxhounds have been recognized in kennels in Michigan (1989) and Alabama (1994) (Schantz, personal communication). Since the discovery of visceral leishmaniasis in the index kennel, the CDC has obtained serologic evidence of leishmanial exposure in hunting dogs from 21 states (Alabama, Connecticut, Florida, Georgia, Iowa, Illinois, Indiana, Kansas, Kentucky, Massachusetts, Maryland, Michigan, Missouri, North Carolina, New Jersey, New York, Ohio, Pennsylvania, South Carolina, Tennessee, and Virginia) and Ontario.[18] Visceral leishmaniasis appears to be pervasive in the North American foxhound population. However, visceral leishmaniasis should be considered a differential diagnosis in any breed of dog with compatible clinical signs. Although visceral leishmaniasis is a zoonotic disease and dogs are the reservoir hosts for human infection, the human infection rate in endemic areas is typically far lower than the canine infection rate.[19] As of yet, visceral leishmaniasis has not been identified in humans associated with any of the kennels in which canine visceral leishmaniasis has been diagnosed.[5–7,18] It is possible that the recognition of canine visceral leishmaniasis in the index kennel represents the emergence of an epizootic that has been smoldering in North American foxhounds for at least 2 decades.

To date, no studies have been undertaken to explore whether or not foxhounds are inherently more susceptible than other breeds to *Leishmania* spp. infection. Factors that might increase the susceptibility of foxhounds to infection with *Leishmania* spp. are unknown. Considerations include population density, breed-related immunodeficiency, and increased environmental exposure. Population density alone seems an unlikely cause for increased susceptibility because direct contact historically has been reported to be an inefficient means of *Leishmania* spp. transmission.[20] Seroprevalence has not been correlated with population density in regions where visceral leishmaniasis is endemic, and with the exception of a single report in a Basenji kennel, visceral leishmaniasis has not been identified in other (non-foxhound) kennel populations.[6,19] Group IV dogs were kept in close proximity to the foxhounds during summer months when sand fly transmission, if present in New York, might occur, and group IV dogs sometimes traveled with the foxhounds to the southeastern United States. However, all 30 Beagles and Basset Hounds were seronegative (all titers $\leq$1 : 16) and PCR negative for visceral leishmaniasis. This observation suggests that genetic susceptibility may play a

role in the prevalence of visceral leishmaniasis in the North American foxhound population. Protective immunity to leishmanial infection is primarily cell mediated and results in the killing of these intracellular pathogens by activated macrophages. The cell-mediated immune response to *Leishmania* spp. in mice has been studied closely because it suggests a relationship between resistance to infection and genetic factors that control helper T-cell (T_H) development. Most studies on the type of T_H-cell response in leishmaniasis have involved mouse strains that differ in their susceptibility to infection with *Leishmania major,* which causes cutaneous leishmaniasis.[21–29] In mice, acquired immunity to *L major* depends on the production of an IL-12–driven CD4+ T_H1-type response, resulting in interferon-γ (IFN-γ) production, whereas susceptibility to *L major* is associated with a CD4+ T_H2-type response and production of IL-4 and IL-10.[21–30] For example, BALB/c mice produce a T_H2-type response and are susceptible to infection with *L major,* whereas other strains of mice such as B10.D2 and C3H produce a T_H1 response and are resistant to infection with *L major*.[24–26,30] Although a clear polarized T_H1 versus T_H2 pattern of disease development has not been demonstrated in visceral leishmaniasis, IL-12 remains an important regulatory cytokine, and resistance to *L donovani* is associated with IFN-γ production.[21,23] Some as yet unidentified genetic factor may predispose foxhounds to visceral leishmaniasis, whereas other breeds are resistant. A clear breed predisposition has not been identified in naturally infected dogs in endemic regions. An increased propensity for hunting dogs, German shepherds, Boxers, and Doberman Pinschers to become infected with visceral leishmaniasis may exist.[31–34] The Ibizian Hound is believed to be genetically resistant to visceral leishmaniasis because this breed consistently produces an effective cell-mediated immune response to infection.[35] Large-scale transmission of *Leishmania* spp. in kennel outbreaks would most likely involve exposure to an insect vector, presumably the sand fly. Although rarely reported in endemic areas, dog-to-dog transmission via direct contact, exposure to blood products from an infected dog, or possibly during parturition may explain infection in some animals.[36,37]

Transmission and maintenance of leishmaniasis in a human or canine population involve a peridomestic or sylvatic cycle between an insect vector and a vertebrate reservoir host. Wild and domestic dogs serve as the primary reservoir hosts for human visceral leishmaniasis in endemic regions.[34,38,39] Rodents are the primary reservoir hosts for cutaneous and mucocutaneous leishmaniasis in humans. Other vertebrates, including cats, horses, and opossums, are susceptible to *Leishmania* spp. infection but are considered to be either secondary or accidental reservoir hosts.

Phlebotomine sand flies (*Lutzomyia* sp. in the New World and *Phlebotomus* sp. in the Old World) are the primary insect vectors for *Leishmania* spp. Transmission also has been reported in vitro by *Rhipicephalus sanguineus* and may occur by direct contact (mechanical transfer).[20,32,34,40] The leishmanial life cycle begins with injection of the infective promastigote into the skin of the vertebrate host by the sand fly. Once in the host, the promastigote is transformed into a nonflagellated form, the amastigote. Amastigote multiplication in macrophages occurs by binary fission either locally (cutaneous or mucocutaneous forms) or throughout the reticuloendothelial system (visceral form). Vectors ingest amastigotes while obtaining a blood meal from the infected host. If ingested by an unsuitable vector, the amastigotes are either destroyed or passed out in the feces. If the vector is suitable, ingested amastigotes transform into promastigotes and attach to the midgut epithelium of the vector. Promastigotes then detach and move cranially to the foregut, where some reattach and others remain free for subsequent transmission by bite and completion of the cycle. Additional complexity is created by the ability of certain sand flies to serve as hosts for multiple *Leishmania* spp. and the difficulty in determining whether or not the presence of *Leishmania* spp. in wild-caught sand flies confirms blood ingestion or competence to transmit the parasite.[41,42]

Although vectorborne transmission by phlebotomine sand flies classically is described as the means by which *Leishmania* spp. infect the host, it is unclear whether this paradigm explains visceral leishmaniasis in foxhounds. Fourteen species of *Lutzomyia* sand flies have been recorded in North America, and at least 3 species (*Lutzomyia anthophora, Lutzomyia diabolica,* and *Lutzomyia shannoni*) are capable of transmitting at least 1 *Leishmania* spp., *Leishmania mexicana,* the cause of cutaneous leishmaniasis in Texas and Mexico.[42–44] *L diabolica* is believed to be the primary vector for *L mexicana* in the New World.[42] The sand fly responsible for transmission of *Leishmania chagasi* (the cause of visceral leishmaniasis in the New World) is *Lutzomyia longipalpis,* and it has not been identified in North America.[42,43] *Lutzomyia vexator,* another North American sand fly, was provisionally identified near an autochthonous focus of visceral leishmaniasis in Oklahoma, but whether or not this sand fly species actually represents the vector of leishmaniasis at this site remains unknown.[43] *L vexator* has been reported in Alabama, Virginia, and Maryland, and *L shannoni* has been identified throughout southeastern North America including Alabama, Georgia, and Florida.[43] *L shannoni, L diabolica,* and *L vexator* have not been reported in New York.[43] If insect vectors are involved in transmission, infected foxhounds most likely were exposed to *Leishmania* spp. in Alabama, Georgia, or Virginia where *L vexator* or *L shannoni* may have served as the vector.

Fifty percent (41 of 81) of the adult foxhounds in groups I, IIa, IIb, IIc, and III were diagnosed with visceral leishmaniasis. By contrast, few young foxhounds (5 of 31, 16%) were diagnosed with visceral leishmaniasis. All except 1 of the adult foxhounds confirmed to have visceral leishmaniasis had traveled to the southeastern United States. Young foxhounds had never left Dutchess County, New York. This observation suggests that transmission is occurring in the index kennel. Most infections, however, presumably were acquired in the southeastern United States. Additional support for this hypothesis is provided by the finding by the CDC that the geographic distribution of hunting dogs with positive leishmanial titers ($\geq$1 : 64) is primarily concentrated in the southeastern United States, particularly Alabama, Georgia, South Carolina, North Carolina, and Virginia.[18]

Isoenzyme electrophoresis is used to classify *Leishmania* spp. into groups termed ''zymodemes.'' *L infantum* MON-

1 is the most frequently reported zymodeme of canine leishmaniasis in the Mediterranean Basin.[45] The presence of 2 zymodemes in 1 foxhound may represent coinfection with 2 strains of *L infantum.*

Although diagnosis of visceral leishmaniasis requires confirmation by serology, molecular methods, histopathology, or culture, a combination of certain clinical signs and laboratory abnormalities should raise suspicion for the disease. Physical examination abnormalities such as poor body condition (wasting), generalized lymphadenopathy, and facial, aural, and periocular dermatitis as observed in the group I foxhounds are typical of, but not specific for, visceral leishmaniasis.[32–34,46,47] Laboratory abnormalities including anemia, hyperglobulinemia, and hypoalbuminemia found in many of the group I and IIa foxhounds also are consistent with visceral leishmaniasis.[33,34] Lymphocytosis or detection of immature and reactive lymphocytes and monocytes was attributed to chronic immune stimulation with failure to eliminate *Leishmania* spp. infection. Exuberant production of nonprotective immunoglobulin by most infected dogs may result in manifestations of immune complex disease such as glomerulonephritis or polyarthritis.[34,46]

One method of confirming a diagnosis of canine leishmaniasis is detection of amastigotes in tissue. With histopathology, macrophages with abundant organisms were readily identified in 7 of 12 (58%) foxhounds from which tissues were examined. Four of the 7 foxhounds in which organisms could be identified were euthanized and examined immediately after death. Even under ideal conditions, it was sometimes difficult to distinguish small fragments of pyknotic nuclei and cellular debris in macrophages from individual organisms. Intact organisms may have been present in the tissues of some foxhounds but could have been overlooked, especially in those dogs not promptly preserved and submitted.

During the initial evaluation of the index kennel, we were uncertain when to declare a foxhound infected with *Leishmania* spp. on the basis of seroreactivity to *Leishmania* antigens. At any point in time, it is difficult to determine the true seroprevalence of *Leishmania* spp. because of cross-reactivity to other protozoa (eg, *Trypanosoma cruzi*), potential delays between infection and seroconversion, failure of some infected dogs to seroconvert, and insufficient antibody concentrations for IFA detection when antibody concentrations have declined despite persistent infection.[48–51] *T cruzi* is enzootic in wild animals in North America and has been reported in domestic dogs in Texas, Louisiana, Oklahoma, South Carolina, and Virginia.[51–54] Titers to *T cruzi* were not performed in all foxhounds, but it is unlikely that cross-reactivity contributed significantly to the seropositive rate in the foxhounds. Foxhounds from the index kennel with high reciprocal titers to *Leishmania* spp. antigens also had variable reciprocal titers to *T cruzi* antigens, but reciprocal titers to *Leishmania* spp. were consistently higher than those to *T cruzi* antigens in infected dogs. In addition, infection in many foxhounds was confirmed by PCR, identification of amastigotes on cytology or histopathology, or culture. Still, it is possible that some reciprocal titers represent false positives due to lack of specificity or false negatives due to lack of sensitivity.

Of the seroreactive foxhounds identified at the initial evaluation, infection was confirmed by PCR detection of *Leishmania* spp. DNA in EDTA-anticoagulated peripheral blood in 78% (26 of 33) of dogs. Peripheral blood was selected as the diagnostic sample for PCR because of convenience, but the sensitivity of PCR to confirm *Leishmania* spp. infection in seropositive dogs or to detect subclinical infection can be enhanced by assay of lymph node, spleen, or bone marrow aspirates rather than blood.[55] Determination of the relative sensitivity of the PCR assay in this study (85%) was biased by selection of foxhounds with high titers against *Leishmania* antigens ($\geq$256) for culture. The sensitivity of the PCR assay used in this study to detect leishmanial infection in the blood of dogs with lower titers is uncertain. Imperfect agreement between serology and PCR in this investigation, particularly during the April, July, and September 2000 sampling periods, likely is multifactorial and could reflect in part the sample chosen for DNA extraction, the treatment of some infected dogs during the investigation, and the inherent sensitivity and specificity of IFA and the modified PCR assays described in this study. The serologic progression of some foxhounds that were originally seronegative or that had low seroreactivity to *Leishmania* antigens to reciprocal titers $\geq$512 with subsequent confirmation of infection by PCR has led to the recognition that any detectable titer may be indicative of exposure or early infection. Other authors also have made this observation.[17,32,48]

Based on serological testing, the foxhounds in this kennel had been extensively exposed to both *Rickettsia* and *Ehrlichia* spp. However, at the time of the investigation, minimal molecular evidence (PCR) of infection was present. Based on PCR testing, 1 dog was coinfected with 2 *Ehrlichia* spp. Coinfection with multiple *Ehrlichia* spp. may not be an unusual phenomenon in kennel dogs with extensive tick exposure.[9] Self-limiting infection, frequent use of doxycycline and imidocarb dipropinate by the attending veterinarian before investigation, or poor PCR sensitivity could account for the discrepancy between the serological and PCR results for *Ehrlichia, Rickettsia,* and *Babesia.* Based on serological testing, minimal evidence was available to support exposure to *B vinsonii* (*berkhoffi*), which is in contrast to seroprevalences ranging from 33 to 93% in dogs from the southeastern United States that are seroreactive to *E canis* antigens.[9,10,56]

Identification of visceral leishmaniasis as the underlying cause of the vague clinical signs and deaths in this kennel was unexpected. As reservoir hosts for human visceral leishmaniasis, infected dogs represent a risk to the human population. Studies are in progress to assess the zoonotic risks as well as the extent of infection in foxhounds and other breeds of dog. Further studies are also in progress to determine a source and route of *Leishmania* spp. transmission and to determine the prevalence and incidence of visceral leishmaniasis in foxhounds and hunting dog populations as well as in pet and stray dogs.

Footnotes

[a] Orange Animal Laboratory, Inc, Goshen, NY
[b] Biocidin, Biobotanical Research, Corralitos, CA

[c] Glucantime, Merial, Lyon, France
[d] Berenil, Hoescht, Frankfurt, Germany
[e] Fluorescein isothiocyanate anti-canine immunoglobulin G conjugate, Cappel, ICN Pharmaceuticals, Inc, Costa Mesa, CA
[f] DNA blood mini-kit, Qiagen, Valencia, CA
[g] *Taq* polymerase, Amplitaq Gold®, Perkin Elmer, Foster City, CA
[h] Model 4200LX DNA sequencer, Li-Cor, Lincoln, NE

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