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Genetic and Antigenic Evidence Supports the Separation of *Hepatozoon canis* and *Hepatozoon americanum* at the Species Level

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Recognition of *Hepatozoon canis* and *Hepatozoon americanum* as distinct species was supported by the results of Western immunoblotting of canine anti-*H. canis* and anti-*H. americanum* sera against *H. canis* gamonts. Sequence analysis of 368 bases near the 3′ end of the 18S rRNA gene from each species revealed a pairwise difference of 13.59%.

Canine hepatozoonosis is an emerging protozoal tick-borne infection of dogs that was first reported in the United States from Texas (8) and Louisiana (10) and according to recent reports has spread to Alabama, Georgia (14), and Oklahoma (17). The causative agent of canine hepatozoonosis in North America is *Hepatozoon americanum*, an apicomplexan parasite of leukocytes and muscular tissues that induces severe myositis and gait abnormalities (20). Before 1997, *H. americanum* was considered a strain of *Hepatozoon canis*, a protozoan with morphologically similar gamonts in leukocytes. *H. canis*, first reported in India in 1905 (12), is the cause of Old World canine hepatozoonosis, which usually causes a milder disease that affects the spleen, lymph nodes, and bone marrow, resulting in anemia and lethargy (1). It has been reported from southern Europe, the Middle East, Africa, and the Far East. The main vector for the disease is the tick *Rhipicephalus sanguineus* (5). In the United States, *H. americanum*-like oocysts were found in hemocoel smears made from *Amblyomma maculatum* ticks found on dogs with hepatozoonosis (20). This tick has recently been shown to be capable of transmitting *H. americanum*, whereas *R. sanguineus* failed to transmit this parasite (15).

The classification of *H. americanum* as a new species was based mainly on the clinical signs, tissue tropism, and pathological and morphological findings from dogs with hepatozoonosis in North America, which were different from those reported for *H. canis* infections from other parts of the world (20). The aims of this study were to provide further evidence at the molecular and antigenic levels for the recent species differentiation between *H. americanum* and *H. canis*.

Blood was drawn in an EDTA tube from a 2-year-old Yorkshire Terrier dog infected with *H. americanum* admitted to the College of Veterinary Medicine at Auburn University in Alabama. In Israel, blood was sampled similarly from a 10-year-old Yorkshire Terrier infected with *H. canis* at the Hebrew University School of Veterinary Medicine. Gamonts of *Hepatozoon* were observed in neutrophils from both dogs by light microscopy of Giemsa-stained blood smears prior to DNA extraction. Genomic DNA from gamont-infected neutrophils was extracted and purified using the IsoQuick Kit (Orca Research Incorporated, Bothell, Wash.). A portion of the 3′ end of the small-subunit (SSU) rRNA gene was amplified by PCR using internal primer 5′-CCAGGTCCAGACATGG-3′ (designated Cocci A) and P3 of Clark and Diamond (6). PCR mixtures consisted of 10 ng of template DNA, 5 mM KCl, 1 mM Tris-HCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ (the last four reagents from Promega, Madison, Wis.), 200 μM (each) deoxynucleoside triphosphate (Pharmacia Biotech, Piscataway, N.J.), 1 μM (each) primer, and 2.5 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies Inc., Gaithersburg, Md.) in 100-μl reaction volumes. PCR was performed by using the following parameters and a thermal cycler (Perkin Elmer Cetus Co., Wellesley, Mass.): 94°C for 30 s (melting), 56°C for 1 min (annealing), and 72°C for 2 min (extension). The resulting PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Product bands were excised from the gel, and DNA was recovered from gel slices using the GeneClean II Kit (Bio 101, Vista, Calif.). The PCR products were cloned using the Zero Background/Kan Cloning Kit (Invitrogen Corporation, Carlsbad, Calif.) and sequenced in both directions with M13 forward and reverse primers using the ABI 377 Prism automated sequencer.

Heparinized peripheral blood (82 ml) was obtained by venipuncture from a dog naturally infected with *H. canis*. The infected blood was layered onto a test tube with a sintered filter (Uni-Sep10+; Novamed, Jerusalem, Israel) that prevents mixing of the blood and the Ficoll-Hypaque density-gradient medium. After centrifugation at 800 × *g* for 20 min at room temperature, a fraction containing leukocytes was collected, suspended in 30 ml of phosphate-buffered saline (PBS) (pH 7.2), and washed three times with PBS by centrifugation at 800 × *g* for 20 min. The leukocytes were then resuspended in 30 ml of PBS, equilibrated in a nitrogen cavitation chamber at 500 lbs/in^2 for 10 min, and disrupted by releasing the pressure (11). The material containing cell-free gamonts and debris was collected in a centrifuge tube and centrifuged for 10 min at 800 × *g*. The pellet containing purified parasites was resuspended in PBS and washed three times with PBS by centrifugation at 800 × *g* for 20 min at 4°C. The final pellet containing released gamonts was resuspended in 1 ml of PBS, and the number of purified parasites was determined in a Neubauer

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hemocytometer with 0.5% trypan blue. The purified and counted gamonts were frozen at −70°C, and at each stage of purification, the material was examined by Nomarski phase microscopy.

Positive anti-*H. canis* serum samples were obtained from a naturally infected dog and an experimentally infected dog with *H. canis* parasitemia from Israel. The serum from the experimentally infected dog was collected 63 days postinfection. Sera from three dogs naturally infected with *H. americanum* from Alabama diagnosed by muscle biopsy (9) were used to test reactivity with *H. canis* gamont antigen by Western blotting and indirect fluorescent-antibody testing (IFAT). Negative-control sera were obtained from a tick-free laboratory-raised dog prior to infection with *H. canis* and from a blood donor dog from the College of Veterinary Medicine at Auburn, Alabama. The experimentally infected dog was inoculated with *H. canis* as previously described (2). Briefly, a 3-month-old laboratory-raised dog was inoculated with 30 adult *R. sanguineus* ticks that were repleted as nymphs on a naturally infected dog. The dog developed hepatozoonosis with parasites which were identified in blood smears and bone marrow aspirates by light microscopy.

The frozen suspension of purified *H. canis* gamonts was thawed at room temperature, and after centrifugation at 800 × *g* for 5 min, the protein concentration of the supernatant was determined by the Bradford method (4). The material was further solubilized in sample buffer (0.025 M Tris-glycine [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate [SDS], 15% [wt/vol] glycerol and bromphenol blue) at 100°C for 3 min. The gamont antigen at 10 μg of protein/lane was subjected to SDS-polyacrylamide gel electrophoresis (7.5 to 17.5% gradient gel) under nonreducing conditions (13). The resolved polypeptides were transferred overnight onto nitrocellulose membranes in Tris-glycine buffer containing 20% methanol at constant 35 mA and 4°C (19). The membranes were blocked with PBS containing 3% casein for 2 h at room temperature, followed by five successive washings with PBS containing 0.5% Tween 20 (PT). Dog sera were diluted in PT, applied to individual strips, and incubated overnight at 4°C. The nitrocellulose strips were washed seven times as described above, and the bound antibody was detected with rabbit anti-dog immunoglobulin G (IgG) conjugated to horseradish peroxidase at 1:3,000 dilution in PBS containing 0.2% bovine serum albumin (BSA). After incubation for 1 h at 37°C, the nitrocellulose strips were washed eight times with PT and developed with 0.005% 3,3′-diaminobenzidine (DAB) (Sigma, St. Louis, Mo.) supplemented with 0.03% cobalt chloride and 0.3% H_2O_2.

Serum samples were analyzed by IFAT for IgG antibodies reactive with *H. canis* antigen (18). Antigen slides were prepared with blood from a naturally infected dog with high parasitemia. The buffy coat was washed with PBS by centrifugation three times, and the final pellet was resuspended in a mixture of equal volumes of PBS and 3% BSA. Thin smears of buffy coat were made on glass slides and dried at room temperature. The slides were immersed for 10 min in acetone and then stored at −80°C. Before use, antigen slides, stored at −80°C, were warmed and dried at 37°C for 30 min. A series of successive twofold dilutions of serum in PBS, from 1:16 to 1:4,096, were applied to the smears and incubated for 30 min at 37°C in a humid chamber. The slides were washed three times in PBS and blotted dry. Fluorescein-conjugated rabbit anti-dog IgG was applied to the wells at 1:70 dilution. The slides were then incubated at 37°C for 30 min, washed as described above, and dried. The smears were mounted under coverslips in PBS-buffered glycerol (pH 8.5) and examined under a fluorescence microscope.

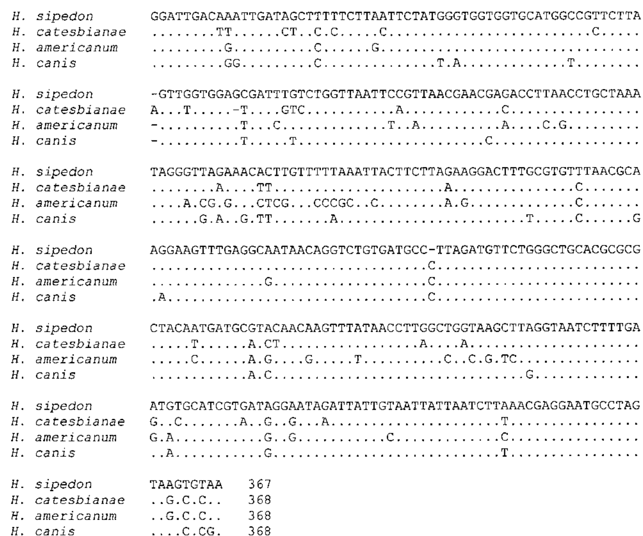

FIG. 1. Alignment of a portion of the 18S rRNA gene from *H. americanum* (United States) and *H. canis* (Israel) and two *Hepatozoon* species, *H. sipedon* from a snake and *H. catesbianae* from a frog. Only the nucleotides that are different from those of *H. sipedon* are shown.

A partial sequence for the 18S SSU rRNA was obtained from *H. americanum* and *H. canis* using cycle sequencing of PCR fragments generated using a combination of a coccidium-specific primer (Cocci A) and a universal primer (6, 16). The sequences have been submitted to GenBank under accession numbers AF206669 (*H. canis*) and AF206668 (*H. americanum*). A region of 368 bases from the two *Hepatozoon* species infecting dogs near the 3′ end of their 18S rRNA genes was used for further analysis. This region spans three hypervariable regions of the 18S rRNA gene. These partial sequences from the 18S rRNA gene from *H. americanum* and *H. canis* were aligned by eye against the same region from two other *Hepatozoon* species, *H. catesbianae* (GenBank accession no. AF206670) and *H. sipedon* (GenBank accession no. AF206671), gamonts of which occur in the blood of frogs and snakes, respectively. The alignment (Fig. 1) demonstrated relatively few differences between the two canine parasites and the *Hepatozoon* species infecting frog and snake hosts. Pairwise differences among taxa in this region of the 18S rRNA gene range from 7.64 to 14.47% (Table 1). The pairwise difference between *H. canis* and *H. americanum* was 50 of 368 bases (13.59%).

Western blot analysis of anti-*H. canis* and anti-*H. americanum* sera reacted with *H. canis* gamont antigens revealed that multiple bands were recognized by sera from dogs infected with either *Hepatozoon* species (Fig. 2). A similarity in the pattern of reactivity of sera from dogs with *H. canis* or *H.*

TABLE 1. Pairwise differences between pairs of *Hepatozoon* species for a 368-base aligned section flanking the 3′ end of the 18S rRNA gene

Species	Pairwise difference[a] (% different) between:		
	H. canis	*H. americanum*	*H. sipedon*
H. americanum	13.59		
H. sipedon	7.64	12.85	
H. catesbianae	10.10	14.47	9.86

[a] These pairwise differences have been adjusted for the presence of gaps in the alignment.

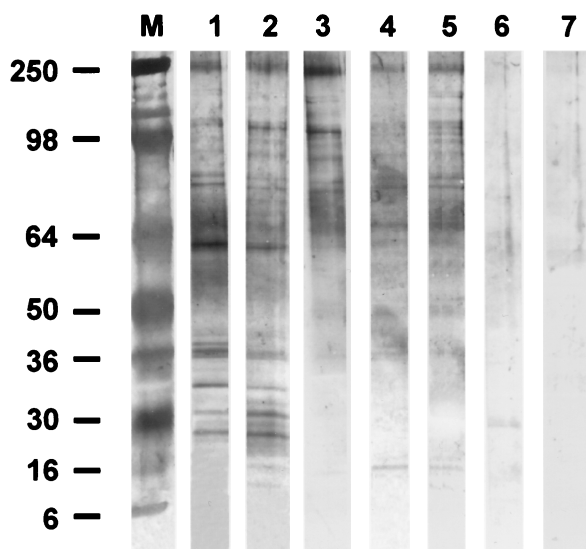

FIG. 2. Western blot analysis of *H. canis* gamont antigens recognized by sera from dogs infected with *H. canis* and *H. americanum*. Reactions of sera from different dogs are shown as follows: lane 1, serum from a dog experimentally infected with *H. canis*; lane 2, serum from a dog with a naturally occurring *H. canis* infection; lanes 3 to 5, sera from three dogs that were naturally infected with *H. americanum*; lanes 6 and 7, control serum from a blood donor dog at Auburn University and from preinfection serum from the experimentally infected dog, respectively. Lane M contains molecular mass markers. Note the triplet of bands with relative rMM of 32 to 28 kDa that are reactive only with anti-*H. canis* sera.

americanum infections was observed for protein bands ranging from 250 to 36 kDa with distinct bands of relative molecular mass (rMM) of 250, 107, 88, 63, and 37 kDa. Reactivity with the 63- and 37-kDa bands was weaker with anti-*H. americanum* sera than with anti-*H. canis* sera. A triplet of antigenic bands with lower rMM of 32, 30, and 28 that were reactive with anti-*H. canis* were not reactive with anti-*H. americanum* sera. Minimal weak nonspecific reactivity was observed with the control and preinfection sera.

By IFAT, serum samples from the two dogs infected with *H. canis* and the three dogs infected with *H. americanum* were all reactive with *H. canis* gamont antigen. Both dogs infected with *H. canis* demonstrated high titers of 1:1,024 for the experimentally infected dog and 1:4,096 for the naturally infected dog, while those infected with *H. americanum* had low titers ranging between 1:32 and 1:64. The two control sera showed no reactivity with *H. canis* antigen by IFAT.

The findings presented in this study demonstrate that the geographically distinct *H. canis* and *H. americanum*, previously thought to be strains of the same species (7, 8) and recently classified separately mainly on the basis of different pathological, clinical, and morphological findings (20), also differ at the genetic and antigenic levels.

The genetic distances among the four *Hepatozoon* species in a portion of the coding region of the 18S rRNA gene were consistent with their recognition as distinct species (20). The pairwise difference between the two parasites of dogs, *H. canis* and *H. americanum*, was 13.59% in the short region of the 18S rRNA gene sampled. This interspecific variation was comparable to the differences between the two *Hepatozoon* species infecting frogs and snakes (e.g., 9.86% between *H. catesbianae* and *H. sipedon*). The differences in this region were comparable to the interspecific variation observed among various *Eimeria* species infecting poultry (3). Since the portion of the coding region sequenced from these four *Hepatozoon* species contains at least three hypervariable regions, the percentage differences between the various *Hepatozoon* species for the entire gene are likely to be considerably lower.

The Western blotting patterns observed in this study for reactivity of anti-*H. canis* and anti-*H. americanum* sera with *H. canis* antigen show that although some similarities in reactivity were seen for higher-molecular-mass proteins, three molecular mass bands in the 32- to 28-kDa region detected by anti-*H. canis* sera were not recognized by anti-*H. americanum* sera. In addition, the anti-*H. canis* serum reacted strongly with the 63- and 37-kDa bands of *H. canis* gamont antigen, while sera from *H. americanum*-infected dogs recognized these antigens weakly. These findings support the hypothesis that *H. canis* and *H. americanum* are antigenically related and show a degree of cross-reactivity by Western blotting, but *H. canis* is substantially different from *H. americanum*, as detected by the Western blotting reactivity to certain protein bands only by anti-*H. canis* serum and the weaker antibody response to other protein bands by anti-*H. americanum* serum. Similarly, high antibody titers (up to 1:4,096) were obtained in homologous IFAT reactions with the sera used for Western blotting and significantly lower titers (up to 1:64) in heterologous reactions. The low cross-reactive IFAT antibody titers to *H. canis* antigen demonstrated in the sera of *H. americanum*-infected dogs are in agreement with the findings of Vincent-Johnson et al. (20), who reported a low to negative reactivity of *H. americanum*-infected canine sera with *H. canis* antigen from Israel by IFAT. The low IFAT titers of anti-*H. americanum* sera may provide another indication that *H. americanum* shares some antigenic epitopes with *H. canis*, as seen by Western blotting, but anti-*H. americanum* serum is distinct and elicits an antibody response that detects only some *H. canis* antigens.

In conclusion, the genetic and antigenic analyses described in this study provide additional support for the differentiation of *H. canis* and *H. americanum* as different species of *Hepatozoon* that are the causative agents of unique and distinct clinical diseases.

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