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Canine leishmaniasis caused by *Leishmania leishmania infantum* in two Labrador retrievers

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Abstract. Canine leishmaniasis, a generally fatal parasitic disease, was diagnosed in 2 dogs with a medical history of foreign travel, lymphadenopathy, emaciation, anorexia, intermittent fever, and cutaneous lesions. Clinically, hyperproteinemia, proteinuria, azotemia, and glomerulopathy were evident. Isolation of *Leishmania* species was done using Schneider's *Drosophila* medium. Syrian hamsters were used for infectivity studies. Clear taxonomic identification was done biochemically by isoenzyme analysis and comparison of zymogram banding patterns with 6 World Health Organization reference strains. Based on the geographic origin of affected dogs, clinicopathologic presentation, visceralization with hepatosplenomegaly in hamsters, and isoenzyme analysis, a diagnosis of *Leishmania leishmania infantum* was made. This study, representing the first taxonomic identification of an isolate from canine leishmaniasis, demonstrates the Zoonotic and epidemiologic implications of this disease.

Dogs and other members of the family *Canidae* are reservoir hosts for members of the *Leishmania donovani* complex in both the New and Old World. In endemic foci of visceral leishmaniasis in the Mediterranean littoral, South America, the Caucasus, and Turkistan, the dog is considered the reservoir of *L. donovani* and is the principal factor in the maintenance of endemicity.[1] Canine leishmaniasis has increased in prevalence in the United States over the last decade. Most dogs from North America with clinical visceral leishmaniasis have had a history of travel to foreign countries.[23,24,26] There are also reports of an endemic focus of canine visceral leishmaniasis in the southwestern United States[2] and of a single autochthonous case in the midwestem United States.[20]

Canine and human visceral leishmaniasis is caused by members of the *L. donovani* complex, which consists of *L. leishmania donovani, L. 1. infantum,* and *L. 1. chagasi.* In addition to this complex, *L. 1. arabica, L. 1. major,* and *L. 1. tropica* have all been isolated from cases of canine cutaneous leishmaniasis.[6,15] Clear identification is based on the newer taxonomic techniques such as isoenzyme characterization.[6]

The parasite that causes leishmaniasis is a dimorphic protozoan transmitted to the mammalian host by sandflies of the genera *Phlebotomus* and *Lutzomyia.* The sandfly injects metacyclic promastigotes, which are phagocytosed by cells of the monocyte macrophage system. They transform and multiply as amastigotes within phagolysosomes in the host cell.[16]

In dogs, leishmaniasis is generally characterized by intermittent fever, splenomegaly, anorexia, progressive weight loss, and cutaneous lesions.[7,18] A considerable amount of information is available on the various clinical syndromes in humans, but remarkably little information on the natural disease in dogs exists, and few canine isolates have been adequately identified. Clinically documented spontaneous canine cases have been diagnosed based on the clinicopathologic presentation and microscopic identification of amastigotes. Most clinical cases have been attributed to members of the *L. donovani* complex without further identification using current taxonomic techniques.

This study was performed to identify the species of *Leishmania* isolated from 1 of 2 clinical cases of canine leishmaniasis using isoenzyme analysis and biological characteristics.[25] The clinicopathologic and necropsy findings in the dogs were correlated with the specific *Leishmania* isolate.

Materials and methods

Case history. Two adult male Labrador retrievers from the same owner were referred at different times to The University of Georgia Veterinary Teaching Hospital with a history of chronic illness characterized by anorexia, intermittent fever, dermatitis, and lymphadenopathy. Both dogs had been acquired in Germany and had lived in both Switzerland and Spain. A physical examination was done upon admission. Blood, serum, and urine samples were collected for complete blood counts, serum chemistry, and urinalysis. Lymph node

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aspirates and skin and kidney biopsies were performed on the first dog (NB8608); whereas bone marrow aspirates and a skin biopsy were performed on the second dog (NB8673). Aspirates for cytologic examination and biopsies for histopathologic evaluation were submitted. Aliquots of sterile bone marrow aspirates from the second dog (NB8673) were used for in vitro inoculation of culture media. Because of lack of response to therapy, altered blood and clinical chemistry values, and renal insufficiency, both dogs were euthanized and necropsied. Representative portions of all major organs were obtained, preserved in 10% neutral buffered formalin solution, and submitted for microscopic examination.

Primary parasite isolation and hamster infection. Aliquots of 0.5 ml of sterile bone marrow from 1 of the infected dogs (NB8673) were inoculated into 25-cm Falcon[a] flasks containing 5 ml of Schneider's *Drosophila* medium[b] supplemented with 20% (v/v) heat-inactivated fetal bovine serum[b] and 50 mg/ml of gentamicin.[c] Cultures were incubated at 25 C and examined for promastigotes daily for 2 wk. Stationary phase promastigotes were subcultured twice at weekly intervals, concentrated, and stored at -190 C. Culture-derived promastigotes were used to infect hamsters and were quantitated and resuspended in isotonic saline solution to deliver approximately 10^6 promastigotes in 0.25 ml. Four male Syrian hamsters were used for infectivity studies. All hamsters were kept and handled following guidelines established by the National Institutes of Health in the Guide for the Care and Use of Laboratory Animals. Each hamster received 0.25 ml of this preparation via the intracardiac injection. Four other hamsters, used as noninfected controls, were inoculated in a similar manner with 0.25 ml of parasite-free saline solution. Two hamsters, one infected and one noninfected control, were euthanized by CO, inhalation when the infected hamsters were moribund at 57, 121, 159, and 168 days postinfection. Liver and spleen weights were recorded, and imprints of these organs were air dried, fixed with methanol, and stained with Giemsa's stain for evaluation of parasite visceralization and organ parasite density estimations. Amastigote densities per gram of organ were determined for the liver and spleen by a previously described method.[19] Heavily infected spleens were then ground in sterile saline solution in a Ten Broeck tissue grinder[d] and diluted to provide the desired numbers of amastigotes. Approximately 4.5 x 10^7 amastigotes were injected intracardially into other hamsters for serial maintenance of the parasite.

Biochemical parasite identification. The *Leishmania* isolate was originally obtained from bone marrow aspirates of 1 of the infected dogs (NB8673). Cryopreserved promastigotes were inoculated into culture flasks (25 cm^2) containing Schneider's *Drosophila* medium supplemented with 20% heat-inactivated fetal bovine serum plus penicillin (250 U/ml), streptomycin (250 μg/ml), and 5-fluorocytosine[c] (500 &ml). After inoculation, the cultures were incubated at 25 C for 6-7 days to allow growth to a stationary phase. Promastigotes were harvested, and lysates were prepared for electrophoresis assays.

Cellulose acetate electrophoresis was performed for all enzymes according to previously described methods.[9,10] The conditions for electrophoresis included the use of cell buffers with either 0.1 M phosphate" or Tris at pH 7.0-7.4 and membrane buffers with 0.05 M Tris/0.05 M NaH_2PO_4, adjusted to pH 8.3 with 40% NaOH.[c] Depending on the enzyme used, a mixture of 1 part cell buffer and 5-14 parts distilled water was used. Reation buffers consisting of 0.05-0.25 M Tris adjusted to different pH levels (6.3-8.0) with 50% HCl[c] were also used. Identification of the isolate was based on comparison of the enzyme zymogram banding patterns with those of 6 World Health Organization reference strains: *L. 1. arabica, L. 1. aethiopica, L. 1. donovani, L. 1. infantum, L. 1. major,* and *L. 1. tropica.* The enzymes used in the assays were malate dehydrogenase, malic enzyme, phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, adenylate kinase, mannose phosphate isomerase, glucose phosphate isomerase, phosphoglucomutase, isocitrate dehydrogenase, and nucleoside hydrolase.

Results

Both dogs had episodes of fever with a maximum temperature of 107 F, which subsided with antipyretic treatment and then relapsed after a few weeks. Hematology, serum chemistry determinations, and urinalysis results at the time of admission are summarized in Table 1. The hemogram revealed a moderate to severe normochromic normocytic nonregenerative anemia in both dogs. The leukogram revealed leukopenia in 1 dog (NB8608) characterized by neutropenia and lymphopenia. The second dog (NB8673) had a normal WBC count with a left shift and lymphopenia. The chemistry profile in both dogs indicated azotemia and hyperproteinemia, which consisted of hypoalbuminemia with hyperglobulinemia. One dog (NB8673) had a mild increase in alkaline phosphatase activity. Urinalysis revealed a proteinuria and mild hematuria in both dogs.

Biopsy evaluation of the kidney from the first dog revealed a proliferative glomerulopathy. The lymph node smear contained plasma cell hyperplasia and mild purulent inflammation. Amastigotes were not evident. Bone marrow smears from the second dog (NB8673) revealed a hypercellular bone marrow with abundant mature and immature megakaryocytes. Numerous amastigotes were present within macrophages or scattered throughout the smear. The myeloid : erythroid ratio was 11:1 with normal myeloid maturation. Erythroid precursors and later stages were infrequently observed. Plasmacytosis was apparent; these cells comprised approximately 25% of bone marrow cell population. A cytologic diagnosis of leishmaniasis was made based on the presence of amastigotes in the bone marrow smears.

At necropsy, both dogs were emaciated and had multiple skin lesions characterized by alopecia, crusts, scaling, nodules, and ulcerations. Both dogs had hepatomegaly with firm enlarged livers and generalized lymphadenopathy. Lymph nodes were enlarged up to 3 times normal size. The kidneys were pale, and 1 dog

Table 1. Laboratory data recorded at the time of admission from 2 dogs naturally infected with *Leishmania 1. infantum.*

Parameter	Dog no. NB8608	NB8673
Hemogram		
Leucocytes (×10³/μl)	1.9	10.4
Segs	1,539	7,592
Nonsegs	57	1,248
Lymphocytes	152	624
Monocytes	152	624
Eosinophils	0	312
Erythrocytes (×10⁶/μl)	3.0	2.54
Hemoglobin	7.8	5.8
Packed cell volume (%)	22	17
Mean corpuscular volume (fl)	73	66
Mean corpuscular hemoglobin concentration (g/dl)	36	23
Mean corpuscular hemoglobin (pg)	26	35
Reticulocytes (%)	0.8	0.8
Platelets (×10³/μl)	2.49	2.9
Serum chemistry		
Blood urea nitrogen (mg/dl)	81	95
Creatinine (mg/dl)	2.6	4.1
Glucose (mg/dl)	102	85
Alanine transaminase (IU/liter)	15	32
Alkaline phosphatase (IU/liter)	16	94
Total protein (g/dl)	8.5	9.3
Albumin (g/dl)	1.7	1.4
Globulin (g/dl)	6.8	7.9
Urinalysis		
Protein (mg/dl)	>1,000	>1,000
Erythrocytes/hpf	2–7	5–7
Leucocytes/hpf	1–4	0
Specific gravity	1.021	1.015
pH	6.0	5.5

(NB8608) had mineralized foci within the adrenal medulla. Histologically, the skin lesions varied from 1 site to another but generally were covered by crusts consisting of keratin, fibrin, cellular detritus, bacterial colonies, leishmanial amastigotes, and occasional areas of mineralization. The epidermis had orthokeratotic hyperkeratosis. The hair follicles were also hyperkeratotic, devoid of any hair shafts, and contained partially mineralized keratin. The dermis had focal to diffuse areas of cellular infiltration consisting predominantly of macrophages, lymphoplasmacytic cells, some multinucleated giant cells, a few neutrophils, and mast cell hyperplasia (Fig. 1). Most of the macrophages contained intracytoplasmic oval-shaped amastigotes with a prominent basophilic nucleus and a characteristic rod-shaped kinetoplast. Several areas of mineralization and granulation tissue were present within some of the sections examined. In other sections, epidermal

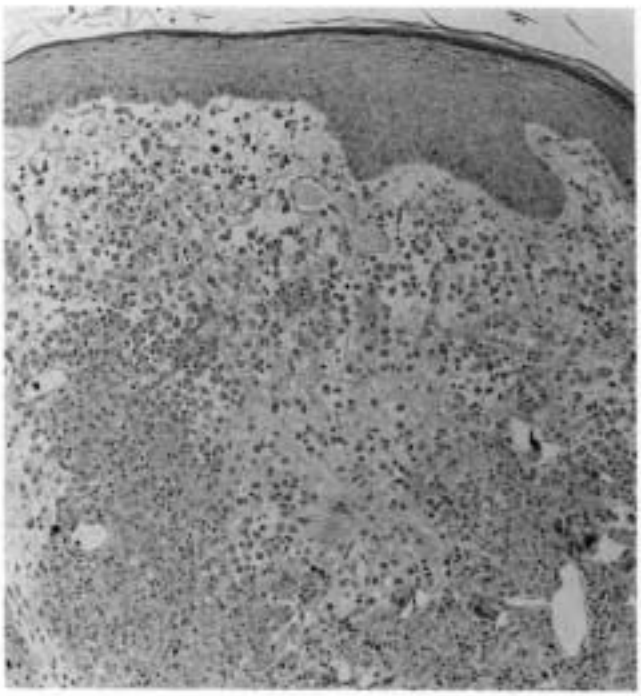

Figure 1. Section of skin of a dog with leishmaniasis, showing focal to diffuse infiltration of macrophages and lymphoplasmacytic cells in the dermis. HE.

ulcerations were present, with free amastigotes on the ulcerated surface.

The liver had numerous randomly scattered to coalescing areas of hepatocellular necrosis and granulomas with epithelioid macrophages containing intracytoplasmic amastigotes and many plasma cells. The splenic red pulp sinuses were densely infiltrated with amastigote-laden macrophages and many plasma cells (Fig. 2). The subcapsular and medullary sinuses of the lymph node contained numerous amastigote-laden macrophages, and erythrophagia was occasionally present. The bone marrow was hypercellular and had a markedly increased proportion of plasma cells and macrophages. *Leishmania* amastigotes were present either within macrophages or free. Amastigote-laden macrophages were also present in the adrenal glands, lungs, kidneys, intestinal submucosa, and in atria subendocardium. The kidneys had periglomerular fibrosis, interstitial nephritis with macrophage and plasma cellular infiltration, glomerular obsolescence, and areas of tubular mineralization (Fig. 3). One dog (NB8608) had mesangial proliferative glomerulopathy with mesangial hyperplasia and increased mesangial matrix. Multifocal areas of visceral dystrophic mineralization was observed in 1 dog (NB8673) and involved the left atrium, coronary arterial media, perithyroid, periadrenal, and glossal arteriolar media. Based on the necropsy and histopathology findings, a disease diagnosis of visceral leishmaniasis was made for both dogs.

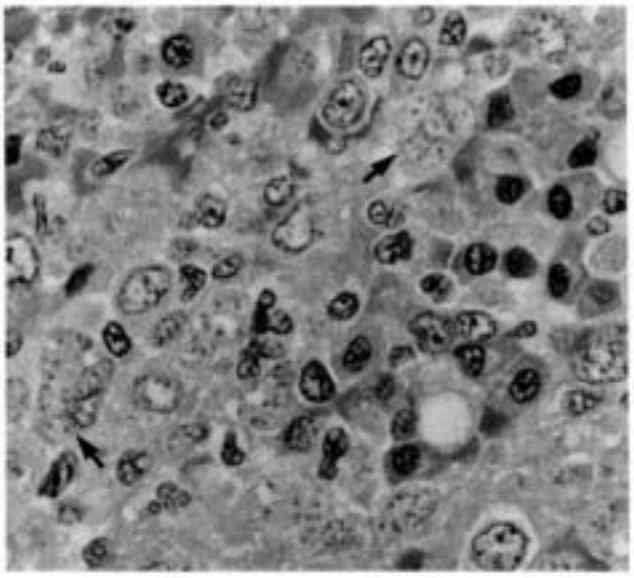

Figure 2. Section of the spleen red pulp sinuses of a dog with leishmaniasis showing amastigote-laden macrophages (arrows) and plasmacytosis. HE.

Cultures inoculated with sterile bone marrow aspirates contained proliferating promastigotes within 48 hours. Giemsa-stained smears of the culture revealed numerous promastigotes with morphologic features characteristic of a kinetoplast and free flagellum. Seven-day-old stationary-phase promastigotes cultured from stabilities obtained from the bone marrow of the second dog (NB8673) were highly infective to hamsters; visceralization in the liver, spleen (Fig. 4), and bone marrow occurred by 57 days postinfection. Hamster organ weights and parasite densities are summarized in Table 2. Moderate splenomegaly and a mild hepatomegaly with high parasite densities were observed at 57, 121, 159, and 168 days postinfection. The highest amastigote densities were observed at 121 days postinfection, when there were 2.05 x 10^{10} and 9.36 x 10^{9} amastigotes in the liver and spleen, respectively. Based on biochemical analysis, the *Leishmania* isolate from the second dog had electrophoretic isozyme patterns indistinguishable from the *L. 1. infantum* reference strains.

Discussion

Canine leishmaniasis was discovered by Nicolle and Compte in 1908.[2] Since then, reports of infected dogs have come from all foci of the Mediterranean area. Away from the endemic areas, imported canine visceral leishmaniasis in Canada, the United States, and the United Kingdom has been documented.[2,11,12,23] These animals present a certain degree of risk to humans, especially children, as the disease can be spread by direct contact.[12,21,22] The human risk is enhanced by the fact that infected dogs can remain clinically normal for extended periods of time.[11] Canine leishmaniasis

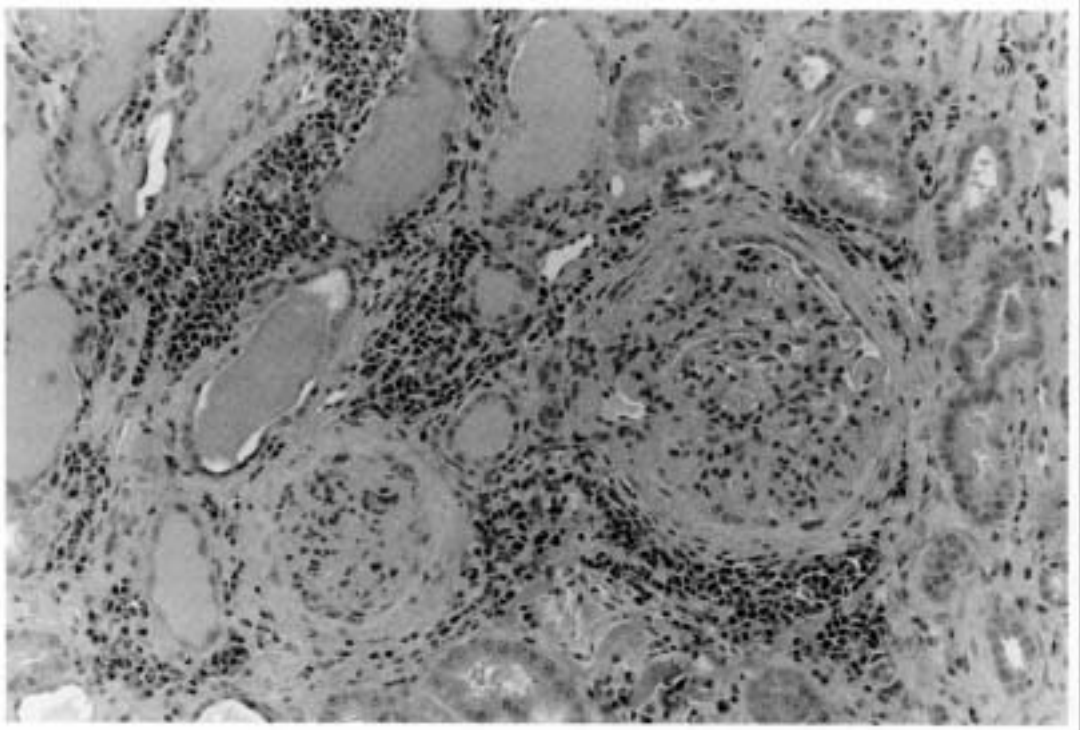

Figure 3. Section of kidney of a dog with leishmaniasis showing periglomerular fibrosis, glomerular obsolescence, and interstitial nephritis. HE.

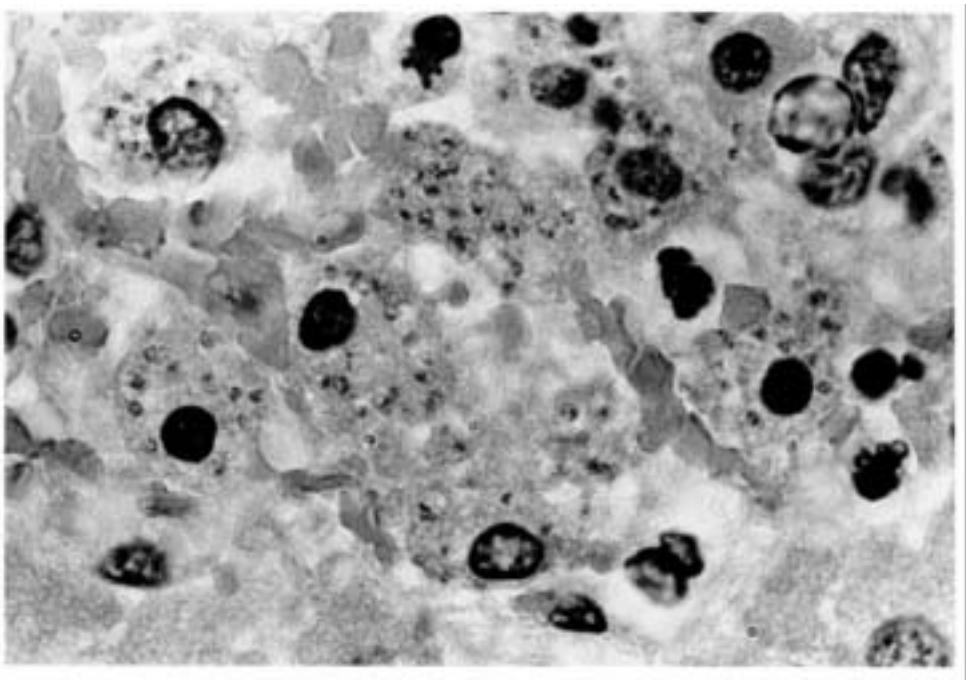

Figure 4. Section of hamster spleen at 57 days postinfection with *Leishmania 1. infantum* from the bone marrow of a dog, showing amastigote-laden macrophages in the red pulp sinuses. HE.

is probably imported considerably more often than it is diagnosed: The disease is often clinically vague, with nonspecific signs. Clinicians may not consider this exotic disease in their differential diagnosis.[11,18] The creation of new endemic foci is possible and may have already happened in Oklahoma[2] and possibly in Ohio.[8,20] Clinically, the chances for correct diagnosis of canine leishmaniasis can be increased by inquiring about possible foreign travel or origin when any dog is presented with lymphadenopathy and other unexplainable problems. In the cases presented at our teaching hospital, although a history of foreign travel and lymphadenopathy was provided, leishmaniasis was only diagnosed in the first dog (NB8608) after necropsy tissues were examined histologically. The owner was then advised to bring the other dog into the hospital, where diagnosis was made by examining bone marrow aspirates.

Some of the characteristic features of canine leishmaniasis are chronic wasting, skin lesions, lymphadenopathy, splenomegaly, anemia, proteinuria, and signs of renal failure. In this and other studies, characteristic clinical abnormalities included intermittent fever, cachexia, lymphadenopathy, anemia, splenomegaly, hypoalbuminemia, hyperglobulinemia, azotemia, and proteinuria.[12,20,26] Diagnostic evidence may be obtained by microscopic examination of lymph node and bone marrow aspirates, which reveal the presence of amastigotes, free or in macrophages. One of the lymph node aspirates from the first dog (NB8608) was negative for any parasites; the bone marrow aspirate from the second dog (NB8673) was positive. Negative lymph node aspirates have been reported previously and are not uncommon, especially in relapsing cases where much searching may produce only a few parasites.[18] Grossly visible lesions in the 2 dogs were similar to those observed in canine leishmaniasis,[1] including emaciation, hepatosplenomegaly, lymphadenopathy, and skin lesions. Ophthalmitis, as reported in other cases, was not observed in these dogs. However, ophthalmitis and leukopenia are variable features and may or may not be present in clinical cases of canine leishmaniasis.[18]

Diagnostic evidence of visceral disease can be further confirmed by histologic examination of the lesion, which is characterized by the accumulation of infected macrophages accompanied by a lymphocytic and plasma cell response.[3] In our study, 2 main cell types were predominant in tissue reactions: macrophages and plasma cell hyperplasia. In dogs, amastigotes multiply

Table 2. Organ weights and parasite densities from 4 hamsters at various intervals after experimental infection with *Leishmania 1. infantum* from bone marrow of an infected dog.

Days postinfection	Organ weights (mg)		Total parasite density	
	Liver	Spleen	Liver	Spleen
57	9,010	1,365	3.00×10^{8}	4.17×10^{8}
121	11,560	2,100	2.05×10^{10}	9.36×10^{8}
159	5,689	336	1.84×10^{8}	1.27×10^{8}
168	5,174	505	1.27×10^{10}	2.59×10^{8}

in macrophages and other cells of the monocyte macrophage system.[4] The infective metacyclic promastigotes target these cells and multiply within the parasitophorous vacuoles.[16] The plasma cell hyperplasia seen in the spleen, lymph nodes, bone marrow, and skin is most likely responsible for the ineffective but profound humoral response seen in canine leishmaniasis and for the subsequent hyperglobulinemia.[5,20] Azotemia and proteinuria observed in this study were probably a result of glomerulopathy. In natural infections with *L. 1. donovani* and *L. 1. infantum,* glomerulomesangial thickening diagnosed as proliferative glomerulonephritis has been reported.[14]

In this study, both dogs presented with alopecia and scaling, ulcerative dermatitis, and nodular dermatitis. Pustular dermatitis was not observed. Skin lesions in canine leishmaniasis are common, and affected dogs can present different dermatologic pictures. Generally, symmetric alopecia and scaling are considered typical for cutaneous leishmaniasis.[11,18] Four different dermatologic patterns have been described in dogs: symmetric alopecia and scaling, ulcerative dermatosis, generalized nodular disease, and pustular dermatitis.[7] The presence of parasitized fixed macrophages in abundance in the dermal layer and the cosmopolitan nature of dogs are important factors in making the dog an efficient reservoir host.[3]

Additional diagnostic tests include culture of tissue explants, infection of laboratory rodents such as hamsters, and electron microscopy. Diagnosis and proper identification of the parasite in this study took into consideration the host species affected, the presenting clinicopathologic features, the geographic origin of the infection, the behavior in the hamster model, and biochemical methods of analysis of isoenzymes by electrophoresis. Spain, where these dogs previously resided, has endemic human and canine leishmaniasis,[25] and *L. 1. infantum* isolates from human cases in the Mediterranean basin visceralize in hamsters.[17] Clear taxonomic identification was obtained by isoenzyme analysis. Based on the above criteria the isolate was *L. 1. infantum*. The identification methods suggested by the World Health Organization expert committee on leishmaniasis[25] make use of both "extrinsic" characteristics (e.g., clinical manifestation, geographic distribution, virulence of clones in rodents) and "intrinsic" attributes (e.g., size, function, molecular structure). The most widely used of the intrinsic and, more particularly, biochemical methods is isoenzyme analysis by electrophoresis. This method is the current baseline of identification. However, newer techniques, such as the use of specific DNA probes and monoclonal antibodies, have been useful for the rapid identification of species without isolation of parasites in culture.[25]

The isolation and subsequent biochemical characterization of *L. 1. infantum* from canine cases in the United States has not been documented previously, and our study presents the first biochemically confirmed isolate within North America. A mixture of several *Leishmania* species can coexist in a single focus, causing clinical presentations that seem identical but occur in different epidemiologic cycles. In the Arabian peninsula, for example, *L. 1. donovani* and *L. 1. infantum* are found together in the same focus, the former only in humans and the latter in humans and dogs.[25] Other species of *Leishmania* can also cause skin lesions in canines without visceralization and are of epidemiologic and Zoonotic importance.[6] These observations highlight the need for exact parasite identification. In the wide geographical range of *L. 1. infantum,* many contrasting situations exist. The recent discovery in both the Old[3,4] and the New Worlds[2,20] of foci of canine leishmaniasis without documented human infection shows how widespread the parasite is. The great versatility of *L. 1. infantum* allows it to adapt to vectors of different species.[25]

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Sources and manufacturers

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b. Grand Island Biological Co., Grand Island, NY.
c. Sigma Chemical Co., St. Louis, MO.
d. Coming Glass Works, Coming, NY.

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