

*CYTAUXZOOM FELIS*: ASSESSING GENETIC VARIABILITY IN AN EMERGING FELINE  
PATHOGEN

by

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ABSTRACT

Although cytauxzoonosis has historically been nearly 100% fatal in domestic cats, increasing numbers of reports of infected cats demonstrating less severe disease suggest the development of different strains of *Cytauxzoon felis*. To test this hypothesis, the genetic variability of *C. felis* in both current and historical samples was assessed, determining if unique genetic sequences varied geographically, in different felid hosts, and in pathogenicity to domestic cats. In addition, the existence of asymptomatic *C. felis* infection in domestic cats was examined. The genetic variability of *C. felis* was evaluated from both surviving and fatally infected domestic cats from Arkansas and Georgia, using the first and second ribosomal internal transcribed spacer (ITS) regions as markers to assess genotypic variability. The identification of unique *C. felis* genotypes obtained from different geographic areas and the association of particular ITS genotypes with outcome of infection support the existence of distinct parasite strains. ITS sequencing of *C. felis* obtained from historical histologic specimens from infected cats diagnosed with cytauxzoonosis at necropsy identified genetically distinct *C. felis* populations and, together with data from contemporary samples, supports a diverse population structure for *C. felis*. Finally, real-time PCR was utilized to identify *C. felis* infection among a

population of domestic cats that were clinically healthy but at higher risk for parasite exposure. Genetically distinct parasite populations that were identical to those detected from clinically ill infected cats were identified within the asymptotically infected domestic cats and were also present in samples from bobcats, the known reservoir host. In total, this investigation demonstrates that *C. felis* sequence diversity exists among infected North American felids. However, sequence variations within the ITS regions do not appear to be useful as markers of *C. felis* pathogenicity in domestic felid hosts. The detection of asymptomatic *C. felis* infection in clinical healthy domestic cats warrants further investigation to determine if these naturally infected domestic cats can serve as an additional reservoir host for *C. felis*, altering the currently accepted paradigm of *C. felis* transmission to domestic cats solely through bobcats as the reservoir host.

INDEX WORDS: Cats, *Cytauxzoon felis*, Genetic variability, Internal transcribed spacer region

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## DEDICATION

To my husband, Justin, and our adorable son, Nate. Thank you both for bringing me so much joy as I completed this work.

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## CHAPTER 1

### INTRODUCTION

*Cytauxzoon felis* is a hemoprotozoal parasite that infects domestic and wild cats.

Infection in domestic cats is generally fatal, with rapid disease progression often resulting in death within one week of initial clinical illness.<sup>7,8,13</sup> The sporadic occurrence, short course of illness, and historic high fatality of cytauxzoonosis in domestic cats has suggested that cats likely serve as aberrant or dead-end hosts of the parasite.<sup>7,9</sup> The bobcat (*Lynx rufus*) has been identified as the natural reservoir host,<sup>2,4</sup> and infection in this species is generally asymptomatic and persistent.<sup>3,5</sup>

Although *C. felis* infection has historically been viewed as uniformly fatal in domestic cats, increasing numbers of reports documenting cats that have survived infection have been published over the past two decades.<sup>6,10,11,12</sup> Supportive care and antiprotozoal drugs have had inconsistent therapeutic results and limited success in treating cytauxzoonosis.<sup>1,6,7,11</sup> Furthermore, some cats surviving *C. felis* infections (both natural and experimental) received no therapy,<sup>10,11,12</sup> suggesting that treatment is an unlikely explanation for the survival of these infected cats. Another speculation is that these cats recovered because they were infected with a less virulent strain of *C. felis*. Survival of infected cats allows for the possibility of persistent infection and the potential for these infected domestic cats to then serve as a new reservoir of infection.

The principle objectives of the research presented herein were:



- 1) to determine if there are genetically distinct *Cytauxzoon felis* populations in infected domestic cats
- 2) to determine if particular *C. felis* genotypes vary temporally, geographically, and in pathogenicity to the domestic cat
- 3) to determine if there is a previously unidentified population of asymptomatic domestic cats that are infected with *C. felis*, and
- 4) to determine if *C. felis* strains present in asymptotically infected domestic cats are genetically unique as compared to those present both in domestic cats that are fatally infected and in the natural reservoir host, the bobcat.

The first two objectives were fulfilled by polymerase chain reaction (PCR) amplification and DNA sequencing of *C. felis*, utilizing the first and second ribosomal internal transcribed spacer (ITS) regions as genetic markers of distinct parasite populations. *C. felis* DNA samples were obtained from clinically ill and fatally infected domestic cats, including samples collected over the past 2 decades, from different geographic areas, and both from surviving and fatally infected cats.

Interestingly, repeated PCR testing of two cats initially presenting with clinical signs consistent with acute cytauxzoonosis, and a third asymptomatic cat that was a housemate of a cat fatally infected with *C. felis*, was positive for *C. felis* DNA at multiple time points, suggesting that persistent, asymptomatic infection may occur in domestic cats.

To further investigate this idea and address the third and fourth objectives of this research, real-time PCR analysis was utilized to screen for occult *C. felis* infection among asymptomatic domestic cats and free-living bobcats. To increase the likelihood of obtaining parasite samples for genetic comparisons, screening samples included those from domestic cats

that had clinically recovered from acute cytauxzoonosis, cats co-habiting with cats previously diagnosed with cytauxzoonosis, and feral cats and free-living bobcats in endemic areas. Again, the *C. felis* ITS sequences were utilized as markers to assess the genotype variability of the parasite between and within the two feline hosts.

The application of the information gained from this research is expected to be multifold. This work is the first investigation of genetic diversity in *C. felis*. While earlier publications have suggested the presence of varying strains of *C. felis*,<sup>8,10,12</sup> the population diversity of *C. felis* genotypes within infected cats has yet to be reported. The detection of variant *C. felis* genotypes described herein supports the existence of distinct parasite strains. In addition, the identification and dissemination of *C. felis* sequencing data, obtained from clinically ill and asymptotically infected domestic cats as well as bobcats, will lay the groundwork for future *C. felis* genetic studies and establish the foundation for further studies on molecular epidemiology and diagnostics of cytauxzoonosis.

This work also details established protocols for DNA extraction, amplification, and sequencing of *C. felis* from blood, fresh-frozen, and formalin-fixed paraffin embedded tissues which will be useful in future genetic and diagnostic studies. In addition, the increased sensitivity of the real-time PCR screening protocol enhances the ability of researchers and veterinarians to identify and diagnose natural occurrences of *C. felis* infection in both domestic and wild cats.

Finally, the data gained from this research also will broaden the current knowledge concerning the epidemiology of cytauxzoonosis. Establishing that there is a significant population of clinically healthy domestic cats that are persistently infected with *C. felis* has important application to treatment and prevention of disease spread. The ability of surviving *C. felis*-infected domestic cats to serve as an additional reservoir host for the parasite would greatly

increase the risk of exposure for other domestic cats and alter the currently accepted paradigm of *C. felis* transmission from bobcats (reservoir host) to domestic cats. Better understanding of the epidemiology of *C. felis* infection will enhance the ability to prevent this highly fatal infectious disease.

## REFERENCES

1. Birkenheuer AJ, Le JA, Valenzisi AM, Tucker MD, Levy MG, Breitschwerdt EB: 2006, *Cytauxzoon felis* infection in cats in the mid-Atlantic states: 34 cases (1998–2004). J Am Vet Med Assoc 228:568–571.
2. Blouin EF, Kocan AA, Glenn BL, Kocan KM, Hair JA: 1984, Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). J Wildl Dis 20:241–242.
3. Blouin EF, Kocan AA, Kocan KM: 1987, Evidence of a limited schizogonous cycle for *Cytauxzoon felis* in bobcats following exposure to infected ticks. J Wildl Dis 23:499-501.
4. Glenn BL, Kocan AA, Blouin EF: 1983, Cytauxzoonosis in bobcats. J Am Vet Med Assoc 183:1155–1158.
5. Glenn BL, Rolley RE, Kocan AA: 1982, Cytauxzoon-like piroplasms in erythrocytes of wild-trapped bobcats in Oklahoma. J Am Vet Med Assoc 181:1251-1253.
6. Greene CE, Latimer K, Hopper E, Shoeffler G, Lower K, Cullens F: 1999, Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. J Am Vet Med Assoc 215:497–500.
7. Greene CE, Meinkoth J, Kocan AA: 2006, Cytauxzoonosis. In: Infectious diseases of the dog and cat, ed. Greene CE, 3rd ed., pp. 722–733. Saunders Elsevier, St. Louis, MO.

8. Hoover JP, Walker DB, Hedges JD: 1994, Cytauxzoonosis in cats: eight cases (1985–1992). J Am Vet Med Assoc 203:455–460.
9. Kier AB, Wagner JE, Kinden DA: 1987, The pathology of experimental cytauxzoonosis. J Comp Pathol 97:415–432.
10. Meinkoth J, Kocan AA, Whitworth L, Murphy G, Fox JC, Woods JP: 2000, Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997–1998). J Vet Intern Med 14:521–525.
11. Motzel SL, Wagner JE: 1990, Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. Vet Parasitol 35:131–138.
12. Walker DB, Cowell RL: 1995, Survival of a domestic cat with naturally acquired cytauxzoonosis. J Am Vet Med Assoc 206:1363–1365.
13. Wightman SR, Kier AB, Wagner JE: 1977, Feline cytauxzoonosis: clinical features of a newly described blood parasite disease. Feline Pract 7:23–26.

## CHAPTER 2

### LITERATURE REVIEW

#### **Historical Perspectives**

The genus *Cytauxzoon* (Greek for increased number of cells in an animal) was first described in an infected duiker (*Sylvicaprae grimmia*) from South Africa in 1948.<sup>46</sup> *Cytauxzoon* species have since been identified in other African ungulates such as the kudu,<sup>45</sup> giraffe,<sup>38</sup> antelope,<sup>58</sup> and tsessebe.<sup>30</sup> *Cytauxzoon*-like organisms were first recognized in the United States in 1973 in four fatally infected domestic cats in Missouri.<sup>60</sup> Histopathology identified numerous schizonts within cells associated with the endothelial lining of major venous channels of all organs and tissues inspected, and piroplasms were detected within erythrocytes in those cases from which stained blood smears were available for review. These findings were characteristic of *Cytauxzoon* spp., and thus the species *Cytauxzoon felis* was created.

It is unknown whether the parasite had been a recent introduction to the United States or if the disease had simply been previously unrecognized, as it was identified from a rural area where veterinary care for domestic cats was uncommon. The latter seems unlikely given the highly fatal nature of the disease and the prominent histologic findings. The emergence of the disease may have represented new exposure of domestic cats to wildlife parasites because of encroachment into wildlife habitats, a change in vector potential or exposure, or a change in pathogenicity of a previously less-virulent parasite.

Since *Cytauxzoon* parasites had been found previously only in African wildlife, further investigations were conducted by the Animal and Plant Health Inspection Service and the Plum

Island Animal Disease Center (PIADC) of the USDA. Their research concluded that cytauxzoonosis was fairly widespread in the United States, and thus not a foreign animal disease, and infectivity studies determined that the parasite was not a major threat to domesticated food animals.<sup>15</sup> Subsequently, research on *C. felis* at PIADC was discontinued.

Since its initial discovery in Missouri, *C. felis* infection in domestic cats has been reported from many areas in the south-central, southeastern, and mid-Atlantic United States, including Texas, Kansas, Oklahoma, Arkansas, Louisiana, Kentucky, Alabama, Georgia, Tennessee, Mississippi, Florida, Missouri, North Carolina, South Carolina, and Virginia.<sup>4,5,20,22,23,25,27,28,64</sup> Organisms genetically similar to *C. felis* have been identified in cats in Spain,<sup>14</sup> France,<sup>13</sup> and South Africa,<sup>2</sup> and piroplasms morphologically indistinguishable from *Cytauxzoon* spp. have been identified in asymptomatic domestic cats in Brazil.<sup>42</sup>

### **Phylogeny and Classification**

Members of the genus *Cytauxzoon* belong to the phylum Apicomplexa, order Piroplasmida and family Theileriidae. Parasites in this family are tick-transmitted protozoans that exist in both an erythrocytic and leukocytic (or tissue) stage in the infected host. The intraerythrocytic piroplasms of *Cytauxzoon* spp. and *Theileria* spp. are indistinguishable. The leukocytic phase of *C. felis*, however, consists of large schizonts that develop within mononuclear phagocytic cells, in contrast to *Theileria* spp., in which schizogony occurs most commonly within lymphocytes. Thus, *Cytauxzoon* was classified separately from *Theileria*.

It has been suggested, however, that the genera *Cytauxzoon* and *Theileria* be synonymized.<sup>58</sup> Schizogony within macrophages has been documented for some *Theileria* spp. (for instance, *Theileria annulata*<sup>50</sup>), and there are strong similarities in resulting clinical signs and pathology in infected African ungulates. A blood parasite infecting eland antelope initially

described as *Cytauxzoon taurotragi* was later shown to be infective to both lymphoid and macrophage-like cell lines, as may be seen for *Theileria* sp.<sup>48</sup> Subsequently, phylogenetic analysis of the sequenced 18S rRNA for the parasites from other African ungulates showed that they cluster within the *Theileria* group; however *C. felis* and *Cytauxzoon* species from other wild Felidae form a separate cluster from *Theileria* spp.<sup>48</sup>

### **Life Cycle of *C. felis***

Infected ticks transmit *C. felis* sporozoites which enter host mononuclear phagocytes and undergo schizogony and binary fission to produce numerous developing merozoites. Ultrastructurally, the parasite appears first as an indistinguishable mass within the cytoplasm of the host cell. As the parasite develops, nuclear division occurs and individual nuclei become more discernable within the large multilobulated syncytium. As the parasite increases in size and complexity, a single nucleus, mitochondria, and related organelles are incorporated into each developing merozoite, and multiple fission generates numerous developing merozoites.<sup>37</sup> The infected and engorged mononuclear cells line the lumens of veins throughout the cat's body and eventually rupture, releasing the merozoites into the blood or tissue fluid. One to three days later, merozoites are observed in host erythrocytes.<sup>22</sup> Free merozoites infect host erythrocytes, most likely by a parasite mediated process, resulting in a parasitemia with intraerythrocytic piroplasms. The observation of tetrads ("Maltese cross" formation) and paired forms support asexual reproduction in the infected erythrocytes.<sup>32</sup> Ultrastructure of the intraerythrocytic stage of *C. felis* reveals round, elongated, and chained organisms with a poorly defined nucleus, rough endoplasmic reticulum, ribosomes, mitochondria, food vacuoles, and a cytostome on its limiting membrane.<sup>37,56</sup>

Ticks ingest the circulating parasitized erythrocytes and the parasites are released into the tick's gut. The parasites differentiate into macro- and micro-gamonts which undergo sexual reproduction and differentiation, ultimately penetrating the gut wall and migrating to the salivary gland. There, asexual reproduction by merogony results in salivary infection and the host is then inoculated by the tick during feeding.<sup>22</sup>

### **Transmission Studies**

Kier *et al.*<sup>34</sup> performed early experimental *C. felis* transmission trials aimed at determining which domestic and wild animal species were susceptible to infection with the newly discovered pathogen. Blood and/or tissue homogenates from domestic cats euthanatized *in extremis* with experimentally transmitted cytauxzoonosis were inoculated into six domestic farm animal species, 17 wildlife species and nine species of laboratory animals. A Florida bobcat (*Lynx rufus floridanus*) developed clinical signs consistent with feline cytauxzoonosis and died of the disease two weeks after inoculation, and an Eastern bobcat (*Lynx rufus rufus*) developed a persistent parasitemia with no overt signs of illness. The death of the bobcat from cytauxzoonosis demonstrated that the lethal form of *C. felis* infection was transmissible not only among domestic cats but also to another species, and that the bobcat was uniquely susceptible to both a fatal and non-fatal form of *C. felis* infection. Susceptibility was not demonstrated in the other animal species tested except for two sheep which developed a transient, low level parasitemia. However, domestic cats that were inoculated with blood from the parasitemic sheep did not develop clinical signs of disease or parasitemia and the cats remained susceptible to infection with virulent inoculum. A subsequent study<sup>59</sup> in which splenectomized sheep were inoculated with parasitemic blood and splenic tissue homogenate from a fatally infected cat



found no evidence for infectivity of *C. felis* to sheep, as no clinical reaction, detectable parasitemia, or *C. felis* antibodies were detected.

In contrast to the parasitemic sheep inoculum, blood and/or spleen homogenate from the experimentally infected Florida bobcat that died of feline cytauxzoonosis inoculated into domestic cats resulted in clinical cytauxzoonosis and death within two weeks after inoculation.<sup>33</sup> However, blood and/or spleen homogenate from the Eastern bobcat with the experimentally transmitted *C. felis* parasitemia inoculated into domestic cats resulted in the development of parasitemia, but no clinical signs and none died. The parasitemic domestic cats were then challenged with lethal inoculum of domestic cat origin and all cats died of cytauxzoonosis. Similarly, intravenous inoculation of peripheral blood from a parasitemic cat into an uninfected cat resulted in a detectable erythroparasitemia in the recipient, though the recipient cat remained asymptomatic.<sup>18</sup> These findings suggest the schizogonous tissue form of the organism, not the erythrocyte piroplasm, appears to be responsible for the fatal systemic disease, and cats receiving the piroplasm stage only remain susceptible to challenge with the tissue form of the organism.

Ticks are believed to be the likely natural vector for *C. felis* because most cases of cytauxzoonosis have been associated with the presence of ticks on the host.<sup>22</sup> Additionally, mature sporozoites have been identified in the salivary glands of feeding *Dermacentor variabilis* adults that were exposed to *C. felis* as nymphs.<sup>36</sup> Blouin<sup>8</sup> fed *D. variabilis* nymphs on splenectomized live-trapped bobcats with parasitemia. The ticks were allowed to molt to the adult stage and then fed on two splenectomized domestic cats. The cats died at 13 and 17 days after tick engorgement, and impression smears of lymph node, lungs, and bone marrow showed numerous large phagocytes containing *C. felis* schizonts, consistent with acute cytauxzoonosis. Subinoculation of parasitemic blood from bobcats to cats, however, appeared to transmit only the

erythrocytic piroplasms resulting in asymptomatic parasitemia. Necropsy performed six months after blood inoculation revealed no schizogonous parasite stages, suggesting that schizonts develop only after tick transmission of the organism.

Despite evidence that, at least experimentally, *D. variabilis* can serve as a transstadial vector of *C. felis*, the occurrence of cytauxzoonosis cases correlates better with the known activity and distribution of *Amblyomma americanum*. In addition, evaluation of Ixodid tick samples from dogs and cats in an endemic area (Missouri) for the presence of *C. felis* using PCR analysis identified *C. felis*-positive samples from *A. americanum* nymphs only and not the other tick species tested, including *D. variabilis*.<sup>10</sup> While an early study by Wightman *et al.*<sup>64</sup> was unable to transmit *C. felis* infection with adult *A. americanum* following feeding of nymphs on experimentally infected domestic cats and bobcats, more recently Reichard *et al.*<sup>52</sup> were more successful. *A. americanum* nymphs were fed on a naturally surviving parasitemic cat, molted to adults, and then were fed on another domestic cat. Clinical signs consistent with acute cytauxzoonosis developed in the recipient cat, and schizonts of *C. felis* were observed in splenic aspirates. Ultimately, there was resolution of clinical signs and the infected cat remained asymptotically parasitemic. *D. variabilis*, *Ixodes scapularis*, and *Rhipicephalus sanguineus* were similarly tested by Reichard *et al.* but did not transmit *C. felis*. These findings confirm that *A. americanum* has the ability to serve as a competent vector of the parasite, though the exact role of *A. americanum* in maintaining and transmitting *C. felis* to domestic cats in enzootic areas has yet to be determined.

### **Cytauxzoonosis in Domestic Cats**

Risk factors for natural *C. felis* infections in domestic cats include warm weather, access to a wooded environment, and exposure to ticks.<sup>5,11,27,51</sup> Geographic clusters of infection have

been observed.<sup>22,41</sup> Interestingly, one report of cytauxzoonosis cases in Oklahoma noted that five of seven infected cats lived in an urban environment and two did not live close to a wooded green belt.<sup>27</sup>

- *Clinicopathologic findings*

*C. felis* infection of domestic cats usually results in rapid and severe disease progression. The most common early clinical findings in cats with acute cytauxzoonosis include anorexia, lethargy, dehydration, depression, and fever (103-107°F), with icterus developing shortly thereafter and dyspnea commonly occurring as the disease progresses.<sup>5,15,22,40,61</sup> Recumbency, hypothermia, and vocalization as if in pain are seen in the terminal stages of disease, and infected cats appear to die from a shock-like state.<sup>22</sup> Most infected cats die within one week of initial clinical illness.<sup>22,27</sup> Rapid, intravascular multiplication of the tissue phase of the parasite coincides with the severe clinical illness seen in domestic cats,<sup>22</sup> suggesting that the schizogonous phase of the organism is responsible for the marked clinical signs of disease, although the exact pathogenesis is unknown.

After experimental infection, intraerythrocytic parasites were first observed on day 10 post-infection (PI), increasing in number thereafter up to 7% infected erythrocytes.<sup>32</sup> Parasitemia levels from natural infection rarely exceed 5%,<sup>64</sup> and 1% is more common. The detection of parasitemia on examination of stained blood smears is considered late-stage in the progression of cytauxzoonosis, usually detected 1-3 days before death. Infected mononuclear cells may also be detected on stained peripheral blood smears and can be as large as 75µm in diameter.

Anemia generally develops after the appearance of erythroparasites.<sup>16</sup> The anemia is commonly nonregenerative<sup>22,27</sup> and mild relative to the degree of icterus. Erythrophagocytosis has been observed in bone marrow aspirates, suggesting the decrease in erythrocytes results

primarily from erythrocyte destruction. Hoover *et al.*<sup>27</sup> proposed that the occurrence of erythrophagocytosis even when only rare parasites are present in blood suggests that much of the destruction of erythrocytes is immune mediated. The leukocyte count may be variable, but profound leukopenia, thrombocytopenia, or both are present, particularly late in the course of disease.<sup>22</sup> Cats with cytauxzoonosis may have prolongation of coagulation values; the activated partial thromboplastin time, in conjunction with thrombocytopenia, is usually most consistently elevated.<sup>21,22</sup> Disseminated intravascular coagulation has been a complication documented in laboratory findings in naturally infected cats.<sup>22</sup>

Hyperbilirubinemia and bilirubinuria are common findings in cats with acute cytauxzoonosis. Serum urea nitrogen and ammonia concentrations and hepatic enzyme activities may be elevated in febrile or comatose animals, but they may not be elevated earlier in the course of disease.<sup>22</sup>

#### - *Antemortem diagnosis*

The antemortem diagnosis of cytauxzoonosis is most commonly made by clinical suspicion and the detection in stained blood smears of intraerythrocytic piroplasms that are morphologically consistent with *C. felis*. Intraerythrocytic piroplasms are generally 1-2.2µm in diameter, and morphology is described most commonly as round ‘signet ring’-shaped bodies and bipolar oval ‘safety pin’ forms.<sup>22,35</sup> However, the development of the schizogonous tissue phase, which is responsible for the clinical signs of disease, precedes the appearance of piroplasms in the peripheral blood by a few days.<sup>22</sup> Therefore, some cats examined early in the course of disease may not be parasitemic on initial evaluation. Antemortem diagnosis may be made by detection of the schizogonous tissue phase in stained aspirates or impressions smears of affected organs, most commonly spleen, lung, liver, lymph nodes or bone marrow.<sup>16</sup> Occasional detection

of mononuclear phagocytes containing tissue-phase schizonts at the feathered edge of a peripheral blood smear has been reported.<sup>22,32</sup>

A sensitive and specific whole blood PCR assay has been developed, targeting a 284 bp fragment of the *C. felis* 18S rRNA gene, which is currently available commercially to aid in the rapid, accurate antemortem diagnosis of infection.<sup>6</sup>

No serological testing for *C. felis* is currently available commercially. Shindel *et al.*<sup>55</sup> used serum from a recovered cat as antibody to demonstrate *C. felis* with an indirect fluorescent antibody test on frozen spleen sections of experimentally infected cats. However, preliminary results indicated that infected cats did not develop detectable antibodies before death or only low titres at a late stage. Later, Uilenberg *et al.*<sup>59</sup> developed an indirect fluorescent antibody test using piroplasm antigen of *C. felis*. The application of the assay, however, was in the detection of antibodies to the parasite in recovered cats, only, and thus has no application to the antemortem diagnosis of infection.

#### - *Postmortem findings*

Splenomegaly with rounded edges and a dark, mottled appearance was the earliest gross lesion observed at necropsy after experimental infection with *C. felis*.<sup>32</sup> Other gross findings include icterus; enlarged, edematous and hemorrhagic lymph nodes; enlarged liver with rounded edges and a mottled appearance; intraabdominal venous distention, most marked in mesenteric and renal veins and in posterior vena cava; and congested and edematous lungs, often with petechial and ecchymotic hemorrhages on the serosal surfaces and throughout the interstitium.<sup>22,32,36,40</sup> In some animals, the pericardial sac is distended with gelatinous and icteric fluid accompanied by petechial and ecchymotic hemorrhages on the epicardium.<sup>36</sup>

Histopathology from formalin-fixed tissues (most commonly lung, liver, lymph node, and spleen) confirms the tissue stage of cytauxzoonosis.<sup>22</sup> *C. felis* schizonts are present within the cytoplasm of large mononuclear phagocytes (15-250µm in diameter) commonly attached to or associated with the endothelium or within the lumens of venous channels of the majority of tissues examined and, for some organs, schizonts may also be observed within the interstitium. After experimental infection, *C. felis* organisms were first observed histologically on day 12 PI as schizonts within mononuclear phagocytes in germinal centers of lymph nodes and spleen and within bone marrow. By day 17 PI, infected mononuclear cells appeared to partially or completely occlude the lumens of venous channels of all major organs.<sup>32</sup>

Stained impression smears made from the affected organs may reveal abundant parasitized mononuclear phagocytes of various sizes with schizonts in different stages of development.<sup>16</sup> Similarly, stained blood smears obtained via cardiac venipuncture may also reveal numerous schizont-laden mononuclear cells.<sup>63</sup>

*In situ* hybridization highlighting the presence of *C. felis* in formalin-fixed tissues from necropsy cases of cytauxzoonosis has identified 2-10 times more infected cells than with routine hematoxylin and eosin stain.<sup>57</sup> As seen in routine histologic sections, parasite-laden cells were identified predominantly within vessels, frequently forming thrombi.

### ***Cytauxzoon felis* Infection in Bobcats and Other Wild Felids**

The Eastern bobcat (*Lynx rufus rufus*) and Florida bobcat (*L. rufus floridanus*) have been identified as the primary reservoirs of *C. felis*.<sup>18,33</sup> Field investigations in Oklahoma have documented infection with a piroplasm indistinguishable from *C. felis* in up to 60% of clinically healthy, wild-trapped bobcats.<sup>19</sup> The degree of parasitemia varied from less than 0.5% to 5% infected erythrocytes but commonly ranged from 1%-3%. Mild anemia was apparent in one

infected bobcat but other ill effects possibly attributable to the parasite were not observed. *C. felis* infection appears to be relatively nonpathogenic in bobcats, with infection most often being subclinical. Fatal disease has been reported sporadically in some bobcats, including natural and experimental infections.<sup>9,34,47</sup>

Examination of liver, spleen, lungs and lymph nodes from 10 bobcats with naturally occurring piroplasm infections did not reveal schizogonous stages.<sup>9</sup> Further investigation determined the schizogonous tissue stage to be short-lived in bobcats, with tissue stages observed in lymph node at 11 days after experimental infection but not at 30 and 60 days following infected tick feeding. Although the schizogonous stage appeared to be short, bobcats are long-term carriers of piroplasms.

Asymptomatic *C. felis* infection also has been detected in other wild felids in the United States including cougars (*Puma concolor couguar*) and Florida panthers (*Puma concolor coryi*).<sup>12,24,54,66</sup> Experimental inoculation of mononuclear cells separated from whole blood of a wild, adult Florida panther into an adult domestic cat resulted in fatal cytauxzoonosis, with clinical signs, gross lesions, and histologic and ultrastructural features consistent with acute *C. felis* infection.<sup>12</sup> Fatal cytauxzoonosis has also been reported in a captive-reared white tiger (*Panthera tigris*) in an endemic area in northern Florida.<sup>17</sup>

Infection with *Cytauxzoon* spp. has been identified in wild felids in other parts of the world as well. Natural *Cytauxzoon* sp. infection has been reported in asymptomatic ocelots, pumas, and jaguars in Brazil, sharing 98% sequence similarity within the 18S rRNA gene to *C. felis* from North American cats.<sup>3</sup> *Cytauxzoon* sp. infection has also been detected in Iberian lynx in Spain<sup>39,43</sup> and captive-reared lions (*Panthera leo*) in Brazil.<sup>49</sup> In Asia, intraerythrocytic piroplasms genetically similar to *C. felis* but with the proposed name *Cytauxzoon manul* have

been detected from asymptomatic Pallas' cats (*Otocolobus manul*) in Mongolia.<sup>31,53</sup> Fatal infection in a Bengal tiger (*Panthera tigris*) that was born and kept in captivity in a German zoo was reported with clinical, gross, histologic and ultrastructural findings consistent with acute cytauxzoonosis.<sup>29</sup> Interestingly, the previous year three young bobcats directly imported from the United States had been introduced into the zoo.

### **Cats Surviving Infection**

Historically, all naturally occurring cases of cytauxzoonosis in domestic cats were reported to be fatal. In 1995, Walker *et al.*<sup>62</sup> described a naturally infected cat that survived *C. felis* infection, having received supportive care with intravenous fluids and enrofloxacin antibiotics but no specific antiprotozoal therapy. An earlier study<sup>44</sup> evaluating the efficacy of the antiprotozoal drugs parvaquone and buparvaquone (both effective in treating the closely related theileriosis of cattle) found them ineffective in treating experimentally induced *C. felis* infection in cats. Unexpectedly, one of the two non-treated, infected control cats in that study survived. Greene *et al.*<sup>21</sup> documented much greater success in treating naturally infected cats; six out of seven cats receiving diminazene aceturate or imidocarb dipropionate antiprotozoal medication and supportive care survived acute cytauxzoonosis, suggesting that treatment was beneficial. However, subsequent efforts to treat cytauxzoonosis with antiprotozoal therapy have been less rewarding, with the majority of cats still succumbing to acute infection.<sup>5</sup>

In 2000, Meinkoth *et al.*<sup>41</sup> documented 18 domestic cats from northwestern Arkansas and northeastern Oklahoma that survived natural infection, most without receiving any specific antiprotozoal treatment. Repeated examination of stained blood smears from 12 of these infected cats revealed that the erythroparasitemia was generally persistent. Survival did not seem dependent on treatment, as only one cat was treated with an antiprotozoal drug, and four cats



received no treatment. The authors suggested that the existence of a less virulent strain of *C. felis* seemed to be a likely explanation for at least some of the survivors, particularly because many of the surviving cats were from a single geographically limited area.

Subsequently, Haber *et al.*<sup>23</sup> determined the prevalence of *C. felis* infection in feral cats that were presented to trap-neuter-return programs in Florida, North Carolina, and Tennessee. Using a *C. felis*-specific PCR assay, they identified a 0.3% prevalence of *C. felis* infection in this population. Clinical signs of cytauxzoonosis were not noted at the time of their capture, although there was limited follow-up information and laboratory testing on these cats to document persistent, subclinical *C. felis* infection. These findings suggest that, despite a low prevalence, apparently healthy *C. felis*-infected cats exist and may have the capacity to serve as a new reservoir for parasitic infection.

A recent study by Reichard *et al.*<sup>52</sup> demonstrated transmission of clinical cytauxzoonosis from a domestic cat that was a natural survivor of *C. felis* infection to a naïve domestic cat using *Amblyomma americanum* as the tick vector. The ability to transmit *C. felis* infection from domestic cats suggests that subclinically infected domestic cats might be a reservoir for further domestic cat infections.

### **Selecting a Genetic Marker**

Sequence analysis of PCR-amplified DNA is a highly sensitive and reproducible method of identifying and typing microorganisms and the most sensitive approach to detect genetic polymorphisms.<sup>65</sup> However, identification of the most suitable loci to sequence in order to detect sufficient variation to allow strain typing can be challenging. The ribosomal DNA (rDNA) of microorganisms provides an attractive target for PCR typing methods. The rRNA genes are functionally constrained and therefore tend to be highly conserved between species and genera.

The small subunit rRNA (SSU rRNA) gene is widely used as a taxonomic marker for similar organisms; but because it is highly conserved, sequencing of the SSU rDNA may not always reliably discriminate between closely related species.

The rRNA internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), which are separated by the 5.8S gene and flanked by the SSU and large subunit rRNA genes in most eukaryotes, are not subject to the same functional constraints as the rRNA genes. As a result, the spacer regions are subject to higher evolutionary rates leading to greater variability in both nucleotide sequence and length.<sup>26</sup> The ITS regions are therefore more likely to show variability among putative strains of *C. felis*. Variability of the ITS regions has been recognized in other closely related apicomplexan species, such as *Theileria* spp.<sup>1</sup> and *Babesia canis*.<sup>67</sup>

### **Current Genetic Information**

Currently, very little is known about the *C. felis* genome. Only sequences for the ribosomal genes have been characterized and submitted to GenBank, and the majority of the *C. felis* sequence comparisons have included only sequences for the structural rRNA genes. One previous study<sup>41</sup> compared sequenced portions of the *C. felis* SSU rRNA gene from a single cat that survived infection with a previously reported gene from *C. felis* and found that the sequences were virtually identical (>99% identity). A second study<sup>10</sup> comparing *C. felis* SSU RNA sequence data obtained from three infected ticks and one naturally infected cat reported >99.8% sequence similarity between each sequence and the previously published sequence. In addition, Birkenheuer *et al.*<sup>5</sup> compared nearly full-length *C. felis* 18S rRNA gene sequences from four cats from the mid-Atlantic states that had died or were euthanized as a result of *C. felis* infection. They shared  $\geq 99.9\%$  sequence identity with the two North American *C. felis* 18S ribosomal DNA sequences known at the time. Only one study<sup>10</sup> has compared *C. felis* ITS1 sequence data.

Two polymorphic bases were found within one sample when comparing ITS1 sequence data from two infected ticks and one naturally infected cat; otherwise, the sequences within the ITS1 region were identical.

## Summary

*C. felis* field studies and experimental transmission studies have provided important information about the epidemiology and pathology of fatal cytauxzoonosis in domestic cats. Description of cats surviving infection, however, suggests there may be varying strains of *C. felis* that differ in pathogenicity to the domestic cat. Prior to this dissertation, there had been no investigation detailing genetic variability in the *C. felis* parasite population to determine whether strain variability played a role in disease transmission and virulence in cats. In addition, the detection of persistent *C. felis* infection in some surviving cats suggests there may be a population of previously unidentified, asymptomatically infected cats that may serve as an additional reservoir for the parasite. Comparisons of the *C. felis* genotypes infecting different feline species (domestic cats and bobcats), in varying geographic locations, and both in clinically ill and asymptomatically infected cats will contribute to a better understanding of the current epidemiology of *C. felis* infection in domestic cats.

## REFERENCES

1. Aktas M, Bendele KG, Altay K, Dumanli N, Tsuji M, Holman, P: 2007, Sequence polymorphism in the ribosomal DNA internal transcribed spacers differs among *Theileria* species. *Vet Parasitol* 147:221-230.
2. Allsopp MTEP, Cavalier-Smith T, DeWall DT, Allsopp BA: 1994, Phylogeny and evolution of the piroplasms. *Parasitology* 108:147-152.

3. Andre MR, Adania CH, Machado Z, Allegretti AM, Felipe PAN, Silva KF, Nakaghi ACH, Dagnone AS: 2009, Molecular detection of *Cytauxzoon* spp. in asymptomatic Brazilian wild captive felids. *J Wildl Dis* 45:234-237.
4. Bendele RA, Schwartz WL, Jones LP: 1976, Cytauxzoonosis-like disease in Texas cats. *Southwest Vet* 29:244-246.
5. Birkenheuer AJ, Le JA, Valenzisi AM, Tucker MD, Levy MG, Breitschwerdt EB: 2006, *Cytauxzoon felis* infection in cats in the mid-Atlantic states: 34 cases (1998–2004). *J Am Vet Med Assoc* 228:568–571.
6. Birkenheuer AJ, Marr H, Alleman AR, Levy MG, Breitschwerdt EB: 2006, Development and evaluation of a PCR assay for the detection of *Cytauxzoon felis* DNA in feline blood samples. *Vet Parasitol* 137:144-149.
7. Birkenheuer AJ, Marr HS, Warren C, Acton AE, Mucker EM, Humphreys JG, Tucker MD: 2008, *Cytauxzoon felis* infections are present in bobcats (*Lynx rufus*) in a region where cytauxzoonosis is not recognized in domestic cats. *Vet Parasitol* 153:126-130.
8. Blouin EF, Kocan AA, Glenn BL, Kocan KM, Hair JA: 1984, Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). *J Wildl Dis* 20:241–242.
9. Blouin EF, Kocan AA, Kocan KM: 1987, Evidence of a limited schizogonous cycle for *Cytauxzoon felis* in bobcats following exposure to infected ticks. *J Wildl Dis* 23:499-501.
10. Bondy Jr. PJ, Cohn LA, Tyler JW, Marsh AE: 2005, Polymerase chain reaction detection of *Cytauxzoon felis* from field-collected ticks and sequence analysis of the small subunit and internal transcribed spacer region of the ribosomal RNA gene. *J Parasitol* 91:458-461.

11. Butt MT, Bowman D, Barr MC, Roelke ME: 1990, Diagnosing erythrocyte parasitic diseases in cats. *Comp Cont Educ Pract Vet* 12:628–638.
12. Butt MT, Bowman D, Barr MC, Roelke ME: 1991, Iatrogenic transmission of *Cytauxzoon felis* from a Florida panther (*Felis concolor coryi*) to a domestic cat. *J Wildl Dis* 27:342-347.
13. Criado-Fornelio A, Buling A, Pingret JL, Etievant M, Boucraut-Baralon C, Alongi A, Agnone A, Torina A: 2009, Hemoprotozoa of domestic animals in France: prevalence and molecular characterization. *Vet Parasitol* 159:73-76.
14. Criado-Fornelio A, Gonzalez-del-Rio MA, Buling-Sarana A, Barba-Carretero JC: 2004, The "expanding universe" of piroplasms. *Vet Parasitol* 119:337–345.
15. Ferris DH: 1979, A progress report on the status of a new disease of American cats: Cytauxzoonosis. *Comp Immun Microbiol Infect Dis* 1:269-276.
16. Franks PT, Harvey JW, Shields RP, Lawman MJP: 1988, Hematological findings in experimental feline cytauxzoonosis. *J Am Anim Hosp Assoc* 24:395-401.
17. Garner MM, Lung NP, Citino S, Greiner EC, Harvey JW, Homer BL: 1996, Fatal cytauxzoonosis in a captive-reared white tiger (*Panthera tigris*). *Vet Pathol* 33:82-86.
18. Glenn BL, Kocan AA, Blouin EF: 1983, Cytauxzoonosis in bobcats. *J Am Vet Med Assoc* 183:1155–1158.
19. Glenn BL, Rolley RE, Kocan AA: 1982, Cytauxzoon-like piroplasms in erythrocytes of wild-trapped bobcats in Oklahoma. *J Am Vet Med Assoc* 181:1251-1253.
20. Glenn BL, Stair EL: 1984, Cytauxzoonosis in domestic cats: Report of two cases in Oklahoma, with a review and discussion of the disease. *J Am Vet Med Assoc* 184:822-825.

21. Greene CE, Latimer K, Hopper E, Shoeffler G, Lower K, Cullens F: 1999, Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. *J Am Vet Med Assoc* 215:497–500.
22. Greene CE, Meinkoth J, Kocan AA: 2006, Cytauxzoonosis. *In: Infectious diseases of the dog and cat*, ed. Greene CE, 3rd ed., pp. 722–733. Saunders Elsevier, St. Louis, MO.
23. Haber, MD, Tucker, MD, Marr, HS, Levy, JK, Burgess, J, Lappin, MR, Birkenheuer, AJ: 2007, The detection of *Cytauxzoon felis* in apparently healthy free-roaming cats in the USA. *Vet Parasitol* 146:316-320.
24. Harvey JW, Dunbar MR, Norton TM, Yabsley MJ: 2007, Laboratory findings in acute *Cytauxzoon felis* infection in cougars (*Puma concolor cougar*) in Florida. *J Zoo Wildl Med* 38: 285-291.
25. Hauck WN, Snider TG 3rd, Lawrence JE: 1982, Cytauxzoonosis in a native Louisiana cat. *J Am Vet Med Assoc* 180:1472-1474.
26. Hillis DM, Dixon MT: 1991, Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 66:411–453.
27. Hoover JP, Walker DB, Hedges JD: 1994, Cytauxzoonosis in cats: eight cases (1985–1992). *J Am Vet Med Assoc* 203:455–460.
28. Jackson CB, Fisher T: 2006, Fatal cytauxzoonosis in a Kentucky cat (*Felis domesticus*). *Vet Parasitol* 139:192-195.
29. Jakob W, Wesemeier H-H: 1996, A fatal infection in a Bengal tiger resembling cytauxzoonosis in domestic cats. *J Comp Path* 114:439-444.
30. Jardine JE: 1992, The pathology of cytauxzoonosis in a tsessebe (*Damaliscus lunatus*). *J S Afr Vet Assoc* 63:49-51.

31. Ketz-Riley CJ, Jazson MV, Van den Bussche RA, Hoover JP, Meinkoth J, Kocan AA: 2003, An intraerythrocytic small piroplasms in wild caught Pallas cat (*Otocolobus manul*) from Mongolia. *J Wildl Dis* 39:424-430.
32. Kier AB, Wagner JE, Kinden DA: 1987, The pathology of experimental cytauxzoonosis. *J Comp Pathol* 97:415-432.
33. Kier AB, Wagner JE, Morehouse LG: 1982, Experimental transmission of *Cytauxzoon felis* from bobcats (*Lynx rufus*) to domestic cats (*Felis domesticus*). *Am J Vet Res* 43:97-101.
34. Kier AB, Wightman SR, Wagner JE: 1982, Interspecies transmission of *Cytauxzoon felis*. *Am J Vet Res* 43:102-105.
35. Kocan AA, Blouin EF: 1985, Hematologic and serum chemical values for free-ranging bobcats, *Felis rufus* (Schreber), with reference to animals with natural infections of *Cytauxzoon felis* Kier, 1979. *J Wildl Dis* 21:190-192.
36. Kocan A, Kocan KM: 1991, Tick-transmitted protozoan diseases of wildlife in North America. *Bull Soc Vector Ecol* 16:94-108.
37. Kocan AA, Kocan KM, Blouin EF, Mukolwe SW: 1992, A redescription of schizogony of *Cytauxzoon felis* in the domestic cat. *Ann N Y Acad Sci* 653:161-167.
38. Krecek RC, Boomker J, Panzorn BL, Scheepers L: 1990, Internal parasites of giraffes (*Giraffa camelopardalis angolensis*) from Etosha National Park, Namibia. *J Wildl Dis* 26: 395-397.
39. Luaces I, Aguirre E, Garcia-Montijano M, Velarde J, Tesouro MA, Sanchez C, Galka M, Fernandez P, Sainz A: 2005, First report of an intraerythrocytic small piroplasm in wild Iberian lynx (*Lynx pardinus*). *J Wildl Dis* 41:810-815.

40. Meier HT, Moore LE: 2000, Feline cytauxzoonosis: A case report and literature review. *J Am Anim Hosp Assoc* 36:493-496.
41. Meinkoth J, Kocan AA, Whitworth L, Murphy G, Fox JC, Woods JP: 2000, Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997–1998). *J Vet Intern Med* 14:521–525.
42. Mendes-de-Almeida F, Labarthe N, Guerrero J, Faria MC, Branco AS, Pereira CD, Barreira JD, Pereira MJ: 2007, Follow-up of the health conditions of an urban colony of free-roaming cats (*Felis catus* Linnaeus, 1758) in the city of Rio de Janeiro, Brazil. *Vet Parasitol* 147:9-15.
43. Millán J, Naranjo V, Rodriguez A, Perez de la Lastra JM, Mangold AJ, de la Fuente: 2007, Prevalence of infection and 18S rRNA gene sequences of *Cytauxzoon* species in Iberian lynx (*Lynx pardinus*) in Spain. *Parasitology* 134:995-1001.
44. Motzel SL, Wagner JE: 1990, Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. *Vet Parasitol* 35:131–138.
45. Neitz, WO: 1957, Theileriosis, gonderioses and cytauxzoonoses. *Onderstepoort J Vet Res* 27:275-430.
46. Neitz WO, Thomas AD: 1948, *Cytauxzoon sylvicaprae* gen. nov., spec. nov., a protozoon responsible for a hitherto undescribed disease in the duiker, *Sylvicapra grimmia* (Linné). *Onderstepoort J Vet Sci Anim Ind* 23:63-76.
47. Nietfield JC, Polluck C: 2002, Fatal cytauxzoonosis in a free-ranging bobcat (*Lynx rufus*). *J Wildl Dis* 38:607-610.



48. Nijhof AM, Pillay V, Steyl J, Prozesky L, Stoltz WH, Lawrence JA, Penzhorn BL, Jongejan F: 2005, Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. *J Clin Microbiol* 43:5907-5911.
49. Peixoto PV, Soares CO, Scofield A, Santiago CD, França TN, Barros SS: 2007, Fatal cytauxzoonosis in captive-reared lions in Brazil. *Vet Parasitol* 145:383-387.
50. Preston PM, Hall FR, Glass EJ, Campbell JD, Darghouth MA, Ahmed JS, Shiels BR, Spooner RL, Jongejan F, Brown CG: 1999, Innate and adaptive immune responses co-operate to protect cattle against *Theileria annulata*. *Parasitol Today* 15:268-274.
51. Reichard MV, Baum KA, Cadenhead SC, Snider TA: 2008, Temporal occurrence and environmental risk factors associated with cytauxzoonosis in domestic cats. *Vet Parasitol* 152:314-320.
52. Reichard MV, Meinkoth JH, Edwards AC, Snider TA, Kocan KM, Blouin EF, Little SE: 2009, Transmission of *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum*. *Vet Parasitol* 161:110–115.
53. Reichard MV, Van Den Bussche RA, Meinkoth JH, Hoover JP, Kocan AA: 2005, A new species of *Cytauxzoon* from Pallas' cats caught in Mongolia and comments on the systematic and taxonomy of piroplasmids. *J Parasitol* 91:420-426.
54. Rotstein DS, Taylor SK, Harvey JW, Bean J: 1999, Hematologic effects of cytauxzoonosis in Florida panthers and Texas cougars in Florida. *J Wildl Dis* 35:613–607.
55. Shindel N, Dardiri AH, Ferris DH: 1978, An indirect fluorescent antibody test for the detection of *Cytauxzoon*-like organisms in experimentally infected cats. *Can J Comp Med* 42:460–465.

56. Simpson CF, Harvey JW, Carlisle JW: 1985, Ultrastructure of the intraerythrocytic stage of *Cytauxzoon felis*. Am J Vet Res 46:1178-1180.
57. Susta L, Torres-Velez F, Zhang J, Brown C: 2009, An *in situ* hybridization and immunohistochemical study of cytauxzoonosis in domestic cats. Vet Pathol 46:1197-1204.
58. Thomas SE, Wilson DE, Mason TE: 1982, *Babesia*, *Theileria* and *Anaplasma* spp. infecting sable antelope, *Hippotragus niger* (Harris, 1838), in southern Africa. Onderstepoort J Vet Res 49:163-166.
59. Uilenberg G, Franssen FFJ, Perie NM: 1987, Relationships between *Cytauxzoon felis* and African piroplasmids. Vet Parasitol 26:21-28.
60. Wagner JE: 1976, A fatal cytauxzoonosis-like disease in cats. J Am Vet Med Assoc 168:585-588.
61. Wagner JE, Ferris DH, Kier AB, Wightman SR, Maring E, Morehouse LG, Hansen RD: 1980, Experimentally induced cytauxzoonosis-like disease in domestic cats. Vet Parasitol 6:305-311.
62. Walker DB, Cowell RL: 1995, Survival of a domestic cat with naturally acquired cytauxzoonosis. J Am Vet Med Assoc 206:1363-1365.
63. Weisman JL, Woldemeskel M, Smith KD, Merrill A, Miller D: 2007, Blood smear from a pregnant cat that died shortly after partial abortion. Vet Clin Pathol 36:209-211.
64. Wightman SR, Kier AB, Wagner JE: 1977, Feline cytauxzoonosis: clinical features of a newly described blood parasite disease. Feline Pract 7:23-26.
65. Williams D, Wilson M, Lewis M: 1999, Deoxyribonucleic acid typing methods for medically important microorganisms. Br J Biomed Sci 56:56-65.

66. Yabsley MJ, Murphy SM, Cunningham MW: 2006, Molecular detection and characterization of *Cytauxzoon felis* and a *Babesia* species in cougars from Florida. J Wildl Dis 42:366-374.
67. Zahler M, Schein E, Rinder H, Gothe R: 1998, Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitol Res 84:544–548.

CHAPTER 3

GENETIC VARIABILITY OF *CYTAUXZOOM FELIS* FROM 88 INFECTED DOMESTIC  
CATS IN ARKANSAS AND GEORGIA<sup>1</sup>

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<sup>1</sup> Brown, H.M., Berghaus, R.D., Latimer, K.S., Britt, J.O., Rakich, P.M., and Peterson, D.S.  
2009. *Journal of Veterinary Diagnostic Investigation*. 21:59-63.  
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**Abstract.** Although cytauxzoonosis has historically been nearly 100% fatal in domestic cats, increasing reports of infected cats demonstrating less severe disease suggest the existence of different strains of *Cytauxzoon felis*. To test this hypothesis, the genetic variability of *C. felis* was examined in blood samples from naturally infected domestic cats from Arkansas and Georgia, using the first and second ribosomal internal transcribed spacer regions (ITS1, ITS2) as markers to assess genotypic variability. In addition, clinical outcome of infection (survival vs. fatal disease) was analyzed. Within the *C. felis* ITS1 region, there were a total of 8 single nucleotide polymorphisms (SNPs) and a single nucleotide insertion. Within the ITS2 region, there were a total of 4 SNPs and a single 40-bp insertion. Taken together, the ITS1 and ITS2 sequence data defined a total of 11 different sequences and 3 unique genotypes. One unique ITS1-ITS2 genotype was detected in samples submitted exclusively from Arkansas, and a second unique genotype was submitted exclusively from Georgia. There was a significant association between infection with *C. felis* containing particular ITS genotypes and survival of the infected domestic cat. The identification of unique *C. felis* genotypes obtained from different geographic areas and the association of particular ITS genotypes with outcome of infection suggest the existence of parasite strains that may vary in pathogenicity to the domestic cat and offer an explanation for the survival of some infected cats in more recent case studies.

**Key words:** Cats; *Cytauxzoon felis*; genetic variability; internal transcribed spacer region.

## Introduction

Cytauxzoonosis is an emerging, highly fatal, tick-borne, hemoprotozoal disease of domestic and exotic cats. The causative organism, *Cytauxzoon felis* (family *Theileriidae*, genus *Cytauxzoon*), is a relatively new pathogen in the United States that was first detected in Missouri in 1973<sup>15</sup> and subsequently throughout the southeastern, south-central, and mid-Atlantic regions of the United States. The bobcat (*Lynx rufus*) has been identified as the natural reservoir host of this parasite.<sup>3,5</sup> In affected domestic cats, the course of disease is usually rapid, and most cats die within 1 week of initial clinical illness.<sup>7,9,17</sup> The sporadic occurrence, short course of illness, and high fatality rate of cytauxzoonosis in domestic cats has suggested that they likely serve as aberrant or dead-end hosts.<sup>7,11</sup>

Although *C. felis* infection historically has been viewed as uniformly fatal in domestic cats, more recent studies document cats that have survived infection.<sup>4,6,12,13,16</sup> However, supportive care and antiprotozoal drugs have had inconsistent therapeutic results and limited success in treating cytauxzoonosis.<sup>2,6,7,13</sup> Furthermore, some cats surviving *C. felis* infections (both natural and experimental) received no therapy,<sup>12,13,16</sup> suggesting that treatment protocol is an unlikely explanation for the survival of these infected cats. Another explanation is that these cats recovered because they were infected with a less virulent strain of *C. felis*. It is notable that the majority of these cats surviving cytauxzoonosis have been documented in a relatively limited geographic area (the Midwest, including northwest Arkansas), which might be expected with the emergence of different genetic strains of the parasite.

An important step in assessing potential strain variation within the *C. felis* population is the use of a suitable gene as a genetic marker of variability. Currently, very little is known about the *C. felis* genome. Only sequences for the ribosomal genes have been characterized and

submitted to GenBank. One previous study<sup>12</sup> compared sequenced portions of the *C. felis* ribosomal RNA (rRNA) gene from a single cat that survived infection with sequences reported in GenBank. All sequences were shown to be virtually identical. The selected genomic target in that study was the small subunit rRNA gene, which is a highly conserved region encoding a functional RNA molecule. In contrast, the first and second internal transcribed spacer regions of the rRNA operon (ITS1, ITS2) are noncoding regions and, thus, do not have the structure-function constraints of the rRNA genes. As such, the ITS regions are likely to evolve more quickly<sup>8</sup> and are more likely to show variability among putative strains of *C. felis*. Variability of the ITS regions has been recognized in other apicomplexan species, such as *Babesia canis*, *Cyclospora cayentanensis*, and *Eimeria* spp.<sup>1,14,18</sup> Three closely related species of *Babesia canis* that vary in pathogenicity for the dog share identical small subunit rRNA sequences but are individually distinguishable by variability in their ITS sequences.<sup>18</sup>

Genetic analysis of the *C. felis* ITS regions from cats with cytauxzoonosis may allow identification of genetically distinct parasite populations and, if present, may offer a possible explanation for the survival of some infected cats in more recent case reports. The aims of the current study were to assess whether the ITS1 and ITS2 regions of rDNA could be used as genetic markers of distinct *C. felis* populations, including those from different geographic areas and those that vary in pathogenicity in the domestic cat, and to establish the foundation for further studies on molecular epidemiology and diagnostics of cytauxzoonosis.

## **Materials and methods**

### **Parasite samples**

*Cytauxzoon felis*-infected blood samples from acutely ill cats diagnosed with cytauxzoonosis from 2005 to 2007 were submitted from collaborating veterinary hospitals and

diagnostic laboratories in Arkansas and Georgia. For each case, the pathologist's diagnosis of cytauxzoonosis was based on the presence of erythrocytic piroplasms that were morphologically consistent with *C. felis* as identified on stained peripheral blood smear review. Samples from Arkansas were submitted by the Veterinary Diagnostic Laboratory at the Arkansas Livestock and Poultry Commission (Little Rock, AR). Samples from Georgia were obtained from the University of Georgia (UGA) Veterinary Teaching Hospital (Athens, GA), the UGA Veterinary Diagnostic Laboratory (Athens, GA), and the UGA Veterinary Diagnostic and Investigational Laboratory (Tifton, GA). Follow-up telephone communication with submitting veterinarians confirmed the presence of clinical disease consistent with acute cytauxzoonosis at the time of the blood sample submission as well as identified the clinical outcome of acute infection for each cat.

#### **DNA extraction and PCR amplification**

DNA was extracted from ethylenediamine tetra-acetic acid (EDTA)-anticoagulated whole blood samples using a commercially available kit<sup>a</sup> according to manufacturer's instructions. Polymerase chain reaction (PCR) analysis was performed on all samples using forward and reverse oligonucleotide primers designed to conserved regions flanking the ITS1 and ITS2 regions of *C. felis*. Primer pairs were designed using sequence information from GenBank. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS1 were 5'-CGATCGAGTGATCCGGTGAATTA-3' and 5'-GCTGCGTCCTTCATCGATGTG-3', respectively. These primers were expected to produce an amplicon of 651 bp from genomic *C. felis* DNA that incorporates the 458 bp ITS1 region plus 18S and 5.8S partial flanking regions. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS2 were 5'-TGAACGTATTAGACACACCACCT-3' and 5'-TCCTCCCGCTTCACTCGCCG-3',



respectively. These primers were expected to produce an amplicon of 431 bp from genomic *C. felis* DNA that incorporates the 265 bp ITS2 region plus 5.8S and 28S partial flanking regions. The PCR components consisted of a 2X hot start *Taq* polymerase master mix<sup>b</sup> containing hot start DNA polymerase, PCR buffer with 3 mmol of MgCl<sub>2</sub>, and 400 µmol of each deoxyribonucleotide triphosphate (dNTP); additional MgCl<sub>2</sub> to bring the final MgCl<sub>2</sub> concentration to 2.0 mmol; 37.5 pmol of each primer; 4 µl of DNA template; and molecular biology grade water to adjust the reaction mixture to a final volume of 50 µl. One known positive template and 2 negative templates (consisting of a known negative sample and molecular biology grade water) were utilized as controls for all amplification reactions.

After initial denaturation at 95°C for 5 min, 35 amplification cycles were performed. Each cycle was comprised of 30 sec at 94°C, 30 sec at 60°C, and 1 min at 72°C, with final extension at 72°C for 10 min. The PCR products were resolved by electrophoresis (100 V, 30 min), using agarose gels that were prestained with ethidium bromide and subsequently visualized with ultraviolet light.

### **DNA sequencing and analysis**

The PCR products were purified using a commercial kit<sup>c</sup> and automated sequencing was then performed at a commercial<sup>d</sup> or university<sup>e</sup> laboratory, utilizing the forward and reverse primers used for PCR. Sequences and chromatogram data were carefully analyzed and forward and reverse sequence data were assembled using the Contig Express module of Vector NTI software<sup>f</sup>. Assembled sequences were then aligned using Vector NTI software<sup>f</sup> to identify any polymorphisms within the ITS regions of the amplicons. Combined ITS1-ITS2 sequence data from each sample were evaluated in regard to state of origin (Arkansas vs. Georgia) to determine if the presence of particular parasite genotypes varied geographically. Using a chi-square test for

independence, the unique *C. felis* ITS genotypes were evaluated in regard to clinical outcome (survival vs. fatal infection) to determine if there was any association between unique parasite genotypes and observed pathogenicity. All tests were performed assuming a 2-sided alternative hypothesis and *p*-values <0.05 were considered statistically significant. When  $\geq 1$  cells in a contingency table had an expected count <5, Fisher's exact test was used. Because many factors other than pathogenicity may affect an owner's decision to euthanize his or her cat, cases in which the infected cat was euthanized were excluded from the analysis.

## **Results**

### **PCR amplification and DNA sequencing**

Amplification of the *C. felis* ITS regions from infected domestic cat blood samples and resolution of amplicons on agarose gel resulted in single bands of PCR product for 108 of 112 (93.4%) samples. Sequencing of all products yielded unambiguous ITS1 and ITS2 sequence data for 88 of 108 (81.5%) samples. Within the 458-bp ITS1 region of the *C. felis* genome, there were a total of 8 single nucleotide polymorphisms (SNPs) and a single nucleotide insertion. Within the 265-bp ITS2 region of the *C. felis* genome, there were a total of 4 SNPs and a single 40-bp insertion. Taken together, the ITS1 and ITS2 sequence data define a total of 11 different sequences and 3 unique genotypes (Table 3.1). The most commonly identified ITS1-ITS2 genotype, designated as ITSA (GenBank accession nos. EU450802 and EU450804) was identified in 48 of 88 (54.5%) samples. The second most common genotype, designated as ITSB (GenBank accession nos. EU450802 and EU450805) and differing by 1 SNP within ITS2, was detected in 21 of 88 (23.9%) samples. The third genotype, designated as genotype ITSC (GenBank accession nos. EU450803 and EU450804) and differing by 4 SNPs and a single nucleotide insertion, was present in 5/88 (5.7%) samples. The remaining ITS1-ITS2 sequences

varied by 1–5 SNPs in which the chromatograms revealed the incorporation of 2 nucleotides at a single position, and 1 sequence contained a 40-bp insertion within ITS2 (GenBank accession nos. EU450802 and EU450806). All samples with variable and mixed nucleotide SNPs and the 40-bp insertion were confirmed by repeated amplification and sequencing reactions.

### **Geographic distribution of genotypes**

Overall, 57 of 88 (64.8%) sequenced *C. felis* samples were submitted from Arkansas, and 31 of 88 (35.2%) samples originated from Georgia. Among the unique ITS1-ITS2 genotypes, ITSA was detected in 48 of 57 (84.2%) Arkansas samples and 1 of 31 (3.2%) Georgia samples. ITSB was detected in 21 of 31 (67.7%) Georgia samples and was not detected in any Arkansas samples, and ITSC was detected in 5 of 57 (8.8%) Arkansas samples and was not present in any Georgia samples. The geographic distribution of the ITS sequences is summarized in Table 3.2.

### **Association of genotype with pathogenicity**

Of the 88 *C. felis* samples analyzed, 12 were obtained from cats that were euthanized and were excluded from the analysis of clinical outcome of infection. Among those remaining, 44 of 76 (57.9%) samples were obtained from cats that survived infection. Among the unique *C. felis* ITS1-ITS2 genotypes, 38 of 48 (79.2%) of those infections identified as having the ITSA genotype survived as compared to 4 of 21 (19.0%) of those infected with the ITSB genotype, and none of the 5 identified as infected with the ITSC genotype ( $P < 0.001$ ). The clinical outcome for *C. felis*-infected cats with different ITS sequences is summarized in Table 3.2.

## **Discussion**

Although cytauxzoonosis has historically been nearly 100% fatal in domestic cats, increasing reports of cats demonstrating less severe disease have led to the hypothesis that different strains of *C. felis* exist that vary in pathogenicity to the domestic cat. The detection of

genetic variability within *C. felis* in the current study supports this hypothesis and reinforces the usefulness of mutation scanning to detect *C. felis* population variation. The level of nucleotide polymorphism detected in the current study is comparable to that detected among different isolates of *Babesia canis* species, a parasite closely related to *C. felis*. A 1998 study<sup>18</sup> analyzing the sequence variability of the ITS1-5.8S-ITS2 rDNA regions among isolates of *Babesia canis canis*, *B. canis vogelii*, and *B. canis rossi* detected intraspecies nucleotide polymorphism on the level of 0.1–2.1% which is similar to findings in the current study. This is a much smaller variability than that found between the different *Babesia* species (18–31%) in the 1998 study, supporting the classification of the different genotypes detected in the current study as strains rather than separate species of *Cytauxzoon*.

Sequencing results of *C. felis* in the present study revealed unique ITS1-ITS2 genotypes that varied geographically. In particular, the ITSB genotype was detected only in samples submitted from Georgia and the ITSC genotype was detected only in samples from Arkansas. The identification of unique genotypes from distinct geographic areas might be expected in the development of different parasite strains.

While clean sequencing data were obtained for the majority of the *C. felis* ITS1 and ITS2 amplicons, samples in which either the ITS1 or ITS2 sequence was ambiguous were excluded from further analysis. Detailed review of the accepted sequencing data identified 14 samples for which the chromatograms revealed the incorporation of 2 nucleotides at a single position within ITS1 or ITS2, though this position varied among samples. This finding is interpreted to reflect coinfection of the cat with multiple *C. felis* genotypes. Alternatively, it is possible that the *C. felis* genome contains multiple copies of the rRNA genes with polymorphic units, as has been described for some isolates of the closely related parasite, *Theileria parva*.<sup>10</sup> However, in the

current study, separate *C. felis* samples reflecting a single infection with each of the 2 different alleles presumed to combine in the mixed infection were detected for some of the mixed nucleotide SNPs. Though not detected for all SNPs, this inconsistency may be a consequence of the study's sample size, and further analysis of more *C. felis* genotypes from these geographic areas may detect infection with each unique parasite allele. Due to the inability to discern between each genotype's influence on clinical outcome in the cats with suspected coinfection with multiple parasites, the sequences containing the nested double peaks in the present study were excluded from the analysis of the association of genotype and pathogenicity.

A strong association was detected between particular *C. felis* ITS1-ITS2 genotypes and survival of domestic cats after acute infection. The *C. felis* ITSA genotype is of special interest given its association with a very high survival rate, particularly when compared to the high fatality rate for infection with other *C. felis* genotypes. Thus, ITS sequencing may be useful to define markers that are able to detect more or less pathogenic strains among *C. felis*-infected cats. Interpretation of the association of *C. felis* genotype and clinical outcome in infected domestic cats, however, is limited in the present study, which does not account for other factors that may affect clinical outcome. For these clinical cases, there was no control for the differences in treatment regimens, and the study design does not account for differences in host factors, such as host genetics and immune response, that may play a significant role in pathogenicity of cytauxzoonosis in infected cats. Still, a statistically significant association of certain ITS genotypes with clinical outcome of infection has been documented.

At present, only the ribosomal genes have been identified and sequenced for *C. felis*, limiting the areas of the genome that can be investigated for sequence variability, and the design of the current study precludes identifying genetic variability outside of the ITS regions.

Ultimately, identification of genetic variability in additional loci, ideally in potential pathogenicity-determining genes, would best test the hypothesis that different *C. felis* strains exist that vary in pathogenicity to the domestic cat. While identification of virulence-associated genes is not a simple task, it should be feasible to utilize genomic data from related piroplasms such as *Babesia bovis* and *Theileria parva* to identify additional loci to be characterized with the assumption that one or more new loci may be more closely linked to potential virulence genes. Further genomic studies of *C. felis* are currently limited by the inability to isolate parasite DNA free from the infected host DNA.

The current study revealed sequence variability in the ITS1 and ITS2 regions of *Cytauxzoon felis* from clinically ill domestic cats diagnosed with cytauxzoonosis. Unique ITS genotypes were identified that varied geographically between Arkansas and Georgia. Additionally, there was a strong association between particular ITS genotypes and outcome of infection. The findings support the hypothesis that there are genetically distinct populations of *C. felis*, which may account for the variability in clinical outcome of domestic cat infections. Better understanding of the epidemiology of *C. felis* infection will enhance the ability to prevent or treat this highly fatal infectious disease.

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### Sources and manufacturers

- a. illustra™ blood genomicPrep™ Mini Spin Kit, GE Healthcare, Little Chalfont, Buckinghamshire, UK.
- b. HotStarTaq® Plus Master Mix, Qiagen Inc., Valencia, CA.
- c. QIAquick® PCR Purification Kit, Qiagen Inc., Valencia, CA.
- d. MWG Biotech Inc., High Point, NC.
- e. University of Georgia Integrated Biotechnology Laboratories, Athens, GA.
- f. Invitrogen Corp., Carlsbad, CA.

### References

- 1. Barta JR, Coles BA, Schito ML, et al.: 1998, Analysis of intraspecific variation among five strains of *Eimeria maxima* from North America. Int J Parasitol 28:485–492.
- 2. Birkenheuer AJ, Le JA, Valenzisi AM, et al.: 2006, *Cytauxzoon felis* infection in cats in the mid-Atlantic states: 34 cases (1998–2004). J Am Vet Med Assoc 228:568–571.
- 3. Blouin EF, Kocan AA, Glenn BL, et al.: 1984, Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). J Wildl Dis 20:241–242.
- 4. Brown HM, Latimer KS, Erikson LE, et al.: 2008, Detection of persistent *Cytauxzoon felis* infection by polymerase chain reaction in three asymptomatic domestic cats. J Vet Diagn Invest 20:485–488.
- 5. Glenn BL, Kocan AA, Blouin EF: 1983, Cytauxzoonosis in bobcats. J Am Vet Med Assoc 183:1155–1158.

6. Greene CE, Latimer K, Hopper E, et al.: 1999, Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. *J Am Vet Med Assoc* 215:497–500.
7. Greene CE, Meinkoth J, Kocan AA: 2006, Cytauxzoonosis. *In: Infectious diseases of the dog and cat*, ed. Greene CE, 3rd ed., pp. 722–733. Saunders Elsevier, St. Louis, MO.
8. Hillis DM, Dixon MT: 1991, Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 66:411–453.
9. Hoover JP, Walker DB, Hedges JD: 1994, Cytauxzoonosis in cats: eight cases (1985–1992). *J Am Vet Med Assoc* 203:455–460.
10. Kibe MK, ole-MoiYoi OK, Nene V, et al.: 1994. Evidence for two single copy units in *Theileria parva* ribosomal RNA genes. *Mol Biochem Parasitol* 66:249–259.
11. Kier AB, Wagner JE, Kinden DA: 1987, The pathology of experimental cytauxzoonosis. *J Comp Pathol* 97:415–432.
12. Meinkoth J, Kocan AA, Whitworth L, et al.: 2000, Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997–1998). *J Vet Intern Med* 14:521–525.
13. Motzel SL, Wagner JE: 1990, Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. *Vet Parasitol* 35:131–138.
14. Olivier C, van de Pas S, Lepp PW, et al.: 2001, Sequence variability in the first internal transcribed spacer region within and among *Cyclospora* species is consistent with polyparasitism. *Int J Parasitol* 31:1475–1487.
15. Wagner JE: 1976, A fatal cytauxzoonosis-like disease in cats. *J Am Vet Med Assoc* 168:585–588.



16. Walker DB, Cowell RL: 1995, Survival of a domestic cat with naturally acquired cytauxzoonosis. J Am Vet Med Assoc 206:1363–1365.
17. Wightman SR, Kier AB, Wagner JE: 1977, Feline cytauxzoonosis: clinical features of a newly described blood parasite disease. Feline Pract 7:23–26.
18. Zahler M, Schein E, Rinder H, Gothe R: 1998, Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitol Res 84:544–548.

Table 3.1. Nucleotide variation and frequency of *Cytauxzoon felis* first and second ribosomal internal transcribed spacer region (ITS1 and ITS2) genotypes and sequences.

Genotype	<u>ITS1 nucleotide site</u>									<u>ITS2 nucleotide site</u>					<i>n</i>	GenBank accession # (ITS1/ITS2)
	75	162	337	338	344	397	415	444	452	180	232	243	249	254		
ITSA	A	A	-	G	T	T	T	A	T	G	T	A	G	-	48	i/ii
ITSB	A	A	-	G	T	T	T	A	T	T	T	A	G	-	21	i/iii
ITSC	A	G	A ins	G	C	T	C	G	T	G	T	A	G	-	5	iv/ii
<b>Sequence ID</b>																
A	A/G†	A	-	G	T	T	T	A	T	G	T	A	G	-	1	i/v
B	A	G/A†	A ins	G	C	T	C	G	T	G	T	A	G	-	1	
C	A	A	-	G	T	T	T	A	T	G/T†	T	A/G†	G	-	2	
D	A	A	-	G	T	T	T	A	T	G	T	A/G†	G	-	4	
E	A	A	-	A/G†	T	C/T†	C/T†	A	T	G/T†	A/T†	A	G	-	2	
F	A	A	-	G	T	T	T	A	T/C†	G	T	A	G	-	2	
G	A	A	-	G	T	T	T	A	T	G	T	A	A/G†	40bp ins	1	
H	A	A	-	G	T	T	T	A	T	G/T†	T	A	G	-	1	

ins = insertion; - = no insertion.

i = EU450802, ii = EU450804, iii = EU450805, iv = EU450803, v = EU450806.

† Chromatograms depict the incorporation of 2 nucleotides at this position.

Table 3.2. State of origin and outcome of infection for *Cytauxzoon felis* first and second ribosomal internal transcribed spacer region (ITS1 and ITS2) genotypes and sequences.

<b>Genotype</b>	<b>N</b>	<b><u>State of origin</u></b>		<b><u>Outcome of infection</u></b>		
		<b>Arkansas</b>	<b>Georgia</b>	<b>Survived</b>	<b>Fatal</b>	<b>Euthanized</b>
ITSA	48	47	1	38	5	5
ITSB	21	0	21	4	10	7
ITSC	5	5	0	0	5	0
<b>Sequence ID</b>						
A	1	1	0	0	1	0
B	1	1	0	0	1	0
C	2	0	2	0	2	0
D	4	0	4	2	2	0
E	2	0	2	0	2	0
F	2	2	0	0	2	0
G	1	1	0	0	1	0
H	1	0	1	0	1	0

CHAPTER 4

GENETIC VARIABILITY OF ARCHIVED *CYTAUXZOOM FELIS* HISTOLOGIC  
SPECIMENS FROM DOMESTIC CATS IN GEORGIA, 1995-2007<sup>2</sup>

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<sup>2</sup> Brown, H.M., Modaresi, S.M., Cook, J.L., Latimer, K.S., and Peterson, D.S. 2009. *Journal of Veterinary Diagnostic Investigation*. 21:493-498.  
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**Abstract.** *Cytauxzoon felis* infection in domestic cats has historically been nearly 100% fatal. However, increasing reports of domestic cats that survive cytauxzoonosis and reports of asymptomatic cats with *C. felis* infections suggest the existence of different parasite strains that vary in pathogenicity. The objective of the current study was to obtain epidemiologic information about cytauxzoonosis through genotypic characterization of archived histologic specimens from domestic cats with *C. felis* infections that were diagnosed in Georgia between 1995 and 2007. Such retrospective data on genetic variability will provide an historic context for current studies of *C. felis* genotype frequencies. *Cytauxzoon felis* DNA was obtained from formalin-fixed, paraffin-embedded tissues from infected cats diagnosed with cytauxzoonosis at necropsy. Genetic characterization of *C. felis* was performed using sequence analysis of the PCR-amplified ribosomal internal transcribed spacer regions 1 and 2 (ITS1, ITS2). Eleven different combined ITS1 and ITS2 sequences were identified, the majority of which were identical to those previously reported in fatally infected cats from Georgia. The findings of the current study document the existence of genetically distinct *C. felis* populations in historical samples and, together with data from contemporary samples, demonstrate a diverse population structure for *C. felis*.

**Key words:** Cats; *Cytauxzoon felis*; genetic variability; internal transcribed spacer region.

## Introduction

Fatal cytauxzoonosis was first described in a domestic cat from Missouri in 1976.<sup>28</sup> Since that time, *Cytauxzoon felis* (family *Theileriidae*) infections in domestic cats have been detected throughout the southeastern, south-central, and mid-Atlantic regions of the United States. Experimental infections have shown both *Dermacentor variabilis*<sup>3</sup> and *Amblyomma americanum*<sup>25</sup> to be competent vectors of the parasite. The bobcat (*Lynx rufus*) is believed to be the reservoir host of this parasite<sup>3,11</sup>; however, asymptomatic infection also has been detected in other wild felids in the United States including panthers and cougars (*Puma concolor coryi*, *P. c. stanleyana* and *P. c. couguar*).<sup>16,27,30</sup> Infection with *Cytauxzoon* spp. has also been identified in felids in other parts of the world, as well. *C. felis* infection has been reported in ocelots<sup>1</sup>, pumas<sup>1</sup>, jaguars<sup>1</sup>, and captive-reared lions<sup>23</sup> in Brazil, in addition to the identification of piroplasms morphologically indistinguishable from *Cytauxzoon* spp. in domestic cats<sup>20</sup>. In Europe, domestic cat and Iberian lynx infection with *Cytauxzoon* spp. have been reported from Spain<sup>8,21</sup>, as well as a single domestic cat infection from France.<sup>7</sup> In Asia, similar intraerythrocytic piroplasms with the proposed name *Cytauxzoon manul* have been detected from Pallas' cats in Mongolia.<sup>26</sup>

In contrast to the common asymptomatic infection of bobcats and cougars in the United States, *C. felis* infection of domestic cats usually results in rapid and severe disease progression, and most infected cats die within one week of initial clinical illness.<sup>13,17</sup> However, more recently published reports have identified cats that have survived *C. felis* infection.<sup>6,12,19,22,29</sup> Supportive care and antiprotozoal drug administration have had inconsistent therapeutic results and limited success in treating cytauxzoonosis.<sup>2,12,13,22</sup> Furthermore, some cats have survived both natural and experimental *C. felis* infections without medical treatment.<sup>6,19,22,29</sup> Thus, treatment protocol

is an unlikely explanation for the survival of these individuals. It is speculated that surviving cats may have recovered because they were infected with a less virulent strain of *C. felis*.

Currently, very little is known about the *C. felis* genome and only sequences for the ribosomal genes have been characterized and submitted to GenBank. One previous study<sup>19</sup> compared sequenced portions of the *C. felis* 18S ribosomal RNA (rRNA) gene between 2 infected cats and found that the sequences were virtually identical. A second study<sup>4</sup> comparing *C. felis* sequence data obtained from 3 infected ticks and 1 naturally infected cat reported >99.8% sequence similarity between each sequence and the previously published sequences.<sup>19</sup> These findings are not unexpected because the 18S region is a highly conserved area of the genome encoding a functional RNA molecule. In contrast, the internal transcribed spacer (ITS) regions are noncoding regions that do not have the structure-function constraints of the rRNA genes. As such, the ITS regions are likely to evolve more quickly and are more likely to show variability among putative strains of *C. felis*. The earlier study<sup>4</sup> comparing *C. felis* sequence data from infected ticks also analyzed data for the *C. felis* first ITS region (ITS1). Two polymorphic bases were found within one sample when comparing ITS1 sequence data from 2 infected ticks and 1 naturally infected cat. Otherwise, the sequences within the ITS1 region were identical. A more recent and comprehensive study<sup>5</sup> of the first and second ITS regions of the *C. felis* rRNA operon has detected unique *C. felis* ITS1 and ITS2 sequences from naturally infected cats in Arkansas and Georgia. The unique ITS1 and ITS2 sequences described in that study varied geographically and had a significant association with clinical outcome (survival vs. fatal infection) in infected domestic cats.

The objective of the current study was to characterize the *C. felis* genome via sequencing of archived paraffin-embedded tissue samples to provide an important historic context for current studies of *C. felis* genotype frequencies in infected domestic cats.

## **Materials and methods**

### **Samples**

A retrospective, computer-generated search was initiated using archived medical records at The University of Georgia (UGA) Veterinary Diagnostic Laboratory (Athens, GA) and the Tifton Diagnostic & Investigational Laboratory (Tifton, GA) to identify domestic cats that were diagnosed at necropsy with cytauxzoonosis. For each individual, the pathologist's diagnosis of cytauxzoonosis was based on the presence of schizonts within phagocytic cells that were morphologically consistent with *C. felis* as identified on formalin-fixed, paraffin-embedded (FFPE), hematoxylin and eosin-stained necropsy tissues. Archived FFPE tissues were retrieved from 98 cats with *C. felis* infections. These diagnostic cases encompassed a time period from 1995 through 2007.

### **DNA extraction**

DNA was isolated from archived FFPE tissues by shaving approximately 15 mg of material from the paraffin blocks. Spleen was preferentially used when present; otherwise, portions of lung, liver, or kidney were collected, and only one tissue sample was used per animal. The paraffin-embedded tissue samples were incubated with xylene overnight followed by a series of ethanol washes. DNA was extracted using a commercially available kit<sup>a</sup>, modified by a prolonged 3-day tissue digestion with an additional 20µl Proteinase K<sup>b</sup> added each day. Extracted DNA was eluted using a 1:2 dilution of the extraction kit's elution buffer in molecular biology grade water. After the elution process, the DNA was stored at 4°C until analyzed.



To prevent cross-contamination, new blades and tools were used for each specimen, and DNA extraction, amplification set-up, and post-amplification steps were each performed in a separate area of the laboratory with separate pipettes.

### **Polymerase chain reaction**

Polymerase chain reaction (PCR) analysis was performed on all samples using forward and reverse oligonucleotide primers designed to anneal to conserved areas flanking the ITS1 and ITS2 regions of the *C. felis* genome. Design of the primer pairs was accomplished using sequence data from GenBank. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS1 were 5' CGA TCG AGT GAT CCG GTG AAT TA 3' and 5' GCT GCG TCC TTC ATC GAT GTG 3', respectively. These primers were expected to produce an amplicon of 651 bp from genomic *C. felis* DNA that incorporated the 458-bp ITS1 region plus 18S and 5.8S partial flanking regions. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS2 were 5' TGA ACG TAT TAG ACA CAC CAC CT 3' and 5' TCC TCC CGC TTC ACT CGC CG 3', respectively. These primers were expected to produce an amplicon of 431 bp from genomic *C. felis* DNA that incorporated the 265-bp ITS2 region plus 5.8S and 28S partial flanking regions.

For samples in which PCR was unable to amplify the entire *C. felis* ITS1 region, PCR analysis was repeated using an additional primer pair designed to produce a smaller, 290-bp amplicon that incorporated 155bp of ITS1 plus the 18S partial flanking region. The sequences of the forward and reverse oligonucleotide primers used to amplify this region within *C. felis* ITS1 were 5' ATA GAG TAA ACG CTT CCT TCG GG 3' and 5' CGC AGA AGT CTG CAA GTC ACA ATG 3', respectively.

Components of the PCR reaction mixture included a 2X hot start *Taq* polymerase master mix<sup>c</sup>, containing hot start DNA polymerase, PCR buffer with 3 mmol of MgCl<sub>2</sub>, and 400 μmol of each deoxyribonucleotide triphosphate (dNTP); additional MgCl<sub>2</sub> to bring the final MgCl<sub>2</sub> concentration to 2.0 mmol; 37.5 pmol of each primer; 4 μl of DNA template; and molecular biology grade water to adjust the reaction mixture to a final volume of 50 μL. One known-positive, whole blood-derived *C. felis* template and two negative templates (consisting of a known *C. felis*-negative feline blood sample and molecular biology grade water) were used as controls for all amplification reactions. Reaction mixtures were assembled in a PCR enclosure with an ultraviolet light<sup>d</sup> so that work surfaces could be decontaminated and trace DNA denatured in between reactions.

After initial denaturation at 95°C for 5 minutes, 38 amplification cycles were performed. Each cycle was comprised of 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C, with final extension at 72°C for 10 minutes. The PCR products were resolved by electrophoresis (100 V, 30 min), using agarose gels that were pre-stained with ethidium bromide and visualized with ultraviolet light.

### **DNA sequencing and analysis**

The PCR products producing single positive bands at the appropriate target lengths were purified using a commercial kit<sup>e</sup>. Automated sequencing was then performed at a commercial<sup>f</sup> or university<sup>g</sup> laboratory, using the forward and reverse primers from the PCR amplifications. Chromatogram data were carefully reviewed for each sample and ambiguous sequence data were excluded from further analysis. Forward and reverse sequence data were assembled and then aligned using Vector NTI software<sup>h</sup> to identify any polymorphisms within the ITS regions of the

amplicons. Sequencing results were then analyzed chronologically to identify temporal relationships among different *C. felis* genotypes.

## **Results**

### ***C. felis* ITS amplification and sequencing**

Initial amplification of the *C. felis* ITS1 region from archived tissues of infected domestic cats yielded high-quality sequence data for 21 of 98 specimens, and amplification of the *C. felis* ITS2 region yielded high-quality ITS2 sequence data for 85 of 98 specimens. Repeat PCR analysis that restricted the length of the target area within ITS1 to a 290-bp region provided high-quality sequence data for an additional 27 specimens. This shorter region within ITS1 was targeted based on increased variability previously detected within this region.<sup>5</sup>

Analysis of the 48 combined *C. felis* ITS sequences (ITS1 and ITS2) revealed 6 single nucleotide polymorphisms (SNPs) within the restricted target region of ITS1 and 5 SNPs within ITS2 (Table 4.1). The most common ITS sequence, labeled here as ITSa and detected in 27 of 48 (56.3%) samples, contained one polymorphic site within ITS2 for which chromatograms revealed the incorporation of two different nucleotides at this single locus (herein referred to as mixed nucleotide positions). The ITSb sequence, detected in 8 of 48 (16.7%) samples, and the ITSd sequence, detected in 3 of 48 (6.3%) samples, had ITS1 nucleotide sequences identical to ITSa and differed only at the same polymorphic site (position 180) within ITS2, where each contained one of the two nucleotides incorporated at the mixed site in ITSa. The ITSc and ITSh sequences, detected in a single sample each, differed from ITSa at 3 SNPs and 2 SNPs, respectively, each at different loci within ITS1. The ITSe sequence was detected in 3 of 48 samples (6.3%) and the remaining ITS sequences (identified here as ITSe, ITSh, ITSi, ITSh, and ITSk) were detected in a single sample each. Each of these sequences differed from those

previously described by the inclusion of mixed nucleotides at 2 - 4 positions, where one of the nucleotides in each case represented the more commonly incorporated nucleotide in the other alleles. All variable and mixed nucleotide SNPs were confirmed by repeating the amplification and sequencing reactions.

### **Chronological distribution of *C. felis* ITS sequences**

The majority of the different *C. felis* sequences were detected in a single sample from a single year and not detected again. Another two sequence types were identified in single samples from 3 different years. The remaining two sequence types, comprising the majority of samples, were detected in specimens scattered throughout the years studied, with no obvious temporal trends. The chronological distribution of the *C. felis* ITS sequences is summarized in Table 4.2.

### **Discussion**

Extraction and PCR amplification of DNA from FFPE tissues poses multiple challenges. Formalin fixation results in cross-linking of protein to nucleic acids, making DNA extraction difficult. In addition, extraction procedures commonly result in DNA fragmentation that limits the size of the target DNA available for PCR analysis. It has been demonstrated that, with increasing age of the specimen and time in formalin fixative, longer DNA fragments are amplified with decreasing success.<sup>10,14,15</sup> Hence, it is not surprising that amplification of the complete ITS1 region (458 bp) was less successful than amplification of the smaller ITS2 region (265 bp). Because DNA compromised by fixation and age may require an amplification strategy that produces smaller PCR products, an additional PCR amplification of a smaller region within ITS1 (290 bp) was performed to obtain additional sequence data for the present study. This shorter region was targeted to amplify and sequence 7 of the 9 variable nucleotide sites identified in an earlier study of *C. felis* ITS1 sequence variability in domestic cats with cytauxzoonosis.<sup>5</sup>

The degree of sequence polymorphism seen in the current set of *C. felis* samples is similar to that which has been observed in previous studies. The majority of the *C. felis* ITS sequence types detected in the current study were identical to genotypes previously detected in domestic cat infections.<sup>5</sup> The *C. felis* ITS sequences unique to the current study were present predominantly in individual samples and differed at 1-3 nucleotides, a level of polymorphism comparable to that detected among all *C. felis* ITS1 and ITS2 sequences available in GenBank.

Detailed review of the sequence chromatograms revealed the incorporation of 2 nucleotides at single, variable positions within ITS1 or ITS2 in the majority of samples. This finding may reflect coinfection of the cat with multiple *C. felis* genotypes. Alternatively, because the DNA sequencing was based on a PCR pool, it could reflect polymorphisms in multiple copies of the rRNA genes that may exist in the *C. felis* genome, as described in some isolates of closely related parasites, such as *Theileria parva*<sup>18</sup> and some *Babesia* spp.<sup>9,24</sup> Resolution of this issue is difficult given the current lack of a cell culture system that would allow establishment of a clonal line of *C. felis*. Alternatively, the use of a Southern blot analysis may allow identification of multiple gene copies, if present.

For the chronologic analysis, there was year-to-year variability in the *C. felis* ITS sequences detected, though with no obvious temporal trend. The most common sequence, ITSa, was found in samples from infected cats scattered among the years analyzed and is identical to the sequence from a single isolate from an infected domestic cat in Georgia reported in a study of more contemporary isolates.<sup>5</sup> Similarly, the second most common ITS sequence, ITSb, was also found scattered throughout the years analyzed, and this sequence is identical to a common sequence in isolates from those more current cat infections in Georgia.<sup>5</sup> Interestingly, the most common *C. felis* ITS sequence described recently<sup>5</sup> in samples from 2005-2007 was identified in

only 3 of the samples in the current study, and this sequence was not detected in samples since 2001. The difference in the ITS genotypes which are most commonly detected suggests that changes have occurred in the *C. felis* population over the time frame covered by the current and previous study<sup>5</sup>.

It is interesting to compare the sequences from the current study to those identified previously as having a fatal or non-fatal outcome of infection in domestic cats. The most common ITS sequence in the current study (ITSa) was only detected in one sample in the previous study (sequence h)<sup>5</sup>, and this cat was fatally infected. The next most common sequence identified in the necropsy cases of the current study (ITSb) was identical to a common genotype (ITSB)<sup>5</sup>, which was significantly associated with a fatal outcome of infection in that study. Conversely, the common genotype in the previous study associated with non-fatal infection (ITSA)<sup>5</sup> was detected in only 3 of the 48 necropsy cases (6.3%) of the current study. Since the population in the current study included all cats that were diagnosed with cytauxzoonosis at necropsy, it is likely these samples represent isolates from both euthanized and fatally-infected domestic cat infections. Since many factors affect an owner's and veterinarian's decision to euthanize an infected cat, it is impossible to make a more direct comparison of the 2 studies' sequencing results, which would require an assumption that those cats presenting to necropsy would have a fatal outcome of natural infection. None-the-less, the *C. felis* ITS sequences associated with fatal outcomes in a previous study<sup>5</sup> were the sequences most commonly detected in the necropsy cases of the current study.

The sample population of the current study was limited to those archived tissues for *C. felis*-infected cats that were available from the State of Georgia, and a previous study<sup>5</sup> identified a much greater percent of fatally-infected cats among those *C. felis* isolates from Georgia versus

Arkansas. A more comprehensive genetic analysis comparing archived *C. felis* samples over a broader geographic area, including states with higher survival rates for infected cats, may better identify sequence diversity and trends expected with the possible emergence of less pathogenic strains of *C. felis*.

In summary, the current study examines the variability of *C. felis* ITS sequences from domestic cats in Georgia that were diagnosed with cytauxzoonosis at necropsy from 1995 to 2007. The identification of 11 different sequences lends further support to the existence of genetically distinct *C. felis* populations. The majority of the *C. felis* ITS sequences identified in the current study were identical to those previously reported in fatally infected cats from Georgia. Identification of other areas of the *C. felis* genome, ideally that encode proteins that affect pathogenicity, and a comparison of sequences from archived samples from infected cats in other geographic areas outside the State of Georgia may provide further support for the existence of different parasite strains that underlie the increased survival of more recently infected cats. Further disclosure of the genetic characterization of the *C. felis* population structure will contribute to a better understanding of the epidemiology of cytauxzoonosis in domestic cats.

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### **Sources and Manufacturers**

- a. QIAamp® DNA Mini-Kit, Qiagen Inc., Valencia, CA.
- b. Sigma-Aldrich, St. Louis, MO.
- c. HotStarTaq® Plus Master Mix, Qiagen Inc., Valencia, CA.
- d. Labconco Corporation, Kansas City, MO.
- e. QIAquick® PCR Purification Kit, Qiagen Inc., Valencia, CA.
- f. MWG Biotech Inc., High Point, NC.
- g. University of Georgia Integrated Biotechnology Laboratories, Athens, GA.
- h. Invitrogen Corp., Carlsbad, CA.

### References

1. André MR, Adania CH, Machado RZ, et al.: 2009, Molecular detection of *Cytauxzoon* spp. in asymptomatic Brazilian wild captive felids. J Wildl Dis 45:234–237.
2. Birkenheuer AJ, Le JA, Valenzisi AM, et al.: 2006, *Cytauxzoon felis* infection in cats in the mid-Atlantic states: 34 cases (1998–2004). J Am Vet Med Assoc 228:568–571.
3. Blouin EF, Kocan AA, Glenn BL, et al.: 1984, Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). J Wildl Dis 20:241–242.
4. Bondy Jr. PJ, Cohn LA, Tyler JW, Marsh AE.: 2005, Polymerase chain reaction detection of *Cytauxzoon felis* from field-collected ticks and sequence analysis of the small subunit and internal transcribed spacer 1 region of the ribosomal RNA gene. J Parasitol 91:458–461.
5. Brown HM, Berghaus RD, Latimer KS, et al.: 2009, Genetic variability of *Cytauxzoon felis* from 88 infected domestic cats in Arkansas and Georgia. J Vet Diagn Invest 21:59-63.



6. Brown HM, Latimer KS, Erikson LE, et al.: 2008, Detection of persistent *Cytauxzoon felis* infection by polymerase chain reaction in three asymptomatic domestic cats. J Vet Diagn Invest 20:485-488.
7. Criado-Fornelio A, Buling A., Pingret JL, et al.: 2009, Hemoprotozoa of domestic animals in France: Prevalence and molecular characterization. Vet Parasitol 159:73–76.
8. Criado-Fornelio A, González-del-Río MA, Buling-Saraña A, et al.: 2004, The “expanding universe” of piroplasms. Vet Parasitol 119:337–345.
9. Dalrymple BP: 1990, Cloning and characterization of the rRNA genes and flanking regions from *Babesia bovis*: use of genes as strain discriminating probes. Mol Biochem Parasitol 43:117-124.
10. Gillio-Tos A, De Marco L, Fiano V, et al.: 2007, Efficient DNA extraction from 25-year old paraffin-embedded tissues: study of 365 samples. Pathology 39:345-348.
11. Glenn BL, Kocan AA, Blouin EF: 1983, Cytauxzoonosis in bobcats. J Am Vet Med Assoc 183:1155–1158.
12. Greene CE, Latimer K, Hopper E, et al.: 1999, Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. J Am Vet Med Assoc 215:497–500.
13. Greene CE, Meinkoth J, Kocan AA: 2006, Cytauxzoonosis. In: Infectious diseases of the dog and cat, ed. Greene CE, 3rd ed., pp. 722–733. Saunders Elsevier, St. Louis, MO.
14. Greer CE, Lund JK, Manos MM: 1991, PCR amplification from paraffin-embedded tissues: Recommendations on fixatives for long-term storage and prospective studies. PCR Methods Appl 1:46-50.

15. Greer CE, Wheeler CM, Manos MM: 1994, Sample preparation and PCR amplification from paraffin-embedded tissues. *PCR Methods Appl* 3:S113-S122.
16. Harvey JW, Dunbar MR, Norton TM, Yabsley MJ: 2007, Laboratory findings in acute *Cytauxzoon felis* infection in cougars (*Puma concolor cougar*) in Florida. *J Zoo Wildl Med* 38:285-291.
17. Hoover JP, Walker DB, Hedges JD: 1994, Cytauxzoonosis in cats: eight cases (1985–1992). *J Am Vet Med Assoc* 203:455–460.
18. Kibe MK, ole-MoiYoi OK, Nene V, et al.: 1994, Evidence for two single copy units in *Theileria parva* ribosomal RNA genes. *Mol Biochem Parasitol* 66:249–259.
19. Meinkoth J, Kocan AA, Whitworth L, et al.: 2000, Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997–1998). *J Vet Intern Med* 14:521–525.
20. Mendes-de-Almeida F, Faria MCF, Branco AS, et al.: 2004, Sanitary conditions of a colony of urban feral cats (*Felis catus linnaeus*, 1758) in a zoological garden of Rio de Janeiro, Brazil. *Rev Inst Med Trop S Paulo* 46:269-274.
21. Millán J, Naranjo V, Rodríguez A, et al.: 2007, Prevalence of infection and 18S rRNA gene sequences of *Cytauxzoon* species in Iberian lynx (*Lynx pardinus*) in Spain. *Parasitol* 134:995–1001.
22. Motzel SL, Wagner JE: 1990, Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. *Vet Parasitol* 35:131–138.
23. Peixoto PV, Soares CO, Scofield A, et al.: 2007, Fatal cytauxzoonosis in captive-reared lions in Brazil. *Vet Parasitol* 145:383–387.

24. Reddy GR, Chakrabarti D, Yowell CA, Dame JB: 1991, Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in erythrocyte culture. *Nucleic Acids Res* 19:3641-3645.
25. Reichard MV, Meinkoth JH, Edwards AC, et al.: 2009, Transmission of *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum*. *Vet Parasitol*, in press.
26. Reichard MV, Van Den Bussche RA, Meinkoth JH, et al.: 2005, A new species of *Cytauxzoon* from Pallas' cats caught in Mongolia and comments on the systematics and taxonomy of piroplasmids. *J Parasitol* 91:420–426.
27. Rotstein DS, Taylor SK, Harvey JW, Bean J: 1999, Hematologic effects of cytauxzoonosis in Florida panthers and Texas cougars from Florida. *J Wildl Dis* 35:613-617.
28. Wagner JE: 1976, A fatal cytauxzoonosis-like disease in cats. *J Am Vet Med Assoc* 168:585–588.
29. Walker DB, Cowell RL: 1995, Survival of a domestic cat with naturally acquired cytauxzoonosis. *J Am Vet Med Assoc* 206:1363–1365.
30. Yabsley MJ, Murphy SM, Cunningham MW: 2006, Molecular detection and characterization of *Cytauxzoon felis* and a *Babesia* species in cougars from Florida. *J Wildl Dis* 42:366-374.

Table 4.1. Nucleotide variation and frequency of *Cytauxzoon felis* internal transcribed spacer region (ITS1 and ITS2) sequences.

Sequence	ITS1 nucleotide site						ITS2 nucleotide site					<i>n</i>	GenBank accession # (ITS1, ITS2)
	338	349	375	397	409	415	117	180	232	243	257		
ITSa	G	A	T	T	T	T	T	G/T*	T	A	C	27	EU450802, FJ536418
ITSb	G	A	T	T	T	T	T	T	T	A	C	8	EU450802, EU450805
ITSc	G	A	T	T	T	T	T	G	T	A	C	3	EU450802, EU450804
ITSd	G	A	T	T	T	T	T	G	T	A/G*	C	3	EU450802, FJ536419
ITSe	G	A	T	T	T	T	C/T*	G	T	A	C/T*	1	EU450802, FJ536420
ITSf	G	A	T	T	T	T	T	G/T*	T	A/G*	C	1	EU450802, FJ536421
ITSg	A	A	T	C	T	C	T	G/T*	T	A	C	1	FJ536423, FJ536418
ITSh	G	A	C	T	C	T	T	G/T*	T	A	C	1	FJ536424, FJ536418
ITSi	A/G*	A	T	C/T*	T	C/T*	T	G/T*	T	A	C	1	FJ536425, FJ536418
ITSj	G	A/G*	T	T	T	T	T	G/T*	T	A	C	1	FJ536426, FJ536418
ITSk	G	A	T	T	T	T	T	G/T*	A/T*	A	C	1	EU450802, FJ536422

\*Chromatograms depict the incorporation of 2 nucleotides at this position.

Table 4.2. Chronologic distribution of *Cytauxzoon felis* internal transcribed spacer region (ITS1 and ITS2) sequences.

Sequence	Specimen year													<i>n</i>
	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	
ITSa	-	-	2	-	3	3	1	4	3	4	5	-	2	27
ITSb	-	-	2	-	-	3	-	1	1	-	-	1	-	8
ITSc	1	-	-	1	-	-	1	-	-	-	-	-	-	3
ITSd	-	-	-	-	-	1	-	-	-	-	1	1	-	3
ITSe	-	-	-	-	-	-	-	-	-	1	-	-	-	1
ITSf	-	-	-	-	-	-	-	-	-	1	-	-	-	1
ITSG	1	-	-	-	-	-	-	-	-	-	-	-	-	1
ITSh	-	1	-	-	-	-	-	-	-	-	-	-	-	1
ITSi	-	-	1	-	-	-	-	-	-	-	-	-	-	1
ITSj	-	-	-	1	-	-	-	-	-	-	-	-	-	1
ITSk	-	-	-	-	1	-	-	-	-	-	-	-	-	1

## CHAPTER 5

### DETECTION OF PERSISTENT *CYTAUXZOOM FELIS* INFECTION BY POLYMERASE CHAIN REACTION IN THREE ASYMPTOMATIC DOMESTIC CATS<sup>3</sup>

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<sup>3</sup> Brown, H.M., Latimer, K.S., Erickson, L.E., Cashwell, M.E., Britt, J.O., and Peterson, D.S. 2008. *Journal of Veterinary Diagnostic Investigation*. 20:485-488. Reprinted here with permission of publisher.

**Abstract.** Repeated polymerase chain reaction (PCR) testing in 3 asymptomatic domestic cats positively detected *Cytauxzoon felis* DNA, suggesting persistent infection. Two cats initially presented with clinical signs consistent with acute cytauxzoonosis and, in both cases, signs of illness resolved after treatment. Parasitemia was detected in peripheral blood smears from these cats upon presentation with illness and at subsequent follow-up appointments, in the absence of clinical illness. PCR analysis was positive for *C. felis* from blood sampled at each time point. A third cat, a housemate of a cat fatally infected with *C. felis*, was preventatively treated for infection at the time of the housemate cat's death. This contact cat, having never shown signs of clinical illness consistent with cytauxzoonosis infection, had no detectable parasitemia but was positive for *C. felis* on repeated PCR testing. Detection of asymptotically infected cats allows for the possibility of a yet unrecognized population of infected domestic cats that may have the capacity to serve as an additional reservoir host for *C. felis*, altering the currently accepted paradigm of *C. felis* transmission to domestic cats through bobcats as the reservoir host. In cases of very low parasitemia, more sensitive means of parasite detection, such as PCR testing, may be necessary to detect infected cats. Increased detection of asymptotically infected cats will aid in understanding the epidemiology of *C. felis* infection and enhance the ability to prevent this highly fatal infectious disease of domestic cats.

**Key words:** Cytauxzoonosis; feline; persistent infection; polymerase chain reaction.

Cytauxzoonosis is an emerging, tickborne, hemoprotezoal disease of domestic and exotic cats that has been characterized with an extremely high fatality rate. The causative organism, *Cytauxzoon felis*, is a relatively new pathogen in the United States that was first identified in Missouri in 1973.<sup>16</sup> Subsequently, this parasite has been reported to infect cats in rural and urban regions throughout the south-central, southeastern and mid-Atlantic United States.<sup>1,2,9,11,12,16,18</sup> The life cycle of *C. felis* involves a tissue (or leukocytic) phase and an erythrocytic phase. Infected ticks transmit the parasite which enters host mononuclear phagocytes and undergoes schizogony and binary fission to produce numerous developing merozoites. These infected mononuclear cells line the lumens of veins throughout the body and eventually rupture, releasing the merozoites into circulation. The free merozoites subsequently infect host erythrocytes, resulting in a parasitemia with intraerythrocytic piroplasms that are visible in routine Romanowsky-stained peripheral blood smears. The bobcat (*Lynx rufus*) has been identified as the natural reservoir host of the parasite.<sup>3,5</sup> In affected cats, the course of disease usually is rapid and most cats die within 1 week of initial clinical illness.<sup>4,7,10,18</sup> The sporadic occurrence, short course of illness, and high fatality rate of cytauxzoonosis in domestic cats has suggested that they likely serve as incidental, dead-end hosts.<sup>7,13</sup>

Although *C. felis* infection has historically been viewed as uniformly fatal in domestic cats, recent studies<sup>6,14,15,17</sup> have documented cats that have survived parasitic infection. Survival of infected cats allows for the possibility of persistent infections. One recent study identified 3 of 961 free-roaming, feral cats tested from trap-neuter-return programs that were polymerase chain reaction (PCR)-positive for *C. felis* infection.<sup>8</sup> Clinical signs of cytauxzoonosis were not noted at the time of their capture, although there is limited follow-up clinical information and laboratory testing on these cats to document persistent, subclinical *C. felis* infection. These findings suggest



that, despite a low prevalence, apparently healthy but *C. felis*-infected cats exist and may have the capacity to serve as a new reservoir for parasitic infection. The present communication describes the clinical history and laboratory testing of 3 asymptomatic, persistently *C. felis*-infected domestic shorthair cats.

A 13-month-old, 3.9-kg, neutered male, indoor/outdoor, domestic shorthair cat from Hot Springs, AR was referred to a private veterinary practice for lethargy, inappetence, and fever that had been first detected 6 weeks previously. On presentation, the cat was febrile (39.4°C) but the physical examination was otherwise unremarkable. The cat had a history of tick exposure and was not on tick preventative. Ethylenediamine tetra-acetic acid (EDTA)-anticoagulated whole blood was submitted to the Veterinary Diagnostic Laboratory of the Arkansas Livestock and Poultry Commission (ALPC; Little Rock, AR) where Wright-Giemsa-stained blood smears revealed intraerythrocytic piroplasms morphologically consistent with *C. felis*. The cat was treated with imidocarb dipropionate<sup>a</sup> (5 mg/kg IM) following premedication with atropine<sup>b</sup> (0.04 mg/kg SQ) 30 min prior. Both treatments were repeated 1 week later. Oral enrofloxacin<sup>c</sup> (3 mg/kg PO q12h) was administered for 14 days. Clinical signs had completely resolved within 2 weeks of presentation, although persistent parasitemia remained. Blood smear review confirmed persistent parasitemia at 5, 10, and 15 months after initial illness, when the cat was clinically healthy (Fig. 5.1).

A littermate of the first cat, a 6.3-kg, neutered male, indoor/outdoor, domestic shorthair cat, also living in the same home in Hot Springs, AR, presented to the same practice 3 months after the first cat was referred. The cat had a 24–48 hr history of decreased appetite, lethargy, and fever. The cat had no known history of tick exposure, although application of tick preventative by the owner was inconsistent. On presentation, the cat was febrile (40.6°C) and lethargic.

EDTA-anticoagulated whole blood was submitted to ALPC, and Wright-Giemsa-stained blood smears revealed intraerythrocytic piroplasms morphologically consistent with *C. felis*. An IV catheter was placed and a constant-rate infusion of lactated Ringer's solution<sup>d</sup> was administered at 15 ml/h for 48 hr. The cat was treated with imidocarb dipropionate<sup>a</sup> (5 mg/kg IM) following premedication with atropine<sup>b</sup> (0.04 mg/kg SQ) 30 min prior. Enrofloxacin<sup>c</sup> (3 mg/kg SQ q24h for 3 days, then 3 mg/kg PO for 11 days) also was administered. Ten days later, at which point clinical signs had resolved, the imidocarb dipropionate<sup>a</sup> treatment with atropine<sup>b</sup> premedication was repeated. Blood smear review performed at 2 and 7 months after the initial diagnosis of cytauxzoonosis revealed persistent parasitemia in an otherwise clinically healthy cat.

EDTA-anticoagulated whole blood samples drawn from the infected cats at each appointment were submitted for further study. Wright-Leishman-stained blood smear reviews of each sample confirmed hemoparasites that were morphologically consistent with *C. felis*. Parasitemia levels at initial diagnosis of cytauxzoonosis and then during follow-up varied between 0.1–0.6% for the first cat and between 0.1–1.0% for the second cat, as quantitated using a Miller ocular and examining 10 100× counting fields.

The third case is a 9-year-old, 4.5-kg, spayed female, domestic shorthair cat from Columbus, GA that was prophylactically treated with a single dose of imidocarb dipropionate<sup>a</sup> (5 mg/kg IM) after another housemate cat died of cytauxzoonosis. While this cat lived predominantly indoors, she did have close contact with several other indoor/outdoor cats, and she did have known tick exposure historically. However, both the owners and attending veterinarian report that this cat had never demonstrated clinical signs consistent with cytauxzoonosis. Wright-Leishman-stained blood smears, prepared 20 and 29 months after the

prophylactic treatment, did not reveal a detectable parasitemia. Blood from each time point also was submitted for PCR analysis.

Polymerase chain reaction analysis for *C. felis* was performed on each EDTA-anticoagulated whole blood sample from all 3 cats at each follow-up appointment. DNA was isolated using a commercially available kit<sup>e</sup> and the *C. felis* internal transcribed spacer region 2 (ITS2) plus 5.8S and 28S partial flanking regions were amplified by PCR. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS2 were 5'-TGAACGTATTAGACACACCACCT-3' and 5'-TCCTCCCGCTTCACTCGCCG-3', respectively. These primers were expected to produce an amplicon of 431 bp from genomic *C. felis* DNA that incorporates the 265 bp ITS2 region. PCR components consisted of a hot start *Taq* polymerase master mix<sup>f</sup> (containing hot start DNA polymerase, PCR Buffer with 3 mM MgCl<sub>2</sub>, and 400 µM each dNTP [deoxyribonucleotide triphosphate]), additional MgCl<sub>2</sub> (bringing the final MgCl<sub>2</sub> concentration to 2.0 mM), 37.5 pmol of each primer, 4µl DNA template, and water, for a final volume of 50 µL. For a positive control, DNA was extracted from the blood of a fatally infected cat that had confirmed cytauxzoonosis on necropsy. Two negative controls included both DNA extracted from a clinically healthy cat with no known history of tick exposure (thus believed to be not at risk for *C. felis* transmission) and water. After initial denaturation at 95°C for 5 min, 38 amplification cycles were performed, each cycle comprised of 30 sec at 94°C, 30 sec at 60°C, and 1 min at 72°C, with final extension at 72°C for 10 min. PCR products were resolved in agarose gels and visualized by ethidium bromide staining (Fig. 5.2). Positive DNA bands of the appropriate length were detected from all blood samples. However, repeated PCR testing of the first blood sample from the cat with the undetectable parasitemia

was inconsistent after long-term storage at  $-20^{\circ}\text{C}$ . These findings may be attributable to the very low level of parasitemia and target DNA available.

Positive PCR products from each blood sample were sequenced by a commercial laboratory<sup>g</sup> and analyzed with Vector NTI software<sup>h</sup>. The ITS2 sequences were aligned with Vector NTI software for sequence comparison. The ITS2 sequences from each isolate in our study (all 3 cases at multiple time points) were identical (GenBank accession no. EU450801). In comparison to the other complete 265 bp ITS2 sequences deposited in GenBank, our consensus sequence was identical to 2 submissions (GenBank accession nos. AY699809 and DQ458797) and differed by either 1 or 2 nucleotides from the other four entries (accession nos. AY695934, AY695935, AY695936 and AY699808).

The present communication reports repeated detection of *C. felis* in the peripheral blood of 3 asymptomatic domestic cats as confirmed by PCR analysis with DNA sequencing. Although 2 cats initially presented with clinical cytauxzoonosis, the third cat had never demonstrated clinical signs consistent with acute *C. felis* infection. This finding allows for a population of yet unidentified domestic cats that may silently harbor the parasite. Due to the inherent insensitivity of blood smear review for the detection of very low levels of parasitemia, more sensitive means of parasite detection, such as PCR analysis, may be necessary to identify asymptomatic carriers of *C. felis*. However, very low levels of parasite DNA may result in inconsistent PCR results. Therefore, repeated PCR testing or more sensitive analyses, like nested or real-time PCR, may be necessary to identify *C. felis*-positive cats.

Asymptomatic, persistently infected domestic cats may have the capacity to serve as an additional reservoir host for *C. felis*, altering the currently accepted paradigm of *C. felis* transmission to domestic cats through bobcats as the reservoir host. Domestic cat reservoirs

would greatly increase the risk of exposure to *C. felis* infection for other cats, though the ability of these cats to serve as a source of infection for fatal disease is unknown. Increased understanding of the epidemiology of *C. felis* infection will enhance the ability to prevent this highly fatal infectious disease of domestic cats.

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### **Sources and manufacturers**

- a. Imizol, Schering-Plough Animal Health, Union, NJ.
- b. IVX Animal Health Inc., St. Joseph, MO.
- c. Baytril, Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, KS.
- d. Hospira, Inc., Lake Forest, IL.
- e. illustra blood genomicPrep Mini Spin Kit, GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK.
- f. HotStarTaq *Plus* Master Mix, Qiagen, Inc., Valencia, CA.
- g. MWG Biotech, Inc., High Point, NC.
- h. Invitrogen Corp., Carlsbad, CA.

### **References**

1. Bendele RA, Schwartz WL, Jones LP: 1976, Cytauxzoonosis-like disease in Texas cats. Southwest Vet 29:244-246.
2. Birkenheuer AJ, Le JA, Valenzisi AM, et al.: 2006, *Cytauxzoon felis* infection in cats in the mid-Atlantic states: 34 cases (1998-2004). J Am Vet Med Assoc 228:568-571.

3. Blouin EF, Kocan AA, Glenn BL, et al.: 1984, Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Derma-centor variabilis* (Say). J Wildl Dis 20:241–242.
4. Ferris DH: 1979, A progress report on the status of a new disease of American cats: cytauxzoonosis. Comp Immunol Microbiol Infect Dis 1:269–276.
5. Glenn BL, Kocan AA, Blouin, EF: 1983, Cytauxzoonosis in bobcats. J Am Vet Med Assoc 183:1155–1158.
6. Greene CE, Latimer K, Hopper E, et al.: 1999, Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. J Am Vet Med Assoc 215:497–500.
7. Greene CE, Meinkoth J, Kocan AA: 2006, Cytauxzoonosis. In: Infectious diseases of the dog and cat, ed. Greene CE, 3rd ed., pp. 722–733. Saunders Elsevier, St. Louis, MO.
8. Haber MD, Tucker MD, Marr HS, et al.: 2007, The detection of *Cytauxzoon felis* in apparently healthy free-roaming cats in the USA. Vet Parasitol 146:316–320.
9. Hauck WN, Snider TG, Lawrence JE: 1982, Cytauxzoonosis in a native Louisiana cat. J Am Vet Med Assoc 180:1472-1474.
10. Hoover JP, Walker DB, Hedges JD: 1994, Cytauxzoonosis in cats: eight cases (1985–1992). J Am Vet Med Assoc 203:455–460.
11. Jackson CB, Fisher T: 2006, Fatal cytauxzoonosis in a Kentucky cat (*Felis domesticus*). Vet Parasitol 139:192-195.
12. Kier AB, Morehouse LG, Wagner JE: 1979, Feline cytauxzoonosis: An update. Missouri Vet 29:15-18.

13. Kier AB, Wagner JE, Kinden DA: 1987, The pathology of experimental cytauxzoonosis. J Comp Pathol 97:415–432.
14. Meinkoth J, Kocan AA, Whitworth L, et al.: 2000, Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997–1998). J Vet Intern Med 14:521–525.
15. Motzel SL, Wagner JE: 1990, Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. Vet Parasitol 35:131–138.
16. Wagner JE: 1976, A fatal cytauxzoonosis-like disease in cats. J Am Vet Med Assoc 168:585–588.
17. Walker DB, Cowell RL: 1995, Survival of a domestic cat with naturally acquired cytauxzoonosis. J Am Vet Med Assoc 206:1363–1365.
18. Wightman SR, Kier AB, Wagner JE: 1977, Feline cytauxzoonosis: Clinical features of a newly described blood parasite disease. Feline Pract 7:23–26.

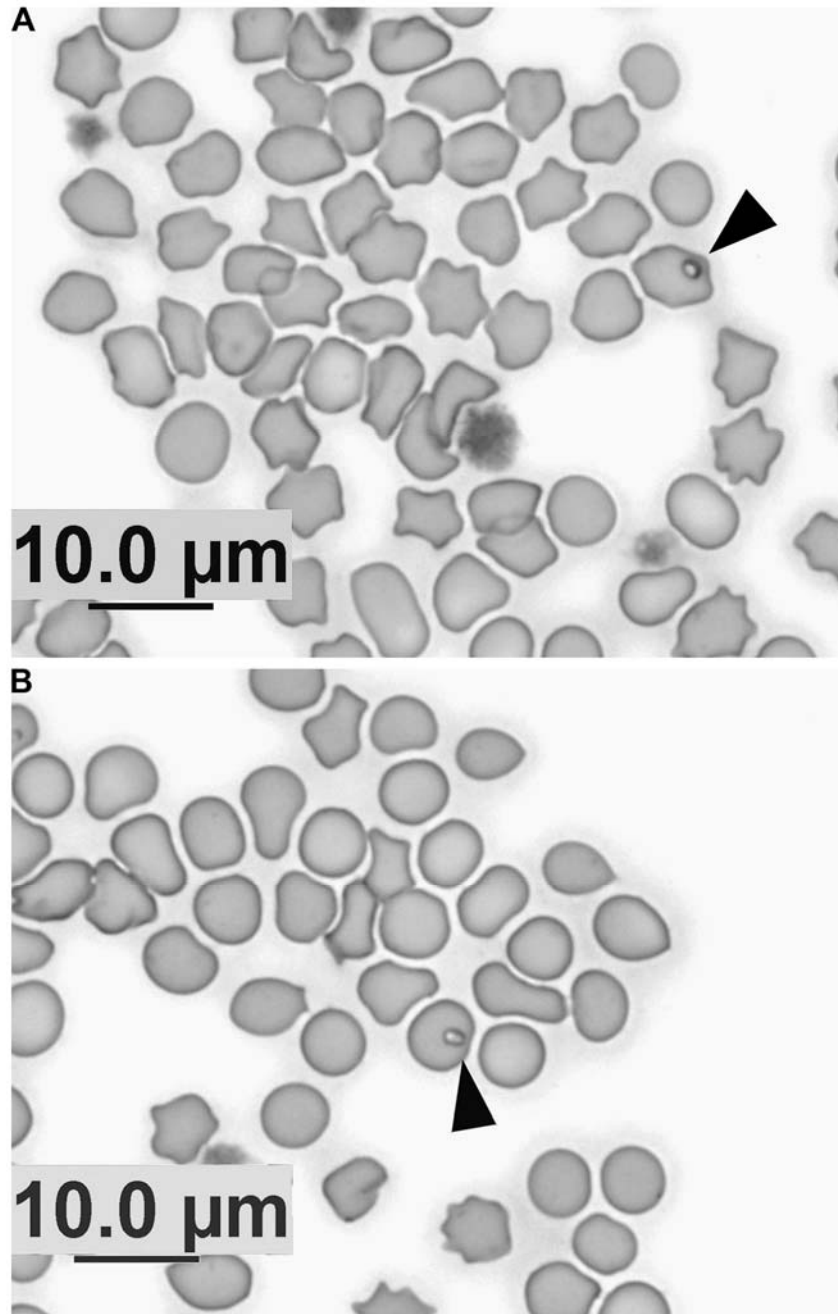


Figure 5.1. Peripheral blood smears from 2 clinically healthy but persistently infected cats prepared 5 months (**A**; first cat) and 2 months (**B**; second cat) after initial diagnosis with acute cytauxzoonosis. Rare *Cytauxzoon felis* piroplasms are present within erythrocytes (arrows). Wright-Leishman stain, 100× oil.



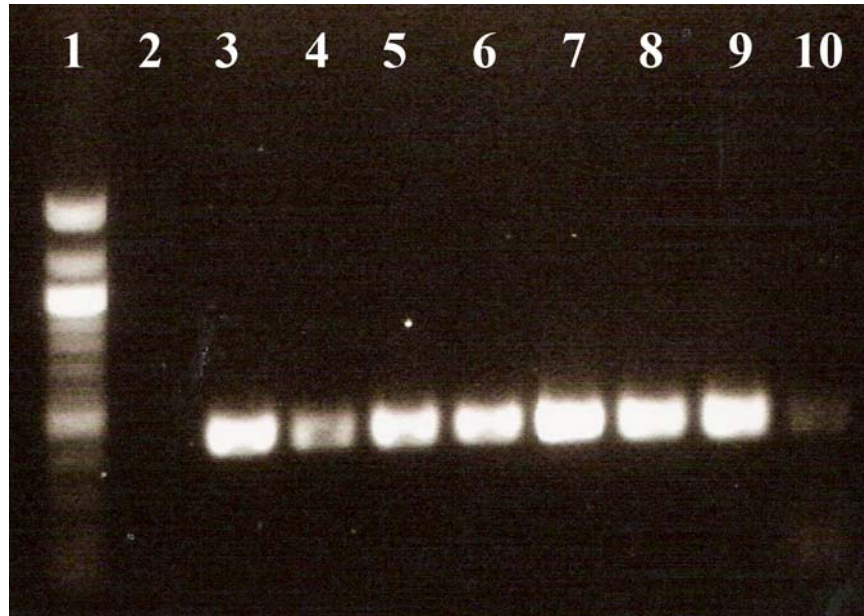


Figure 5.2. Amplification of the *Cytauxzoon felis* internal transcribed spacer region 2 (ITS2) gene from 3 asymptomatic, persistently infected cats. Lane 1 = DNA 100-bp ladder; lane 2 = negative control (no DNA); lane 3 = the first cat on presentation, when clinically ill; lanes 4, 5, 6 = the first cat 5, 10, and 15 months, respectively, after acute cytauxzoonosis, when clinically healthy; lane 7 = the second cat on presentation, when clinically ill; lanes 8, 9 = the second cat 2 and 7 months, respectively, after acute cytauxzoonosis, when clinically healthy; lane 10 = the third cat, asymptomatic, 9 months after initial detection of *C. felis* DNA by polymerase chain reaction.

## CHAPTER 6

### IDENTIFICATION AND GENETIC CHARACTERIZATION OF *CYTAUXZOOM FELIS* IN ASYMPTOMATIC DOMESTIC CATS AND BOBCATS<sup>4</sup>

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<sup>4</sup> Brown, H.M., Lockhart, J.M., Latimer, K.S., and Peterson, D.S. 2010. *Veterinary Parasitology*. In press.  
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## Abstract

The objectives of the current study were to assess the prevalence of *Cytauxzoon felis* infection among a population of domestic cats that were clinically healthy but at higher risk for parasite exposure and to determine if the strains present in these asymptotically infected cats were genetically unique as compared to those present both in domestic cats that were fatally infected and in the natural reservoir host, the bobcat. Using real-time PCR analysis targeting a portion of the parasite 18S rRNA gene specific for *C. felis*, 27/89 (30.3%) high-risk asymptomatic domestic cats from Arkansas and Georgia, and 34/133 (25.6%) bobcats from Arkansas, Georgia and Florida, were identified as positive for *C. felis* infection. Conventional PCR analysis was performed on all positive samples, targeting the *C. felis* ribosomal internal transcribed spacer regions 1 and 2 (ITS1, ITS2) in order to utilize the ITS sequences as markers to assess the genotype variability of the parasite population. Within the asymptotically infected domestic cat samples, 3 genetically distinct parasite populations were identified. The *C. felis* ITS sequences from asymptomatic cats were identical to those previously reported from clinically ill infected cats, and 2 of the 3 sequence types were also present in infected bobcat samples. While sequence diversity exists, evaluation of the ITS region does not appear to be useful to verify pathogenicity of *C. felis* strains within host species. However, the presence of asymptomatic *C. felis* infections in clinical healthy domestic cats warrants further investigation to determine if these cats can serve as a new reservoir for *C. felis* transmission.

**Key words:** Cat; *Cytauxzoon felis*; internal transcribed spacer region; molecular diagnostics; parasite; piroplasms; reservoir host

## Introduction

Cytauxzoonosis is an emerging, tickborne, hemoprotozoal disease of domestic and exotic cats which has historically been considered highly fatal. The causative organism, *Cytauxzoon felis*, is a relatively new pathogen in the United States which was first detected in Missouri in 1973 (Wagner, 1976) and subsequently throughout southeastern and south-central states. The bobcat (*Lynx rufus*) has been identified as the natural reservoir host of the parasite (Glenn et al., 1983; Blouin et al., 1984). In the United States, asymptomatic *C. felis* infection of free-ranging Florida panthers (*Puma concolor coryi*) and Texas cougars (*Puma concolor stanleyana*) transplanted to Florida has also been reported (Rotstein et al., 1999).

In acutely infected domestic cats, the course of disease is rapid and most cats die within 1 week of initial clinical illness (Hoover et al., 1994; Wightman et al., 1977; Greene et al., 2006). The sporadic occurrence, short course of illness, and historic high fatality of cytauxzoonosis in domestic cats has suggested that they likely serve as aberrant or dead-end hosts (Kier et al., 1987; Greene et al., 2006).

Although *C. felis* infection has historically been viewed as uniformly fatal in domestic cats, recent studies have documented cats that have survived infection (Motzel and Wagner, 1990; Walker and Cowell, 1995; Greene et al., 1999; Meinkoth et al., 2000). Survival of infected cats allows for the possibility of persistent infection, and studies have identified parasitemic cats following treatment for acute cytauxzoonosis in which clinical signs resolved (Walker and Cowell, 1995; Meinkoth et al., 2000; Brown et al., 2008). Some cats have remained detectably parasitemic greater than 1 year after resolution of clinical signs (Meinkoth et al., 2000; Brown et al., 2008) and PCR testing on blood from some clinically healthy cats with undetectable parasitemias has identified *C. felis* DNA (Haber et al., 2007; Brown et al., 2008). Persistently

infected cats could serve as new foci of infection, thus greatly increasing the risk of exposure to *C. felis* for other domestic cats.

It is unknown whether persistent *C. felis* infections in domestic cats are genetically distinct from those causing fatal infections. Genetic characterization of *C. felis* from asymptotically infected domestic cats would allow a comparison of the parasite genotypes infecting both clinically ill and asymptomatic persistently infected cats, as well as genotypes present in the known reservoir wild cat hosts. Very little is currently known about the *C. felis* genome. One previous study compared a sequenced portion of the *C. felis* 18S rRNA gene derived from an asymptomatic experimentally infected cat with a GenBank archived sequence from a cat with fatal infection; both sequences were virtually identical (>99% sequence identity) (Meinkoth et al., 2000). Subsequent studies comparing the non-coding internal transcribed spacer regions 1 and 2 (ITS1, ITS2) of *C. felis* from infected domestic cats in Arkansas and Georgia found nucleotide variability in the range of 0-1.8% (Brown et al., 2009a). In addition, this same study identified a significant association between particular parasite genotypes and whether infected cats survived or died. The detection of genotype variability within the *C. felis* parasite population, and the association of particular genotypes with the clinical outcome of infection, supported the hypothesis that *C. felis* strains varied in virulence, with some strains causing less severe disease and increased survival of infected domestic cats.

Haber et al. (2007) tested free-roaming domestic cats using a *C. felis*-specific PCR assay and found that 3/961 (0.3%) cats were PCR test positive. Although clinical signs in the positive cats were not noted at the time of their capture, there was limited follow-up or other clinical information on these cats to unambiguously document persistent, subclinical infections. Despite the low prevalence, the presence of apparently healthy, infected, free-roaming cats suggested that

these cats may have had the capacity to serve as an additional reservoir host for *C. felis*. A recent study by Reichard et al. (2009) demonstrated transmission of clinical cytauxzoonosis from a domestic cat that was a natural survivor of *C. felis* infection to a naïve domestic cat using *Amblyomma americanum* as the tick vector. The ability to transmit *C. felis* infection from domestic cats suggested that subclinically infected domestic cats might be a reservoir for further infection of domestic cats. Previously, it was presumed that domestic cats that became infected after other household cats demonstrated clinical signs of cytauxzoonosis had been exposed to similarly infected ticks in the shared environment that had fed on infected bobcats. In light of Reichard's most recent experimental infection of domestic cats, support now exists for the possibility of cat-to-cat transmission via tick vectors. However the role of chronically infected cats in disease transmission in areas in which cytauxzoonosis appears hyperendemic remains unknown.

The ability of surviving *C. felis*-infected domestic cats to serve as an additional reservoir host for the parasite would alter the currently accepted paradigm of *C. felis* transmission from bobcats (reservoir host) to domestic cats. The objectives of the current study were to assess the prevalence of *C. felis* infection among a population of domestic cats that were clinically healthy but at higher risk for parasite exposure and to determine if the strains present in these asymptotically infected cats were genetically unique as compared to those present both in domestic cats that are fatally infected and in the natural reservoir host, the bobcat. Establishing that there are clinically healthy domestic cats that are persistently infected with *C. felis* and may have the potential to serve as a reservoir host for the parasite has important application to treatment and prevention of disease spread. Unique *C. felis* strains in infected domestic cats that

were not detected in bobcats would suggest that the bobcat may not be necessary in the currently accepted paradigm of *C. felis* transmission where wild felids are the only known reservoir hosts.

## **Materials and methods**

### **Animals and sample collection**

Ethylenediamine tetra-acetic acid (EDTA)-anticoagulated whole blood samples from domestic cats were obtained by routine phlebotomy at private veterinary practices with written consent from the cats' owners, when applicable. Participating private practices were identified from previous studies as clinics in counties from which multiple *C. felis*-positive samples were submitted to our collaborating diagnostic laboratories over the previous 3 years. Cases, identified as those considered to be at high risk for *C. felis* exposure, included cats that had clinically recovered from acute cytauxzoonosis, contact cats that co-habitated with cats previously diagnosed with cytauxzoonosis, and feral cats in endemic areas. In each case, the submitting veterinarian found no clinical evidence for infection or illness on physical exam.

Bobcat tissues samples were obtained from multiple sources in Georgia, Arkansas, and Florida. Fresh frozen spleen sections were submitted from bobcats collected by the United States Department of Agriculture Wildlife Services as part of a study to evaluate the effects of mesomammalian predator removal on bobwhite-quail reproduction in Georgia and Florida. Fresh-frozen spleen samples were also obtained postmortem from the diagnostic service at the Southeastern Cooperative Wildlife Disease Study (Athens, GA) and a private wildlife rehabilitator in Florida. Finally, formalin-fixed paraffin-embedded (FFPE) splenic tissue and fresh-frozen spleen sections were obtained from the Veterinary Diagnostic Laboratory at the Arkansas Livestock and Poultry Commission (Little Rock, AR) after routine diagnostic necropsy was performed.

## DNA extraction

DNA was extracted from EDTA-anticoagulated whole blood samples using the illustra™ blood genomicPrep™ Mini Spin Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturer's instructions. DNA was extracted from fresh-frozen splenic tissue samples using the QIAamp DNA Mini-Kit (Qiagen, Inc., Valencia, CA). DNA from the FFPE tissue was also extracted using the QIAamp DNA Mini-Kit (Qiagen Inc., Valencia, CA), with a preliminary overnight 56°C incubation with proteinase K (Sigma-Aldrich, St. Louis, MO).

### 2.2 Real-time polymerase chain reaction (real-time PCR) amplification

Using multiple sequence alignments (Vector NTI, Invitrogen, Valencia, CA) to compare the 18S rRNA gene sequences from *C. felis* and related piroplasms (*Babesia* spp., *Theileria* spp.), primer sequences were selected to specifically amplify an 82-bp fragment of the *C. felis* 18S rRNA gene. The selected forward primer was 5' TGC ATC ATT TAT ATT CCT TAA TCG 3' and the reverse primer was 5' CAA TCT GGA TAA TCA TAC CGA AA 3'. In order to perform multiplex real-time PCR analysis, a linear fluorescent-labeled probe was designed to anneal within the targeted 18S amplicon. The 18S probe sequence was 5' TTA TTT ATG TTG TGG CTT TTT CTG GTG ATT 3' and was labeled with 5'-HEX fluorescence and 3'-BHQ1 quencher. The real-time PCR components consisted of a 2X *Taq* polymerase master mix (iQ Multiplex Powermix, Bio-Rad Laboratories, Hercules, CA) containing DNA polymerase, PCR buffer with 11 mM MgCl<sub>2</sub>, and deoxyribonucleotide triphosphates (dNTPs); 6 pmol of the forward primer, 4 pmol of the reverse primer, and 2 pmol of the Hex-labeled probe; 1 µl of DNA template; and molecular biology grade water to adjust the reaction mixture to a final volume of 20 µl. A negative template from an uninfected cat and a non-template control consisting of molecular biology grade water were utilized as controls for all amplification reactions. After an



initial activation and denaturation step at 95°C for 3 min, 40 cycles of a 2-step real-time amplification were performed. Each cycle was comprised of 30 sec at 95°C and 1 min at 54°C, after which point fluorescence data were collected.

### **Standard PCR amplification and DNA sequencing**

All real-time PCR-positive samples were further analyzed by conventional PCR analysis using forward and reverse oligonucleotide primer sets designed to conserved regions flanking both the ITS1 and ITS2 regions of *C. felis*. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS1 from the domestic cat samples were 5' CGA TCG AGT GAT CCG GTG AAT TA 3' and 5' GCT GCG TCC TTC ATC GAT GTG 3', respectively. An alternate reverse primer utilized to amplify *C. felis* ITS1 from the bobcat samples was 5' GGA GTA CCA CAT GCA AGC AG 3'. These primer sets were expected to produce amplicons of 651 bp and 746 bp, respectively, from genomic *C. felis* DNA that incorporated the 458-bp ITS1 region plus 18S and 5.8S partial flanking regions. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS2 from all samples were 5' AGC GAA TTG CGA TAA GCA TT 3' and 5' TCA GCC GTT ACT AGG AGA 3', respectively. This primer set was expected to produce an amplicon of 475 bp from genomic *C. felis* DNA that incorporated the 265-bp ITS2 region plus 28S and 5.8S partial flanking regions.

For all reactions, the PCR components consisted of a 2X hot start *Taq* polymerase master mix (HotStar Taq Plus Master Mix, Qiagen Inc., Valencia, CA) containing hot start DNA polymerase, PCR buffer with 3 mmol of MgCl<sub>2</sub>, and 400 µmol of each deoxyribonucleotide triphosphate (dNTP); 37.5 pmol of each primer; 5 µl of DNA template; and molecular biology grade water to adjust the reaction mixture to a final volume of 50 µl. A negative template

consisting of molecular biology grade water was utilized as a control for all amplification reactions. After initial denaturation at 95°C for 5 min, 35 amplification cycles were performed, each consisting of 30 sec at 94°C, 30 sec at 60°C, and 90 sec at 72°C, with a final extension at 72°C for 10 min. The PCR products were resolved by electrophoresis (100 V, 30 min), using agarose gels that were prestained with ethidium bromide and subsequently visualized with ultraviolet light.

The PCR products were purified using a commercial kit (QIAquick® PCR Purification Kit, Qiagen Inc., Valencia, CA) and automated sequencing was performed at a university laboratory (Georgia Genomics Facility, University of Georgia, Athens, GA) or a commercial laboratory (MACROGEN USA, Rockville, MD). The forward and reverse primers used for standard PCR were utilized for all sequencing reactions except for the bobcat ITS1 amplicons. For these sequencing reactions, an additional reverse primer was designed to anneal further downstream than the amplification primer, providing higher quality sequencing results. The sequence for this alternate ITS1 reverse primer was 5' TTC GCA GAA GTC TGC AAG TC 3'.

Sequences and chromatogram data were carefully analyzed and forward and reverse sequence data were assembled and aligned using Geneious Pro 4.6.5 software (Biomatters Ltd.; Auckland, NZ) to identify any polymorphisms within the ITS regions of the amplicons. ITS1 and ITS2 sequence data obtained from *C. felis*-infected asymptomatic domestic cats were compared to those from obtained from bobcats and to *C. felis* ITS sequences previously reported from clinically ill domestic cats with cytauxzoonosis (Brown et al., 2009a) to determine if the presence of particular parasite sequences varied in pathogenicity or by host species.

## Results

Whole blood samples from 89 domestic cats (43 from Arkansas, 46 from Georgia) and tissue samples from 133 bobcats (73 from Georgia, 6 from Arkansas, and 54 from Florida) were included in the study. Using real-time PCR analysis targeting a portion of the parasite 18S rRNA gene specific for *C. felis*, 27/89 (30.3%) blood samples from high-risk domestic cats (Table 6.1) and tissue samples from 34/133 (25.6%) bobcats were identified as positive for *C. felis* infection.

Conventional PCR analysis and amplicon sequencing performed on all real-time PCR-positive samples resulted in unambiguous *C. felis* ITS1 and ITS2 sequence data for 25/27 (92.6%) infected domestic cats. Within the 458-bp ITS1 region of the *C. felis* genome, all sequences were identical except for one sample with 4 single nucleotide polymorphisms (SNPs) and a single nucleotide insertion. Within the 265-bp ITS2 region of the *C. felis* genome, there was 1 SNP. Taken together, the *C. felis* ITS1 and ITS2 sequence data defined a total of 3 different genetically distinct parasite populations present within asymptotically infected domestic cats (Table 6.2).

Unambiguous *C. felis* ITS1 and ITS2 sequence data was obtained for 25/34 (73.5%) infected bobcats. Within the 458-bp ITS1 region of the *C. felis* genome, a total of 5 SNPs and a single nucleotide insertion were identified. Within the 265-bp ITS2 region of the *C. felis* genome, there were a total of 3 SNPs. Taken together, the *C. felis* sequence data from bobcat samples defined a total of 11 different ITS sequence types (Table 6.3). For several of the SNPs within ITS1 and the majority of the SNPs within ITS2, the chromatogram data revealed the incorporation of 2 nucleotides at a single position. Polymorphic sites were designated with IUPAC codes.

The *C. felis* ITS1-ITS2 sequences derived from infected asymptomatic domestic cats were identical to those previously reported from clinically ill infected cats (Brown et al., 2009a). The *C. felis* ITSa genotype (GenBank accession nos. EU450802 and EU450804) was detected most commonly in both asymptotically infected cats and clinically ill cats that survived infection, and for both cat populations, the majority of the parasite infections were acquired in Arkansas. The second most common genotype, designated as ITSb (GenBank accession nos. EU450802 and EU450805) was also previously reported in clinically ill domestic cats, the majority of which were fatal infections and, for both cat populations, infection was far more commonly detected in samples from Georgia. The third genotype, designated as genotype ITSc (GenBank accession nos. EU450803 and EU450804) and present in a single asymptotically infected cat from Arkansas, was previously reported from clinically ill domestic cats from Arkansas, all of which died.

The most common *C. felis* ITSa sequence reported from asymptotically infected domestic cats was also present in the infected bobcats. In addition, there were two other ITS sequence types reported in bobcats (ITSf, GenBank accession nos. EU450802 and FJ536421; ITSi, GenBank accession nos. EU450802 and FJ536419) that had been previously reported from histologic tissues of *C. felis*-infected domestic cats presented for necropsy (Brown et al., 2009b). The most common *C. felis* ITS sequence type present in bobcat samples, as well as an additional 7 sequences present infrequently in bobcats, had not been previously reported from infected domestic cats.

## **Discussion**

A real-time PCR survey of blood samples from domestic cats considered at high risk for *C. felis* exposure detected a significant population of asymptotically infected animals. The

cats included in the survey were selected to increase the likelihood of obtaining parasite samples for genetic comparisons. As cats surviving clinical cytauxzoonosis may remain persistently infected, blood samples from survivors were included when available. Eleven of 14 (78.6%) cats remained *C. felis* positive after clinical treatment for acute cytauxzoonosis. One of these cats has presumably remained infected for greater than 4 years because it has been kept strictly indoors since the initial illness. Domestic cats that co-habitated with those previously diagnosed with infection were included in the study, as they would likely share exposure to the same environment and infected ticks. Having never exhibited clinical signs consistent with infection, it is surprising that 6 of 14 (15%) contact cats were real-time PCR positive for *C. felis*. As Reichard et al. (2009) has shown, cat-to-cat transmission of infection may be possible via a tick vector, which may also contribute to the infection rate of these contact cats. Finally, feral cats from endemic regions identified in our previous work were included in the sample population as they would have a higher likelihood of tick exposure due to their outdoor lifestyle and lack of preventative treatment with acaricides. The previous study by Haber et al. (2007) of free-roaming cats in Tennessee, North Carolina, and Florida detected a *C. felis* infection prevalence of 0.3%. Among feral cats from Arkansas and Georgia included in the current study, 10 of 35 (28.6%) were *C. felis* positive. Geographic differences in the feral cat populations tested may account for higher prevalence of *C. felis* infection among feral cats in the current study. In addition, increased detection may be attributable to a difference in sensitivity of the different PCR testing methods (real-time PCR used in the current study versus conventional PCR used previously).

The overall prevalence of *C. felis* in high-risk cats from Arkansas (41.9%) was twice that of similar cats tested from Georgia (19.6%). These results are not surprising, given that our

previous study (Brown et al., 2009a) identified a far greater number of cats from Arkansas surviving acute cytauxzoonosis compared with those infected in Georgia. Although host factors cannot be excluded, geographic disparity in the number of asymptomatic *C. felis*-infected cats likely results from the survival of cats infected with less pathogenic strains of the parasite. If these surviving cats remain persistently infected, further transmission of these selected parasite strains may occur in a more localized area. Conversely, infections with more highly pathogenic strains are likely to result in host fatality. These highly pathogenic *C. felis* genotypes would not be detected among asymptomatic cats and would be unavailable for further transmission among the domestic felid population.

The degree of ITS sequence polymorphism seen in the current set of *C. felis* samples is comparable to that detected among all *C. felis* ITS1 and ITS2 sequences available in GenBank. As observed in previous studies (Brown et al., 2009a,b), detailed review of the sequencing chromatograms revealed the incorporation of 2 nucleotides at a single position within ITS1 or ITS2 for many bobcat samples, though this position varied among samples. This finding is interpreted to reflect coinfection of the host with multiple *C. felis* genotypes, a finding not unexpected in the wild reservoir host which is suspected to be exposed to various parasite strains over a wider geographic area. Alternatively, the mixed nucleotide loci may reflect polymorphisms in multiple copies of the rRNA genes that may exist in the *C. felis* genome.

The *C. felis* ITS sequence types detected in the asymptomatic cats in the current study were identical to genotypes previously detected in clinically ill domestic cats. An earlier study (Brown et al., 2009a) demonstrated a strong association between particular *C. felis* ITS1-ITS2 genotypes and survival of domestic cats after acute infection, suggesting that ITS sequencing may be useful to define markers that are able to detect more or less pathogenic strains among *C.*

*felis*–infected cats. While the most common *C. felis* ITS sequence detected in the asymptomatic cats was that also reported previously in cats surviving acute infection, the second most common ITS genotype in the asymptomatic cats had been previously detected in predominantly fatal infections. In both studies, however, the geographic distribution of the ITS genotypes varied greatly among Arkansas and Georgia samples, as might be expected in the development of different parasite strains. While suitable for examining genetic diversity among *C. felis* populations, the presence of common ITS sequences in asymptomatic, clinically ill, and fatally infected domestic cats demonstrates that ITS1 and ITS2 rDNA does not appear to be useful for discriminating among variably pathogenic strains of the parasite, at least when comparing geographically distributed isolates.

Increased *C. felis* ITS sequence diversity was present in the infected bobcat samples, as might be expected within the wild reservoir host presumed to be exposed to variable parasite strains over a wider geographic area. The most common *C. felis* ITS sequence detected in bobcats had not been previously described in infected domestic cats. While this finding may reflect the identification of a strain that is incapable of infecting domestic cats, it is more likely a reflection of the geographic variability of the sample populations, as this sequence was detected only in bobcats from a particular trapping site in northern Florida from which we had no domestic cat samples.

At present, only the ribosomal genes have been identified and sequenced for *C. felis*, limiting the areas of the genome which can be investigated for sequence variability. Ultimately, identification of genetic variability in genes affecting pathogenicity of the parasite would best test the hypothesis that genetically distinct strains of *C. felis* are capable of establishing persistent infections in the domestic cat and help better define the epidemiology of infection.

Asymptomatically infected cats that are carriers of the parasite may serve as reservoirs for infection and thus increase the risk of exposure for other domestic cats. Additional studies are warranted to evaluate the potential for these naturally infected cats to serve as a reservoir for further disease transmission. Better understanding of the epidemiology of *C. felis* infection will enhance the ability to prevent this highly fatal infectious disease.

### **Conclusion**

The current study identified a population of domestic cats that were asymptomatically infected with *C. felis*. While *C. felis* ITS sequence diversity existed among infected cats and bobcats, utilization of this non-coding region for detection of parasite strain variability by host species and pathogenicity did not appear to be useful. Establishing that there was a population of clinically healthy domestic cats that were asymptomatically, and presumably persistently, infected with *C. felis* has important implications in feline health. Further evaluation of the potential for these asymptomatically infected cats to serve as a new reservoir for *C. felis* transmission is warranted.

### **Conflict of interest**

The authors have no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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### References

- Blouin, E.F., Kocan, A.A., Glenn, B.L., Kocan, K.M., Hair, J.A., 1984. Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). J. Wildl. Dis. 20, 241-242.
- Brown, H.M., Latimer, K.S., Erikson, L.E., Cashwell, M.E., Britt, J.O. Peterson, D.S., 2008. Detection of persistent *Cytauxzoon felis* infection by polymerase chain reaction in three asymptomatic domestic cats. J. Vet. Diagn. Invest. 20, 485-488.
- Brown, H.M., Berghaus, R.D., Latimer, K.S., Britt, J.O., Rakich, P.M., Peterson, D.S., 2009. Genetic variability of *Cytauxzoon felis* from 88 infected domestic cats in Arkansas and Georgia. J. Vet. Diagn. Invest. 21, 59-63.
- Brown, H.M., Modaresi, S.M., Cook, J.L., Latimer, K.S., Peterson, D.S., 2009. Genetic variability of archived *Cytauxzoon felis* histologic specimens from domestic cats in Georgia, 1995–2007. J. Vet. Diagn. Invest. 21, 493-498.
- Glenn, B.L., Kocan, A.A., Blouin, E.F., 1983. Cytauxzoonosis in bobcats. J. Am. Vet. Med. Assoc. 183, 1155-1158.
- Greene, C.E., Latimer, K., Hopper, E., Shoeffler, G., Lower, K., Cullens, F., 1999. Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. J. Am. Vet. Med. Assoc. 215, 497-500.
- Greene, C.E., Meinkoth, J., Kocan, A.A. 2006. Cytauxzoonosis. In: Greene CE (Ed), Infectious Diseases of the Dog and Cat, 3rd ed. Saunders Elsevier, St. Louis, MO, pp. 722-733.

- Haber, M.D., Tucker, M.D., Marr, H.S., Levy, J.K., Burgess, J., Lappin, M.R., Birkenheuer, A.J., 2007. The detection of *Cytauxzoon felis* in apparently healthy free-roaming cats in the USA. *Vet. Parasitol.* 146, 316-320.
- Hoover, J.P., Walker, D.B., Hedges, J.D., 1994. Cytauxzoonosis in cats: eight cases (1985-1992). *J. Am. Vet. Med. Assoc.* 203, 455-460.
- Kier, A.B., Wagner, J.E., Kinden, D.A., 1987. The pathology of experimental cytauxzoonosis. *J. Comp. Pathol.* 97, 415-432.
- Meinkoth, J., Kocan, A.A., Whitworth, L., Murphy, G., Fox, J.C., Woods, J.P., 2000. Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997-1998). *J. Vet. Intern. Med.* 14, 521-525.
- Motzel, S.L., Wagner, J.E., 1990. Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. *Vet. Parasitol.* 35, 131-138.
- Reichard, M.V., Meinkoth, J.H., Edwards, A.C., Snider, T.A., Kocan, K.M., Blouin, E.F., Little, S.E., 2009. Transmission of *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum*. *Vet. Parasitol.* 161, 110-115.
- Rotstein, D.S., Taylor, S.K., Harvey, J.W., Bean, J., 1999. Hematologic effects of cytauxzoonosis in Florida panthers and Texas cougars in Florida. *J. Wildl. Dis.* 35, 613-607.
- Wagner, J.E., 1976. A fatal cytauxzoonosis-like disease in cats. *J. Am. Vet. Med. Assoc.* 168, 585-588.
- Walker, D.B., Cowell, R.L., 1995. Survival of a domestic cat with naturally acquired cytauxzoonosis. *J. Am. Vet. Med. Assoc.* 206, 1363-1365.
- Wightman, S.R., Kier, A.B., Wagner, J.E., 1977. Feline cytauxzoonosis: Clinical features of a newly described blood parasite disease. *Feline Pract.* 7, 23-26.

Table 6.1. Real-time PCR results for high-risk cats tested for *Cytauxzoon felis*, including type of parasite exposure and state of origin.

Real-time PCR	<i>n</i>	Type of <i>C. felis</i> exposure			State of origin	
		Survivor	Contact	Feral	Arkansas	Georgia
Positive	27	11	6	10	18	9
Negative	62	3	34	25	25	37
Total	89	14	40	35	43	46

Table 6.2. *Cytauxzoon felis* internal transcribed spacer region (ITS1 and ITS2) sequences obtained from asymptotically infected domestic cats characterized by nucleotide variation, frequency, and state of origin of *C. felis* infection.<sup>a</sup>

Sequence	ITS1					ITS2	<i>n</i>	State of origin		GenBank accession # (ITS1, ITS2)
	162	337	344	415	444	180		AR	GA	
ITSa	A	-	T	T	A	G	16	15	1	EU450802, EU450804
ITSb	A	-	T	T	A	T	8	1	7	EU450802, EU450805
ITSc	G	A ins	C	C	G	G	1	1	0	EU450803, EU450804

<sup>a</sup> ins = insertion; - = no insertion.

Table 6.3. *Cytauxzoon felis* internal transcribed spacer region (ITS1 and ITS2) sequences obtained from infected bobcats characterized by nucleotide variation, frequency, and state of origin of *C. felis* infection.<sup>a</sup>

Sequence	ITS1						ITS2			<i>n</i>	State of origin			GenBank accession # (ITS1, ITS2)
	338	344	397	415	444	457	180	204	243		AR	GA	FL	
ITSa	G	T	T	T	A	-	G	G	A	5	2	0	3	EU450802, EU450804
ITSd	A	T	T	T	A	-	T	G	A	1	0	0	1	GU581166, EU450805
ITSe	G	C	C	T	G	C ins	G	A	A	1	0	0	1	GU581167, GU581170
ITSf	G	T	T	T	A	-	G/T <sup>b</sup>	G	A/G <sup>b</sup>	1	0	1	0	EU450802, FJ536421
ITSg	G	C	C	T	G	C ins	G	A/G <sup>b</sup>	A	8	0	1	7	GU581167, GU581171
ITSh	G	T	T	T	A	-	G	A/G <sup>b</sup>	A/G <sup>b</sup>	1	0	1	0	EU450802, GU581172
ITSi	G	T	T	T	A	-	G	G	A/G <sup>b</sup>	3	0	1	2	EU450802, FJ536419
ITSj	A/G <sup>b</sup>	T	C/T <sup>b</sup>	C/T <sup>b</sup>	A	-	G	G	A	2	0	0	2	FJ536425, EU450804
ITSk	A/G <sup>b</sup>	T	C/T <sup>b</sup>	C/T <sup>b</sup>	A	-	G	A/G <sup>b</sup>	A	1	0	0	1	FJ536425, GU581171
ITSl	G	C	C/T <sup>b</sup>	T	A/G <sup>b</sup>	C ins	G	A/G <sup>b</sup>	A	1	0	0	1	GU581168, GU581171
ITSm	G	C/T <sup>b</sup>	C/T <sup>b</sup>	T	G	C ins	G	A/G <sup>b</sup>	A	1	0	0	1	GU581169, GU581171

<sup>a</sup> ins = insertion; - = no insertion.

<sup>b</sup> Chromatograms depict the incorporation of 2 nucleotides at this position.

## CHAPTER 7

### SUMMARY AND CONCLUSIONS

The principle objective of the studies presented herein was to determine if there existed genetic evidence for strain variability of *Cytauxzoon felis* in North American felids. In order to do so, an extensive sampling network was created to obtain blood and tissue samples from *C. felis*-infected domestic cats and bobcats; included were both current and historical samples, samples from diverse geographic areas, and samples from cats with both fatal and non-fatal infections. Additional objectives were to determine if there existed a population of previously unidentified, asymptotically infected domestic cats and if the *C. felis* genotypes identified in the asymptomatic cats were genetically unique compared to those detected in the clinically ill cats and the bobcats. These objectives were cumulatively met by PCR amplification and nucleotide sequencing of the *C. felis* ITS regions as a genetic marker, allowing for sequence comparisons among and between the different groups sampled.

The first investigation evaluating sequence variability among clinically ill, infected domestic cats identified unique ITS genotypes that varied geographically between Arkansas and Georgia. Additionally, there was a strong association between particular ITS genotypes and outcome of infection (fatal versus non-fatal). The findings support the hypothesis that there are genetically distinct populations of *C. felis*, which may account for the variability in clinical outcome of domestic cat infections. The identification of unique genotypes from distinct geographic areas might be expected in the development of different parasite strains, as well.

The subsequent identification of numerous *C. felis* ITS sequences from historical specimens from fatally infected domestic cats in Georgia lends further support to the existence of genetically distinct *C. felis* populations. The difference in the ITS genotypes that were most commonly detected suggests that changes have occurred in the *C. felis* population over time. The documentation of genetically distinct *C. felis* populations in historical samples, together with data from contemporary samples, demonstrates a diverse population structure for *C. felis*. Such retrospective data on genetic variability will provide an historic context for future studies of *C. felis* genotype frequencies.

After confirming with repeated PCR testing the persistence of *C. felis* infection in 3 domestic cats, the prevalence of asymptomatic *C. felis* infection among high risk domestic cats was investigated. The domestic cats tested included cats that survived clinical cytauxzoonosis, domestic cats that co-habitated with those previously diagnosed with infection, and feral cats from endemic regions. Remarkably, 30% of these cats from Arkansas and Georgia were real-time PCR positive for occult *C. felis* infection. Although host factors cannot be excluded, geographic disparity in the number of asymptotically *C. felis*-infected cats, with more detected in Arkansas, suggests survival resulted from infection with less pathogenic strains of the parasite. The ability of surviving *C. felis*-infected domestic cats to serve as an additional reservoir host for the parasite would increase the risk of exposure for other domestic cats and alter the currently accepted paradigm of *C. felis* transmission from bobcats (reservoir host) to domestic cats. Genetically distinct parasite populations were identified within the asymptotically infected domestic cat samples; however, they were identical to those detected from clinically ill infected cats and were also present in infected bobcats.

This research provides an extensive description of the population structure of *C. felis* as measured by sequence polymorphisms in the ribosomal ITS regions. It does not appear, however, that these loci will be useful for distinguishing *C. felis* strains based upon pathogenicity to the domestic cat. The next important step in *C. felis* research will be to identify additional genetic markers, ideally those more closely linked to pathogenicity of the organism, in order to determine whether strain variability plays a role in disease transmission and virulence in cats. The paucity of sequence data from this pathogen is a limiting factor when considering future studies. While whole genome sequences are available from closely related organisms such as *Theileria* spp. and *Babesia* spp., nothing is known of *C. felis* genome sequences and organization outside of the rRNA locus.

Another important finding of this work is the detection of *C. felis* infection in previously unidentified, asymptotically infected cats. Further investigation into the role these persistently infected cats play in disease transmission in endemic areas is warranted, and more in-depth studies of the prevalence of *C. felis* in domestic cat populations is necessary to better define this potential reservoir. Better understanding of the epidemiology of *C. felis* infection will enhance the ability to prevent or treat this highly fatal infectious disease.