J Vet Intern Med 2002;16:642–649

Molecular Evidence Supporting *Ehrlichia canis*–Like Infection in Cats

Edward B. Breitschwerdt, Anthony C.G. Abrams-Ogg, Michael R. Lappin, Dorothee Bienzle, Susan I. Hancock, Sara M. Cowan, Jennifer K. Clooten, Barbara C. Hegarty, and Eleanor C. Hawkins

Currently, the pathogenic role of *Ehrlichia canis* in cats has been proposed predominantly on the basis of the serologic evidence of natural infection and the infrequent detection of morulae-like structures within the cytoplasm of leukocytes in cats. The purpose of this report was to provide molecular evidence supporting *E canis*–like infection in 3 cats that had clinical manifestations consistent with canine ehrlichiosis but lacked antibodies to *E canis* antigens. Serum from all 3 cats contained antinuclear antibodies (ANAs). The predominant disease manifestation was polyarthritis in 1 cat and bone marrow hypoplasia or dysplasia, accompanied by pancytopenia or anemia and thrombocytopenia, in 1 cat each. The alignment of *E canis* partial 16S ribosomal DNA (rDNA; 382 nucleotide positions), amplified from EDTA blood samples from each cat, was identical to each other and was identical to a canine isolate of *E canis* (GenBank accession number AF373613). In 1 cat, concurrent treatment with corticosteroids may have interfered with the therapeutic effectiveness of doxycycline for the elimination of *E canis*–like infection. To further define the spectrum of ehrlichiosis in cats, polymerase chain reaction (PCR) testing may be necessary until serologic testing is thoroughly validated in experimentally or naturally infected cats. In addition, until *E canis* has been isolated from cats and several tissue culture isolates are available from disparate geographic regions for detailed comparative genetic study, the molecular evidence presented in this study supporting *E canis*–like infection in cats must be interpreted with caution.

Key words: Antinuclear antibodies; Leukemia; Polyarthritis; Thrombocytopenia.

Although various *Ehrlichia* spp. have been reported to cause disease in cows, sheep, dogs, horses, and human beings, the role of any specific *Ehrlichia* spp. as a pathogen in cats remains less clearly defined.[1–3] The 1st evidence for naturally occurring ehrlichiosis in cats was provided in 1986 by Charpentier and Groulade in France.[4] Ehrlichiosis in cats was subsequently reported in 1989 by Buoro et al[5] when they described intracytoplasmic inclusions in monocytes and lymphocytes derived from 3 cats in Kenya. By both light and electron microscopy, the inclusions were morphologically similar to *Ehrlichia* sp. *morulae,* as observed on blood smears obtained from other animals. Subsequently, morulae were described in stained blood smears obtained from cats in the United States,[6] France,[7] Brazil,[8] and Sweden.[9] To date, no *Ehrlichia* spp. has been cultured from the blood of a cat; however, Bjoersdorff et al[9] amplified and sequenced 16S rDNA from an EDTA anticoagulated blood sample obtained from a 14-month-old shorthaired cat from Sweden that was 100% identical to canine and equine granulocytotropic *Ehrlichia* strains from the same region.

Antibodies to *E canis* antigens have been detected by indirect immunofluorescent antibody testing in cat sera from North America[10,11]; however, to our knowledge, *E canis*–like DNA has not previously been amplified and sequenced from cat blood or tissues. The purpose of this report was to describe the clinicopathologic, serologic, and molecular findings in 3 cats that were naturally infected with an *E canis*–like organism. Because these cats represent the 1st cases of *E canis*–like infection in North America identified on a molecular basis, a brief history reflecting the clinical complexity of each case will be provided.

From the Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC (Breitschwerdt, Cowan, Hancock, Hawkins, Hegarty); the Department of Clinical Studies and the Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada (Abrams-Ogg, Bienzle, Clooten); and the Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO (Lappin). *Previously presented as an abstract at the ACVIM Forum, Denver, CO, May 2001.*

Reprint requests: Edward B. Breitschwerdt, CVM, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606; e-mail: ed_breitschwerdt@ncsu.edu.

Submitted April 29, 2002; Revised July 8, 2002; Accepted July 8, 2002.

0891-6640/02/1606-0001/$3.00/0

Materials and Methods

Microimmunofluorescent Antibody Test

The accurate diagnosis of an infectious disease relies on the clinician's evaluation of the patient's history, a physical examination, and clinicopathologic abnormalities, in conjunction with supportive clinopathologic data (isolation, serology, or molecular testing). Because our efforts to provide serologic corroboration supporting *E canis* infection in these 3 cats were problematic (ie, we were unable to detect *E canis*–specific antibodies), additional research studies were initiated in conjunction with the evaluation of these cats in an attempt to validate serologic testing for *E canis* infection in cats. Each serum was tested against *E canis* (strain Florida) antigens by methods previously described[12] but was modified by the substitution of goat anti-feline immunoglobulin G (IgG) fluorescein isothiocyanate (FITC) at a dilution of 1:200 for goat anti-canine IgG FITC. *Ehrlichia canis* was propagated in vitro in 030 or DH82 canine cell lines as described previously.[12] Infected cultures were harvested when 80–100% of the cells were infected, and cell suspensions were affixed to 30-well Teflon-coated slides.[a] The conjugate of cats was known to be reactive with other antigen preparations routinely used for indirect fluorescent antibody (IFA) testing in the North Carolina State University, College of Veterinary Medicine, Vector Borne Diseases Diagnostic Laboratory (NCSU-CVM-VBDDL). In an effort to find an appropriate positive control sample, 28 sera from cats were provided by the Colorado State University (CSA; source, M. Lappin). Serum from an uninfected cat was used as a negative control, but a positive control has yet to be identified in our laboratory. An additional 32 clinical accessions received by the NCSU-CVM-VBDDL from cats with clinical signs suggestive of ehrlichiosis and sera from the 3 cats described in this report were tested against *E canis* antigens, as described above.

DNA Extraction

With a commercially available QIAmp Blood kit,[b] DNA was extracted from 200 μL of EDTA anticoagulated whole blood that had been stored frozen at −80°C according to the manufacturer's protocol.

Polymerase Chain Reaction Amplification

Initially, polymerase chain reaction (PCR) testing was performed with *Ehrlichia* genus-specific primers as previously described.[12,13] To increase sensitivity and to obtain a larger amplicon for gene sequence analysis, a modified *Ehrlichia* genus PCR was developed as follows: *Ehrlichia* genus PCR was performed in a 50-μL reaction volume containing 10 μL DNA template, 200 μM (each) deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP); 25 pmol of primers designated ECC-mod (5′-AGAACGAACGCTGGCGGCAAG-3′) and HE3-Rmod (5′-CTTCTATAGGTACCGTCATTATCTTCCCTATTG-3′); 2 mM $MgCl_2$; and 1.25 U of AmpliTaq Gold.[c] Amplifications were performed in a Techne Progene[d] as follows: 7 minutes at 95°C, followed by 45 cycles with 45 seconds of denaturation at 95°C and 45 seconds at 72°C. Culture-grown *E canis* was used as a positive control. PCR products were identified by ethidium bromide on a 1% agarose gel.

Culture-grown *E canis* was used as a positive control. PCR products (400 bp in size) were identified by ethidium bromide staining after electrophoresis in a 1% agarose gel.

Cloning and Sequencing

Ehrlichia genus PCR products were cloned into the pCR2.1 cloning vector, and the *E coli* TOP10 strain was transformed by the supplier's protocol.[e] Recombinants were selected by the blue-white color of colonies, and plasmid DNA from several clones was isolated with the QIAprep Plasmid Kit.[f] Recombinant plasmid DNA was sequenced bidirectionally with infrared fluorescently labeled primers M13R-700 (5′-CAGGAAACAGCTATGACCATG) and T7-800 (5′-TAATACGACTCACTATAGGGCGA) with the Sequitherm EXCEL II DNA Sequencing Kit-LC as recommended by the supplier.[g] The sequencing reaction conditions were as follows: 2 minutes at 92°C, followed by 30 amplification cycles (30 seconds at 92°C, 15 seconds at 55°C, and 30 seconds at 72°C). The sequencing reactions were analyzed by polyacrylamide gel electrophoresis (3.75%) on a LI-COR 4200 automated DNA sequencer.[h]

Case Descriptions and Results

Cat 1

Historically, this male castrated domestic shorthaired cat was rescued by the owners as a stray kitten. He was whelped and remained feral in the wooded natural area adjacent to the house. The owner did not recall finding ticks or fleas on the cat, which was subsequently maintained as a predominantly indoor pet with supervised walks in a fenced backyard. In June 1998, at approximately 1 year of age, the cat developed an acute illness, characterized by anorexia, lethargy, fever (40.5°C), joint pain, and prolapse of the left 3rd eyelid. After 2 weeks of medical therapy, including antibiotics (enrofloxacin and clindamycin), corticosteroids, and SC fluids, which failed to induce remission of the clinical signs, the cat was referred to the NCSU Veterinary Teaching Hospital (NCSU-VTH) for additional evaluation. At presentation, the cat was febrile (40.2°C), reluctant to stand and lame, with pain in both rear legs. When forced to stand, the cat would carry the left rear leg. Subsequently, during hospitalization, the cat developed a shifting leg lameness involving the front and rear legs, suggesting bone or joint pain. There was mild rear limb muscle atrophy and swelling in the ventral neck region. An ophthalmic examination did not identify fundic lesions. A neurologic examination revealed asymmetrical rear limb weakness, with tremors and apparent pain on muscle palpation. There was a neutrophilic leukocytosis (segmented neutrophils, 47,142/μL), without a left shift. Creatine kinase was normal (33 IU/L; reference range, 26–243 IU/L). Serologic test results for toxoplasmosis, coronavirus, feline immunodeficiency virus (FIV), and feline leukemia virus (FeLV) were negative. Thoracic radiographs showed a subtle interstitial pattern in the dorsocaudal lung lobes and a slightly enlarged heart. The abdominal ultrasound was unremarkable, and an echocardiogram did not detect vegetative lesions on the mitral or aortic valves. A urine culture failed to grow bacteria. No spinal column abnormalities were found on abdominal radiographs. The lumbar cerebrospinal fluid was normal, except for a mild increase in protein (52.5 mg/dL; reference range, 1–45 mg/dL). A serum cryptococcal antigen test was positive (reciprocal titer, 256), whereas the same test performed on cerebrospinal fluid was negative, and a focal site of cryptococcal infection was never localized. Aspiration cytology of the left stifle and the right and left tarsal joints confirmed neutrophilic polyarthritis. Antibodies were detected to nuclear antigens on 2 separate occasions (reciprocal antinuclear antibody [ANA] titers, 160 and 80) during hospitalization. Because of the positive cryptococcus antigen test, the cat was treated with fluconazole 50 mg PO q12h for 2 months. On July 20, 1998, reciprocal titers to cryptococcus and antinuclear antigens were 64 and 80, respectively. After fluconazole treatment, the cat remained clinically normal until shortly before the 2nd referral.

On May 12, 2000, the cat was again referred to the NCSU-VTH for the evaluation of unilateral conjunctivitis, severe lethargy, anorexia, an occasional cough, and a fever (rectal temperature, 41.1°C) of 2 weeks' duration. Before referral, these signs were poorly responsive to treatment with amoxicillin, enrofloxacin, and fluids. Despite a negative cryptococcal antigen test result, fluconazole 50 mg PO q12h was begun on May 11, 2000, after which the cat again developed ventral neck swelling, salivation, and difficulty breathing. The administration of dexamethasone 10 mg IV induced resolution of the swelling in the ventral neck region. At presentation, the cat weighed 5.4 kg. Physical examination abnormalities included fever (rectal temperature, 41.05°C), tachycardia (210 bpm), and a 2/6 apical systolic heart murmur with a gallop rhythm. The gait was stilted and short strided, and the cat preferred to remain recumbent during the examination. There was moderate effusion in both stifle joints. A complete neurologic examination failed to detect abnormalities. Radiographs of the right and left stifles showed joint mice bilaterally. Chemosis and protrusion of the left 3rd eyelid resolved spontaneously and then temporarily occurred in the right eye; this was presumptively associated with herpes virus infection. The right retina contained a small elliptic hyperreflexive lesion, consistent with taurine deficiency; the plasma taurine concentration was normal (79 nmol/mL; reference range, 60–120 nmol/mL). Conjunctival cytology showed neutrophilic in-

flammation with epithelial hyperplasia; however, cultures failed to isolate *Mycoplasma* spp., *Calicivirus*, or herpesvirus I. Mild cardiomegaly was again noted on thoracic radiographs. By echocardiography, there was increased left ventricular contractility but no evidence of cardiomyopathy. Laboratory abnormalities included a neutrophilic leukocytosis with a left shift (segmented neutrophils, 39,130/μL; band neutrophils, 2,580/μL), moderate neutrophil toxicity, and mild hyperglycemia (serum glucose, 156 mg/dL; n = 72–144 mg/dL). Platelet numbers could not be counted (including an attempted manual platelet count) because of excessive platelet clumping on this and several subsequent CBCs. Both stifle joints contained moderate numbers of nondegenerate neutrophils. Cultures of the joint fluid failed to grow bacteria, *Mycoplasma* spp., or fungal organisms. ANAs were again detected at a reciprocal titer of 320. *Toxoplasma gondii* (immunoglobulin M [IgM] and IgG antibodies), *Bartonella henselae* IgG, FIV/FeLV enzyme-linked immunosorbent assay (ELISA), and heartworm antigen and antibody tests were negative. Urine-specific gravity was 1.014 with a 3+ protein reaction on a urine dipstick. The urine protein : creatinine ratio was normal (0.34; normal, <0.8), and the urine culture failed to grow bacteria. Cryptococcal capsular antigen testing results with both urine and serum samples were negative.

During the next 8 days of hospitalization, the cat lost 0.5 kg and had a persistent fever that was not responsive to fluconazole, butorphenol, azithromycin, amoxicillin IV, or, eventually, imipenem. The cat's appetite remained poor, but it improved on those days when carprofen was administered. There was no change in the character of the heart murmur. The cat developed a progressive nonregenerative anemia (PCV decreased from 41% before referral to 22%) and a profound left shift (on hospital day 8, the segmented neutrophil count was 26,000/μL with 17,000 band neutrophils/μL and 3,700 immature granulocytes/μL, with moderate neutrophil toxicity and atypical neutrophil segmentation).

On hospital day 10, EDTA anticoagulated blood was submitted for *Ehrlichia* and *Bartonella* PCR. Fluconazole, azithromycin, amoxicillin, and carprofen were discontinued. Imipenem was continued, and doxycycline and dexamethasone were begun. Appetite increased, and fever and joint pain resolved. On hospital day 14, the PCV was 18%, the aggregate reticulocyte count by flow cytometry was 3.3%, and the segmented neutrophil count was 35,728/μL, with 6,032 band neutrophils/μL, 464 immature granulocytes/μL, 2,320 monocytes/μL, and 464 reactive lymphocytes/μL. Despite continued clinical improvement, there was an abrupt increase in alanine aminotransferase activity (1,634 IU/L; reference range, 5–134 IU/L). The ultrasonographic appearance of the liver was normal. The degree of neutrophilic inflammation was markedly improved in both stifle joints. The cat remained stable and was discharged from the hospital 2 days later, with instructions to administer prednisone 5 mg PO q12h for 21 days, doxycycline 25 mg PO q12h for a total duration of 21 days, and imipenem 25 mg SC q12h for a total duration of 14 days.

Recheck examinations at 1, 3, and 7 weeks postdischarge revealed a continued improvement in clinical status and a total resolution of all CBC and biochemical abnormalities. During this time, no joint distention was discernible, the repeat joint fluid cytology remained normal, and the prednisone dose was gradually tapered. Six weeks after discharge, the PCR results became available. PCR amplification failed to detect *Bartonella* spp. DNA; however, *E canis*–like DNA was amplified and subsequently sequenced from an EDTA blood sample obtained on May 15, 2000. *E canis*–like DNA was later amplified from EDTA blood samples obtained on August 28 and October 30, 2000, during which time the cat was being treated with prednisone only (2.5 mg q24h). After the October 30 PCR result (*E canis* DNA still detectable) became available, corticosteroids were discontinued, and doxycycline (25 mg q12h for 3 weeks) was begun on November 21. On January 8 and April 25, 2001, PCR amplification failed to detect *Ehrlichia* DNA. During this time, 2 CBCs, a serum biochemical profile, and a urinalysis were unremarkable, and ANAs were no longer detectable. Between May 2000 and April 2001, the cat gained 1.7 kg, and there were no additional reports of illness through December 2001. In early January 2002, the cat developed febrile illness accompanied by lethargy and lameness. Three months later, the cat was again referred to the NCSU-VTH, at which time there was mild neutrophilic polyarthritis, a sinus bradycardia of undetermined origin, and retinal degeneration. The cat died within 12 hours of being placed on a ventilator, with a presumptive diagnosis of acute respiratory distress syndrome. EDTA anticoagulated blood samples were not obtained before starting doxycycline therapy, and subsequent attempts to amplify *E canis* by PCR from posttreatment samples were not successful. Postmortem examination diagnoses included moderate to marked neutrophilic and histiocytic myocarditis with focal myocardial fibrosis; diffuse, severe neutrophilic and histiocytic pneumonia; multifocal, lymphoplasmacytic interstitial nephritis; and multifocal lymphoplasmacytic pancreatitis with focal nodular hyperplasia. Although an infectious etiology was suspected, no organisms were observed.

Cat 2

A 1-year-old spayed female lynx-point Siamese crossbred cat (3.0 kg) was referred to the Ontario Veterinary College, Guelph, Canada, on March 31, 2000, for the evaluation of inappetence, lethargy, and lymphadenopathy of 2 days' duration. The cat was obtained as a kitten by the owner from a private source, and she had traveled extensively with the owner in eastern Canada. The owner, a veterinarian, reported that popliteal lymphadenopathy had been present since the cat was obtained. Physical examination abnormalities included moderate peripheral lymphadenopathy and fever (rectal temperature, 41.1°C [106.0°F]). The CBC on admission showed severe pancytopenia. The hematocrit was 17% with no evidence of reticulocytosis, the segmented neutrophil count was 50/μL, the band neutrophil count was 40/μL, and the platelet count was 36,000/μL. The platelet count on day 3 was <1,000/μL. Bone marrow aspiration and core biopsies showed marked erythroid and megakaryocytic hypoplasia and myeloid hyperplasia with a marked left shift, consistent with either myelodysplasia or myeloid leukemia. Bone marrow

biopsy results were also consistent with recovery from myelosuppression, but this differential consideration was not supported by the clinical history. Lymph node cytology showed reactive hyperplasia. Serum was reactive with nuclear antigens (reciprocal ANA titer, 320). A serum chemistry profile and a urinalysis were unremarkable. Microbial cultures of blood, bone marrow, and urine failed to grow bacteria. ELISA test results for FeLV/FIV were negative, and FeLV was not detected in the bone marrow by IFA testing. Antibodies were not detected to *B henselae* or *E canis* antigens by IFA testing. PCR amplification failed to yield *Ehrlichia* genus or *Hemobartonella felis* DNA. Thoracic radiographs showed sternal lymphadenopathy. Abdominal ultrasound showed splenomegaly. The cat was treated with packed red cell and fresh whole-blood transfusions and with ampicillin and enrofloxacin for a presumptive diagnosis of sepsis secondary to severe neutropenia. A tentative diagnosis was made of immune-mediated myelodysplasia (immune-mediated myeloid maturation arrest with ineffective myelopoiesis), and the cat was treated with dexamethasone and cyclosporine. Fever resolved by day 4, segmental neutrophils were normal (8,720/μL) by day 5, and platelets were normal (145,000/μL) by day 7, at which time neutrophils peaked at 13,940/μL. The cat was discharged with instructions to give enrofloxacin 15 mg q24h for 28 days, prednisone 10 mg q24h indefinitely, and cyclosporin indefinitely (dose adjusted to maintain a whole-blood concentration between 200 and 400 ng/mL). Bone marrow aspiration and core biopsies 1 month after discharge showed normal to mildly increased cellularity, normal erythropoiesis and megakaryopoiesis, and persistent mild myeloid left shift. The cat remained healthy until October, except for an additional episode of lymphadenopathy in June (lymph node cytology showed pyogranulomatous inflammation), which resolved spontaneously. Between April and October, the hematocrit stabilized at approximately 34%, the platelet counts remained normal, and the neutrophil counts varied from low to normal (1,280–5,610/μL). The daily dose of prednisone was increased to 15 mg in July, because the neutrophil count had decreased to 1,470/μL. A reciprocal ANA titer in August was 320.

On October 27, the cat again presented with an inappetence of 2 days' duration, a fever (rectal temperature, 41.1°C [106.0°F]), peripheral lymphadenopathy, and pancytopenia. The hematocrit on admission was 25% with no evidence of reticulocytosis, the segmented neutrophil count was 50/μL, and the band neutrophil count was 10/μL. The platelet count on day 3 was 6,000/μL. Bone marrow aspiration and core biopsies showed similar, but less severe, changes than the biopsies on 1st presentation. Colony-forming unit granulocyte-macrophage assays were performed in an effort to distinguish between maturation arrest and myeloid leukemia, but results were equivocal. There were increased numbers of colonies compared to bone marrow cells from a normal cat in cultures established both with and without a granulocyte-macrophage colony-stimulating factor. A serum chemistry profile showed hyperglobulinemia (6.9 mg/dL; reference range, 2.8–5.2 mg/dL), which was determined by electrophoresis to be due to increased alpha globulins and polyclonal gammopathy. Lymph node cytology showed plasma cell hyperplasia. Urinalysis was unremarkable. The cat was treated in a manner similar to that in the 1st presentation. The fever resolved on day 4, and neutrophil and platelet counts were increasing, but still low, on day 6 when the cat was discharged with instructions to administer doxycycline 12.5 mg q12h in the event there was an undetected ehrlichial infection. Cyclophosphamide (12.5 mg; 4 days on, 3 days off) was added to the treatment regimen of prednisone and cyclosporine to enhance the degree of immunosuppression. The cat was clinically normal when rechecked 1 week later; the hematocrit was 22%, the neutrophil count was 6,780/μL, and the platelet count was 400,000/μL. The reciprocal ANA titer was 160. At this time, by means of modified *Ehrlichia* genus PCR primers, *E canis*–like DNA was amplified and sequenced by the NCSU-CVM-VBDDL from an EDTA blood sample archived from the initial presentation.

Over the next 2 months, the cat remained healthy, the hematocrit stabilized at approximately 35%, and the platelet counts remained normal, but once again, the neutrophil count fell from the previous peak during recovery from myelosuppression and remained low to low normal, with counts ranging from 740 to 2,730/μL. Cyclophosphamide therapy was discontinued in the event that it was contributing to neutropenia. Doxycycline, cyclosporine, and prednisone were continued. Over the next year, the hematocrit ranged from 35 to 42%, the platelets remained normal, and the neutrophil counts ranged from 2,360 to 6,160/μL and were usually normal. Further bone marrow evaluation was declined. The daily dose of prednisone was reduced to 10 mg in April 2001 and to 5 mg in July 2001. ANA titers remained positive, with reciprocal values of 640 in July, November, and December 2001. *E canis*–like DNA was not detected by PCR in July and November 2001. This finding notwithstanding, the owner elected to continue doxycycline therapy as well as prednisone and cyclosporine. Multifocal dermatophytosis developed in April 2001, which has been successfully managed with lufenuron. The cat was otherwise healthy during this follow-up period and is clinically normal at the time of writing (April 2002).

Cat 3

A 1-year-old-male neutered domestic shorthaired cat (3.9 kg) was referred to the Ontario Veterinary College, Guelph, Canada, on October 17, 2000, for the evaluation of lethargy of 3 days' duration. The cat was adopted as a stray by the present owner and traveled extensively with the owner in a long-distance transport truck in eastern Canada. Physical examination abnormalities included pale mucous membranes that contained a few petechial hemorrhages. The rectal temperature was 38.5°C, the pulse was 180 bpm, and the respiratory rate was 44 breaths/min. Fundic examination revealed bilateral retinal detachments, with accompanying subretinal fluid, suspected retinal tears, and vitreous hemorrhage, which were presumptively related to anemic hypoxia and thrombocytopenia. On the day of admission and before blood transfusion, the Doppler systolic blood pressure was 125 mmHg. The cat was blind in 1 eye and had minimal vision in the other. There was a grade III-IV/VI systolic heart murmur and radiographic and ultrasonographic evidence of cardiomegaly, both of which were presump-

tively associated with severe anemia. CBC abnormalities included severe nonregenerative anemia (hematocrit, 6%; reticulocyte count, 0%) and severe thrombocytopenia (platelet count, 2,000/μL). A Coombs' test was negative. The segmented neutrophil count was 5,900/μL, with no band neutrophils reported on the differential cell count. A cytologic examination of an aspiration bone marrow biopsy and a histologic examination of a core bone marrow biopsy were both indicative of marked erythroid and megakaryocytic hypoplasia. There was a synchronous development of the myeloid series. The serum erythropoietin concentration was 600 mIU/mL (reference range, 1–15 mIU/mL, assay performed by the Hematology Laboratory, University of Pennsylvania). Serum was reactive with nuclear antigens (reciprocal ANA titer, 160). ELISA test results for FeLV/FIV were negative. PCR amplification failed to yield FeLV in the bone marrow or *H felis* DNA in the blood. Antibodies were not detected to *E canis* antigens by IFA testing. Serum chemistries and abdominal radiographs were unremarkable. A tentative diagnosis was made of immune-mediated erythroid and megakaryocytic hypoplasia. Initial treatment consisted of the transfusion of 1 U of fresh whole blood (60 mL/U) and prednisone 12.5 mg q24h. The hematocrit increased from 6 to 13% after the transfusion. Cyclosporin (dosage adjusted to maintain a whole-blood concentration between 200 and 400 ng/mL) and doxycycline 25 mg q24h (2-week course) were added to the treatment regimen. One week later, the hematocrit had decreased to 6%, the platelet count was 4,000/μL, and another unit of blood was administered by transfusion.

During the following week, the platelet count increased to 1,094,000/μL and then stabilized into the reference range over the next 2 weeks. However, 2 additional transfusions of fresh whole blood were given, 1 week apart, because of persistent anemia (hematocrit, 11–13%). Neither of these transfusions increased the posttransfusion hematocrit, which was perhaps due to extravascular hemolysis, because there were no signs of a transfusion reaction, and the cat did not become icteric. Cyclophosphamide (12.5 mg; 4 days on, 3 days off) was added on November 6 to intensify the immunosuppression due to persistent anemia, after which there was evidence of regeneration within 1 week. Anemia and cardiomegaly resolved during the next month. *Ehrlichia* PCR results became available on December 7, at which time CBC cell values were within reference ranges. *E canis*–like DNA was amplified and sequenced from an EDTA blood sample collected in October 2000. To ensure the therapeutic elimination of ehrlichial infection, doxycycline therapy was reinitiated at a dose of 25 mg q12h for 4 weeks. One month later, serum was tested for ANAs; the reciprocal titer was 160. The hematocrit and platelet numbers were normal, but neutropenia had developed (810/μL). Neutropenia was attributed to cyclophosphamide therapy, which was discontinued. Doxycycline, cyclosporine, and prednisone were continued. Over the next 14 months, the hematocrit ranged from 30 to 44%, the platelet numbers remained within the reference range, and the neutrophil counts ranged from 920 to 4,180/μL. Further bone marrow evaluation was declined. The daily dose of prednisone varied from 5 to 10 mg. ANA titers in April and November 2001 were negative, but the reciprocal titer in February 2002 was 80. *Ehrlichia* PCR was negative for samples obtained in January, March, October, and November 2001. This finding notwithstanding, the owner elected to continue doxycycline therapy, because the withdrawal of the antibiotic in March and December 2001 was associated with a reported decrease in activity. The cat was otherwise healthy during this follow-up period and is clinically normal at the time of writing (April 2002), except for persistent visual deficits.

Results

Microimmunofluorescent Antibody Test

Serum samples from the 3 cats described in this manuscript were tested at the NCSU, and multiple samples from cat 1 and a sample from cat 2 were tested at the CSU for seroreactivity to *E canis* antigens. Titers were $<1:16$ on all sampling dates in both laboratories. Of the 32 cats that were tested since 1995 in the NCSU-VBDDL, seroreactivity was not detected to *E canis* whole-organism antigens by IFA. Of the 28 serum samples from cats provided by the CSU, 5 of 28 tested positive by the CSU, and 1 tested positive by the VBDDL. Each of these cats had titers against *Anaplasma phagocytophilia* (previously *Ehrlichia phagocytophilia*, *Ehrlichia equi*, or human granulocytic ehrlichiosis), suggesting exposure to *A phagocytophilia* rather than exposure to *E canis.*

PCR Amplification and DNA Sequencing

With 2 different 16S rDNA *Ehrlichia* genus primer sets, amplicons of the anticipated size were obtained from EDTA blood samples derived from all 3 cats. Subsequently, one 16S rDNA *Ehrlichia* genus amplicon obtained from each cat, which includes the variable region of the 16S ribosomal RNA (rRNA) gene for *Ehrlichia* spp., was cloned and sequenced. The resulting sequences were identical to each other and were 100% (382 of 382 nucleotide positions) similar to the sequence of a Venezuela canine isolate of *E canis* (GenBank accession number AF373613). The sequences derived from the cats also had 99% homology to *E canis*, Florida strain (M73226), and *E canis*, Oklahoma strain (M73221), with the difference being a guanine rather than an adenine at nucleotide position 199. Subsequently, attempts to amplify *Ehrlichia* DNA from 90 stored EDTA blood samples from cats with undefined diseases or from additional clinical accessions of cats sent to the NCSU-VBDDL originating from North America have not been successful. However, identical *E canis* DNA sequences were subsequently detected in the blood of 2 cats from France (Beaufils and Breitschwerdt, personal communication).

Discussion

Infection with an *E canis*–like organism was confirmed in the 3 cats in this report by PCR amplification of the variable portion of the 16S rRNA gene from an EDTA anticoagulated blood sample, after which the respective amplicons were cloned and sequenced. All 3 DNA sequences were identical to each other and were 100% similar to a canine isolate of *E canis* (GenBank accession number

AF373613). Attempts to amplify the entire 16S rRNA gene (approximately 1,400 bases) were not successful because of technical limitations associated with small quantities of *E canis*–like template DNA in the blood samples of the cats. A retrospective attempt to isolate an organism in tissue culture from a small quantity of stored blood from cat 1 was not successful. This is not unexpected, because the isolation of *E canis* from naturally infected dogs under optimal conditions is rarely successful. On the basis of the currently available *Ehrlichia* 16S rDNA sequences in GenBank (ie, the region of the 16S rRNA gene containing phylogenetically relevant information), the sequences obtained from these cats would be consistent with *E canis* infection. However, it should be noted that 2 of these cats reside in a region of Canada in which *E canis* infection is considered uncommon in dogs. Although tick infestation was not reported historically, all 3 cats had ample opportunity for exposure to a variety of insect vectors. Two of the cats in this report were adopted as strays, and the 3rd cat traveled extensively by truck with the owner throughout eastern Canada. Therefore, it is possible that an *Ehrlichia* genotype capable of infecting cats with an identical partial 16S rDNA sequence homology to *E canis* may have evolved with different antigenic properties (ie, lack of serologic cross-reactivity to canine *E canis* antigens), as well as a nontick mode of transmission. Until *E canis* has been isolated in tissue culture from cats and several isolates are available from disparate geographic regions for detailed genetic study, the molecular evidence presented in this study supporting *E canis*–like infection in cats must be interpreted with caution.

For cat 1, *E canis*–like DNA was repeatedly amplified from EDTA blood samples that were collected and processed in the laboratory on 3 separate occasions during a 4-month period. Samples from cats 2 and 3 were also processed on different testing dates. In all instances, numerous other patient samples from dogs and cats, evaluated during the same period, as well as all negative controls yielded negative PCR results. In addition, a new set of *Ehrlichia* genus PCR primers was introduced into the laboratory at the time of the evaluation of cat 1 to obtain a larger segment of 16S rDNA for sequencing purposes. Therefore, 2 different *Ehrlichia* genus primers were used to independently amplify *Ehrlichia* DNA from the blood of these cats. Ultimately, the newly developed primers have proven to be of enhanced sensitivity compared to previous *Ehrlichia* genus primers used in our laboratory.[12,13] This most probably accounts for the initial negative PCR result in cat 2, which, on retesting of the same sample with the new primer set, was PCR positive. Collectively, these factors decrease the potential that the PCR results reported in this study might reflect laboratory contamination with PCR amplicons.

All 3 cats in this report appear to represent chronic *E canis*–like infections. We were unable to retrospectively amplify *E canis* DNA from a stored EDTA blood sample from cat 1 obtained during the initial evaluation in 1998, despite a very similar clinical presentation, compared to the May 12, 2000, presentation, when *E canis* DNA was amplified. However, cat 1 was treated with enrofloxacin for at least 2 weeks before obtaining the 1998 sample, which would most probably decrease or eliminate template DNA in the blood sample. In addition, a negative PCR result will never eliminate the possibility that this cat was chronically or recurrently infected with an *E canis*–like organism. *E canis*–like DNA was retrospectively amplified from an EDTA blood sample from cat 2 that was stored frozen in Guelph 6 months earlier, along with serum that was submitted for *E canis* IFA testing. Because *E canis* antibodies were not detected, PCR was not performed until there was disease recurrence. Therefore, before PCR diagnosis, cats 1 and 2 were each examined at a university teaching hospital 2 years or 6 months earlier, respectively. Despite severe anemia with associated cardiac hypertrophy, cat 3 presented with only mildly decreased activity and appetite, again suggestive of chronic compensated anemia. In all 3 cats, an infectious cause was suspected, but extensive screening for other infectious diseases, including FIV, FeLV, toxoplasmosis, bartonellosis, and hemobartonellosis, were negative, with the exception of a positive cryptococcal antigen test in cat 1.

At the time of disease onset, cats 2 and 3 were approximately 1 year of age. Although cat 1 was 3 years old, it is possible that this cat was infected with an *E canis*–like organism 2 years earlier, when evaluated for a very similar pattern of disease manifestations. The Swedish cat reported by Bjoersdorff et al[9] was 14 months old when *E equi* (now reclassified as *A phagocytophilia*) was amplified and sequenced from an EDTA blood sample. Recently, Lappin et al (personal communication) have identified *A phagocytophilia* infection detected by PCR amplification and DNA sequencing in 2 cats from Massachusetts that were 1 and 3 years old. Collectively, these results suggest that clinical disease might be expected more frequently in young cats. However, these results do not preclude infection in older cats or the possibility that PCR amplification of *Ehrlichia* or *Anaplasma* spp. DNA from the blood of an older cat might be a less sensitive diagnostic modality because of lower quantities of target DNA for PCR amplification. Although imperfect because of less than optimal sensitivity, PCR, as used in these cats, illustrates the potential utility of molecular diagnostic testing for the identification of intracellular pathogens in patients with complex and poorly defined disease presentations.

On the basis of the diagnostic complexity of these patients, the concurrent use of doxycycline and immunosuppressive drugs, the ultimately fatal outcome for cat 1, and the long-term treatments employed in cats 2 and 3, it is impossible to clearly ascribe the clinical abnormalities in these cats to *E canis*–like infection. With the exception of seroreactivity to nuclear antigens, an extensive array of diagnostic tests failed to implicate another specific cause for the clinical or laboratory abnormalities in these cats. As with dogs having *E canis* infections, cats may not develop disease manifestations or may experience only a mild illness after the initial infection. The predominant clinical abnormality in cat 1 was neutrophilic polyarthritis accompanied by fever, which in dogs has been most frequently associated with *Ehrlichia ewingii* infection.[14,15] Neutrophilic polyarthritis was confirmed by cytologic analysis of joint fluid at both 1 and 3 years of age. In a previous study, there was an epidemiologic association between polyarthritis in cats and *E canis* antibody detection.[3] Other nonspecific clin-

ical abnormalities, including lethargy, anorexia, conjunctivitis, swelling in the ventral neck region, and mild interstitial lung disease, were reported in these cats, all of which can be observed in association with canine ehrlichiosis.[13–16]

CBC abnormalities (pancytopenia in cat 2 and anemia and thrombocytopenia in cat 3) accompanied by hypoplastic changes in the bone marrow are also found in some dogs with ehrlichiosis.[14] Of substantial prognostic importance, myeloid leukemia or myelodysplasia was considered a potential differential diagnosis for cat 2. Myeloid leukemia and normal early myeloid expansion in response to an immune-mediated maturation arrest may result in similar bone marrow findings, because both conditions are characterized by the proliferation of blastlike cells. Given the historically poor prognosis associated with leukemia or myelodysplasia, which is generally associated with FeLV infection, and the potentially good prognosis associated with the immune-mediated maturation arrest, the decision was made to treat for the more likely responsive condition. Although myeloid leukemia has not been definitively ruled out, the positive ANA titer and long-term response to doxycycline and immunosuppression are supportive of an immune-mediated disorder. In dogs and humans, bone marrow cytopathology in patients with ehrlichiosis can vary substantially, particularly in relation to the duration of infection before sampling.[14,17,18] The mechanisms by which ehrlichial organisms induce changes in the bone marrow, particularly hypoplasia or myelofibrosis, remain poorly understood. The extent to which immunosuppression should be used concurrently with doxycycline when *E canis*–like infection is suspected in cats remains unclear; however, ehrlichial infection should be considered a differential diagnosis in cats with bone marrow hypoplasia, particularly when accompanied by dysplastic changes.

ANAs were detected in the serum of all 3 cats on multiple occasions in the immunology laboratories at NCSU and Guelph, respectively. In addition, when retested at NCSU, the serum of cats 2 and 3 were again reactive to antinuclear antigens. In cats 1 and 2, the attending clinician requested an ANA titer as a component of the initial diagnostic evaluation. In cat 1, ANAs were found several times during the initial and subsequent evaluations, but ANAs were no longer detected after the presumptive (on the basis of the negative PCR amplification of *Ehrlichia* DNA) therapeutic elimination of *E canis*–like infection. In cat 3, the test was performed retrospectively, because ANAs had been previously detected in cats 1 and 2. In contrast to persistent seroreactivity to antinuclear antigens in cat 2, concurrent treatment with doxycycline and immunosuppression resulted in decreased ANA titers in cat 3. The extent to which ANA testing has been validated for use in cats is unclear. However, there is increasing evidence from human medicine that ANAs can be detected in patients with chronic bacterial infections,[19,20] which perhaps include infections caused by intracellular pathogens, such as *Ehrlichia* spp.[21] Although the detection of ANAs is consistent with a clinical diagnosis of systemic lupus erythematosus, this study shows that autoantibodies elicited by exposure to the epitopes of infectious agents as well as other possibilities should be considered in patients with antibody reactivity to nuclear antigens.

The failure to detect *E canis* antibodies in the serum samples obtained from the cats in this study at multiple times renders questionable the routine use of serology to implicate exposure to or infection with *E canis.* It is possible that cats infected with *E canis* are immunologically anergic. Alternatively, the *E canis*–like strains infecting these cats may be antigenically different from the *E canis* strain (Florida) used in our laboratory for IFA testing; therefore, antibodies were not detected. However, serum from 2 of these cats was also negative at the CSU when a different *E canis* strain for IFA testing was used. More work is certainly warranted to attempt to validate a serologic test for the diagnosis of *E canis* infection in cats. Until a validated serologic test is available, PCR amplification of *Ehrlichia* DNA from EDTA blood samples can be used in an effort to confirm a diagnosis. Although more sensitive than culture, a negative PCR result would not rule out a diagnosis of *Ehrlichia* spp. infection. To optimize the chances of detecting ehrlichial DNA in patient blood samples, it is critically important to obtain the EDTA blood sample for diagnostic testing before the initiation of antibiotics. Obtaining samples before treatment would also facilitate a successful isolation of *Ehrlichia* spp. in tissue culture, which is a critically important need in order to clarify those species that can infect cats. Although definitive studies in cats are not available, the treatment of dogs with an antibiotic such as enrofloxacin, as was used in cat 1 before the initial referral, will decrease the concentration of ehrlichemia below the level of PCR detection without eliminating *E canis* infection.[22]

Although the duration and dose of doxycycline that will result in the therapeutic elimination of *E canis* in dogs remain somewhat controversial, data from treatment trials involving experimentally or naturally infected dogs suggest that 3 weeks of doxycycline at a dosage regimen of 5 mg/kg q12h should result in therapeutic elimination in most dogs.[12,23–25] On the basis of clinical response, the partial normalization of CBC abnormalities, and the failure to detect DNA by PCR in posttreatment EDTA blood samples, doxycycline does appear to be effective for the therapeutic elimination of *E canis*–like infection in cats. Of potential therapeutic importance, cat 1 remained PCR positive for *E canis* DNA on 2 additional occasions, spanning nearly 4 months, while receiving corticosteroids. After the cessation of corticosteroids and 3 weeks of doxycycline, we were unable to detect *E canis* DNA on 2 subsequent occasions, during which time the cat remained healthy without CBC abnormalities. The cause of fatal illness in cat 1 after a 16-month disease-free interval remains unresolved but may suggest treatment failure or coinfection with other known or unknown pathogens, as has been reported in dogs infected with *E canis*.[12,13,16] Despite negative posttreatment PCR results, the owners were reluctant to stop either doxycycline or immunosuppressive drug therapy for cats 2 and 3.

On the basis of the data that can be derived from the 3 cats in this report, a differential diagnosis of ehrlichiosis in cats due to an *E canis*–like infection should be considered in young cats with a fever of unknown origin, polyarthritis, hyperglobulinemia, the plasma cell infiltration of lymph nodes, or unexplained cytopenias accompanied by bone

marrow hypoplasia or dysplasia. The detection of ANAs in a cat with an undefined illness should also stimulate the consideration of ehrlichiosis in cats as a differential diagnosis. On the basis of the sequential analysis of bone marrow samples from cat 2, infection with an *E canis*–like organism may induce cytopathic changes that could be misinterpreted as myeloid leukemia. IFA testing with a canine *E canis* isolate as the antigen source may not result in the detection of *E canis*–specific antibodies in cats. When used concurrently, corticosteroids or other immunosuppressive drugs may interfere with the effectiveness of doxycycline for the therapeutic elimination of *E canis*–like infection in cats. To further define the spectrum of ehrlichiosis in cats, PCR testing may be necessary until serologic testing is validated in experimentally or naturally infected cats.

Footnotes

[a] Teflon-coated slides, Cel-line Associates, Erie Scientific, Portsmouth, NH
[b] QIAmp Blood kit, Qiagen, Chatsworth, CA
[c] AmpliTaq Gold, Applied Biosystems, Foster City, CA
[d] Progene, Techne Inc, Princeton, NJ
[e] *E coli* TOP10 strain, Invitrogen, Paisley, UK
[f] QIAprep Plasmid Kit, Qiagen, Chatsworth, CA
[g] Sequitherm EXCEL II DNA Sequencing Kit-LC, Epicentre Technologies, Madison, WI
[h] DNA sequencer, 4200 series, LI-COR Biosciences, Lincoln, NE

Acknowledgments

This research was supported by the State of North Carolina and in part by a donation by Merial Limited, Duluth, GA. The authors would like to acknowledge the assistance of the owners of these cats for facilitating repeated testing and long-term evaluation. We also acknowledge Drs Janis Milliken, Amelie Leclerc, Wendy Foster, and Amanda Gaskin for assistance in the management of these cats and Julie Bradley and Allison Rogala in the NCSU-CVM-VBDDL for technical assistance.

References

1. Rikihisa Y. The tribe *Ehrlichieae* and ehrlichial diseases. Clin Microbiol Rev 1991;4:286–308.

2. Walker DH, Dumler JS. Emergence of the Ehrlichioses as human health problems. Emerg Infect Dis 1996;2:18–29.

3. Stubbs CJ, Holland CJ, Reif JS, et al. Feline ehrlichiosis; literature review and serologic survey. Compend Cont Educ Pract Vet 2000;22:307–317.

4. Charpentier F, Groulade P. Probable case of ehrlichiosis in a cat. Bull Acad Vet France 1986;59:287–290.

5. Buoro IBJ, Atwell RB, Kiptoon J, et al. Feline anemia associated with *Ehrlichia*-like bodies in three domestic shorthaired cats. Vet Rec 1989;125:434–436.

6. Bouloy RP, Lappin MR, Holland CH, et al. Clinical ehrlichiosis in a cat. J Am Vet Med Assoc 1994;204:1475–1478.

7. Beaufils JP, Marin-Granel J, Jumelle P. *Ehrlichia* infection in cats: A review of three cases. Pratique Med Chir Anim Compagnie 1995;30:397–402.

8. Almosny NRP, Massard CL. Feline ehrlichiosis—Review. Clin Vet 1999;4:30–32.

9. Bjoersdorff A, Svendenius L, Owens JH, et al. Feline granulocytic ehrlichiosis—A report of a new clinical entity and characterisation of the new infectious agent. J Small Anim Pract 1999;40:20–24.

10. Mattheman LA, Kelly PJ, Wray K, et al. Antibodies in cat sera from southern Africa react with antigens of *Ehrlichia canis.* Vet Rec 1996;138:364–365.

11. Peavy GM, Holland CJ, Dulta SK, et al. Suspected ehrlichial infection in five cats from a household. J Am Vet Med Assoc 1997; 210:231–234.

12. Breitschwerdt EB, Hegarty BC, Hancock SI. Sequential evaluation of dogs naturally infected with *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia equi, Ehrlichia ewingii,* or *Bartonella vinsonii.* J Clin Microbiol 1998;36:2645–2651.

13. Kordick SK, Breitschwerdt EB, Hegarty BC, et al. Coinfection with multiple tick-borne pathogens in a Walker Hound kennel in North Carolina. J Clin Microbiol 1999;37:2631–2638.

14. Neer TM. Canine monocytic and granulocytic Ehrlichiosis. In: Greene CE, ed. Infectious Diseases of the Dog and Cat, 2nd ed. Philadelphia, PA: WB Saunders; 1998:139–147.

15. Stockham SL, Schmidt DA, Curtis KS. Evaluation of granulocytic ehrlichiosis in dogs of Missouri, including serologic status to *Ehrlichia canis, Ehrlichia equi* and *Borrelia burgdorferi.* Am J Vet Res 1992;53:63–68.

16. Suksawat J, Xuejie Y, Hancock SI, et al. Serologic and molecular evidence of coinfection with multiple vector-borne pathogens in dogs from Thailand. J Vet Intern Med 2001;15:453–462.

17. Pearce CH, Conrad ME, Nolan PE, et al. Ehrlichiosis: A cause of bone marrow hypoplasia in humans. Am J Hemtol 1988;28:53–55.

18. Abott KC, Vukelja SJ, Smith CE, et al. Hemophagocytic syndrome: A cause of pancytopenia in human ehrlichiosis. Am J Hematol 1991;38:230–234.

19. Pisetsky D, Drayton D, Wu ZQ. Specificity of antibodies to bacterial DNA in the sera of healthy human subjects and patients with systemic lupus erythematosus. J Rheumatol 1999;26:1934–1938.

20. Neujahr DC, Reich CF, Pisetsky DS. Immunostimulatory properties of genomic DNA from different bacterial species. Immunobiology 1999;200:106–119.

21. Wong SJ, Thomas JA. Cytoplasmic, nuclear and platelet autoantibodies in human granulocytic ehrlichiosis patients. J Clin Microbiol 1998;36:1959–1963.

22. Neer TM, Eddlestone SM, Gaunt SD, et al. Efficacy of enrofloxacin for the treatment of experimentally induced *Ehrlichia canis* infection. J Vet Intern Med 1999;13:501–504.

23. Sainz A, Tesouro MA, Amusategui I, et al. Prospective comparative study of 3 treatment protocols using doxycycline or imidocarb dipropionate in dogs with naturally occurring ehrlichiosis. J Vet Intern Med 2000;14:134–139.

24. Breitschwerdt EB, Hegarty BC, Hancock SI. Doxycycline hyclate treatment of experimental canine ehrlichiosis followed by challenge inoculation with two *Ehrlichia canis* strains. Antimicrob Agents Chemother 1998;42:362–368.

25. Harrus S, Waner T, Aizenberg I, et al. Amplification of ehrlichial DNA from dogs 34 months after infection with *Ehrlichia canis.* J Clin Microbiol 1998;36:73–76.