Feline cytauxzoonosis:
Reconsideration of pathophysiologic mechanisms

by

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Abstract

Feline cytauxzoonosis is a frequently fatal disease of domestic cats in the United States caused by the tick-transmitted protozoal hemoparasite *Cytauxzoon felis*. Feline cytauxzoonosis was first identified in southwestern Missouri in the early 1970's and has since expanded into more northern regions of the United States. Clinical symptoms and death are largely attributed to *C. felis* schizogony within vascular-associated mononuclear cells. Yet, in-depth investigation of the pathophysiologic mechanisms of feline cytauxzoonosis have been hindered by ethical concerns with experimental infection and an inability to induce in vitro *C. felis* schizogony. Early reports attributed anemia with associated hyperbilirubinemia to extravascular hemolysis and death to vascular occlusion by *C. felis* schizont-laden macrophages, ischemic injury, and disseminated intravascular coagulation. Furthermore, some publications suggest a link between infecting *C. felis* strain with patient morbidity and mortality. Recently, immunohistochemical and serologic evaluation of localized and systemic pro-inflammatory cytokine responses in acute feline cytauxzoonosis prompted nuanced reconsideration of pathophysiologic mechanisms for illness and death in affected cats. Here, serum biochemical abnormalities were utilized as a method of assessing pathophysiologic mechanisms in acute feline cytauxzoonosis. In addition, several projects aimed at understanding the pathophysiologic mechanisms of disease in feline cytauxzoonosis are proposed.

Assessment of hematologic and serum biochemical abnormalities is routinely employed in veterinary medicine. Alterations in concentrations of individual analytes and patterns involving several analytes provide clinicians guidance on underlying pathophysiologic mechanisms. Thorough descriptions and assessments of serum biochemical abnormalities in domestic cats infected with *C. felis* are lacking, or sometimes contradictory, in published

literature. Here, serum biochemical abnormalities were retrospectively assessed in 28 naturally infected domestic cats to determine common abnormalities and patterns of acute feline cytauxzoonosis. Results were then compared to those of two feline disorders which may have similar clinical presentations in domestic cats, hepatic lipidosis and cholangiohepatitis.

Acute feline cytauxzoonosis was characterized by nonregenerative anemia (20/28, 71.4%), leukopenia (23/28, 82%), thrombocytopenia (23/23, 100%), hyperbilirubinemia (27/28, 96.6%), hypoalbuminemia (26/28, 93%), reduced (18/28, 64%) or low normal (10/28, 36%) serum ALP activity, and hyponatremia (23/28, 82%). No correlation between severity of anemia and magnitude of hyperbilirubinemia in feline cytauxzoonosis patients was identified. Feline cytauxzoonosis was associated with statistically significant ($p<0.05$) decreases in hemoglobin, total leukocyte, and platelet concentrations when compared to hepatic lipidosis and cholangiohepatitis patients. Reduced ALP activity was unique to cats with feline cytauxzoonosis and the decreased serum ALP activity was significantly lower ($p<0.05$) than in hepatic lipidosis and cholangiohepatitis. Furthermore, feline cytauxzoonosis patients had higher total bilirubin concentrations ($p<0.05$) than those with hepatic lipidosis and cholangiohepatitis.

The overall pattern of hematologic and serum biochemical profile changes in cats with feline cytauxzoonosis is similar to those reported in feline bacterial sepsis, including nonregenerative anemia, hyperbilirubinemia, and reduced serum ALP activity. As feline cytauxzoonosis is simply a form or protozoal sepsis with similar pro-inflammatory cytokine responses as those reported in feline bacterial sepsis, these similarities are not unexpected. However, the decoupling of the degree of anemia and magnitude of hyperbilirubinemia in feline cytauxzoonosis patients suggests that extravascular hemolysis, while present, is not likely the only pathophysiologic mechanism for development of hyperbilirubinemia in septic cats. Reduced

hepatocellular bile excretion mediated by pro-inflammatory cytokines, a process known as functional or inflammation-induced cholestasis, may be occurring in septic cats. Previous publications have excluded functional cholestasis as a possibility due to the absence of increased serum ALP activity in cats, as observed in humans and dogs. Furthermore, reduced serum ALP activity is often ignored in serum biochemical profile panels as "clinically insignificant". Yet, the repeatability of this abnormality in both bacterial and protozoal sepsis warrants reconsideration of the diagnostic utility of low serum ALP activity and the expectation that cats should have increased serum ALP activity with functional cholestasis.

Future research stemming from questions regarding the pathophysiologic mechanisms of feline cytauxzoonosis based on serum biochemical abnormalities are proposed. The first proposal is an investigation into histologic and immunohistochemical evidence of functional cholestasis in formalin-fixed, paraffin-embedded liver sections of cats afflicted with feline cytauxzoonosis. This proposal will use immunohistochemical labeling for the pro-inflammatory cytokines, IL-6, TNF-α, and IL-1β, known to induce functional cholestasis in cats as well as the two transport proteins these cytokines down-regulate, multidrug resistant protein 2 (MRP2) and bile salt export pump (BSEP). The second proposed project will look at the hematologic and serum biochemical profiles of a larger cohort of feline patients with bacterial, protozoal, fungal, and viral sepsis and non-infectious systemic inflammatory response syndrome to determine if there are similar serum biochemical patterns between all causes of systemic inflammation in feline patients. Finally, evaluation of the association between *C. felis* strain and clinical outcomes is proposed comparing *C. felis* strains identified in formalin-fixed tissues of deceased domestic cats to those of a large population of recently identified *C. felis* carriers in Kansas.

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Chapter 1 - Feline cytauxzoonosis: A review

Introduction

In the United States, feline cytauxzoonosis is a rapidly progressive, often-fatal disease of domestic cats (*Felis catus*), and rarely wild felids, caused by the tick-transmitted protozoal hemoparasite, *Cytauxzoon felis*. Since identified in 1976 in southwestern Missouri, USA, the geographic distribution of *C. felis* in the U.S. has expanded in a northeasterly direction now encompassing about one third of the contiguous United States (Figure 1).[8,10-13] Despite a nearly 100% mortality rate[14], published reports of North American cats surviving natural infection, sometimes without treatment, sparked identification of molecularly unique *C. felis* strains of potentially varying pathogenicity.[4,15,16] Surviving cats become persistent *C. felis* carriers maintaining a low number of circulating intra-erythrocytic organisms and serving as reservoirs for transmission.[4] As the incidence of feline cytauxzoonosis increases in nonendemic areas, enhancing clinical recognition of disease, understanding pathophysiologic mechanisms, evaluating potential prognostic indicators, and developing new treatment strategies targeting the organism or host responses to infection will be needed.

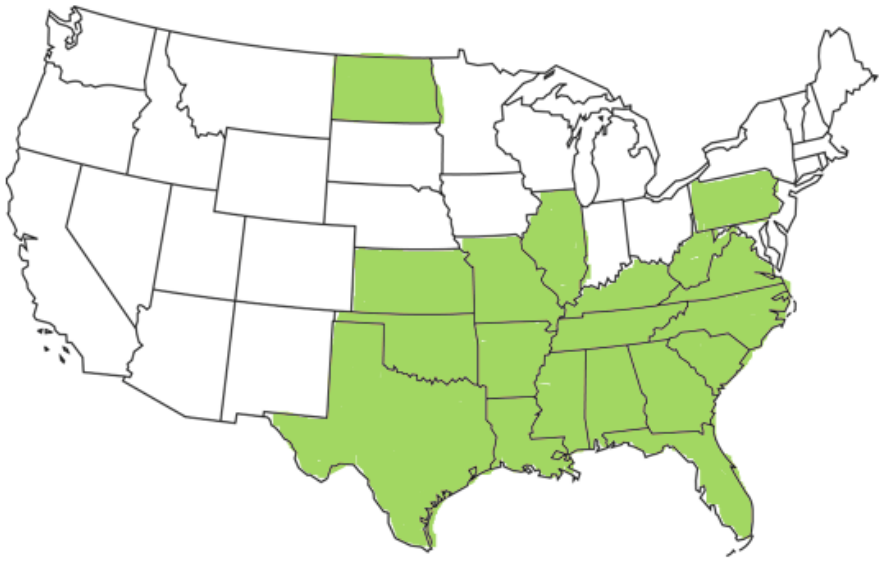


Figure 1: Geographic distribution of *C. felis* in the United States based on molecular sequences or microscopically observed organisms detected in domestic cats, reservoir hosts, or tick vectors.

Cytauxzoon felis, the causative agent

Taxonomy

Cytauxzoon. felis, the causative agent of feline cytauxzoonosis, has a unique classification and taxonomic history as the only well-characterized protozoal organism within the genus *Cytauxzoon*. *Cytauxzoon* organisms are closely related to *Theileria* and *Babesia* species with similar morphologies and life cycles[17]. These organisms belong to the order *Piroplasmida* of the phylum *Apicomplexa*. Hemoparasites in the order *Piroplasmida* are characterized by their morphology, invasion of preferred host cell types, host species, and, often, a reliance on tick-transmission. Considerable debate remains on the taxonomic classification of the genus *Cytauxzoon.* However, recent mitochondrial analysis of organisms within the order *Piroplasmida* suggests maintaining the genus *Cytauxzoon* while reconsidering classification from a distinct clade into one shared with the genus *Theileria*, excepting *Theileria equi* (Figure 2).[17] As comparative molecular analysis continues within this order, reclassifications may still occur.

New species within the *Cytauxzoon* genus have recently been described or are emerging. In 2005, microscopic visualization of piroplasms, 18s rRNA gene sequencing, and phylogentic analysis resulted in the discovery of *C. manul* in Mongolian Pallas' cats.[18] In the last decade, molecular sequences similar to *C. felis* have been identified in European felids with a few case reports of a fatal cytauxzoonosis.[19-26] Until recently,these organisms were classified as *Cytauxzoon sp.*, closely related, but not identical, to *C. felis*. *Cytauxzoon sp*. was recently renamed *Cytauxzoon europaeus*.[27] In at least one report, transmission via blood transfusion occurred.[25] Research on this emerging disease of European felids is underway. Further discussion of other *Cytauxzoon* species is beyond the scope of this review.

History

The genus *Cytauxzoon* was first introduced in 1948 by Neitz et al. after identifying *Theileria*-like organisms in an African gray duiker (*Sylvicapra grimmia*), a small antelope-like ungulate.[28] Histologic evaluation of affected tissues revealed protozoan organisms within vascular associated histiocytes, rather than lymphocytes expected of *Theleria spp*., prompting creation of a unique species with a distinct life cycle. Similar organisms were later identified in other African ungulates including a greater kudu (*Tragelaphus strepsiceros*)[29], eland

(*Taurotragus oryx pattersonianunus*)[30], giraffe (*Giraffa camelopardalis*)[31], roan antelope (*Hippotragus equines*)[32], and sable antelope (*Hippotragus niger*)[32]. Molecular analysis has since reclassified these infectious protozoans of south African ungulates into the genus *Theileria*.[33,34] This reclassification of ungulate *Cytauxzoon* organisms has left only those *Cytauxzoon* organisms selectively infecting felids within the genus.

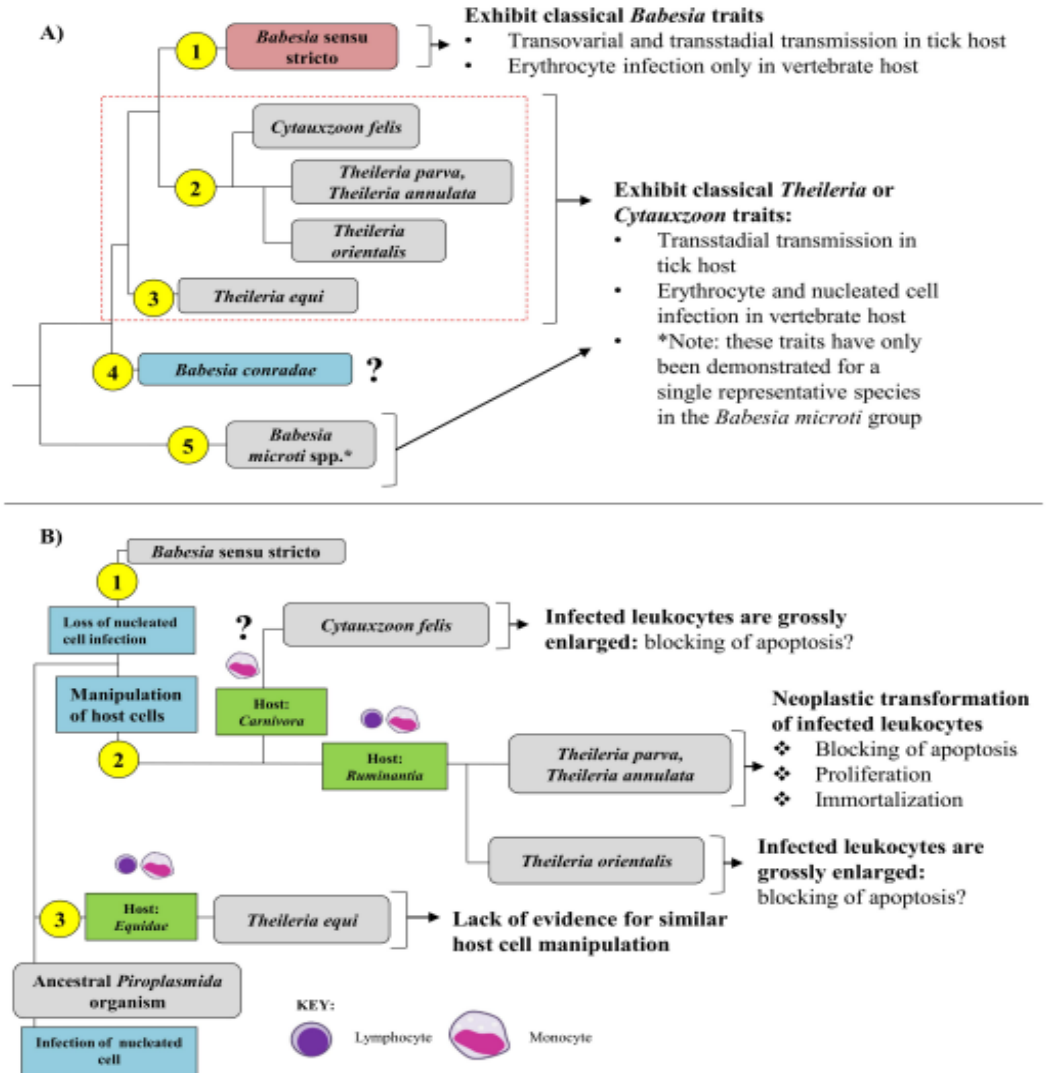


Figure 2: Proposed taxonomic classification of the genus Cytauxzoon within the order Piroplasmida by Schreeg et al. correlating biologic behavior with mitochondrial and 18s sequences.[17] (Image reproduced with open access permission.)

In 1976, Wagner published the first descriptions of fatal *Cytauxzoon felis* infection in four domestic cats in southwestern Missouri, USA.[8] Lesions were similar to those described by Neitz et al. in 1948. Protoazoal organisms within erythrocytes and vascular-associated histiocytes of the liver, spleen, lungs, and lymph nodes of affected cats were microscopically evident. Subsequently, bobcats (*Lynx rufus*)[35], and potentially jaguars (*Panthera onca*)[36], have been identified as the natural reservoir host of *C. felis* with fatal infection reported in North and South American domestic cats (*Felis catus*)[37], South American lions (*Panthera leo*)[38], few North American bobcats (*Lynx rufus*)[39,40], a captive-reared white tiger (*Panthera tigris*)[41] in Florida, USA, and a captive-reared jaguar (*Panthera onca)*[42] in Brazil.

Life Cycle

The *C. felis* lifecycle is complex, involving both sexual and asexual reproduction in a competent arthropod vector and asexual reproduction in the felid reservoir or aberrant host (Figure 3). In the United States, competent arthropod vectors are the brown dog tick (*Dermacentor variabilis*) and the Lone Star tick (*Amblyomma americanum*).[43] The bobcat (*Lynx rufus*) is considered the primary reservoir host in the United States. Florida panthers (*Puma concolor coryii*), Texas cougars (*Puma concolor stanleyana),* and persistently infected *C. felis* carrier domestic cats (*Felis catus*) may also be reservoir hosts. Domestic cats are dead-end, or aberrant, hosts with frequently fatal infections. *In vitro* studies of the *C. felis* lifecycle have proven difficult due to the inability to culture the organisms through schizogony in the tissue phase (discussed below). Thus, much of the knowledge regarding the *C. felis* lifecycle is extrapolated from histologic observations and studies of closely related *Theileria* organisms.[44]

Transmission of *C. felis* from the tick vector to the vertebrate felid host occurs during acquisition of a tick blood meal. *C. felis* sporozoites are released from tick salivary glands and phagocytized by host monocytes/macrophages. Within the monocyte/macrophage, sporozoites undergo schizogony, forming numerous intracytoplasmic schizonts. In contrast to *Theileria* sp., *C. felis* schizogony has been confirmed to occur in macrophages/monocytes rather than lymphocytes.[45] These *C. felis* schizont-laden macrophages line blood vessels throughout the vertebrate host body, known as the tissue phase of the *C. felis* lifecycle. Infected monocytes/macrophages rupture releasing merozoites to infect nearby intravascular erythrocytes, initiating the erythrocytic phase of the *C. felis* lifecycle. Intra-erythrocytic merozoites mature

into trophozoites or gametocytes. Within the erythrocyte, trophozoites may also undergo asexual merogony. Microscopically visible intra-erythrocytic forms are collectively termed piroplasms as merozoites, trophozoites, and gametocytes cannot be differentiated with standard light microscopy.

Intra-erythrocytic gametocytes are ingested during subsequent tick feedings of infected vertebrate hosts. Once in the tick gut lumen, gametocytes develop into macrogametes and microgametes and undergo sexual gamogony to form zygotes. These zygotes penetrate tick gut epithelium, undergo meiosis, and form haploid *C. felis* kinetes within the epithelial cells. Kinetes are released into the tick hemolymph and travel to the salivary glands where asexual sporogony occurs. These sporozoites are now ready for transmission to the next felid host.

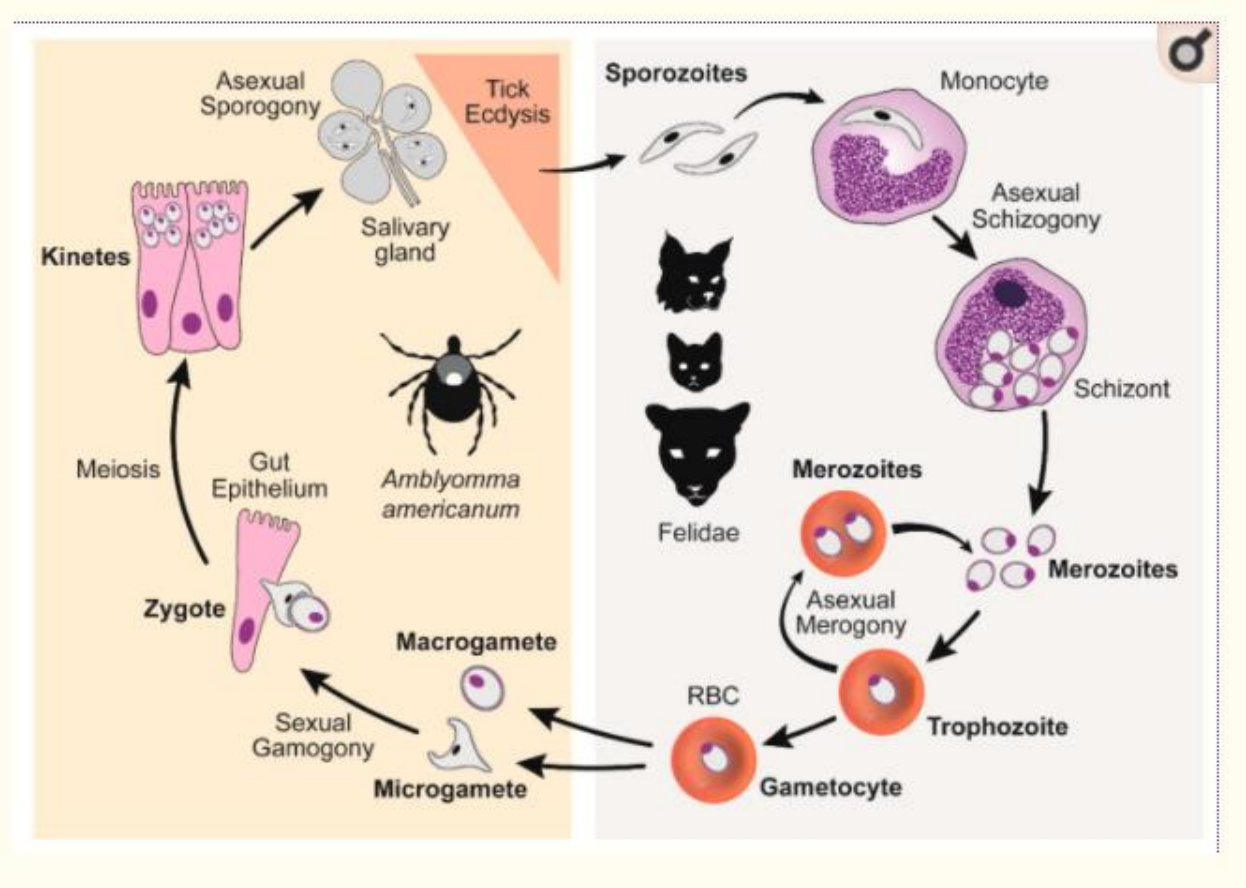


Figure 3 *C. felis* life cycle as represented by Wikander et al.[2] (Image reproduced with open access permission.)

North American C. felis strains

Initial experimental infections of cats indicated a near 100% mortality rate with only one survivor reported in approximately 500 experimentally infected cats.[14] In the two decades following these initial studies, only one case report of a cat surviving natural infection was published.[46] That changed in 2000 when Meinkoth et al. published a case series of 18 cats surviving natural infection with *C. felis*, some without treatment, in an enzootic area of northeastern Oklahoma, USA and northwestern Arkansas, USA.[4] Interestingly, these locations are in the same small geographical region as the first identified case of *C. felis* in 1973 (Figure 4).[8] Amplification and sequencing of nuclear small subunit ribosomal ribonucleic acid (NSS rRNA) gene revealed a 1730 bp product with >99% identity with NSS rRNA gene products of known *C. felis*, ruling out infection with a different protozoal species. Due to the sudden appearance of multiple survivors within a limited geographic range over a short 18-month period, emergence of a less pathogenic *C. felis* strain was postulated. Shortly afterward, numerous asymptomatic *C. felis* domestic feline carriers were identified in endemic areas including Arkansas, Georgia, Tennessee, and Florida, USA.[15,47]

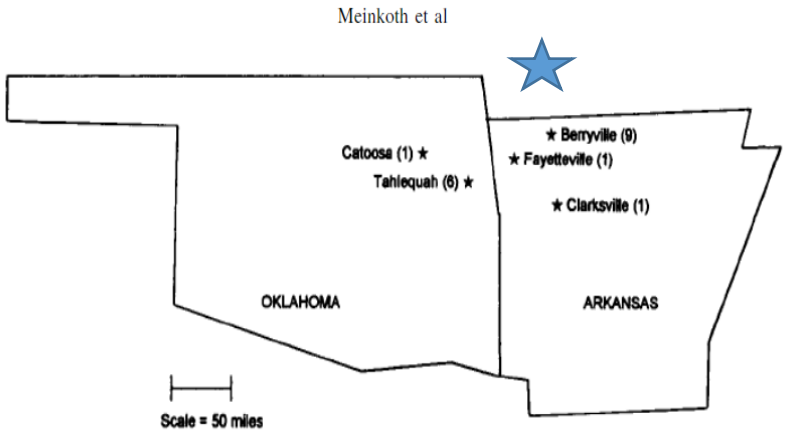


Figure 4: Geographic location of cats surviving natural *C. felis* infection in northwest Arkansas and Oklahoma.[4] Blue star = location of first reported *C. felis* cases by Wagner in 1976.[8] (Image reproduced with open access permission.)

As the NSS rRNA is a highly conserved gene encoding a functional RNA molecule, variations within the gene sequence are unlikely to identify unique *C. felis* strains. Thus, sequencing of the noncoding first and second internal transcribed spacer regions of rRNA operan

(ITS1 and ITS2, respectively) have been targeted for molecular identification of North American *C. felis* strains.[6] These noncoding regions are more susceptible to mutations which do not hinder RNA function making them prime targets for detections of intraspecies strains.[6] Sequencing of the ITS1 and ITS2 regions initially revealed unique genomic sequences among naturally infected cats from Arkansas and Georgia.[6,48] These gene sequence combinations in the ITS1 and ITS2 genes identified eleven unique *C. felis* strains dubbed ITSa-k.[13,48-50] The ITSa *C. felis* strain predominated in cats from Arkansas and was significantly associated with survival[6], supporting the hypothesis of Meinkoth et al. that a potentially less pathogenic *C. felis* strain was emerging in this geographic region.[6] A retrospective analysis of historical *C. felis* genotypes on formalin-fixed paraffin-embedded tissues from fatal *C. felis* infections in Georgia indicated no change in the predominate genotype over 12 year period; however, ITSa was not the predominate genotype of surviving cats in Georgia nor had the number of surviving cats in Georgia increased as much as in Arkansas.[6,48] Unfortunately, similar retrospective histologic specimen studies of changing strains in other *C. felis* endemic areas with reportedly high survival rates, such as Arkansas and Oklahoma, have not been reported to the author's knowledge.

Curiously, further associations of unique ITS1/ITS2 gene sequences with survival of cats have not been found in other geographic regions of the United States. For instance, ITS1/ITS2 genotypes isolated from asymptomatic *C. felis* carrier cats in Arkansas, USA are also identical to those known to cause fatal illness in both Arkansas and Georgia.[6,50,51] The ITSa *C. felis* genotype is also the most common genotype isolated in wild felid populations throughout other enzootic states, including Florida, Kentucky, Louisiana, Missouri, North Carolina, and Oklahoma, USA.[16,49,51] Yet, no further associations between the ITSa genotype and domestic cat survival have been published in local feline populations outside of Arkansas. Additional studies designed for evaluation of treatment efficacy identified no association between strain and survival or response to treatment; however, case numbers were quite low.[52] Further investigations regarding the association between *C. felis* strains and domestic cat survival, with and without treatment, in other enzootic regions are needed. Pathophysiologic interactions between domestic felids and *C. felis* populations in this fairly localized geographic region may be playing a role in the localized increase in survival rate. Because ITS1 and ITS2 gene sequences may be associated with, but not predictive of, survival in only a limited geographic region, ITS1 and ITS2 sequencing is not currently recommended as tool to predict survival in domestic cats.[6]

Expanding geographic range

Over the last two decades, the ecological range of *C. felis* has expanded both within the United States and into South America.[2,10-13,53-56] This expanding range seems to be the result of complex environmental, tick vector, reservoir host, and aberrant host interactions. Coinciding with this geographical spread, the reported incidence and prevalence of feline cytauxzoonosis has increased in both endemic and new geographic locations.[10,57,58]

Molecularly confirmed identification of *C. felis* in reservoir hosts and/or aberrant hosts combined with published reports of microscopically visible *C. felis* organisms in felids from Brazil and the United States are increasing (Figure 1).[10-13,36,38,42,56] *C. felis* genomic sequences are evident in bobcats (*Lynx rufus*) within geographic areas of the United States, such as North Dakota and Pennsylvania, without current published reports of fatal feline cytauxzoonosis.[12,13] As known tick vectors are present in these geographic regions[13], reports of fatal transmission to locally predisposed domestic cats seem inevitable. The absence of published data on *C. felis* in assumed naïve regions, such as the American Midwest and Northern Plains, does not preclude the presence of *C. felis* in these regions, particularly those areas with known tick vector populations.[54]

Published case reports of *C. felis* in Brazilian felids date to the early 2000's with recognition of *Cytauxzoon*-like organisms in a wild mountain lion.[59] Three years later, molecular confirmation and identification of *C. felis* in a captive-reared lion was reported.[38] Since then, *C. felis* has been identified in healthy and ill domestic cats (*Felis catus*) [37,53], jaguars (*Puma onca*)[36,42], ocelots (*Leopardus pardalis*)[60], and pumas/cougars (*Puma concolor*)[60]. The massive swathes of Central America between the United States and Brazil, currently the apparent epicenters of *C. felis* transmission, are likely harboring as yet unevaluated *C. felis* carriers within wild, and possibly domestic, felid populations. Known *C. felis* tick vectors have not yet been identified in South America.[61]

Interestingly, *C. felis* molecular sequences have also been reported in cats from eastern and western Asia.[62,63] A small cohort of 16 asymptomatic and 3 clinically ill cats were identified in Iran.[63] *C. felis* molecular sequences of the highly variable ITS1 and ITS2 sequences were identified in 21.5% of domestic and wild felids in Yunnan Province, China[62]; however, clinical reports of disease with microscopic confirmation of organisms are lacking from this region. As

in Brazil, tick species with a known capacity for *C. felis* transmission have not yet been identified in eastern or western Asia.[64] As molecular characterization of *C. felis* and these identified strains continues, these organisms may be reclassified.

Evidence linking climate change, rising ambient temperatures, and expanding ecological ranges of tick vectors in North America is ample.[54] Expansion of tick vector ranges relies not only on a favorable climate, but also on availability of preferred host species. The two known tick vectors of *C. felis*, the American dog tick (*Dermacentor variabilis*) and the lone star tick (*Amblyomma americanum*), are susceptible to range expansion. Currently, *D. variabilis* is found throughout the eastern and midwestern United States extending from the Gulf of Mexico to New England. Predictive models indicate future expansion throughout New England and into Canada.[65] More troubling is the already expanding range of *A. americanum*, considered to be the primary vector for *C. felis* transmission.[12,13,54] The northern borders of *A. americanum* territories once ended at the Ohio River Valley and southern New Jersey, but now they extend from just south of the eastern USA-Canada border to northern Michigan, New England, Pennsylvania, and New York (Figure 5).[54] Furthermore, genetically distinct subpopulations of *A. americanum* ticks exist and this may have implications for *C. felis* transmission.[54] With the movement of tick

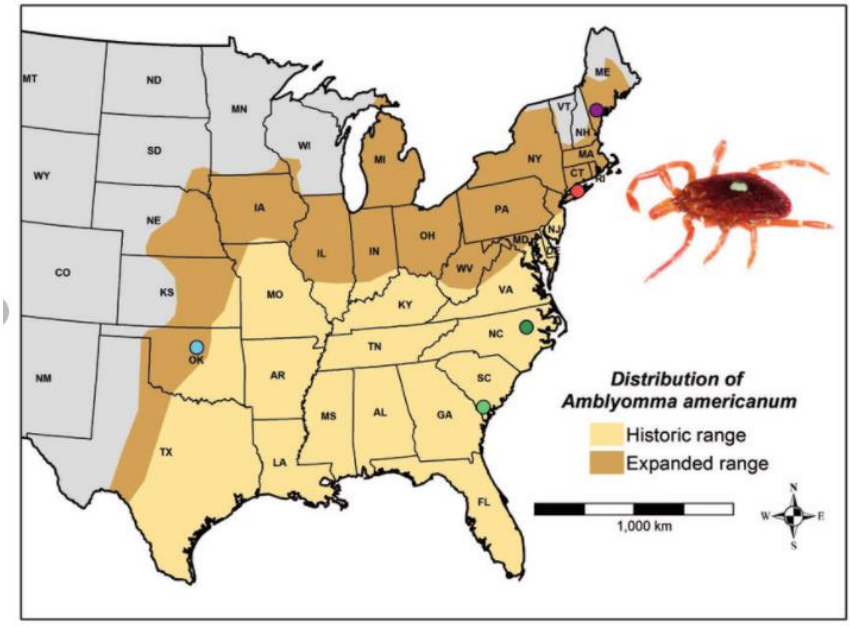


Figure 5. Expanding geographical range of *Amyblomma americanum* in the United States.[5] (Image from the open access journal Genome Biology and Evolution)

vectors, the incidence of feline cytauxzoonosis in new and existing geographic areas may increase.

Vertebrate hosts, both reservoir and aberrant, may also be contributing to the expansion of disease distribution. Currently, the bobcat (*Lynx rufus*) is widely accepted as the natural reservoir host for *C. felis* in North America. *C. felis* infections in bobcats tend to be asymptomatic, although a few reports of fatal disease have been reported.[13,35,39,40] Additional reservoir hosts may also exist. *C. felis* genomic sequences have also been identified in asymptomatic cougars in Brazil (*Puma concolor*) and Florida panthers (*Puma concolor coryii*) and Texas cougars (*Puma concolor stanleyana)* in the United States suggesting that free-ranging cougars may also be natural reservoir hosts.[13,66] However, since Florida panthers are currently endangered in the United States, they are unlikely to be contributing significantly to *C. felis* transmission.[67] The role of domestic cats (*Felis catus*), particularly persistently infected *C. felis* carriers, in the increasing incidence and expanding geographical range is unclear. The impact of environmental change and urbanization on vertebrate host populations may also alter *C. felis*'s future geographic distribution in North and South America.

Clinical Manifestations and Pathophysiologic Mechanisms of Disease in Domestic Cats

Clinical signs of acute feline cytauxzoonosis, a form of protozoal sepsis, are compatible with those of bacterial sepsis and systemic inflammatory response syndrome (SIRS), which are both associated with multiple organ dysfunction, disseminated intravascular dissemination, and often death.[68-71] Early reports of acute feline cytauxzoonosis attributed death to vascular occlusion by schizont-laden macrophages, thrombosis, and subsequent ischemia.[14,68,71,72] Although altered coagulation, post-mortem hemorrhage, and hypoxic injury are well-documented, recent literature has suggested that the systemic inflammatory response spurred primarily by schizont-laden macrophages in the tissue phase of the *C. felis* lifecycle may play a more significant role in organ failure and acute death of affected domestic cats.[3,71,73,74] Clinical, laboratory, and histologic manifestations of acute feline cytauxzoonosis with a primary focus on underlying pathophysiologic mechanisms are discussed below.

Signalment, history, and physical exam findings

C. felis may affect domestic cats of any age, sex, or breed, but male cats > 1 year of age with outdoor access are over-represented in retrospective analyses.[57] The seasonal case incidence is bimodal. The first peak occurs in late spring to early summer as adult arthropod vectors emerge and begin acquiring blood meals. Cases decline in late summer followed by a second smaller peak in early fall as infected tick vector nymphs begin feeding.[57,75] Domestic cats with outdoor access to heavily wooded tick vector habitats, even in urban locations, are at greater risk of infection.[75]

Figure 6: Visible icterus in inner pinna (blue arrow) of a domestic cat infected with *C. felis*. Photo courtesy of Dr. Heather Austin, Skiatook, OK

Experimental *C. felis* infection indicates clinical signs develop approximately 5-14 days following infection.[1,14,72] Importantly, the schizogonous tissue phase is required for development of clinical signs.[74] Early symptoms include lethargy, depression, and anorexia following a period outdoors.[76,77] Physical examination of acutely ill cats often reveals icterus (Figure 6), pyrexia, dehydration, and slowed capillary refill times.[76] In experimental infections, fever usually coincides with the onset of parasitemia approximately 6-10 days post-infection and ranges from about 102.5-106◦F.[1,72] Resistance to handling interpreted as generalized or abdominal pain has also been described.[77,78] With disease progression, tachypnea (>/= 60 bpm), tachycardia,

lymphadenomegaly, and hepatosplenomegaly develop.[10,79] Prior to death, hypothermia (95-96◦F) and tachypnea, sometimes with open mouth breathing, are profound.[1,76] Death or euthanasia usually occur within 5 days of presentation and 1-2 weeks of initial *C. felis* infection.[1,76]

Clinical symptoms in domestic cats with acute feline cytauxzoonosis are compatible with the clinical diagnostic criteria of SIRS, sepsis, or severe sepsis. Several publications have set forth cut-off values for the clinical diagnosis which may vary slightly.[69,70,78] Generally, two or more of the following criteria must be present: pyrexia (>/= 103.5◦F) or hypothermia (</= 100.0◦F); tachycardia (>/=225 bpm) or bradycardia (</= 144 bpm); tachypnea (>/= 40 bpm); and leukocytosis (>/= 19,500 WBC/μL) or leukopenia (</= 5,000 WBC/μL) or >/= 5% band neutrophils.[78] Clinical criteria for sepsis are usually the same as those for SIRS with the addition of an identifiable source of infection.[78] Severe sepsis has previously been defined as sepsis with evidence of organ dysfunction (such as acute kidney injury or acute respiratory distress syndrome), hypoperfusion, and hypotension.[69] At minimum, acute feline cytauxzoonosis meets the clinical diagnostic criteria for sepsis and, often, severe sepsis just prior to death.

Clinical Laboratory Abnormalities

The hematologic abnormalities of acute feline cytauxzoonosis have been well-described for decades. Acute feline cytauxzoonosis is characterized almost uniformly by a nonregenerative anemia with concurrent hyperbilirubinemia, leukopenia, and thrombocytopenia.[1,10,76,77,80,81] In contrast to other causes of pancytopenia, bone marrow failure is not a feature of feline cytauxzoonosis.[1] Multiple pathophysiologic mechanisms have been hypothesized for these hematologic changes.

Nonregenerative anemia in acute feline cytauxzoonosis is primarily attributed to extravascular immune-mediated hemolysis with death occurring prior to mounting a full regenerative response.[1] Findings in experimental *C. felis* infection have supported this hypothesis of extravascular hemolysis as the cause for anemia in acutely affected domestic cats. Anemia first develops at approximately 6-8 days post-infection coinciding with the onset of parasitemia and progressively worsens with clinical disease up until death (Figure 7).[1] Furthermore, mononuclear cells containing phagocytosed erythrocytes have been described in experimentally infected cats.[1,72,74] Early experiments in feline cytauxzoonosis demonstrated an inverse relationship between high concentrations of anti-erythrocytic-piroplasm antibodies and decreased

packed cell volume suggesting antibody dependent erythrolysis also occurs.[80] Furthermore, positive membranous immunocytochemical labeling for IgM has been demonstrated in blood smears of acutely infected feline patients.[71] Other than progressive anemia, published clinical laboratory evidence of antibody-dependent erythrolysis is scarce. Only one case report of a naturally infected cat with direct antiglobulin (Coomb's) and saline agglutination testing is available.[81] Both tests were negative suggesting a lack of antibody mediated erythrocyte destruction in acute feline cytauxzoonosis, at least in this patient.

Extravascular hemolysis should induce erythrocyte regeneration within the bone marrow resulting in release of immature erythrocytes, particularly reticulocytes, into the peripheral blood stream within 3-4 days of onset of anemia.[82] In cats, the resulting peripheral reticulocytosis peaks at 4 days post-hemolytic insult.[82] Erythrocyte regeneration is evident in the bone marrow as erythroid hyperplasia, an increased number of erythroid precursors, and a decreased myeloid-to-erythroid ratio approximately 12-24 hours prior to a reticulocytosis in peripheral blood. However, myeloid-to-erythroid ratios of experimentally infected cats are within normal reference intervals for the species supporting the absence of an marrow erythroid hyperplasia in preparation for an erythrocytic regenerative response.[1] This lack of erythrocyte regeneration has been speculated to be due to the rapid morbidity and mortality of afflicted cats before a regenerative response could develop. In data available by Franks et al., at least 3 of 7 experimentally infected cats survived 4-5 days beyond the development of anemia and the expected time frame for at least evidence of regeneration in the bone marrow. Yet, no reticulocytosis or evidence of erythroid hyperplasia was reported in the marrow of any of the cats in this publication. This suggests alternate mechanisms may be contributing to the failed regenerative response in acutely affected domestic cats (See Chapter 2, Discussion).

In addition, mild to moderate anemia (hematocrit 14-26%) without concurrent hyperbilirubinemia has been reported in persistently infected *C. felis* carrier cats.[4] One report of cats surviving natural infection following treatment with antiprotozoal agents and supportive care reported no hematologic abnormalities with resolution of parasitemia.[83] Although extravascular hemolysis may still be responsible for the removal of infected erythrocytes, a regenerative response and subsequently normal hematocrit would still be expected in these persistently infected carriers. In persistently infected cats, anemia of chronic disease (A.K.A. anemia of chronic inflammation or anemia of inflammatory disease) mediated by pro-

inflammatory cytokines, such as IL-6, may be inhibiting a regenerative response. These same inflammatory cytokines may also hinder a regenerative response in acute inflammation.[84,85] Further experimental studies on the potential role of pro-inflammatory cytokines on the erythroid regenerative responses in acute feline cytauxzoonosis are needed.

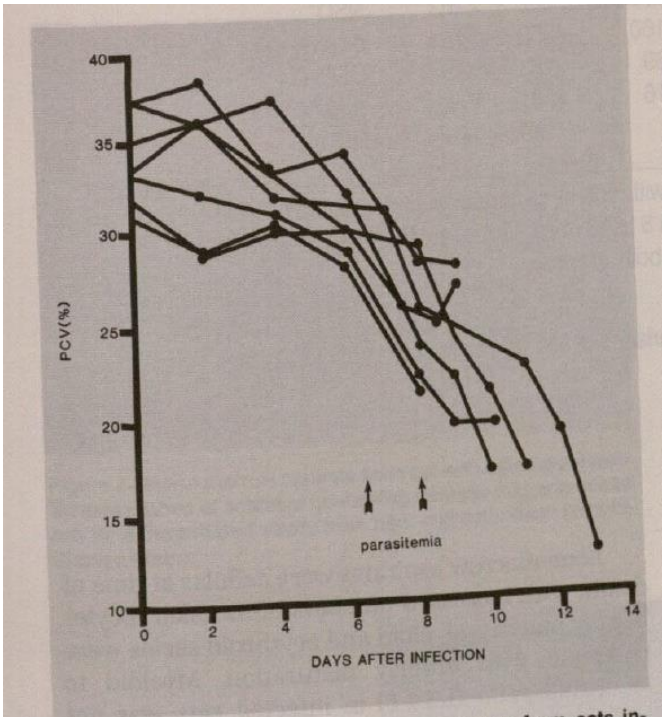


Figure 3—Packed cell volume measurements from cats infected with *C. felis* by intraperitoneal injection at time 0. Arrows indicate days that parasitemias were first recognized six to eight days after infection.

Figure 7: Development of anemia as determined by repeated packed cell volume measurement in experimental *C. felis* infection by Franks et al.[1] (Image reproduced with permission from the Journal of the American Animal Hospital Association.)

Icterus, as expected with both intravascular and extravascular immune-mediated hemolysis, does not develop until just prior to death.[1] The temporal relationship between the onset of anemia, hyperbilirubinemia, and icterus is unclear as data linking the exact time of onset with the severity of anemia in experimentally infected cats is lacking. Due to the near certainty of death and profound morbidity of acutely afflicted cats, future experimental infection studies with *C. felis* will be limited. Reviewing the data of Franks et al., it appears that some (4 of 7) experimentally affected cats were anemic for ~4-6 days prior to death suggesting a lag of several

days between the onset of anemia and hyperbilirubinemia (Figure 7). This may simply be due to the low number of piroplasm-infected erythrocytes early in the erythrocytic phase of feline cytauxzoonosis. As more erythrocytes are infected with disease progression, more erythrocytes are expected to be removed via extravascular hemolysis subsequently worsening the hyperbilirubinemia. If this hypothesis is accurate, the magnitude of anemia and hyperbilirubinemia would hypothetically correlate well with each other. Under this same hypothesis, persistently infected cats are unlikely to be hyperbiliburinemic as only a low number of erythrocytes are infected and targeted for removal. However, virtually nothing is known about the mechanisms for persistent *C. felis* infection and the apparent ability of these piroplasm-infected erythrocytes to escape immune destruction for an extended period of time.[77] Further evaluations on the correlation between hyperbilirubinemia and anemia in both acutely infected and persistently infected domestic cats are needed.

Marked leukopenia, often due to lymphopenia and neutropenia, is common in acute feline cytauxzoonosis.[1,10,72,76,86,87] Leukopenia is most commonly attributed to a pronounced lymphopenia which begins shortly after the onset of parasitemia.[1] Lymphopenia has historically been attributed to corticosteroid-mediated stress response[1,88]; however, evidence now indicates that pro-inflammatory cytokines induce lymphopenia of acute inflammation.[82] Pro-inflammatory cytokines (e.g. IL-6, TNF-α, and IL-β) are known to be increased both locally and systemically in clinically ill feline cytauxzoonosis patients, supporting acute inflammation as the cause for lymphopenia rather than corticosteroid-mediates stress.[3,71] In addition to lymphopenia, both neutropenia and neutrophilia have been reported in acute feline cytauxzoonosis.[77,87,89] Toxic changes in neutrophils and granulocytic precursor cells, indicative of accelerated bone marrow neutropoiesis due to increased peripheral demand, have been identified in peripheral blood films and bone marrow samples of affected cats.[77,89] Changes in neutrophil concentrations and morphology are the result of pro-inflammatory cytokine effects on neutropoiesis within the bone marrow as expected with protozoal sepsis.[82]

Acutely affected cats are typically thrombocytopenic[1] with a single case report of thrombocytosis.[81] In experimental *C. felis* infections, thrombocytopenia develops approximately 8 days post-infection, coinciding with the onset of parasitemia, fever, and early clinical signs and worsens with disease progression.[1] Thrombocytopenia is primarily attributed to platelet consumption associated with disseminated intravascular occlusion (DIC) with some

consideration for immune-mediated destruction.[1,72,79,83] The diagnosis of DIC in feline patients requires 3 or more of the following criteria: thrombocytopenia, prolonged prothrombin time (PT), prolonged activated partial thromboplastin time (PTT), increased D-dimer or fibrin degradation product (FDP) concentration, and low fibrinogen concentration.[79,90] In a small case series of five acutely affected cats reported by Conner et al., all five cats had thrombocytopenia, prolonged PT, and increased D-dimer concentrations meeting clinical criteria for DIC. Four of five patients also had prolonged PTT. Thromboelastograms in all five cats indicated hypocoagulability. The increased PT and PTT results reported by Conner et al. were in direct contrast to early evaluations of experimentally infected cats indicating no significant difference in the PT and PTT results when comparted to control cats.[1] However, gross necropsy and histologic evidence of hemorrhage corroborates the presence of DIC.[72] Despite laboratory and post-mortem evidence of DIC, clinical evidence of active pre-mortem hemorrhage has not been reported in the literature.[1,79]

Descriptions of serum biochemical abnormalities and urinalysis findings in acute feline cytauxzoonosis are limited to individual case reports or small case series with little in depth analysis or discussion of pathophysiologic mechanisms. Commonly reported serum biochemical abnormalities include hyperbilirubinemia, hyperglycemia, hypoalbuminemia, increased hepatic enzyme activities, azotemia, and altered electrolyte concentrations. One clinical case report of feline cytauxzoonosis diagnosed at the Kansas State University Veterinary Health Center (but not included as part of the retrospective review in Chapter 2), indicated no serum biochemical or urinalysis abnormalities at presentation despite a leukopenia characterized by a neutropenia and lymphopenia.[87]

Hyperbilirubinemia (indicated by increased total bilirubin concentrations or visible icterus) is commonly[1,10,76,83,89], but not uniformly, present.[76,86,87] When total bilirubin values are reported, hyperbilirubinemia values are wide-ranging from mild to marked increases in total bilirubin concentrations (4.6 +/- 3.7 mg/dL [Ref. range 0.0 -0.5 mg/dL]).[10] Concurrent bilirubinuria and the presence of bilirubin crystals on a urine sediment have also been reported.[76,83]

Hyperbilirubinemia is largely attributed to extravascular hemolysis of infected erythrocytes with phagocytosis by macrophages in bone marrow, spleen, and liver.[1,77,83,91] Pathophysiologic mechanisms for hyperbilirubinemia secondary to extravascular hemolysis are

well-established.[82] Hemoglobin breakdown in these removed/hemolyzed erythrocytes forms unconjungated bilirubin which is transported to the liver bound to albumin. Unconjugated bilirubin is taken up by hepatocytes and enzymatically conjugated to glucuronide before excretion into the biliary tract. The rate limiting steps in this process are conjugation and biliary excretion, which may become overwhelmed resulting in backflow of conjugated bilirubin into the serum and subsequent excretion in urine causing bilirubinuria.[82] Thus, extravascular hemolysis as a mechanism for hyperbilirubinemia is supported by microscopically visualized erythrophagia in the bone marrow and spleen of experimentally infected cats along with concurrent anemia, hyperbilirubinemia, and bilirubinuria.

Additional contributing mechanisms to hyperbilirubinemia have been alluded to in some publications and personal communications but supporting evidence in peer-reviewed publications is lacking. For instance, Bondy et al indicated that hyperbilirubinemia is a result of "both intrahepatic infiltration of schizont-laden macrophages as well as hemolysis".[91] but the role of intrahepatic schizont-laden macrophages is unspecified in either publication.

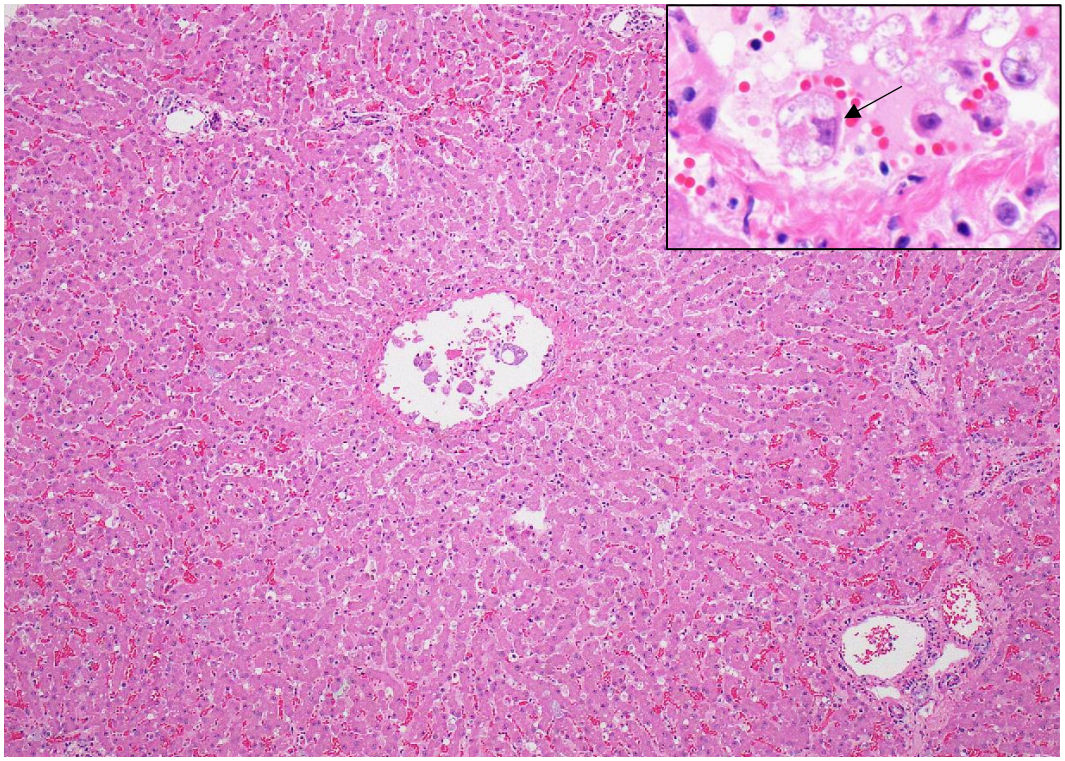


Figure 8: Liver, Hematoxylin & Eosin stain, 10x objective. Hexagonal hepatic lobule with portal triads at the margin (lower right corner) and a central vein in the middle of a randomly selected *C. felis*-affected cat. Central vein contains *C. felis* schizont-laden macrophages. Distention biliary canaliculi with bile pigments is not evident. Inset: Schizont-laden macrophage in central vein (black arrow), 50x oil objective, H&E stain.

Compression of bile ducts by intra-hepatic infiltration of schizont-laden macrophages has been postulated in hypothetical clinical rounds discussions.[92] Histologically, this should result in dilation of bile canaliculi and accumulation of bile pigments within biliary canilicular lumens, neither of which have been reported in publications on hepatic lesions of feline cytauxzoonosis (Figure 8).[10,40,61,72,74,77,83,86,89] Schizont-laden macrophages may also indirectly result in decreased bilirubin excretion into the biliary tract via their release of pro-inflammatory cytokines.[3,71,93,94]

Hyperglycemia is common and often attributed to a physiologic stress response.[1,10,76,83] Physiologic hyperglycemia results from the direct and indirect actions of increased cortisol, norepinephrine, and epinephrine concentrations from the adrenal glands.[82] Cortisol directly promotes hepatocellular gluconeogenesis and decreases hepatocellular glucose uptake by reducing the number and efficacy of hepatocellular membrane GLUT-4 (glucose transporter type 4) transporters. Indirectly, glucocorticoids also stimulate glycogenolysis and decrease glycogenesis via increased glucagon activity. Catecholamines, such as epinephrine and norepinephrine, stimulate adrenergic receptors to increase glycogenolysis, stimulate growth hormone, and suppress insulin secretion from pancreatic β-cells – all of which reduce cellular glucose uptake and increase serum glucose concentrations. Epinephrine also indirectly induces hypercortisolemia via increased adrenocorticotropin stimulating hormone (ACTH), thus, inducing the downstream effects of increased serum cortisol on serum glucose concentrations. In people, the pro-inflammatory cytokines, TNF-α and IL-1β, also inhibit insulin release enhancing the hyperglycemic effects in patients with systemic inflammation.[95] No publications evaluating hormone concentrations in natural or experimental feline cytauxzoonosis are currently available confirming these hormonal effects on serum glucose concentrations.

Hypoproteinemia, often characterized by a selective hypoalbuminemia, has also been reported with acute feline cytauxzoonosis.[76,83] Selective hypoalbuminemia in the face of inflammation is also known as an inflammatory dysproteinemia. Albumin is a negative acute phase protein, meaning serum concentrations of albumin decrease in the face of acute inflammation. Serum protein electrophoretograms in a small group of naturally infected *C. felis* domestic cats revealed an increased concentration in the α- and β-globulin fractions supportive of an acute inflammatory response.[71] Inflammatory hypoalbuminemia is the result of decreased hepatocellular albumin production resulting from the effects of the pro-inflammatory cytokines, IL-6, TNF-α, and IL-1β.[96] Increased clearance rate of albumin may also be occurring with acute

inflammation.[96] In people, pro-inflammatory mediators increase vascular permeability resulting in leakage of albumin into the perivascular spaces.[97] As vascular leakage and perivascular edema may occur in feline cyatuxzoonosis, inflammation-induced leakage of albumin into the perivascular areas may be an additional mechanism of hypoalbuminemia.

Renal loss of albumin may also be considered. In one publication of acute feline cytauxzoonosis, proteinuria without concurrent hematuria, hemoglobinuria, or evidence of active urinary inflammation (i.e. a quiet urine sediment) was reported.[76,83] Although this reported proteinuria is likely due to albumin loss, no testing, such as a urine albumin concentration, urine protein electrophoresis, or urine albumin:creatine ratio, was performed to confirm this. As with the leakage of albumin into perivascular spaces discussed above, pro-inflammatory mediators may increase renal glomerular permeability resulting in increased urine losses of albumin that overwhelm the proximal renal tubular albumin resorption capacity leading to albuminuria.[98] Further studies on pathophysiologic mechanisms of acute kidney injury and albuminuria in cats with SIRS, as present in acute feline cytauxzoonosis, are necessary to fully understand the pathophysiologic mechanisms.

Increased serum activity of the hepatocellular enzymes alanine transaminase (ALT) and lactate dehydrogenase (LDH) has been reported in a small case series of 8 domestic cats naturally infected with *C. felis* in Oklahoma from 1985-1992.[98] Increased ALT and LDH were present in 4/8 affected cats, but it is unclear if increases were present in the same four cats. The average serum ALT concentration was 79.8+/-56.5 IU/L (reference interval 10-30 IU/L) meanwhile the average LDH concentration was 937.8+/-635.5 IU/L (reference interval 16-79 IU/L). Both ALT and LDH are primary indicators of hepatocellular injury. Currently, ALT is a considered a more sensitive marker of hepatocellular injury in cats. Such hepatocellular injury may result from ischemia, hypoxia, metabolic disease, neoplasia, nutritional disorders, infectious and noninfectious inflammation, toxins, and trauma.[82] In feline cytauxzoonosis, hypoxic injury secondary to anemia and vascular occlusion by schizont-laden macrophages is likely the primary insult causing release of ALT from damaged hepatocytes. While severe muscle injury and iatrogenic hemolysis have also been reported to increase both serum ALT and LDH activity, hemolysis severe enough to interfere with ALT concentrations should cause red-tinged serum. In these cats, grossly evident serum hemolysis was not indicated. In contrast, even mild hemolysis

may increase serum LDH activity results. The combination of increased ALT and LDH without grossly visible serum hemolysis likely indicates hepatocellular injury is present.

Alterations in blood urea nitrogen (BUN) and creatine concentrations have also been described. Two affected cats had a reported decrease in BUN and one had a selective increase in creatinine concentration compatible with clinical dehydration.[83,86] Pre-renal azotemia characterized by increased BUN concentration without a concurrent increase in creatinine concentration is relatively common in publications on the largest cohorts of naturally infected domestic cats.[10,76,83] No publications have postulated that cause of this azotemia beyond dehydration and hypovolemia. This type of pre-renal azotemia is often associated with gastrointestinal hemorrhage. Given the previously discussed evidence of hypocoagulation, thrombocytopenia, and DIC in affected cats, clinically undetected gastrointestinal hemorrhage may be occurring. In a single published case, large blood clots in the duodenum and jejunum were noted.[76] This type of hemorrhage would contribute to the anemia in affected cats and cause a nonselective hypoproteinemia. However, a concurrent hypoglobulinemia and reported common necropsy or histologic evidence of gastrointestinal hemorrhage are lacking. Gross and histologic evidence of gastrointestinal hemorrhage may be present, but simply unpublished. Further descriptive studies linking the presence of increased BUN, fecal occult blood test results, with gross and histologic evidence of gastrointestinal hemorrhage are needed to provide supporting evidence for this form of pre-renal azotemia in acutely affected domestic cats.

Azotemia characterized by an increased BUN concentration without a concurrent increase in creatinine concentration may also occur with hypovolemia or enhanced antidiuretic hormone (ADH) activity in the distal nephron.[82] Azotemia secondary to hypovolemia is often, but not always, accompanied by an increase in both BUN and creatinine concentrations. The reduced renal tubular flow of urine which accompanies hypovolemia increases the rate of tubular BUN resorption increasing the serum BUN concentration. In addition, hypovolemia stimulates ADH release which promotes BUN resorption in the distal nephron. The actions of ADH can increase BUN resorption rate by nearly four times the normal basal rate.[82] Notably increased urine concentrations reported in affected cats with increased serum BUN concentrations (urine specific gravity >1.051[83] and >1.058[76]) support hypovolemia as a primary mechanism for increased serum BUN without a concurrent increased creatinine concentration. Pro-inflammatory cytokines may also be playing a role in the selective BUN increase. IL-6 and IL-1β have been

shown to stimulate ADH release and indirectly enhance its action in the distal nephron of rats and people.[99,100] As discussed above, increased ADH activity can profoundly influence BUN resorption rate altering increased BUN concentrations. Furthermore, IL-6 may directly alter renal epithelial function.[101] With increased local and systemic pro-inflammatory cytokine levels in domestic cats with feline cytauxzoonosis[3,71], similar mechanisms may be occurring. Histologic evidence of mild periglomerular and interstitial inflammation in kidneys of affected cats may support the hypothesis of inflammation driven acute kidney injury in these patients. Further immunohistochemical and/or molecular studies evaluating the potential correlations between ADH production in the hypothalamus and hypothalamic/renal IL-6 tissue expressions may be helpful to further elucidate the role of systemic proinflammatory mediators in renal and electrolyte disturbances associated with acute feline cytauxzoonosis.

Electrolyte abnormalities and acid-base disturbances of acute feline cytauxzoonosis have been reported.[91] Evaluation primary literature sources identified hyponatremia and hypokalemia in acutely affected cats. Review articles on feline cytauxzoonosis have indicated acid-base disturbances are present, but no references to primary literature sources was made.[76] In addition, no acid-base disturbances were identified in the primary literature sources reviewed here. Pathophysiologic mechanisms for the electrolyte and reported acid-base abnormalities have not been postulated in the reviewed published reports. Hypokalemia in four cats was relatively mild averaging 3.38+/-0.23 mEq/L (reference interval 3.8-5.0 mEq/L)[76] and likely associated decreased potassium intake due to anorexia. Further discussion of hypokalemia is not warranted, but discussion of potentially marked hyponatremia is.

Reported hyponatremia in three affected domestic cats was mild to marked with an average of 138.8+/-10.2 mEq/L (reference interval 120-150 mEq/L) in three acutely affected cats.[76] Hyponatremia in the face of clinically described dehydration and laboratory evidence of hypovolemia (increased BUN and high urine specific gravity) is unusual. Hypovolemia should induce sodium resorption in the collecting tubules via the action of aldosterone. Thus, acute feline cytauxzoonosis is associated with a hypovolemic hyponatremia due to ongoing sodium losses, likely via the renal tubules or third-spacing. With sodium losses exceeding sodium resorption, hyponatremia may be further exacerbated by the dilutional effects of water intake via drinking or fluid administration.[82] Increased ADH activity secondary to systemic inflammation in acute feline cytauxzoonosis may also be playing a role in the development of hyponatremia.

As discussed with mechanisms of action for the selective increase in BUN, pro-inflammatory cytokines IL-6 and IL-1β can stimulate ADH release.[99,100] ADH promotes water resorption in the distal nephron which can further dilute serum sodium concentrations. ADH also has a minor role in stimulating sodium and chloride resorption in the renal tubule[82]; however, this may not be enough to counteract the dilutional effects of water resorption. Finally, hyponatremia may occur secondary to third-space accumulation of fluid in body cavities, including the pleural, pericardial, and peritoneal spaces.[82] In the cases of acute inflammation, perivascular accumulation of fluid, as discussed above for hypoalbuminemia, may also be occurring. Pericardial effusion and perivascular edema have both been reported on post-mortem examination of domestic cats with acute feline cytauxzoonosis.

Gross and histologic lesions

Gross lesions for feline cytauxzoonosis are well-established for most organ systems and characterized by evidence of icterus, vascular leakage, and hemorrhage at the time of death. At post-mortem examination, affected individuals are reportedly in good body condition reflecting the acutely progressive nature of feline cytauxzoonosis.[76,102] Generalized icterus in mucus membranes, skin, and adipose tissues is common, but not always evident.[10,76,102] Yellow or red-tinged serosanguinous cavitary effusions, primarily in the pericardial and pleural spaces, may be present.[1,14,72,74,102] In experimentally infected domestic cats, grossly visible and progressive distention of the renal veins and caudal vena cava was observed by day 15 post-infection.[72] The reserve dilatory capacity in large, distended abdominal veins may explain why peritoneal fluid accumulation is less common than in the pericardial and pleural spaces. Petechiation and ecchymosis are common in various tissues including the lungs, lymph nodes, epicardium, subepicardium, meninges, and urinary bladder.[14,72,102] A single affected cat was also reported to have large blood clots within the lumen of the duodenum and jejunum.[76]

Histologically, feline cytauxzoonosis is characterized by *C. felis* schizont-laden macrophages and monocytes occupying, and sometimes occluding, blood vessels throughout the body.[14,72,74] In both experimentally and naturally infected domestic cats, the highest numbers of schizont-laden macrophages are present in the pulmonary, splenic, and hepatic vasculature.[10,14,72,74,76] Infected macrophages have also been reported in the vascular spaces of the lymph nodes, bone marrow, kidneys, brain, pancreas, gastrointestinal tract, and eyes.[1,76,102,103]

Schizont-laden macrophages appear to have a predilection for venous vasculature, such as minor veins, sinusoids, venules, as well as shared capillary beds, as no reports of schizont-laden macrophages in the arterial vasculature were identified in this literature review.[14] Schizont-laden macrophages attach to vessel walls[45,72], the slower blood flow and often larger lumens of venous spaces may be more hospitable for schizont-laden macrophage attachment to vessel walls. In addition, the correct adhesion molecules for attachment to vessel walls may be preferentially expressed on the endothelial cell surfaces of veinous, rather than arterial, blood vessels.

With intravascular parasitism, vasculitis is intuitively expected in acute feline cytauxzoonosis. Histologically vasculitis is characterized by inflammatory cells attacking, surrounding, or invading vascular endothelium with frequent fibrin deposits and occasional foci of endothelial necrosis. Perivascular hemorrhage and edema may also be present. Interestingly, despite the close association of infected macrophages with vessel walls, only mild histologic evidence of vasculitis has been identified.[45] In few reports, low numbers of neutrophils have been described surrounding smaller vessels containing schizont-laden macrophages.[3] Perivascular hemorrhage and edema are commonly described and seem to be most evident near pulmonary, splenic, and hepatic vascular spaces.[3,45,72] Furthermore, both infected macrophages and adjacent endothelial cells have significantly increased positive cytoplasmic immunolabeling for inducible nitric oxide synthase (iNOS), an enzyme responsible for producing nitric oxide, a potent vasodilator.[3] Vascular dilation can promote fluid leaking and perivascular edema. Despite the absence of pronounced vascular invasion with inflammatory cells, fibrin, and necrosis, vascular injury is still histologically and immunohistochemically evident in acute feline cytauxzoonosis.

Pulmonary failure associated with a shock-like state mediated by pro-inflammatory cytokines (i.e. acute respiratory distress syndrome) has been postulated as a significant contributing factor to death in acute feline cytauxzoonosis.[73] The progressive tachypnea, dyspnea, and hypothermia observed prior to death along with the histologic and known localized immune responses support this theory. At necropsy, lungs are edematous, congested, or reddened with multifocal hemorrhage.[14,72,74,76,102] Experimental infections indicate that edema and congestion begin at approximately 15 days post-infection and progressively worsen until death.[72] Histologically, moderate to severe vascular occlusion by schizont-laden macrophages, moderate interstitial pneumonia, mild intra-alveolar hemorrhage, and moderate pulmonary edema are

present in affected lungs. Furthermore, immunohistochemical labeling of lung tissue from cats naturally infected with *C. felis* reveals increased tissue expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 along with iNOS and MHCII (Figure 9).[3] These histologic and immunohistochemical findings are compatible with those expected during the early exudative phase of acute respiratory distress syndrome.[45] In addition, feline cytauxzoonosis patients develop interstitial pneumonia characterized by alveolar septal thickening, hypercellularity, protein-rich edema, occasional hyaline membrane formation, and frequent neutrophilic

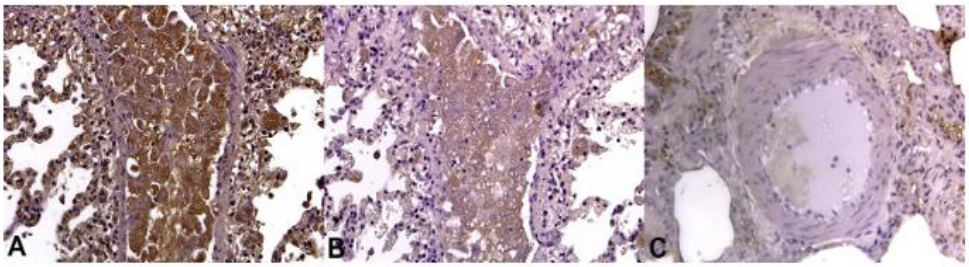


Fig. 2. Immunohistochemistry for TNF-α. (A) Section of lung from a *C. felis*-infected cat, using anti-TNF-α antibody. Note high intensity of immunoreactivity, particularly within the cytoplasm of infected, intravascular monocytes and interstitial leukocytes. (B) Section of lung from a *C. felis*-infected cat, using isotype antibody. Note background staining of leukocytes, most likely due to endogenous peroxidases. (C) Section of lung from an uninfected cat, using anti-TNF-α antibody. Note occasional immunoreactive interstitial leukocytes.

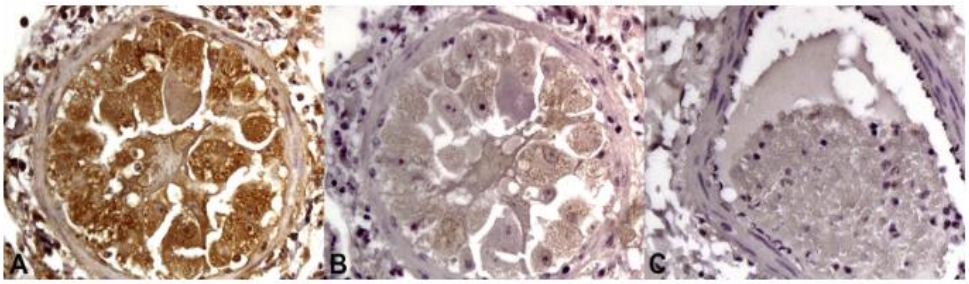


Fig. 3. Immunohistochemistry for IL-1β. (A) Section of lung from a *C. felis*-infected cat, using anti-IL-1β antibody. Note high intensity of immunoreactivity, particularly within the cytoplasm of infected, intravascular monocytes and interstitial leukocytes. (B) Section of lung from a *C. felis*-infected cat, using isotype antibody. Note slight background staining of infected, intravascular monocytes. (C) Section of lung from an uninfected cat, using anti-IL-1β antibody. Note absence of immunoreactivity.

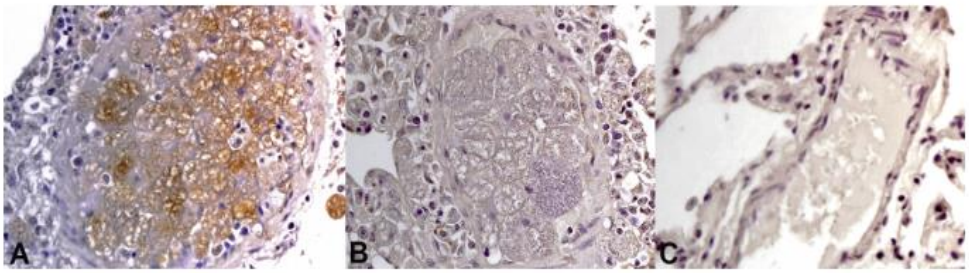


Fig. 4. Immunohistochemistry for IL-6. (A) Section of lung from a *C. felis*-infected cat, using anti-IL-6 antibody. Note high intensity of immunoreactivity, particularly within the cytoplasm of infected, intravascular monocytes. (B) Section of lung from a *C. felis*-infected cat, using isotype antibody. Note slight background staining of infected, intravascular monocytes and interstitial leukocytes. (C) Section of lung from an uninfected cat, using anti-IL-1β antibody. Note absence of immunoreactivity.

Figure 9: Immunohistochemical labeling for pro-inflammatory cytokines in lungs from cats naturally infected with *C. felis* as represented by Frontera-Acevedo et al.[3] (Image reproduced with permission from Science Direct publishing.)

infiltrates.[45,73] Necrotic foci and schizont-laden macrophages within the pulmonary parenchyma may also be observed.[45,72] On occasion, alveolar spaces contain foamy macrophages sometimes with intracytoplasmic phagocytized erythrocytes.[73]

C. felis schizont-laden macrophages are commonly reported in the spleen and liver; yet, in depth discussions regarding lesions are relatively sparse and immunohistochemical studies are lacking. Splenomegaly and congestion are the first gross abnormality identified in experimental infections[72,74] and are frequently, but not always, observed in naturally infected domestic cats.[10,76] In contrast to other publications[14,72], in situ hybridization by Susta et al revealed that the spleen, rather than lungs, is the most heavily parasitized organ.[45] Phagocytosis of erythrocytes by macrophages is prominent in the splenic red pulp and marginal zones.[72] By day 15 post-infection, hepatomegaly characterized by accentuated hepatic lobules and a mottled appearance is evident.[10,14,72,74] Histologically, the parasite burden in the liver varies.[14,45] Schizont-laden macrophages are most commonly observed in central veins and hepatic vein tributaries but rarely in portal veins.[72,74] Despite the presence of increased serum hepatocellular enzyme activity, no histologic evidence of hepatocellular injury has been reported; however, increased hepatocellular enzyme activity from hepatocyte injury can occur prior to, or without, supporting histologic evidence.

Lymphadenomegaly and congestion are common, but not always present, and may be localized near the site of infection/inflammation or generalized, usually by day 12 post-infection.[1,10,14,72,74,76] Schizont-laden macrophages accumulate within and along nodal sinusoids.[14] Progressively increasing numbers of "mononuclear phagocytes" have been described in the germinal centers of lymphoid follicles, but it is unclear if these "mononuclear phagocytes" contained replicating *C. felis* organisms.[72] The single publication reporting in situ hybridization in affected lymph nodes did not describe the distribution of organisms within the node.[45] The presence of schizont-laden macrophages within germinal centers may play an important in understanding humoral immune responses to natural infection. Furthermore, inhibition of infected macrophages extravasation has been suggested.[45] If schizont-laden macrophages are present in nodal germinal centers, infected macrophage extravasation and migration are occurring, at least in affected lymph nodes. Evaluating these cell trafficking patterns may be important to understand the development of humoral immunity in surviving and persistently infected patients.

The kidneys may be slightly swollen beginning around day 15 post-infection.[72] Renal histologic descriptions beyond the presence of both intravascular and interstitial schizont-laden macrophages are sparse. Susta et al described the renal changes of only two naturally infected cats in some detail. Mild mononuclear inflammation comprised of lymphocytes, plasma cells, and, in one cat, macrophages was observed in the renal cortical interstitium and periglomerular region, respectively. The cat with periglomerular mononuclear inflammation, including macrophages, also had evidence of a membranoproliferative glomerulonephritis and numerous tubular protein casts suggesting glomerular injury associated with *C. felis* infection. However, the multifocal lymphoplasmacytic inflammation, as in the second cat, is also a feature of chronic kidney disease and common in cats. Further descriptions of histologic renal lesions with knowledge of the full clinical history of affected patients and immunohistochemical evaluation of tissue inflammatory cytokine profiles are needed. Too little is described of the renal changes in acute feline cytauxzoonosis to definitively link any evidence of inflammation or injury with the theorized mechanisms of acute kidney injury discussed above as a potential mechanism for pre-renal azotemia in affected domestic cats.

Cardiac lesions can be grossly and histologically evident. Thinning of the right ventricle and rounding of the cardiac apex has been described in experimentally infected domestic cats.[72] Visible moderator bands in the left ventricle were described in one naturally infected cat with concurrent heartworm infection. In that case, left ventricular changes were likely secondary to increased pulmonary arterial pressures associated with the presence of *Dirofilaria immitus* adults. Histologic descriptions of cardiac lesions are limited to a single cat in one small case series.[45] Multifocal and focally extensive myocardial degeneration with distension of perivascular spaces and extravasated schizont-laden macrophages. These lesions resulting in altered cardiac function may contribute to the development of pleural and pericardial effusion in some affected cats. No currently available published descriptions mention if cardiac lesions and effusions are present in the same individuals. It is also unclear what percentage of affected individuals develop cardiac lesions or if these lesions are associated with ischemic injury and/or systemic inflammatory responses.

Although clinical signs associated with neurologic and ocular lesions are uncommon, gross and histologic lesions in the brain and ocular/periocular structures have been evaluated. Neural lesions include parenchymal grey and white matter vacuolation, microhemorrhages, and

random necrosis are present, consistent with hypoxic-ischemic injury.[104] Diffuse reactive astrogliosis (i.e. astrocyte hyperplasia) and positive immunohistochemical labeling of for cleaved caspase-3 (indicative of apoptosis) were interpreted as evidence of early hypoxic-ischemic injury in the brain parenchyma.[103] There was no statistical association between the number of infected cells and degree of inflammation in the brain.[103] The majority of parasitized cells have been documented in the choroid plexus and choroid villi.[45] Gross ocular lesions include scleral and nictitating membrane icterus, episcleral vessel injection, and elevated third eyelids.[102] Histologically, schizont-laden macrophages were visible in the retinal vessels and throughout the uveal tract, most heavily within the ciliary body. Vitreal and subretinal hemorrhage with retinal detachment were also noted.[102]

Host immune responses

Investigations of experimental and, more recently, natural *C. felis* infection have repeatedly demonstrated the importance of schizogony and innate immune responses as the cause of morbidity and mortality in acute feline cytauxzoonosis. The association between schizont-laden macrophages of the tissue phase and pro-inflammatory cytokines in disease progression is an emerging area of research focus.[3,73,103] Little is known regarding humoral immune responses of surviving domestic cats following experimental and natural infection.

Early experimental studies on *C. felis* transmission confirmed the association of schizogony with clinical symptoms and death. In bobcats, the natural reservoir host, significant schizogony rarely develops and appears self-limiting while intra-erythrocytic *C. felis* piroplasms are persistent.[35,45] Those bobcats that develop a pronounced schizogonous tissue phase invariably become ill or die.[39,45] Similar experiments in cats demonstrate that experimental infection with tissue homogenates or whole blood containing schizont-laden macrophages or transmission via a competent tick vector with subsequent schizogony development are required for severe clinical illness and subsequent death to occur.[40,74,77] In contrast, domestic cats infected directly with peripheral blood of persistently infected bobcats lacking schizont-laden macrophages develop persistent parasitemia with no evidence of schizogony.[74]

Although schizogony was assumed to occur in macrophages in early reports, doubts lingered about the true origin of the schizont-laden cell. Schizogony of *Theileria* species occurs within lymphocytes and some authors expressed concerns that lymphocytes could still be the

infected cell with *C. felis*.[45] Positive cytoplasmic immunolabeling with an anti-lysozyme antibody in schizont-laden cells of histologic sections from affected cats finally confirmed that *C. felis* schizogony occurs in histiocytes, including macrophages.[45] The association between clinical illness and schizogony in cells of histiocytic lineage specifically, is a critical step in understanding the localized and systemic pathophysiologic steps in acute feline cytauxzoonosis.

Identification of activated, phagocytic intravascular macrophages is unusual. Under typical conditions, monocytes derived from bone marrow stem cells are released into circulation; exit blood vessels via the leukocyte adhesion cascade under the direction of inflammatory cytokines and chemokines; and, are fully activated into mature phagocytic cells within the extravascular spaces of inflamed tissues. In the case of acute feline cytauxzoonosis, infected macrophages do appear to adhere to vascular endothelium based on light and transmission electron microscopy findings. Evaluation of CD18, a leukocyte adhesion molecule highly expressed in cells of macrophage-monocyte lineage, provides supporting evidence of vascular adhesion. Membraneous immunohistochemical labeling for CD18 on infected intravascular macrophages in lung sections was notably higher when compared to macrophages in the pulmonary interstitium of non-infected domestic cats.[71] Upregulation of CD18 expression was subsequently confirmed with quantification of CD18 mRNA transcripts in the peripheral blood of infected cats. Acutely ill cats infected with *C. felis* had 9.0 times higher CD18 expression than un-infected cats, while those that died from acute feline cytauxzoonosis had 11.6 times greater expression of CD18 than un-infected cats. This increased CD18 expression likely plays an important role in promoting further macrophage activation and cytokine release, as discussed below. While macrophage vascular adhesion is evident, host-parasite interactions with *C. felis* may also be hindering transendothelial migration of infected macrophages into surrounding tissues. Infected schizont-laden macrophages lacked immunolabeling for calprotectin, a superficial adhesion molecule important for diapedesis and extravasation of cells of the monocyte-macrophage lineage.[45] The accumulation of schizont-laden macrophages within vascular lumens may support the theory of hindered transendothelial migration; yet, extravascular schizont-laden macrophages within tissue parenchyma have been reported in histologic descriptions.[45,72]

Furthermore, *C. felis* infection may alter pro-apoptotic and cellular anabolic pathways in schizont-laden macrophages prolonging survival and proliferation of infected cells. Increased

cytoplasmic immunolabeling for protein 53 (p53) has been described in *C. felis* infected macrophages.[45] Under normal circumstances, p53 is rapidly removed from the cytoplasm via ubiquitination. When p53 production increases or escapes ubiquitination, p53 is translocated to the cell's nucleus where it stimulates pro-apoptotic responses inducing cell death. The accumulation of p53 within the cytoplasm of infected macrophages may indicate up-regulation of p53 production or altered translocation into the nucleus. Regardless, this finding may indicate for delayed apoptosis of infected cells. In addition to delayed apoptosis, schizont-laden macrophages also have immunohistochemically detectable nuclear PCNA expression indicative of active DNA replication and organelle duplication.[45] Thus, *C. felis* may also be inducing mitosis of infected macrophages or high-jacking cellular organelle for its own metabolic demands during schizogony. Further evaluations of infected macrophage expression of apoptotic proteins and p53-induced transcription factors may provide additional detail on alterations in apoptosis induced by *C. felis* infection.

Systemic and localized cytokine profiles in natural *C. felis* infections indicate infected macrophages undergo classical T_H1-mediated activation. Classically activated macrophages respond to the IFN-γ and TNF-α to promote inflammation, tissue injury, and microbial death via production and release of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, upregulated MCHII expression, and increased NO production. Further downstream effects of classically activated macrophages include activation of the arachidonic acid cascade and altered coagulation processes. Imbalanced and excessive production of these inflammatory mediators of classically activated macrophages can lead to the clinically observed evidence of sepsis, DIC, and death that are well-documented in cases of acute feline cytauxzoonosis. Supporting the presence of classically activated macrophages, increased expression of IL-6, IL-1β, TNF-α, iNOS, and MHCII in lung sections from infected domestic cats relative to un-infected domestic cats has been demonstrated (Figure 9).[3] Inflammatory responses localized in the pulmonary tissues appear to be more pronounced than those observed systemically. Naturally infected, acutely ill cats have increased serum concentrations of IL-1β and TNF-α; however, no statistical differences in serum concentrations were detected between patients that survived and died. Quantification of tissues expressions of IL-6, IL-1β, TNF-α, iNOS, and MHCII via reverse transcriptase-PCR (RT-PCR) from surviving and deceased cats may provide additional support for the importance of localized pulmonary inflammatory to patient survival.

Little is known about the humoral immune responses in naturally infected domestic cats. With so few cats surviving the rapid and pronounced initial inflammatory response to the shizogonous tissue phase, development of protective humoral immunity seems exclusive to surviving natural infection without treatment. In experimental infections, cats that survived the schizogonous tissue phase fail to produce clinical symptoms following re-exposure to tissue homogenates and whole blood containing infective schizont-laden macrophages.[14,45] In contrast, cats infected only with erythrocytes containing *C. felis* piroplasms never develop an active schizogonous tissue phase and succumb to disease upon subsequent challenge with schizont-containing tissues.[77] This suggests that the humoral responses formed during the schizogonous tissue phase provide protective immunity against reinfection. Relative to healthy feline patients, serum IgM levels appear to be higher relative to healthy cats based on positive immunocytochemical labeling of blood smears in acutely ill *C. felis* patients.[71] This small series of 5 cats also demonstrated that those surviving natural infection were more likely to have positive immunocytochemical labeling for IgM on blood smears suggesting a potential protective role of IgM in early infection. The presence of IgM in surviving cats indicates antigen-independent activation of B-lymphocytes in nodal germinal centers, compatible with some histologic lymph node descriptions of late stage experimental infections.[14,72] Comparison of antibody responses via radioimmunodiffusion on serologic samples from a greater number of naturally infected cats that survive and succumb to infection may prove beneficial in the initial characterization of antibody responses in acute infections. Improving treatment strategies and identification of more carrier cats may provide future populations ripe for researching the humoral immune responses of acutely infected domestic cats.

Laboratory Diagnosis

Light Microscopy

Antemortem diagnosis of feline cytauxzoonosis relies on accurate microscopic identification of the characteristic intra-erythrocytic *C. felis* piroplasms on a standard blood film. These characteristic piroplasms are ~1.0-2.0 μm in diameter, round to ovoid, signet rings with a darkly staining peripheralized nucleus adjacent to a centralized internal clearing (Figure 10A-B). Piroplasms are usually present indvidually, but may also be present in couplet and tetrad (i.e.

Maltese cross) formations.[72] Piroplasms are microscopically identical to those of *Babesia felis* and *Cytauxzoon europaeus*, but neither of these organisms have ever been identified in the United States. Thus, microscopic identification of intra-erythrocytic piroplasms in domestic cats from the United States is considered pathognomonic for feline cytauxzoonosis.

Accurate diagnosis is particularly important for veterinarians in geographical locations with newly emerging feline cytauxzoonosis cases. Experimental infection suggests an intra-erythrocytic parasitism rate usually >5 piroplasms per 1,000 erythrocytes (approximately one 50x oil objective field); however, piroplasms are infrequent in the early erythrocytic phase and may be missed despite the presence of clinical signs in affected cats.[72,87] Intra-erythrocytic piroplasms and common artifacts may be misconstrued for each other by less experienced blood film evaluators. Howell-Jolly bodies are intra-erythrocytic remnants of erythrocyte nuclei which can be similar in size but lack a centralized clearing. Refractile drying artifacts, stain precipitate, and small platelets overlying erythrocytes may also be confused with intra-erythrocytic *C. felis* piroplasms.

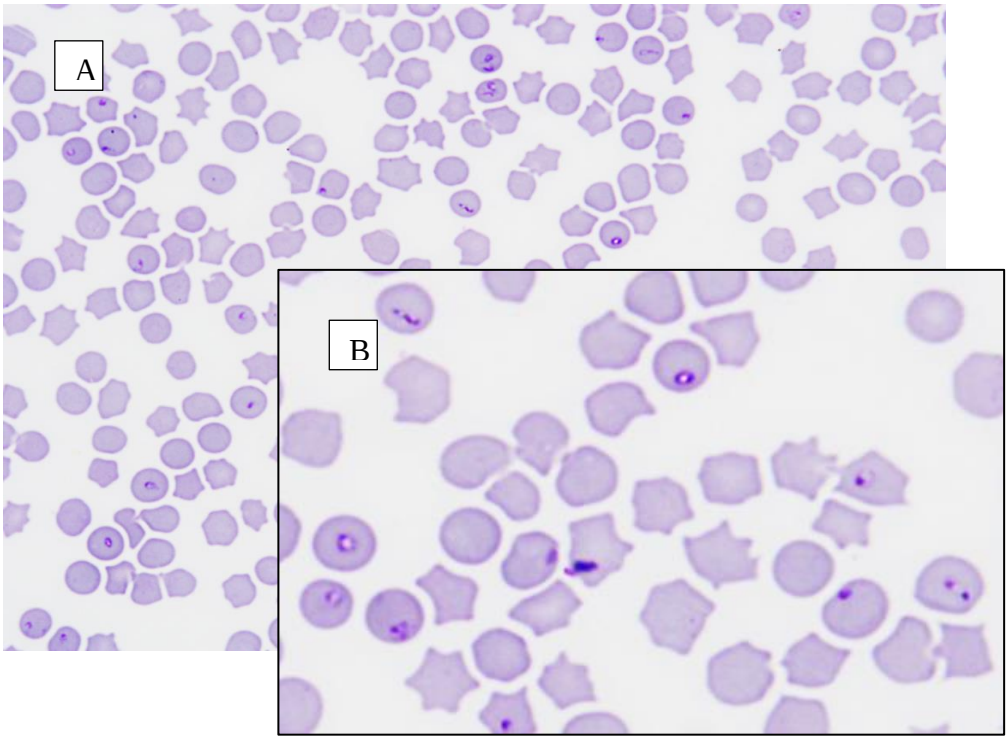


Figure 10: A) Peripheral blood smear. Modified Wright stain. 100x oil objective. Intra-erythrocytic *C. felis* piroplasms. B) Magnified Image A.

Visualization of *C. felis* schizont-laden macrophages may also be used in the antemortem diagnosis of feline cytauxzoonosis. Schizont-laden macrophages may be present in blood smear preparations or fine needle aspirate biopsies of the liver, spleen, bone marrow, or lymph node from affected individuals.[76,77,86] Macrophages containing organisms of unknown origin have even been described in cytologic evaluation of transtracheal wash from a domestic cat later diagnosed with feline cytauxzoonosis on necropsy.[87] Mature schizont-laden macrophages are large (~50-100 μm diameter) and filled with numerous variably sized (~0.75-2.0 μm diameter)

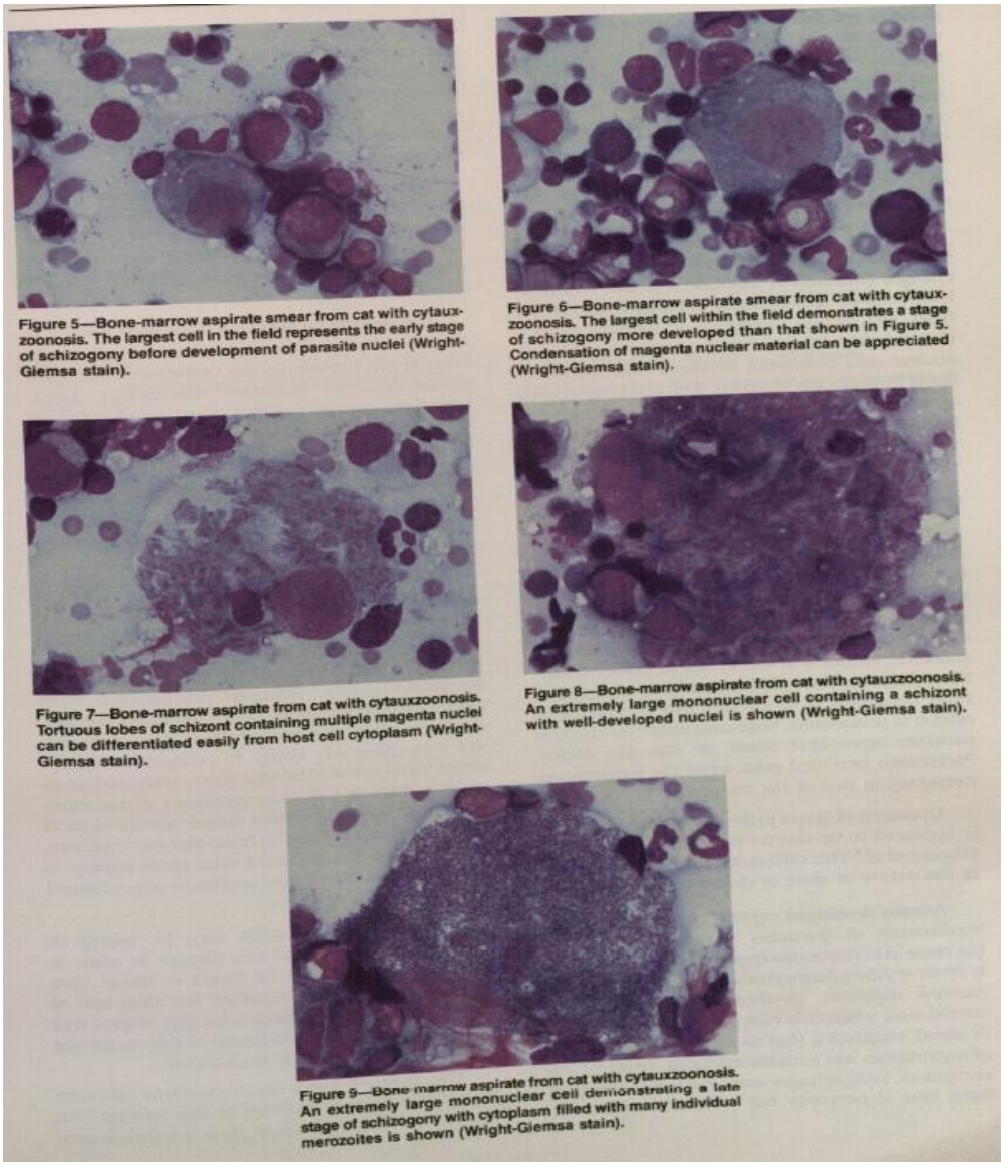


Figure 5—Bone-marrow aspirate smear from cat with cytauxzoonosis. The largest cell in the field represents the early stage of schizogony before development of parasite nuclei (Wright-Giemsa stain).

Figure 6—Bone-marrow aspirate smear from cat with cytauxzoonosis. The largest cell within the field demonstrates a stage of schizogony more developed than that shown in Figure 5. Condensation of magenta nuclear material can be appreciated (Wright-Giemsa stain).

Figure 7—Bone-marrow aspirate from cat with cytauxzoonosis. Tortuous lobes of schizont containing multiple magenta nuclei can be differentiated easily from host cell cytoplasm (Wright-Giemsa stain).

Figure 8—Bone-marrow aspirate from cat with cytauxzoonosis. An extremely large mononuclear cell containing a schizont with well-developed nuclei is shown (Wright-Giemsa stain).

Figure 9—Bone marrow aspirate from cat with cytauxzoonosis. An extremely large mononuclear cell demonstrating a late stage of schizogony with cytoplasm filled with many individual merozoites is shown (Wright-Giemsa stain).

Figure 11: Morphologic progression of C. felis schizont-laden macrophages in the bone marrow of experimentally infected domestic cats as represented by Franks et al.[1] (Image reproduced with permission from the Journal of the American Animal Hospital Association.)

granular parasites (Figure 11). Macrophage nuclei are notably marginalized with stippled chromatin patterns and single, large, prominent nucleolus. In experimental infections, progressive changes in the morphology of infected macrophages have been documented.[1,14]

Molecular Analysis

Molecular assays performed on whole blood are also available to aide clinical diagnosis when *C. felis* piroplasms are present in low circulating numbers, such as in early infection or carrier states. The PCR targets a 284 bp segment of the *C. felis* 18s rRNA gene and is specific enough to differentiate *C. felis* from other *Babesia* species.[105] No published literature on the specificity relative to *Cytauxzoon europaeus*. or *Cytauxzoon manul* was found during this literature review. The PCR assay was able to reliably detect as few as ten 18s rRNA gene copies in 1 microliter of feline whole blood with detection of 0.01 18s rRNA gene copies/µL about 20% of the time.[105] Limitations of PCR include delayed diagnosis associated with sample collection, shipping, and processing and the inability to differentiate acute and chronic infections.

In addition to PCR, in situ hybridization in histologic sections of *C. felis*-infected tissues has been described, but may not be commercially available.[45] In this case, the molecular target was a ribosomal RNA sequence of Babesia microti that was 91% identical to the equivalent *C. felis* rRNA sequence. In situ hybridization was able to detect ~2-3 times more C. felis-infected macrophages, particularly in smaller vessels, than light microscopy alone. Although in situ hybridization is not necessary for a definitive histologic diagnosis of feline cytauxzoonosis, it may prove beneficial in further studies characterizing pathophysiologic responses in affected tissues.

Treatment, Survival Predictors, and Prevention

Despite the high mortality rate of acute feline cytauxzoonosis, quick institution of treatment strategies is still recommended. Current treatment of feline cytauxzoonosis is two-pronged, including antiprotozoal medications and supportive care. Prevention strategies focus on reducing tick-vector exposure and there is ongoing vaccine development for at-risk domestic cats

Treatment

Various antiprotozoal medications have been evaluated, mostly in small case series of naturally and experimentally infected cats. Currently, combination therapy with the antioprotozaol drug atovaquone and the antibiotic azithromycin has been shown to have the best survival rate in naturally infected cats and is considered the standard of care. Despite this recommendation, 40% of treated cats are still expected to succumb to infection.[52] Successful treatment with combination atovaquone and azithromycin therapy may also be hindered by a cytochrome b genotype conferring atovaquone resistance in some *C. felis* organisms.[81,106] Diminazene aceturate in combination with imidocarb diproprionate successfully led to survival of natural infection in 5/6 domestic cats; however, patients remain parasitemic following treatment.[83,107,108] Use of diminazene aceturate is limited in the United States by lack of FDA approval and difficulty attaining the medications. Imidocarb diproprianate administration alone resulted in a 26% survival of naturally infected domestic cats.[52] Paravaquone and buparavaquone, historically used for the treatment of bovine theileriosis, are ineffective.[109] Supportive therapy for clinical symptoms, including intravenous fluid administration, analgesics, oxygen supplementation, and parental nutrition, are necessary for hospitalized patient comfort, but may not improve clinical outcomes.[88] In fact, outside of anecdotal experiences, no published data exists indicating supportive care improves clinical outcomes at all in naturally infected domestic cats. A better understanding of pathophysiologic mechanisms and host-organism interactions could uncover future therapeutic targets.

Predictors of Survival

With no large-scale prospective studies on feline cytauxzoonosis, information on potential predictors of survival is limited and widely dispersed among disparate publications. Furthermore, it is unclear if survival is a primary function of host immune responses, infecting *C. felis* genotype, treatment strategies, or a combination of all these. This lack of reliable predictors for survival is not only a function of protozoal sepsis caused by feline cytauxzoonosis, but also other forms of sepsis and SIRS in feline patients.

Anecdotal evidence suggests that host immune responses in cats surviving natural infection are less pronounced than those in patients succumbing to the disease. Meinkoth et al.

documented the perception by primary care veterinarians that survivors failed to develop hypothermia or respiratory distress as typically expected just prior to death. Frontera-Acevedo et al. concluded that systemic cytokine responses are higher in cats that die from natural infection, but treatments were not documented. In cats treated with atovaquone/azithromycin therapy, higher white blood cell concentrations, lower parasite burdens, and lower bilirubin concentrations were all associated with a greater chance of survival.[52] As leukopenia and total bilirubin concentrations may be affected by cytokine responses, these surviving cats may have had a less pronounced systemic inflammatory response. However, the effects of treatment on reducing parasite burden and subsequent inflammatory responses are unclear.

Many questions remain about the association of *C. felis* genotypes with survival. Initial reports of surviving cats suggested a potential correlation between ITS1-ITS2 sequences and survival. Yet, similar sequences have been identified in domestic cats that survive and succumb to natural infection. Despite publications suggesting a lack of an association between strain and survival, recent published reviews continue to perpetuate the possibility of association. A single publication dedicated to assessing strain in both deceased and persistently infected patients seems necessary to fully address this question. Regardless of infecting *C. felis* strain, evaluation of the *C. felis* cytochrome b gene does seem warranted in naturally infected cats to predict response to atovaquone/azithromycin therapy.[81] Other as yet undiscovered features of the *C. felis* organism and genotype may prove to be associated with patient survival in the future.

Prevention

As with most infectious disease control strategies in feline patients, limiting exposure to infectious has been the mainstay of disease prevention. Maintaining cats as strictly indoor pets in addition to consistent acaricide application is often recommended in endemic areas. Limit cat exposure to heavily tick-infested outdoor areas, particularly in the spring and fall months when tick feeding peaks. Topical acaricidal agents, including selamectin+soralaner and imidacloprid+flumethrin, have been proven to prevent *C. felis* infection by killing attached tick vectors before *C. felis* transmission can occur.[110,111] As more carrier cats are identified, tick control will be necessary to reduce transmission, especially in multi-cat households.

Development of an effective vaccine targeting highly conserved *C. felis* proteins have also been proposed for high-risk feline populations. Ideal protein targets should be recognized by

the immune system, conserved across all *C. felis* strains, and critical to the schizogonous tissue phase associated with patient morbidity and mortality.[112] As schizogony has not been successfully cultivated *in vitro*, genetic targeting of homologous protein sequences and protein microarrays have been used to determine 33 candidate proteins for vaccine targeting.[112,113] At least one of these proteins encoded by the "cytauxzoon felis 76" (cf76) gene was determined to be highly expressed during schizogony via *in situ* hybridization of affected lung tissue.[112] Early evaluation of vaccines developed from these protein targets failed to prevent infection and, in 3/7 challenged cats, failed to prevent death even in the face of experimental treatment.[113] However, one surviving cat did demonstrate reduced symptom severity. The failed vaccine response may have been the result of vaccine production procedures or inappropriate candidate proteins. Further research regarding the function of these highly conserved proteins in the *C. felis* life cycle, pathophysiology, and vaccine candidacy is warranted.

Conclusions

Many challenges in feline cytauxzoonosis remain providing opportunities for future research endeavors. As the ecological range of tick vectors expands, increasing case numbers are expected in geographical regions unfamiliar with the clinical presentation of acute feline cytauxzoonosis. Raising awareness of this disease among veterinary practitioners in historically nonendemic areas will be needed. In depth understanding of the pathophysiologic mechanisms, host-parasite interactions, and immune responses is lacking. The inability to successfully cultivate *C. felis* through the schizogonous phase in a laboratory setting has limited the tools available for uresearching pathophysiologic mechanisms and immune responses. Other techniques will need to be devised until successful cultivation is achieved. Acute feline cytauxzoonosis as a model for understanding the unique pathophysiologic mechanism of SIRS and other forms of sepsis in cats may prove useful in furthering our understanding of acute inflammation in this species. Many questions also remain regarding the pathophysiologic responses and epidemiologic role of *C. felis* carrier cats. Further research in these areas may prove beneficial in identifying potential treatment targets and improving clinical outcomes.

Chapter 2 - Hematologic and biochemical profiles of cats naturally infected with *Cytauxzoon felis*: 28 cases (2007-2018)

Objective: The geographical distribution of feline cytauxzoonosis is expanding in the United States. Clinical signs of feline cytauxzoonosis include lethargy, anorexia, and icterus, similar to hepatic lipidosis and cholangiohepatitis. Identifying hematologic and serum biochemical abnormality patterns may assist practitioners in recognizing and differentiating feline cytauxzoonosis from other causes of feline icterus.

Samples: Hematology and serum biochemistry profiles of 28 parasitemic cats naturally infected with *Cytauxzoon felis*

Procedures: Retrospective search of the Kansas State Veterinary Diagnostic Laboratory records database between January 2007 and June 2018 for intraerythrocytic *Cytauxzoon felis* piroplasms and concurrent complete blood counts and serum biochemical profiles.

Results: Feline cytauxzoonosis patients present with nonregenerative anemia (20/28, 71.4%), leukopenia (23/28, 82%), thrombocytopenia (23/23, 100%), hyperbilirubinemia (27/28, 96.6%), hypoalbuminemia (26/28, 93%), reduced (18/28, 64%) or low normal (10/28, 36%) serum ALP activity, and hyponatremia (23/28, 82%). No correlation between severity of anemia and magnitude of hyperbilirubinemia was identified. Reduced ALP activity was unique to cats with feline cytauxzoonosis, relative to hepatic lipidosis and cholangiohepatitis. Schizont-laden macrophages were visible on peripheral blood smears in 21.4% (6/28) of cases.

Conclusions and Clinical Relevance: Hematologic and serum biochemical abnormalities of feline cytauxzoonosis are similar to feline bacterial sepsis. The combination of non-regenerative anemia, leukopenia, thrombocytopenia, hyperbilirubinemia, and reduced serum ALP activity in icteric cats should increase the clinical suspicion, but is not pathognomonic, for feline cytauxzoonosis. Hyperbilirubinemia is more common than anemia. Blood smear evaluation for intra-erythrocytic *Cytauxzoon felis* piroplasms, tissue aspirates for schizont-laden macrophages, or molecular testing are necessary for diagnostic confirmation.

Introduction

In the United States, feline cytauxzoonosis is a rapidly progressive and often fatal condition of domestic cats caused by *Cytauxzoon felis* (CF). *Cytauxoon felis* is a tick-transmitted protozoal hemoparasite endemic to the southeastern and southcentral United States.[77] Asymptomatically infected bobcats (*Lynx rufus*) are the primary reservoir host for CF, which is subsequently transmitted to domestic cats via the tick vectors *Ambylomma americanum* and *Dermacentor variabilis.* Over the past two decades, published reports identifying CF in northern nonendemic areas of the United States have increased, coinciding with the northern expansion of tick vector ecological range. [10,11,13,112] Familiarizing practitioners, especially those in nonendemic areas, with the hematologic and biochemical profile changes associated with CF could facilitate improved diagnostic recognition, quicker initiation of appropriate treatment, and reduced patient suffering.

Cytauxzoon felis has a classical protozoan lifecycle with two distinct infectious phases in domestic cats: 1) the tissue phase and 2) the erythrocytic phase.[77] Briefly, during the tissue phase, CF organims undergo schizogony within blood-vessel associated mononuclear cells throughout the body, particularly within the spleen, liver, lungs, and lymphoid organs. Schizont-laden macrophages accumulate in and around blood vessels causing vascular injury, thrombosis, and possibly ischemic injury.[72] The erythrocytic phase begins as infected macrophages rupture, releasing numerous CF merozoites, also known as piroplasms, into the blood stream where they infect circulating erythrocytes. A tick vector then acquires a blood meal of merozoite-infected erythrocytes from an ill or persistently infected felid. During blood meal digestion, merozoites are freed from erythrocytes and undergo gametogenesis within the tick gastrotintestinal and salivary gland epithelium. The resultant CF sporozoites are then transmitted to the next felid host during future tick feedings completing the organism's life cycle.[112]

Clinical symptoms develop during the late tissue phase due to inflammation and vascular damage facilitated by schizont-laden macrophages.[77,114] Early symptoms, such as lethargy, anorexia, and general malaise, are nonspecific. Pyrexia is common and appears to coincide with the onset of the erythrocytic phase.[1] Affected cats are frequently icteric, mimicking other feline conditions such as hemolytic anemia, hepatic lipidosis, and cholangiohepatitis. Patients may become dehydrated with lymphadenomegaly, hepatosplenomegaly, and tachypnea. Within days of diagnosis, most infected cats succumb to infection or are euthanized due to disease

progression.[1,8,10,87] A small percentage survive natural infection, sometimes without treatment.[4,47] Survivors maintain a low number of ciruclating intra-erythrocytic CF piroplasms, appear clinically healthy, and may serve as additional CF reservoir hosts.

Diagnosis of CF relies on accurate identification of intraerythrocytic CF piroplasms on a routine blood film. As clinical symptoms of CF may overlap with other causes of icterus, practitioners less familiar with cytauxzoonosis may not routinely evaluate blood films from icteric cats. Cats in the early erythrocytic phase of the disease may have a low number of easily overlooked circulating intraerythrocytic piroplasms.[77] Finally, common drying artifacts, Howell-Jolly bodies, or platelets overlapping erythrocytes have similar microscopic appearances that can be misidentified as CF piroplasms. Molecular assays for the detection of CF gene sequences are available but may not be rapidly employed. As CF continues to move into nonendemic areas, overcoming these diagnostic challenges is necessary.

Hematologic and serum biochemical profile pattern recognition is already familiar and well-established in veterinary medicine. We hypothesized that CF patients have common hematologic and serum biochemical abnormality patterns that may alert clinicians to CF in clinically ill, icteric cats. Hematologic changes associated with experimentally induced CF have been described for decades; however, serum biochemical changes are often minimally described as secondary findings.[1,10,66,76,77] Our study retrospectively describes both the hematologic and serum biochemical profile patterns in a larger cohort of naturally infected CF cats. These results were compared to biochemical patterns in cats with hepatic lipidosis and histologically confirmed cholangiohepatitis, which may have similar clinical presentations.

Case Selection Criteria

The Kansas State Veterinary Diagnostic Laboratory database[a] was searched for cats with CF between January 2007 and June 2018. CF was diagnosed via microscopic identification of characteristic intraerythrocytic CF piroplasms in peripheral blood smears by a board-certified or board-eligible veterinary clinical pathologist. (Fig. 7) Only animals with both a complete blood count and serum biochemistry profile at the time of diagnosis were included. If multiple complete blood count and/or serum biochemical profiles were performed on a single animal, only the profile completed at initial diagnosis was included. For patients of the Kansas State

University Veterinary Health Center, records were reviewed to determine the survival status following diagnosis.

In addition, the Kansas State University Veterinary Health Center medical records were searched for cats diagnosed with hepatic lipidosis (HL) or cholangiohepatitis (CH) during the same timeframe. HL was confirmed by either cytologic or histologic evaluation of hepatic samples. Only those cats with histologic confirmation of CH were included. All cats received a complete blood count and serum biochemical profile at initial diagnosis. If more than one profile was performed on a single animal, only those results reported on the date closest to the cytologic or histologic sample collection were used.

In 2014, the Clinical Pathology Laboratory at the Kansas State Veterinary Diagnostic Laboratory replaced a Cell Dyn® 3700[b] hematology analyzer with a Siemens ADVIA® 2120i[c] and a Hitachi® 911[d] serum biochemical analyzer with a Roche Cobas® c501[e]. In accordance with American Society for Veterinary Clinical Pathology guidelines[115], unique analyzer-specific feline reference intervals were developed for both analyzers. Utilizing Graph Pad® Prism 7[g], each analyzer's datasets were assessed for normal distribution with the Shapiro Wilk test and an estimate of central tendency and distribution (means ± SD or median and range). As the estimate of central tendency was significantly different for some analytes, descriptive statistics expressed as percentages above and below the analyzer-specific reference interval was deemed the best method for reporting comparative results. Proportions of analyte values below, within, and above reference interval were evaluated via Fischer Exact Test or the Spearman Rank test (Graph Pad Prism 7) between cats with CF, HL, and CH for selected analytes.

Results

Database analysis yielded 52 mentions of "Cytauxzoon felis" in feline blood smear reports between January 2007 and June 2018. Intraerythrocytic piroplasms were visible in 48/52 cases. Only 29 of these 48 cases had both a concurrent complete blood count and serum biochemical profile. One of these 29 cats received two complete blood count and serum biochemical profiles within a 48-hour period. Only the first of these was included in data analysis. Thus, 28 cases met our inclusion criteria.

Male cats (19/28, 68%) predominated with 14 neutered (50%) and 5 unaltered (18%). Eight spayed females (29%) and one cat with an unspecified sex (3%) completed the CF sample

set. Age was provided in 26/28 cases with an average of 4.75 years ranging from 4 months to 11 years. The majority were domestic shorthairs (20/28, 71%) with fewer domestic longhairs (4/28, 14%), and one each of the following: domestic medium hair, American Shorthair, Maine coon, and unspecified. All cases occurred between March and October. Of 24 cases with known outcomes, all died or were euthanized within 48 hours of admission. Testing for 18/28 (64%) CF patients was performed between 2007 and 2013 utilizing the Cell Dyn® 3700 and Hitachi® 911 while the remaining 10/28 (36%) patient samples were analyzed using the Siemens ADVIA® 2120i and Roche Cobas® c501.

The majority of parasitemic CF cats were anemic based on test values below the lower limit of the reference interval for packed cell volume (PCV), calculated hematocrit (Hct), hemoglobin concentration (Hgb), and/or erythrocyte concentration (RBC) (Table 1). Visibly icteric serum was present in 27/28 cases. The single cat without icteric serum was not anemic. Anemias were non-regenerative in all cases due to lack of polychromasia noted on blood smear evaluation (28/28) and automated reticulocyte concentrations ≤ 20,000 reticulocytes/uL for 10/10 samples analyzed with the Seimens ADVIA® 2120i. Reticulocyte concentration was not measured on samples analyzed by the Cell Dyn® 3700 and manual reticulocyte concentrations were not performed on any of the remaining 18 samples. Red blood cell indices supported non-regenerative anemia as the majority of cats had MCV and MCHC results within reference interval (Table 1). Nucleated erythrocytes were present in 35.7% of parasitemic cats (10/28). Schizont-laden macrophages (Figure 11) were observed on the peripheral blood smear in 21.4% (6/28), all with a concurrent inappropriate rubricytosis.

Leukocyte changes indicated acute inflammation. Leukopenia, characterized by a marked lymphopenia and neutropenia with left shift, was common (Table 1). Mild to marked toxic changes were often present in neutrophils (25/28, 89%) even when a left shift was not observed (12/25, 48%). Reactive lymphocytes were observed in approximately half of cases (13/28, 46%) while granular lymphocytes were observed in one case.

Table 1. Proportions of Cytauxzoon felis infected cats with hematologic analytes below, above, and within reference interval. *Analytes having a lower reference limit of zero (0) are denoted as not applicable (N/A)

Hemogram	Below RI*	Within RI	Above RI
RBC	12/28 (43%)	16/28 (57%)	0/28 (0%)
Hb	14/28 (50%)	14/28 (50%)	0/28 (0%)
Hct	19/28 (68%)	9/28 (32%)	0/28 (0%)
PCV	19/28 (68%)	9/28 (32%)	0/28 (0%)
MCV	2/28 (7%)	25/28 (89%)	1/28 (4%)
MCH	1/10 (10%)	7/10 (70%)	2/10 (20%)
MCHC	0/28 (0%)	21/28 (75%)	7/28 (25%)
Retic	N/A	10/10 (100%)	0/10 (0%)

Leukogram	Below RI*	Within RI	Above RI
WBC	23/28 (82%)	5/28 (18%)	0/28 (0%)
Neut	14/28 (50%)	14/28 (50%)	0/28 (0%)
Band	N/A	15/28 (54%)	13/28 (46%)
Lymph	27/28 (96%)	1/28 (4%)	0/28 (0%)
Mono	N/A	26/28 (93%)	2/28 (7%)
Eos	N/A	28/28 (100%)	0/28 (0%)
Baso	N/A	28/28 (100%)	0/24 (0%)

Thrombogram	Below RI	Within RI	Above RI
Plt	24/24 (100%)	0/24 (0%)	0/24 (0%)

Prior to 2014, standard operating procedures in the Kansas State Veterinary Diagnostic Laboratory Clinical Pathology Laboratory dictated invalidation of platelet concentrations and blood smear platelet estimates when platelet clumping was present. Thus, for 5/18 CF patients evaluated between 2007 and 2013, no platelet concentration or platelet estimate values were available due to platelet clumping. All samples evaluated between 2014 and 2020 received either an automated platelet concentration or semiquantitative platelet estimate. Based on available platelet estimates or measured concentrations, all cats (23/23, 100%) were thrombocytopenic. Concurrent platelet clumping was documented in 15/24 cats with platelet estimates or automated measurements, confounding interpretation.

Frequently observed serum biochemical abnormalities in the CF cats included hyperbilirubinemia, hypoalbuminemia, often with a concurrent hypoproteinemia, and hyperglycemia (Table 2). No statistical correlation between the magnitude of the total bilirubin concentration and severity of anemia was identified. Serum hepatic and muscle enzyme activities were similar in CF cats. Most CF cats (64.2%, 18/28) had serum ALP results below the reference interval or within the lower quartile of the reference interval (35.7%, 10/28). ALT activity levels were within the reference interval for the majority of evaluated cases (Table 2). Of the five cats with ALT enzyme activity below the reference interval, three concurrently had low ALP enzyme activity. AST and GGT enzyme activities are not included in the standard feline serum biochemical profile at the Kansas State Veterinary Diagnostic Laboratory; thus, AST and GGT pattern data is not available. One half of parasitemic cats (14/28) had increased serum CK activity levels.

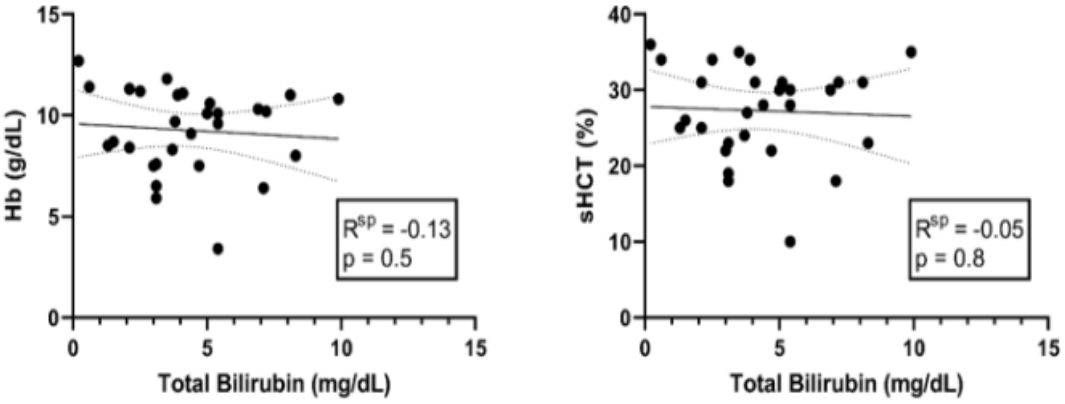


Figure 12: No correlation between serum hemoglobin, spun hematocrit (PCV), and serum total bilirubin concentration in cats naturally infected with *C. felis*

Table 2. Proportion of Cytauxzoon felis infected cats with serum biochemical analyte results below, above, and within reference interval. *Analytes having a lower reference limit of zero (0) are denoted as not applicable (N/A). BUN = blood urea nitrogen; ALT = alanine transaminase; ALP = alkaline phosphatase

Serum Biochemical Analyte	Below RI*	Within RI	Above RI
Glucose	0/28 (0%)	5/28 (18%)	23/28 (82%)
BUN	3/28 (11%)	10/28 (36%)	15/28 (53%)
Creatinine	1/28 (4%)	23/28 (82%)	4/28 (14%)
Total Protein	20/28 (71%)	8/28 (29%)	0/28 (0%)
Albumin	26/28 (93%)	2/28 (7%)	0/28 (0%)
Globulins	1/28 (4%)	26/28 (92%)	1/28 (4%)
Calcium	15/28 (54%)	13/28 (46%)	0/28 (0%)
Phosphorus	2/28 (7%)	17/28 (61%)	9/28 (32%)
Sodium	23/28 (82%)	5/28 (18%)	0/28 (0%)
Potassium	17/28 (61%)	11/28 (40%)	0/28 (0%)
Chloride	22/28 (79%)	6/28 (21%)	0/28 (0%)
Bicarbonate	3/28 (11%)	25/28 (89%)	0/28 (0%)
ALT	5/28 (18%)	23/28 (82%)	0/28 (0%)
ALP	18/28 (64%)	10/28 (36%)	0/28 (0%)
Total bilirubin	N/A	1/28 (4%)	27/28 (96%)
Cholesterol	4/28 (14%)	24/28 (86%)	0/28 (0%)
Creatine Kinase	1/28 (4%)	14/28 (50%)	13/28 (46%)

Common trends in renal and electrolyte values were also present amid the CF cats. Azotemia, usually manifesting only with increased BUN concentration, was present in approximately half of the CF cats (Table 2). Only 4 azotemic cats had a concurrent increase in serum creatinine concentration. Hyponatremia (23/28, 82%), hypochloremia (22/28, 79%), and hypokalemia (17/28, 61%) were common, but not always concurrent. Although blood gas profiles were not performed, 3/28 cats had evidence of a metabolic acidosis (decreased serum [HCO3-]). Hypocalcemia was present in approximately half (15/28, 54%) of CF cats and always in patients with a concurrent hypoalbuminemia, suggesting a decreased protein bound calcium fraction. Few cats were hyperphosphatemic (9/28, 32%), all with concurrent azotemia indicating decreased renal excretion as the likely pathophysiologic mechanism for hyperphosphatemia.

We hypothesized that anemia, leukopenia, thrombocytopenia, and decreased ALP activity could be useful parameters to help prioritize CF over other causes of hyperbilirubinemia and icterus in cats, particularly hepatic lipidosis and cholangiohepatitis. Based on our medical records search parameters, 72 cases of cytologically or histologically confirmed hepatic lipidosis and 8 cases of histologically confirmed cholangiohepatitis were identified. Proportions of cats with analyte values below, within, or above RI were compared and found to be significantly different between groups for anemia (determined by assessing hemoglobin concentration, Figure 13A, as it is less influenced by preanalytical errors), leukopenia (Figure 13B), and thrombocytopenia (Figure 13C). Although the majority of cats in all three groups were hyperbilirubinemic, hyperbilirubinemia was more common in CF cats (Figure 4A). As expected, the distribution of ALP activities between CF, HL, and CH cats was also significantly different (Figure 4B). ALP activities below reference interval were present almost exclusively in CF cats. Only one cat with HL had ALP activity below RI.

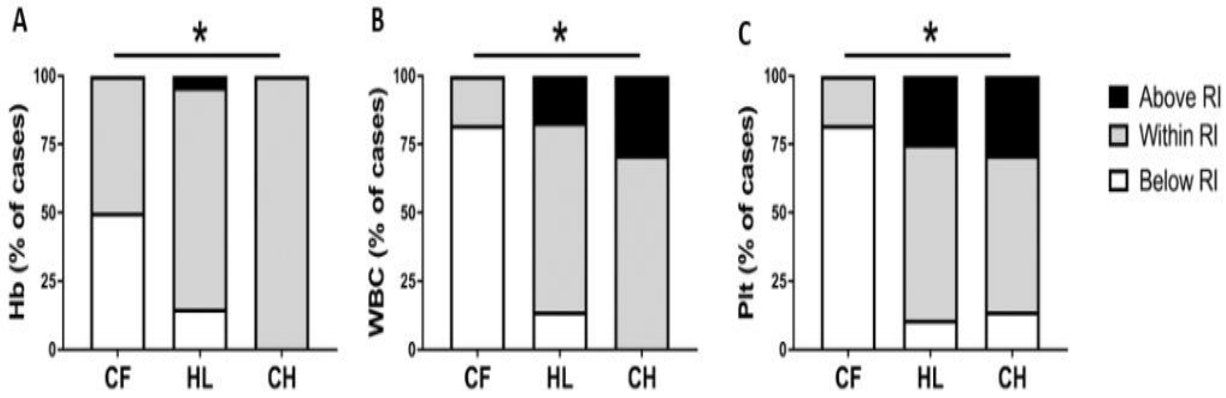


Figure 13: Statistically significant differences in proportion of cats with hemoglobin, leukocyte (WBC), and platelet (Plt) concentrations below, within and above reference interval in cats with feline cytauxzoonosis (CF), hepatic lipidosis (HL), and cholangiohepatitis (CH)

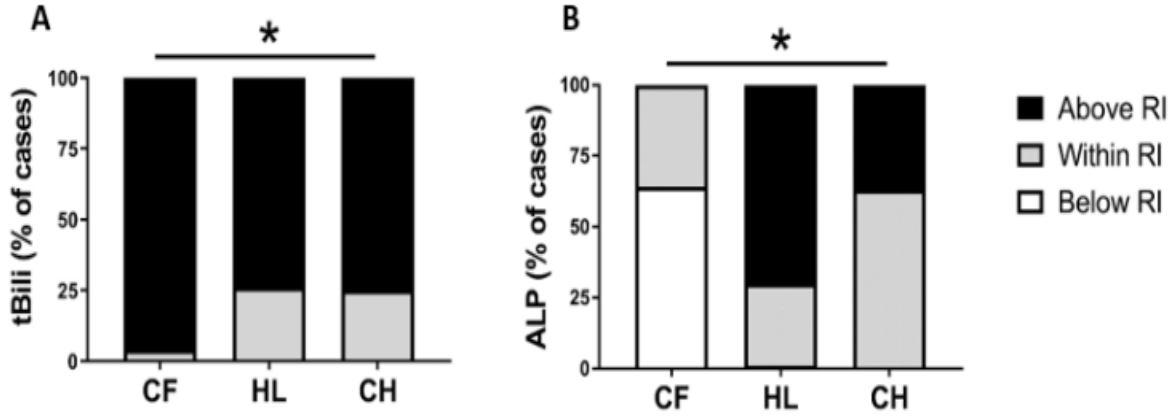


Figure 14: Statistically significant differences in proportion of cats with total bilirubin and serum ALP concentrations below, within, and above reference interval in cats with feline cytauxzoonosis (CF), hepatic lipidosis (HL), and cholangiohepatitis (CH)

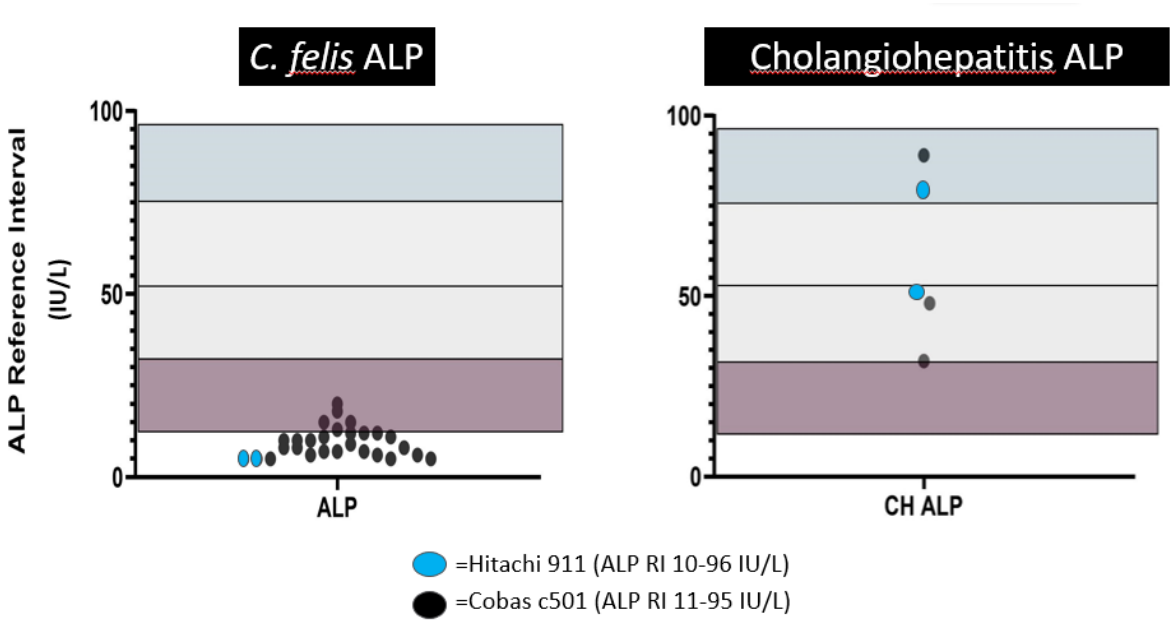


Figure 15: Distribution of serum ALP activity results for *C.felis* infected domestic cats and cats with histologically confirmed cholangiohepatitis. HL not shown due to magnitude of ALP increase altering the proportions in the y-axis values. Purple = lowest quartile of reference interval. Gray = two middle quartiles of reference interval. Blue = top quartile of reference interval.

Discussion

Hematologic and serum biochemical parameters in naturally infected CF patients are similar to those reported with other forms of systemic sepsis in cats.[70,116] Nonregenerative anemia, leukopenia, and thrombocytopenia are usually, but not always, present. Hyperbilirubinemia, hypoalbuminemia, and hyperglycemia coincides with findings reported in a smaller case series of CF patients.[10,76,79] Pre-renal azotemia and electrolyte disturbances were common. The low or within reference interval serum ALP and ALT activities contrasted with prior publications[76,91] indicating that liver enzyme activities in CF patients "can often be increased". Serum ALP activities for CF, HL, and CH significantly differed and may help prioritize differentials for icteric cats at presentation.

Hematologic and serum biochemical abnormalities in CF cats, including lower serum ALP enzyme activity, resemble those reported in cats with systemic bacterial sepsis[70,105,116]. These similarities are not unexpected as clinical sepsis is simply a systemic inflammatory response to an infectious agent - bacterial, fungal, protozoal, viral, or algal. CF is simply systemic protozoal sepsis. Both CF and feline systemic bacterial sepsis patients have increased

serum or tissue expression of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β.[3,70,71] These pro-inflammatory cytokines are responsible for hematologic and serum biochemical changes associated with acute inflammation.[82] In both CF and systemic bacterial sepsis, observed inflammatory changes include leukopenia characterized by lymphopenia and concurrent neutropenia often with a left shift. Decreased serum concentrations of negative acute phase proteins, including albumin and some lipoproteins, resulting in hypoalbuminemia and hypocholesterolemia, respectively, are well-known. Stress hyperglycemia due to high cortisol, norepinephrine, and epinephrine is not uncommon[69] and, in people, exacerbated by TNF-α and IL-1β inhibition of insulin release.[95] Because of these similarities with feline systemic bacterial sepsis, evaluation of a peripheral blood smear, tissue aspirates, and/or molecular testing are necessary for CF diagnosis.

Hematologic findings of CF patients were similar to those previously reported[1,76,77,87]. Leukopenia, neutropenia, left shifted neutrophils, and toxic neutrophil changes were common. In contrast to other published reports[61], identification of granular lymphocytes in blood smears was rare (1/28, 3.5%). This discrepancy in the significance of circulating granular lymphocytes may be due to subjective variability between veterinary professionals reviewing and reporting blood film observations. Based on this data, the presence of granular lymphocytes does not appear to be associated with the presence of *C. felis* piroplasms. Although thrombocytopenia was present in 24/24 cats with an automated or estimated platelet concentration, confirming true thrombocytopenia in CF patients proved difficult due to concurrent platelet clumping in 15 of these 24 cats. Platelet clumping falsely decreases automated platelet counts and blood smear platelet concentration estimates as fewer individualized platelets are available for counting.[82] This pseudothrombocytopenia phenomenon is particularly common in cats. Yet, pseudothrombocytopenia was not as common in the HL or CH populations. True thrombocytopenia, likely due to platelet consumption, was suspected for all CF patients by the board-certified or board-eligible clinical pathologist reviewing blood films. When evaluating a blood film for intra-erythrocytic CF piroplasms, clinicians should be aware of platelet clumping and its effects on automated platelet counts.

Nonregenerative anemia was common (71.4%, 20/28) and defined as a PCV, HCT, Hgb, and/or RBC less than the lower limit of the RI in the presence of a normal automated reticulocyte concentration or lack of visible polychromasia. Despite the absence of regeneration, circulating

nucleated erythrocytes (i.e. rubricytosis) were present in approximately 1/3 of CF patients (35.6%, 10/28). This inappropriate rubricytosis is suspected to result from CF-induced vascular endothelial inflammation in the bone marrow and spleen causing premature leakage of nucleated erythrocytes into circulation. Inappropriate rubricytosis is also present in 35% of cats with feline bacterial sepsis due to similar pathophysiologic mechanisms.[116] Interestingly, all 6/28 CF cases with visible schizont-laden macrophages on the peripheral blood film had concurrent rubricytosis supporting the hypothesized pathophysiologic association between schizont-laden macrophages, vascular inflammation, and inappropriate rubricytosis. Inappropriate rubricytosis should prompt closer examination for schizont-laden macrophages on the peripheral blood film of cats with suspected acute cytauxzoonosis.

The pathophysiologic mechanism of anemia in parasitemic CF cats is widely accepted as piroplasm-induced extravascular immune-mediated hemolysis facilitated by macrophages in the spleen, liver, and bone marrow.[1,77,86] This is supported by histologically observed erythrophagocytic macrophages in the bone marrow of cats experimentally infected with CF.[1] The lack of regeneration has been attributed to the rapid clinical decline of CF patients before the bone marrow mounts a regenerative response.[1] Although mild anemia has been reported in persistently infected CF carriers[4], published reports of continued or repeated hemolytic crises are not available, raising questions about the intensity and longevity of piroplasm-induced hemolytic immune responses. In one author's clinical experience (BK), persistently infected CF cats have not been anemic and do not have other clinical evidence of continued hemolysis such as serum hyperbilirubinemia or icterus. Other factors may also be contributing to the failed regenerative response in acutely ill CF patients. Inflammatory cytokines, particularly IL-6, can exacerbate a nonregenerative response by limiting iron availability, altering erythropoietin responsivity, and reducing erythrocyte longevity.[84,85] Inflammation-induced anemia in cats may be evident as early as 3 days following identification of systemic inflammation.[84] Although an unlikely primary mechanism for CF anemia, inflammatory cytokines may be exacerbating the failed regenerative response in acutely ill CF patients.

Notably, about one third of parasitemic CF patients (8/28, 28.6%) were not anemic at presentation, potentially due to reference intervals or patholphysiologic mechanisms. The majority of nonanemic cats were identified between 2007 and 2013 prior to the reference interval change. If newer reference intervals for feline PCV are applied to all samples, the number of

anemic CF cats increases to 25/28 (98.2%). This calls attention to the importance of analyzer-specific reference intervals in clinical interpretations. Alternatively, non-anemic CF cats may have presented prior to developing anemia or with concurrent dehydration masking anemia. Marked pre-renal azotemia (urea nitrogen 89 mg/dL [16-35 mg/dL]; creatine 4.6 mg/dL; [0.8-2.1 mg/dL]; urine specific gravity 1.054) was present in one non-anemic CF cat indicating potential clinical dehydration. As complete clinical history and follow up data were not available for all CF patients, non-anemic CF carriers may have been included here. Recently, the prevalence of CF carrier cats in eastern Kansas was determined to be 25.8%, raising some concern for inclusion of potential CF carriers in this retrospective analysis.[2] However, all cats included in this study presented with clinical signs compatible with acute feline cytauxzoonosis. Ultimately, the absence of anemia should not exclude CF as a differential at the time of presentation.

As with anemia, hyperbilirubinemia in CF patients is attributed to piroplasm-induced immune-mediated hemolysis. Most CF patients (27/28, 96.4%) were hyperbilirubinemic at presentation, yet not all were anemic (6/27, 22.2% of hyperbilirubinemic CF cats). In several individuals, the magnitude of hyperbilirubinemia was disproportionately high for a relatively mild anemia. Among all individuals, no correlation between the magnitude of anemia, determined by hematocrit or hemoglobin concentration, and hyperbilirubinemia was evident. This decoupling of anemia and hyperbilirubinemia suggests that hyperbilirubinemia in CF patients is likely multifactorial.

Intra-hepatic pathophysiologic mechanisms may be playing a role in development of hyperbilirubinemia. Feline hepatocytes have reduced glucuronidation activity relative to other species; thus, the rate of intra-hepatic bilirubin conjugation and subsequent excretion via the biliary tract is slower.[117] This may slow the rate of bilirubin excretion in bile. Histologically, hepatic vessels and sinusoids are distended by schizont-laden macrophages which may theoretically compress nearby bile ducts. However, bile canaliculi dilation and intra-canicular bile accumulation have not been described in the livers of CF patients.[73,77] Finally, a functional cholestasis initiated by pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β, must be considered.[73,94] In people, these pro-inflammatory cytokines reduce hepatocyte excretion of bilirubin into bile canaliculi causing accumulation of bilirubin within the hepatocyte cytoplasm. Functional cholestasis is known to cause increased serum ALP concentrations in dogs and people. However, serum ALP activity is not a sensitive indicator of cholestasis in cats[82] and may

not be increased with functional cholestasis in feline patients. As discussed, the pathophysiologic effects of these pro-inflammatory cytokines are evident histologically and systemically in CF patients.[3,71,73,82] Recently, the magnitude of hyperbilirubinemia, but not the degree of anemia, was associated with a higher mortality rate in experimentally infected CF patients.[52] If functional cholestasis is contributing to hyperbilirubinemia in CF patients, hyperbilirubinemia may be an indirect indicator of systemic inflammation mediated by IL-6, TNF-α, and IL-1β. Further research is necessary before ruling out a functional cholestasis in septic cats based on the absence of increased serum ALP activity alone.

Unexpectedly, serum hepatic enzyme activities were not increased in CF patients, contradicting prior publications.[37,91] One report evaluating the efficacy of anti-protozoal medications for treatment of acute feline cytauxzoonosis also reported a lack of increased serum ALT activity.[52] We considered analytic error; however, similar results utilizing two serum biochemical analyzers, each with unique reference intervals, indicates analyzer error is unlikely here. In cats, serum ALT activity is an important marker of hepatocyte injury secondary to hypoxia, inflammation, mechanical trauma, and hepatocellular toxins among others.[82] Histologically, vascular occlusion and inflammation by schizont-laden macrophages in hepatic vessels and sinusoids induces thrombosis, ischemia, and subsequent hepatocyte injury or death creating an environment ideal for increasing serum ALT activity results.[72,73,77] Yet, serum ALT activity was normal or decreased in all 28 cats. Similarly, normal or low serum ALT activity is reported in feline bacterial sepsis.[116] As serum ALT activity increases secondary to hepatocellular injury or rupture, it is theoretically possible that, despite the histologic lesions, insufficient hepatocellular injury is present in CF patients for a substantial serum ALT increase. Other enzymes associated with hepatocellular injury, such as AST, SDH, or GLDH, may have a greater sensitivity for detecting hepatocellular injury in septic feline patients. Comparison of hepatocellular injury enzyme activities in a large number of cats with various forms of sepsis is needed to confirm this unexpected pattern.

Serum ALP activity was also lower than expected, similar to reported results in feline bacterial sepsis.[116] Traditionally, low serum ALP activity is considered clinically insignificant. Yet, the repeatability of low or low normal serum ALP activity in feline patients with bacterial and protozoal sepsis challenges that dogma. As discussed above, cytokine-induced functional cholestasis seems likely in CF patients. In people and dogs, functional cholestasis results in

increased serum ALP activity via indirect effects of IL-6, TNF-α, and IL-1β.[94,118] Pro-inflammatory cytokines reduce hepatocellular bile salt excretion indirectly inducing increased ALP release from hepatocyte membranes into plasma.[94] As increased serum ALP activity is not a sensitive indicator of feline cholestatic disease, feline hepatocytes may not respond to intracellular bile acid accumulation with increased ALP activity as in dogs and people. Alternatively, the lack of increased ALP activity in septic cats may indicate hepatocellular enzyme responses to pro-inflammatory cytokines in functional cholestasis are unique in feline patients. Evaluation of other cholestatic enzymes, particularly GGT, may provide some insight into cholestatic enzyme patterns in septic cats. Unfortunately, GGT was not included on the standard feline serum biochemistry panel and was not evaluated here. Further studies comparing cholestatic enzyme patterns in cats with inflammation-induced functional cholestasis and other causes of cholestasis are needed.

The significant difference in the ALP value of CF cats versus HL and CH cats may help practitioners prioritize differentials in icteric cats. Increased serum ALP activity can be present with both HL and CH, particularly as these disorders may occur simultaneously, but is more pronounced in HL patients.[119] Here, ALP activity above the reference interval was also more common in HL compared CH patients (Figure 4). The statistically significant difference between serum ALP activities in HL and CH patients when compared to the CF group was striking. All patients in the CF group had an ALP result lower than or within the bottom quartile of the RI. Both HL and CH results for ALP followed well-established serum ALP activity patterns suggesting that these smaller cohorts are statistically representative of the larger population.[119,120] Thus, serum ALP activity results may be beneficial to practitioners in quickly prioritizing between CF , HL, and CH.

Azotemia characterized by increased BUN (15/28, 53%) often without a concurrent creatinine increase (4/15 cats with increased BUN, 27%) was fairly common. All cats with increased creatinine concentrations had concurrently increased BUN concentration. This selective BUN increase is consistent with previous reports[10,76], although decreased and normal BUN concentrations have also been described.[52,76,83] A disproportionate BUN increase relative to creatinine indicates a pre-renal azotemia, typically associated with a high protein diet or gastrointestinal hemorrhage in small animals. CF patients have evidence of hemorrhage on post-mortem examination with one report of a single cat with blood clots identified in the intestinal

lumen.[72,76,79] Although less common, this selective increase in BUN can occur with hypovolemia.[82] Hypovolemia slows renal tubular flow rates allowing more time for BUN resorption in the renal tubule. In addition, hypovolemia stimulates antiduretic hormone (ADH) secretion. ADH promotes BUN resorption in the distal nephron by up to four times baseline resulting in increased serum BUN without a concurrent increase in creatinine concentration.[82] Acute kidney injury associated with sepsis may also be considered, although a concurrently increased creatinine concentration is usually expected. In septic people with clinical evidence of acute kidney injury, the pro-inflammatory cytokine IL-6 has been linked to increased ADH activity in the distal nephron and altered tubular epithelial function in the face of adequate renal perfusion.[100] With known increases in IL-6 in CF and septic cats, a similar pathophysiologic mechanism may be enhancing distal nephron resorption of urea in feline patients. Further evaluation of the effects of pro-inflammatory cytokines on renal function and ADH concentrations in septic cats is necessary.

Electrolyte disturbances including hyponatremia (23/28, 82%), hypochloremia (22/28, 79%), and hypokalemia (17/28, 61%) were common, but not always concurrent. Similar electrolyte patterns are evident in cats with bacterial sepsis.[69,70,116] Hyponatremia and hypokalemia have been reported previously in CF patients.[76] Hyponatremia of CF patients is likely due to renal loss of sodium. If CF patients are similar to septic people, IL-6-induced ADH release can potentiate sodium loss in the renal tubule.[100] Due to histologic and gross necropsy evidence of pulmonary edema, pleural, and/or pericardial effusion, perivascular third-spacing of sodium may also be considered.[72,73] In most CF patients, hypochloremia and hyponatremia were proportionally decreased indicating the same pathophysiologic mechanism for both abnormalities. The three CF patients without proportional chloride and sodium changes (data not shown) had a concurrent metabolic acidosis evidenced by decreased serum bicarbonate concentrations. Complete blood gas profiles were not available in this retrospective case series. Finally, hypokalemia was often mild and attributed to anorexia prior to presentation although renal potassium loss may also be present.

CF was diagnosed via visualization of intraerythrocytic CF piroplasms on peripheral blood films evaluated by a board certified or board-eligible veterinary clinical pathologist; however, blood film evaluation has limitations. Other feline protozoal hemoparasites, such as *Babesia felis* and a European *Cytauxzoon sp.*, have similar microscopic appearances.[121] No

known published or anecdotal reports of these organisms in domestic cats residing in the United States were identified by the authors. Thus, the observed intraerythrocytic piroplasms in clinically affected cats in the United States are presumed to be CF. Confirmation of CF with molecular assays was not performed. Because of similarities between CF piroplasms, common artifacts, and Howell-Jolly bodies, the diagnostic accuracy of blood film evaluation likely depends upon the experience of the veterinarian reviewing the smear. Regardless, up to 50% of affected cats may have no visible circulating piroplasms at the time of initial presentation[114] making repeated blood film evaluation necessary. With these diagnostic limitations, hematologic and serum biochemical abnormalities in conjunction with the patient's clinical presentation should prompt an initial and repeated blood film evaluation by an experienced veterinarian or clinical pathologist.

The restricted geographical region of CF patients here may have affected our results. First, the familiarity of local practitioners with CF coupled with strict inclusion criteria may have contributed to the small sample size. Clinicians in CF endemic areas may be more likely to suspect feline cytauxzoonosis at presentation and achieve a diagnosis via a point-of-care blood film evaluation without submitting samples to a diagnostic laboratory. In addition, there are conflicting reports on associations between regional CF strains and patient survival.[16,48,50] We cannot rule out potential effects of local CF strains on the hematologic and serum biochemical results described here. Additional multi-institutional prospective studies identifying strains, cytokine responses, blood work abnormality patterns, and clinical outcomes are needed.

To achieve higher case numbers, the timeframe for sample selection was extended resulting in the utilization of two hematology and two serum biochemical analyzers. As instrument performance and reference intervals are unique to each analyzer, direct comparisons between analyte results are not recommended. In some individuals, RI altered clinical interpretations. To address the lack of direct comparison, descriptive statistics relative to analyzer-specific RI were deemed the most reliable way to present data. This limited our ability to provide clinical decision limits for CF patients. However, the overall patterns observed were similar between both hematology analyzers and both serum biochemical analyzers. This indicates that the observed patterns may be repeatable among the wide range of hematologic and serum biochemical analyzers used in veterinary practices and laboratories. However, additional large scale, multi-institutional studies are needed to confirm this hypothesis.

Conclusion

Feline patients with intraerythrocytic CF piroplasms often present with a nonrgenerative anemia, leukopenia, thrombocytopenia, hyperbilirubinemia, reduced serum ALP activity, and hyponatremia. This constellation of hematologic and serum biochemical changes may alert practitioners to prioritize CF as a differential for critically ill, icteric cats. Hematologic and serum biochemical patterns in CF patients are similar to these reported in feline bacterial sepsis. Thus, patterns are not specific for feline cytauxzoonosis and should not replace other diagnostic tests. A diagnosis of feline cytauxzoonosis requires accurate identification of intraerythrocytic CF piroplasms via blood film evaluation, observing schizont-laden macrophages in tissue or blood samples, and/or molecular testing for CF nucleic acid sequences.

Chapter 3 - Proposals for future research in the pathophysiology of acute feline cytauxzoonosis

Little published data exists on the detailed pathophysiologic mechanisms of feline cytauxzoonosis beyond standard descriptions of gross, histologic, hematologic, and serum biochemical findings, as discussed in Chapter 1 and Chapter 2. Experimental *in vitro* studies have been hindered by the inability to successfully cultivate *C. felis* through the schizogonous phase. A deeper understanding of the pathophysiologic mechanisms of this disease may present opportunities for new prognostic determinants, prevention strategies, and potential treatment targets for acutely affected domestic cats. Below are research proposals stemming from the literature review of Chapter 1 and retrospective evaluation of hematologic and serum biochemical analyses in Chapter 2. Each proposal includes a truncated introduction, general methods overview, justification, and anticipated study design limitations.

Evidence of functional (inflammation-induced) cholestasis in feline cytauxzoonosis

Domestic cats naturally infected with *C. felis* have increased serum levels and pulmonary tissue expression of the same pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) known to induce functional (i.e. inflammation-induced) cholestasis in people.[3,71,94] In dogs and people, this functional cholestasis results in both increased serum total bilirubin concentrations and ALP enzyme activities.[94]As outlined in Chapter 2, the serum biochemical profiles of domestic cats naturally infected with *C. felis* demonstrated a hyperbilirubinemia which did not correlate with the degree of anemia and low or low-normal serum ALP activity. This same pattern of increased pro-inflammatory cytokines and hyperbilirubinemia, but the absence of increased serum ALP activity, has been reported in cases of feline systemic bacterial sepsis and non-infectious causes of systemic inflammatory response syndrome (SIRS).[69,122] Traditionally, reduced serum ALP enzyme activity has been viewed as clinically insignificant. Yet, the repeatability of reduced serum ALP enzyme activity in published reports of systemic bacterial sepsis, SIRS,[69,70,116], and now protozoal sepsis (see Chapter 2) in feline patients may warrant reconsideration of this dogma. While the hyperbilirubinemia in *C. felis* is likely multifactorial, I hypothesize that a

functional cholestasis is contributing to the development of hyperbilirubinemia but is not associated with increased serum ALP activity as expected in other species.

A brief review of the pathophysiologic mechanisms of functional cholestasis is warranted (Figure 13).[94] Although both total bilirubin and serum ALP increases are observed in functional cholestasis in humans and dogs, the pathophysiologic pathways differ. The mechanism for hyperbilirubinemia will be discussed first. Unconjugated bilirubin, a product of red cell hemoglobin degradation, is transported to hepatocytes bound to albumin and then freely diffuses across the hepatocyte cell membrane. Once inside the hepatocyte, bilirubin is conjugated to glucuronide via the enzymatic activity of uridine glucuronyl transferase to form conjugated bilirubin. Feline hepatocytes have decreased glucuronidation capabilities relative to other species, which may contribute to hyperbilirubinemia due to unconjugated bilirubin accumulation in hepatocytes or serum.[117] Conjugated bilirubin is then actively transported into the bile canaliculi via multidrug resistant protein 2 (MRP2), the rate limiting step of bilirubin excretion. In sepsis, IL-6, TNF-α, and IL-1β all downregulate MRP2 expression resulting in intracellular accumulation of both conjugated and unconjugated bilirubin leading to hyperbilirubinemia. This intracellular accumulation of bilirubin is protective in acute inflammation as bilirubin is a free-radical scavenger and modulates inflammatory cytokine responses.[123] In contrast, increased serum ALP activity results from intracellular accumulation of bile acids. IL-6, TNF-α, and IL-1β downregulate the bile salt export pump (BSEP) responsible for exporting bile salts into the bile canaliculi. The resulting increase in intrahepatocyte bile acids induces hepatic production of ALP and, indirectly, release of ALP into the plasma via cytoplasmic blebbing. Increased serum alkaline phosphatase activity may play a role in reducing renal oxidative injury associated with systemic inflammation and sepsis.[98]

Previous publications on feline bacterial sepsis and SIRS ruled out the possibility of a functional cholestasis as a contributing pathogenic mechanism for hyperbilirubinemia due to the absence of an increased serum ALP concentration and a “lack of histologic evidence of cholestasis”.[69,116] Relative to dogs, feline hepatocytes normally have lower cytoplasmic concentrations of ALP. Furthermore, increased serum ALP activity is not a sensitive indicator of feline cholestatic disease.[82] Because serum ALP increases are not always evident in cats with cholestasis, the absence of increased serum ALP activity alone should not rule out cholestasis. This lack of ALP increase in the face of possible functional cholestasis may be due to a

decreased response to intracellular bile salt accumulation in feline hepatocytes; however, no published studies were identified that confirm this hypothesis.

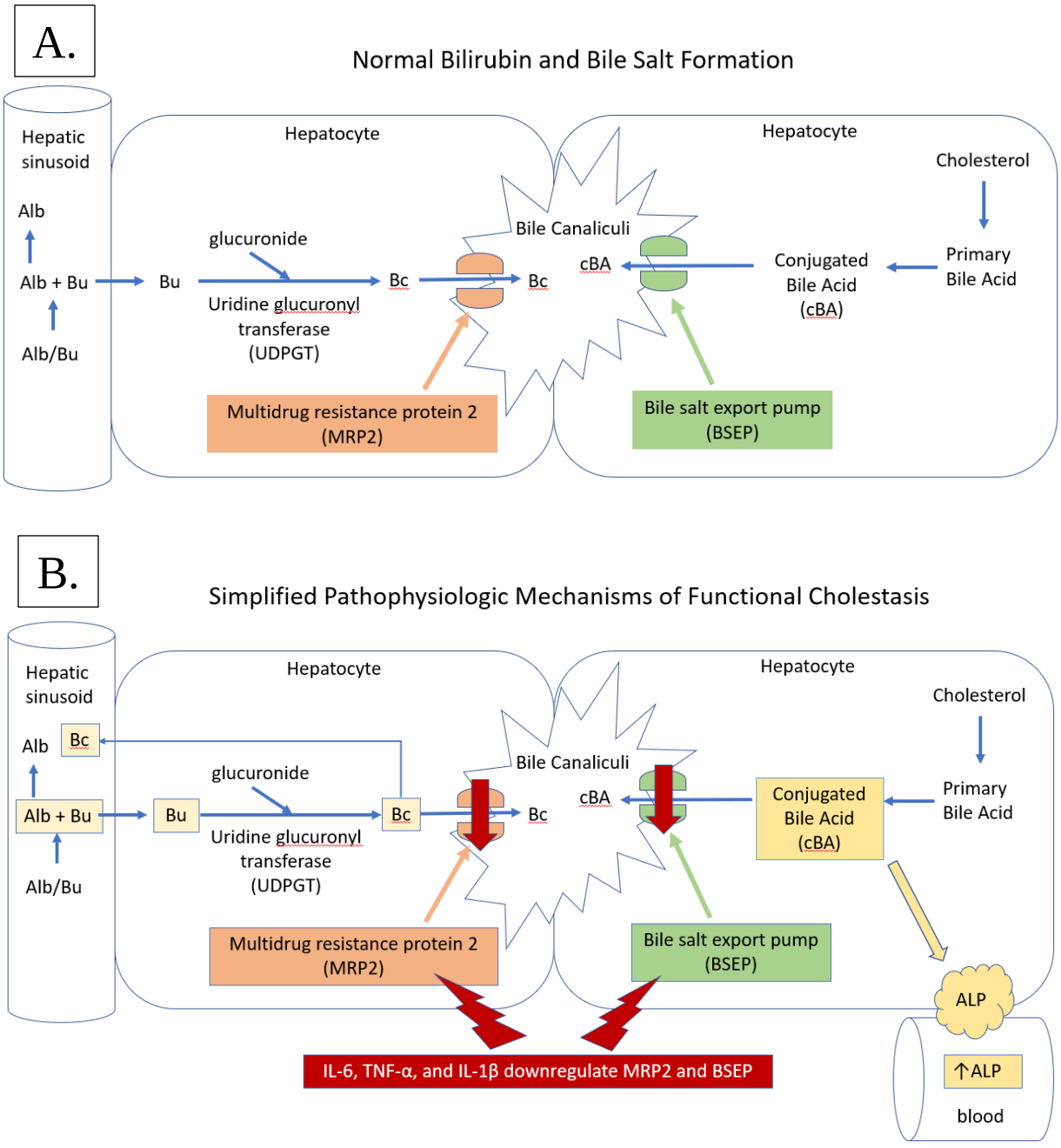


Figure 16: A) Normal bilirubin and bile salt formation in hepatocytes. B) Simplified diagram of the pathophysiologic mechanisms of functional (inflammation induced) cholestasis.

Additional analytic and pathophysiologic mechanisms for the decreased serum ALP activity must also be considered. Hyperbilirubinemia may exert a clinically significant negative interference effect on ALP measurement in human samples utilizing the Olympus AU4500 chemistry analyzer.[124] A similar negative interference in feline samples has not been reported in either the Hitachi 911® or Cobas c501® utilized in Chapter 2. Technical publications provided by Roche, the manufacturer of both the Hitaci 911® and the Cobas c501®, indicate no detectable interference in serum ALP measurement with bilirubin concentrations up to 60 mg/dL, a concentration far higher than reasonably expected in pathologic hyperbilirubinemia.

Furthermore, other feline disorders with marked hyperbilirubinemia, such as cholangiohepatitis evaluated in Chapter 2, do not result in a similar decrease in measured serum ALP activity. Anorexia has been anecdotally associated with decreased serum ALP activity in rats[125] and malnutrition and eating disorders in people.[126] Most septic cats are anorexic at presentation and a similar decrease in serum ALP activity may be present in these cats. Evaluating this may prove difficult as even short periods of anorexia in feline patients can induce hepatic lipidosis, which is characterized by a high serum ALP activity as observed in the Chapter 2 results.

Understanding the effects of pro-inflammatory cytokines on feline hepatocellular responses in acute feline cytauxzoonosis first requires 1) confirmation of intracytoplasmic bile accumulation in larger cohort of naturally infected domestic cats, 2) confirmation of localized increases in pro-inflammatory cytokines in hepatic tissues, and 3) evaluation of MRP2 and BSEP immunoreactivity in affected hepatocytes. Immunohistochemistry would assess for changes in in MRP2 and BSEP protein localization (e.g. cytoplasmic v. membranous) and intensity of membrane protein immunoreactivity relative to normal feline livers. However, full evaluation of protein expression (i.e. protein production quantification) would require additional testing modalities such as quantitative reverse transcriptase PCR for to assess MRP2 and BSEP gene expression and Western blot quantification of total protein concentration. Below are three proposed study designs to begin addressing these questions utilizing formalin-fixed, paraffin-embedded tissues sections from deceased cats with histologically evident *C. felis* schizonts. A retrospective search of the KSVDL database yielded 57 potential candidate cases between 2006 and 2021.

Future studies stemming from these results may include: 1) including other forms of sepsis/SIRS; 2) a combination of retrospective and prospective correlation with serum hepatic enzyme activities; and 3) comparison with other common causes of hepatic disease in cats such as hepatic lipidosis and cholangiohepatitis. As pathophysiologic mechanisms of disease are similar in other forms of sepsis/SIRS, hepatic tissue expression of inflammatory cytokines is expected to be similar. Correlating the immunohistochemical evidence of functional cholestasis with the serum biochemical abnormality patterns in the same patients could provide supporting evidence that functional cholestasis may not induce increased serum ALP activity in cats. Understanding localized inflammatory cytokine patterns in hepatic diseases other than feline cytauxzoonosis/sepsis/SIRS may provide additional valuable information on pathogenesis and potential treatment targets.

Histologic evidence of intracytoplasmic hepatocyte accumulation of bile in acute feline cytauxzoonosis

If pro-inflammatory cytokines are inhibiting excretion of bile salts into the bile ducts causing intra-hepatocyte accumulation of bile, traditional histologic evidence of obstructive cholestasis (i.e. distended bile ducts) would not be expected and it was not observed in this single representative liver. Instead, accumulation of golden intracellular pigments within the cytoplasm of Zone 3 hepatocytes surrounding the central vein would be expected. Yet, no descriptions of visible intracellular pigments in Zone 3 hepatocytes were found in published histologic descriptions of liver sections from *C. felis* patients. In depth descriptions beyond the presence of intravascular schizont-laden macrophages are generally lacking. Intracyotoplasmic pigments in Zone 3 hepatocytes may represent not only accumulation of bile but also lipofuscin, copper, or iron. These can be differentiated with the special stains Hall's bile, Schmorl's, rhodanine, and Perl's Prussian blue, respectively.

To examine my hypothesis of a functional cholestasis in cats, I retrospectively evaluated a hematoxylin-eosin-stained section of formalin-fixed, paraffin-embedded liver from a single randomly selected case of feline cytauxzoonosis. Golden intracytoplasmic pigment was evident in Zone 3 hepatocytes surrounding central veins that contained characteristic schizont-laden macrophages. A series of special stains was requested on serial sections of the affected liver. All

photos represent an area surrounding the same central vein known to contain *C. felis* schizont-laden macrophages.

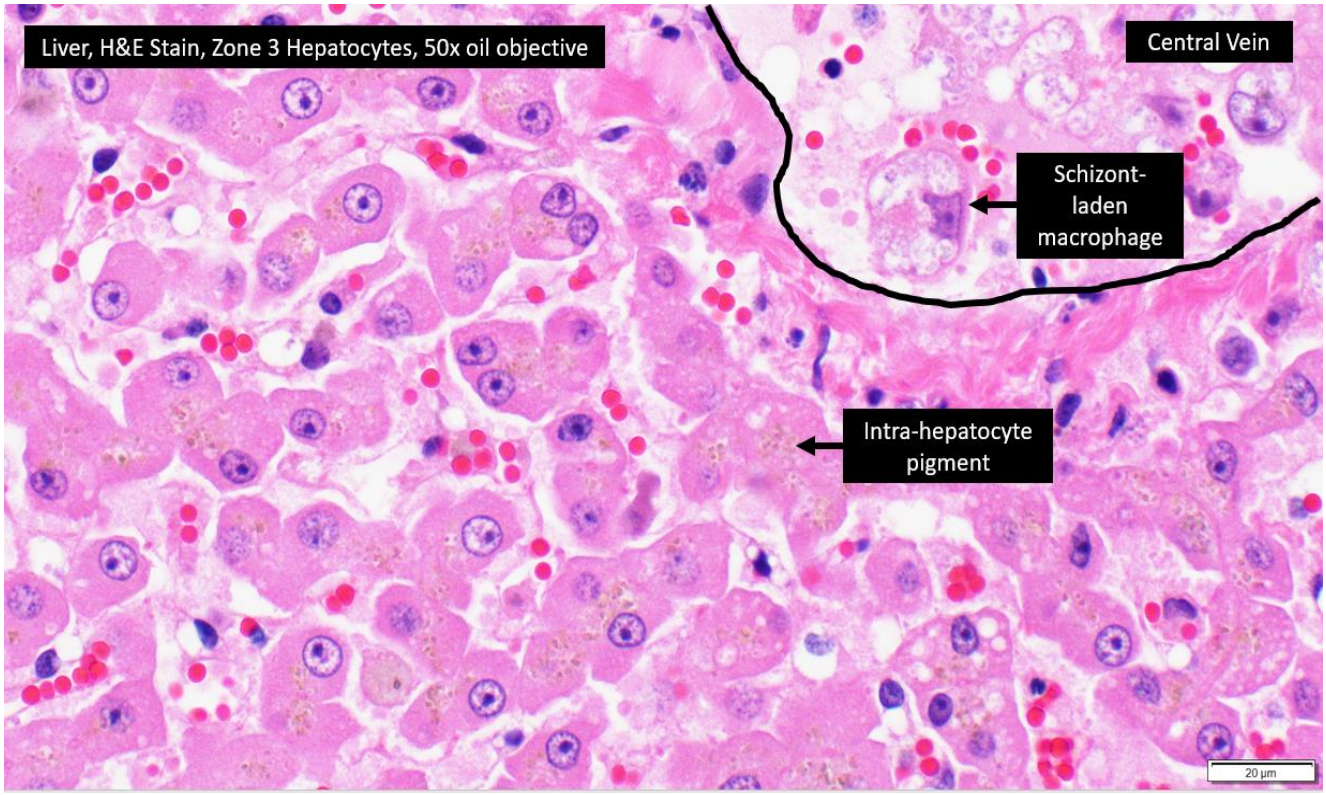


Figure 17: Hematoxylin & eosin stained FFPE section of liver from a cat with *C. felis*. Intracytoplasmic pigments are evident in Zone 3 (centrolobular) hepatocytes surrounding a central vein containing *C. felis* schizont-laden macrophages.

First, the Hall's Bile (or bilirubin) stain was performed. This stain oxidizes bilirubin into biliverdin producing a green/yellow color change in bile pigment. Intracytoplasmic bile pigments are visible in Zone 3 hepatocytes of Hall's-stained sections when compared to the serial H&E stained section (Figure 17). This intracytoplasmic bile pigment may be conjugated bilirubin accumulating due to a functional cholestasis or unconjugated bilirubin accumulating due to the decreased glucuronidation activity of feline hepatocytes and accelerated immune-mediated hemolysis. Initial staining in this single case (Figure 18) provides supporting evidence that the hypothesis of a functional cholestasis in acute feline cytauxzoonosis is plausible.

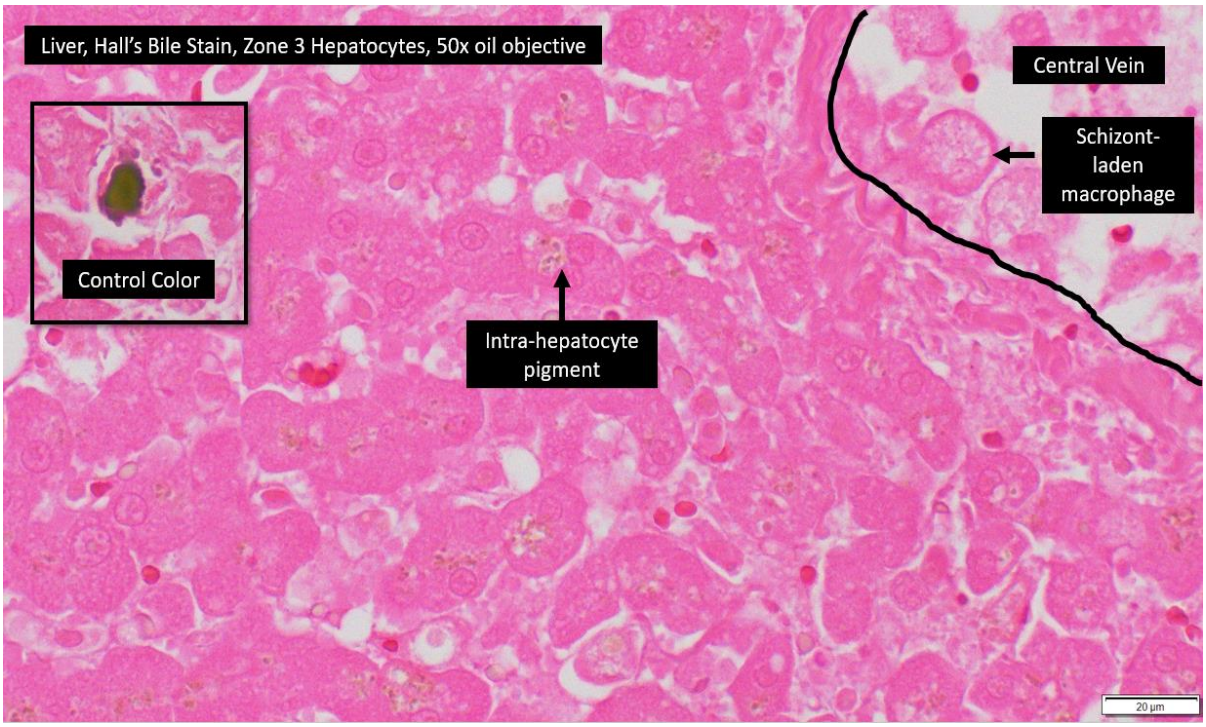


Figure 18: Tangential liver sections of a randomly selected case of feline cytauxzoonosis demonstrating the presence of cytoplasmic pigments staining positive for bile via a Hall's stain**.**

Despite the presence of intrahepatocyte bile with the Hall's stain, no normal feline hepatic tissue was evaluated concurrently for comparison with the single affected cat liver. Published data on the expected intensity of Hall's staining for intrahepatocyte bile accumulation is lacking. In a personal communication, one anatomic pathologist has not appreciated notable accumulation of intrahepatocyte bile in histologic sections of normal feline liver.[127] The small amount of intrahepatocyte pigment accumulation may have been presumed lipofuscin with no additional stains performed to confirm this assumption. However, Hall's stain is not sensitive at detecting small amounts of bile accumulation and significant accumulation must be present for a positive interpretation of this stain. Thus, intrahepatocyte bile may likely be present in normal feline samples, yet unreliably detected by Hall's staining and light microscopy.

Perl's prussian blue, Ziehl-Neelson, and rhodamine stains were also performed on serial sections to evaluate for intracellular iron, lipofuscin, and copper accumulation, respectively. Intracellular iron accumulation is expected in hemolytic and inflammatory processes, like acute feline cytauxzoonosis. Mild multifocal intracellular iron pigment accumulation was evident by the faint blue staining with Perl's Prussian blue in the cytoplasm of some hepatocytes (Figure

19); however, the majority of the intra-hepatocyte pigment is negative for iron. Lipofuscin is typically evaluated with a Schmorl's or modified Ziehl-Neelson stain; however, neither of these are available in the KSVDL histology laboratory. Instead, a standard Ziehl-Neelson was performed. Although the Ziehl-Neelson stain was negative (Figure 20), lipofuscin must still be considered a possible differential for some of the pigment observed in the H&E stained block. Copper accumulation in feline hepatocytes associated with hepatic failure and cirrhosis, heritable enzyme dysfunctions, or copper toxicity is quite rare. Rhodanine staining of the pigment was negative (Figure 21).

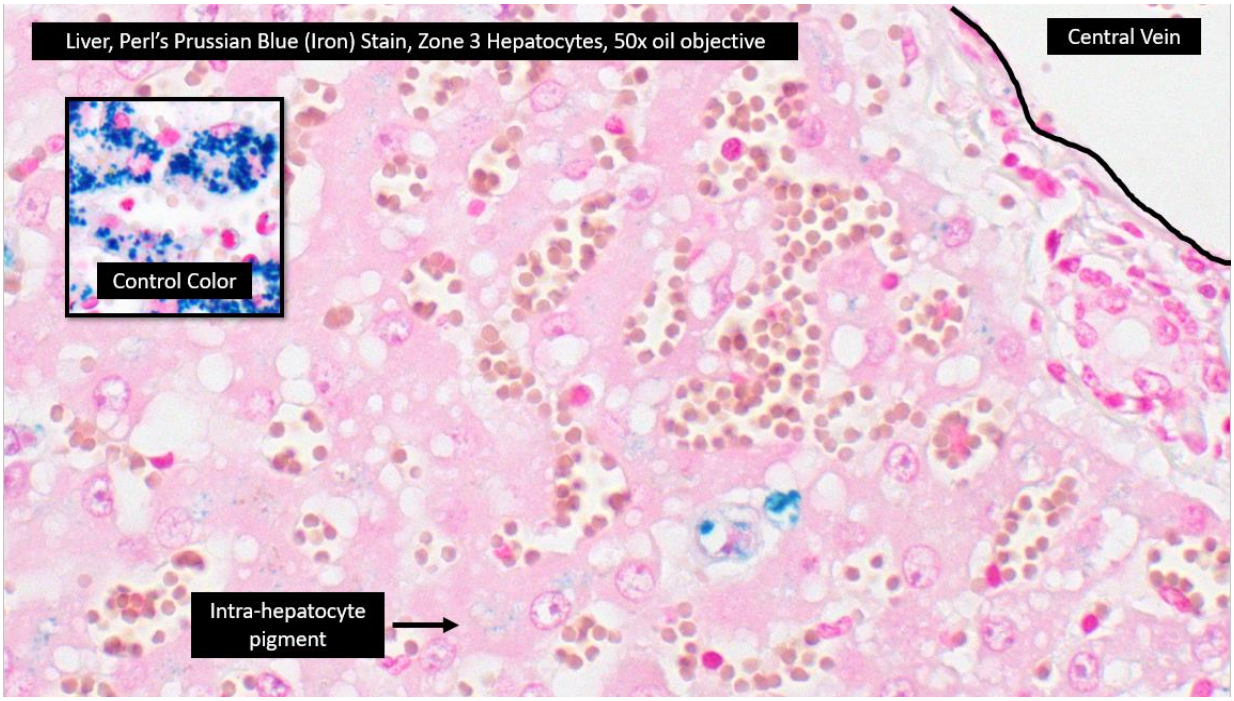


Figure 19: Perl's prussian blue stain for iron on FFPE section of liver from a cat with feline cytauxzoonosis

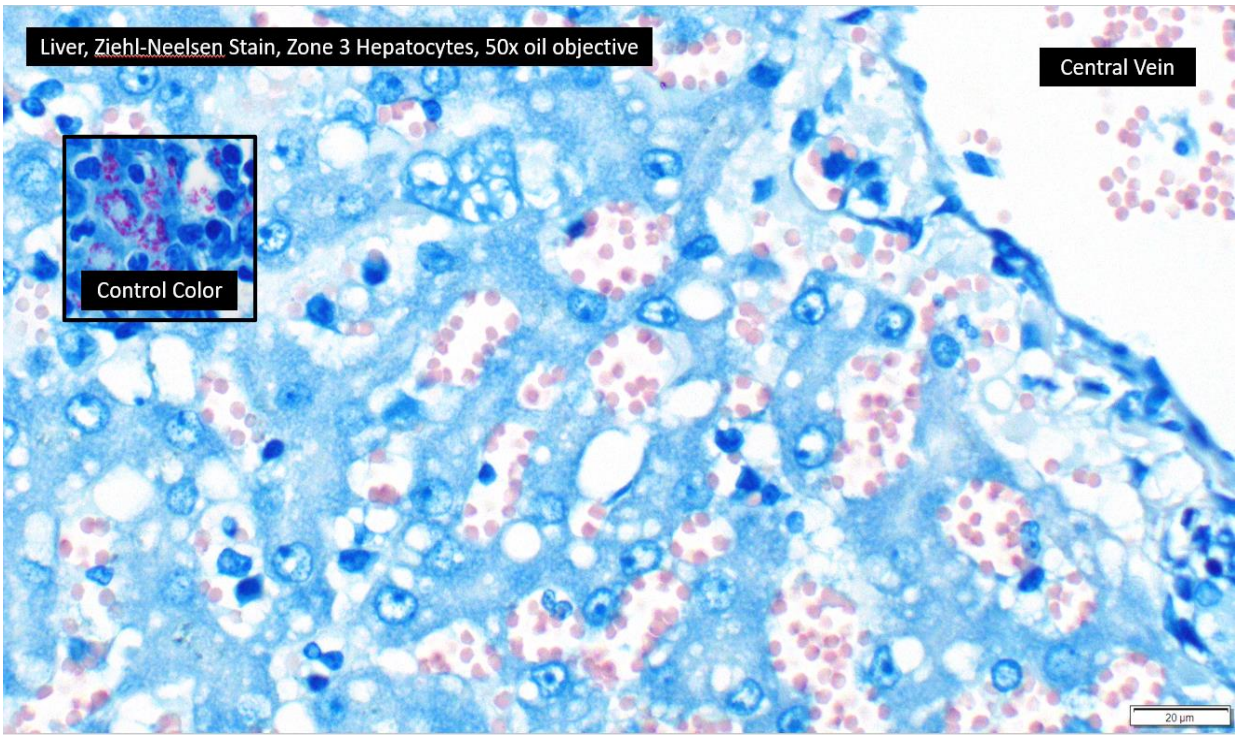


Figure 20: Ziehl-Neelsen stain in leiu of a Schmorl's stain for lipofuscin on a FFPE histologic section of liver from a cat with feline cytauxzoonosis

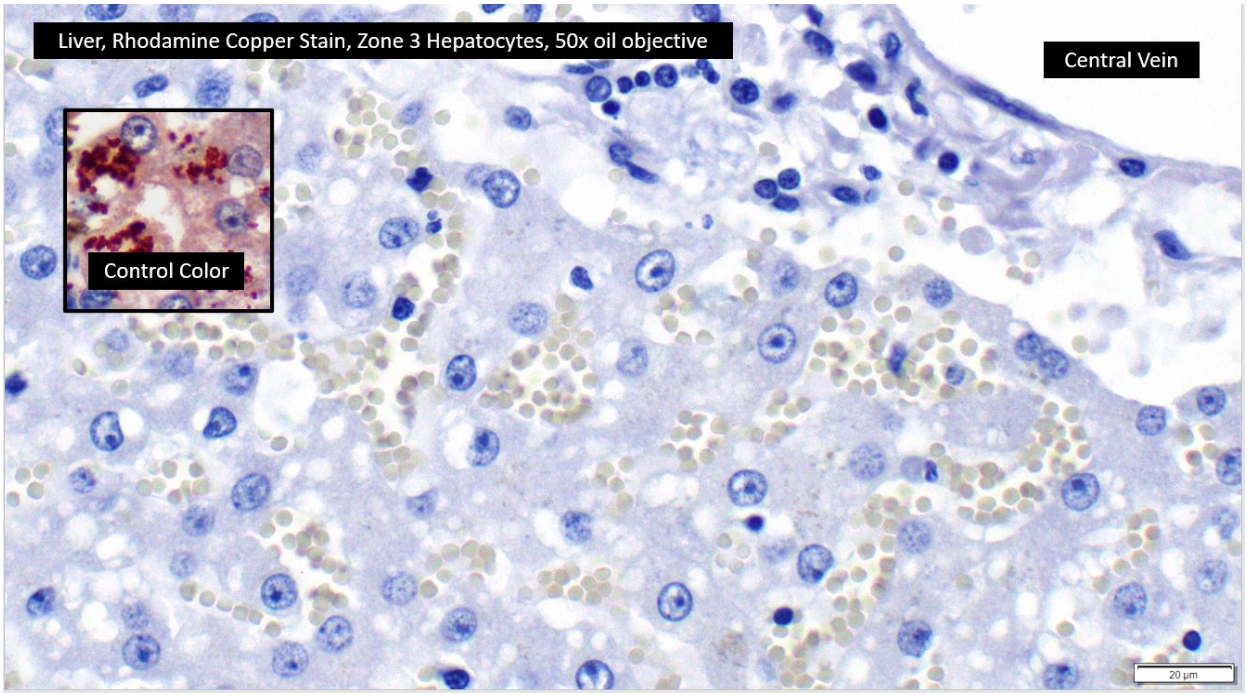


Figure 21: Rhodamine stain for copper on a FFPE liver section from a cat with feline cytauxzoonosis

Based on these preliminary results, I propose performing a Hall's bile stain, Perl's Prussian blue, and true Schmorl's/modified Ziehl-Neelson on serial tissue sections of each of the previously identified 57 prospective *C. felis* cases in the KSVDL database between the years of 2007 and 2021. Further in-depth descriptions of histologic abnormalities in H&E sections may also be competed at the time of evaluation. These stains provide evidence for the accumulation of the end products of cellular processes, but no information on the cause for their accumulation. Consequently, additional immunohistochemical evaluations are necessary to confirm the presence of functional cholestasis in acute feline cytauxzoonosis.

Hepatic immunohistochemical expression of IL-6, TNF-α, and IL-1β in feline cytauxzoonosis

Confirming the presence of functional cholestasis requires characterizing localized pro-inflammatory cytokine expression in hepatic tissues from infected cats. No descriptive studies of pro-inflammatory cytokine levels in feline hepatic tissues were found in a preliminary literature search. However, pro-inflammatory cytokine responses in affected lung tissue have been characterized by Frontera-Acevedo et al.[3] I propose evaluation for tissue expression of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β in FFPE liver sections in fatal cases of feline cytauxzoonosis and comparing the results to FFPE sections of domestic cats without hepatic disease. Increased hepatic tissue immunolabeling for IL-6, TNF-α, and IL-1β relative to normal cats would provide evidence in support of the hypothesis presented in Chapter 2 that hepatic microenvironmental conditions in cats with acute feline cytauxzoonosis, and likely other causes of sepsis/SIRS, are ideal for development of functional cholestasis even if increases in serum ALP activity are not observed. All three pro-inflammatory cytokines, IL-6, TNF-α, and IL-1β, are produced and expressed by hepatocytes; thus, cytoplasmic immunolabeling in hepatocytes is expected.[128-130]

Frontera-Acevedo et al. utilized the following antibodies for immunohistochemical evaluation if affected lung tissues: 1) goat anti-mouse TNF-α polyclonal antibody (Clone M-18, Santa Cruz Biotechnology), 2) goat anti-feline IL1β polyclonal antibody (Catalog Number AF1796, RnD Systems), and 3) mouse anti-feline IL-6 monoclonal antibody (Clone 341031, RnD Systems). Both the IL-6 and IL-1β anti-feline antibodies are specific and validated for feline tissues. The anti-TNF-α antibody is polyclonal but shown to cross-react with feline tissues in prior publications.[131] However, neither these manuscripts provides full validation information

(e.g. western blot analysis) and no control tissues were discussed. Based on the potential for background staining and ubiquity of these cytokines (see Figure 9), an isotype control seems warranted.

Tissues sections were incubated with these primary antibodies overnight at 4◦C. Because this incubation period creates challenges for potential automation, IHC's will need to be performed manually. Primary antibody dilutions, antigen retrieval, and detection methods are listed in the table of Figure 22. Frontera-Acevedo et al. utilized a subjective scoring system for immunoreactivity intensity with evaluation via light microscopy by multiple individuals, including a board certified veterinary anatomic pathologist. Digital scanning and pixel quantification systems, such as that provided by Aperio, can produce more consistent and reliable determinations of immunoreactivity.

Table 3:Primary immunohistochemical antibody methods reported by Frontera-Acevedo et al.[1] (Table reproduced with permission from Science Direct publishing.) L.A.B = undefined in primary publication; ABC = avidin-biotin complex kit

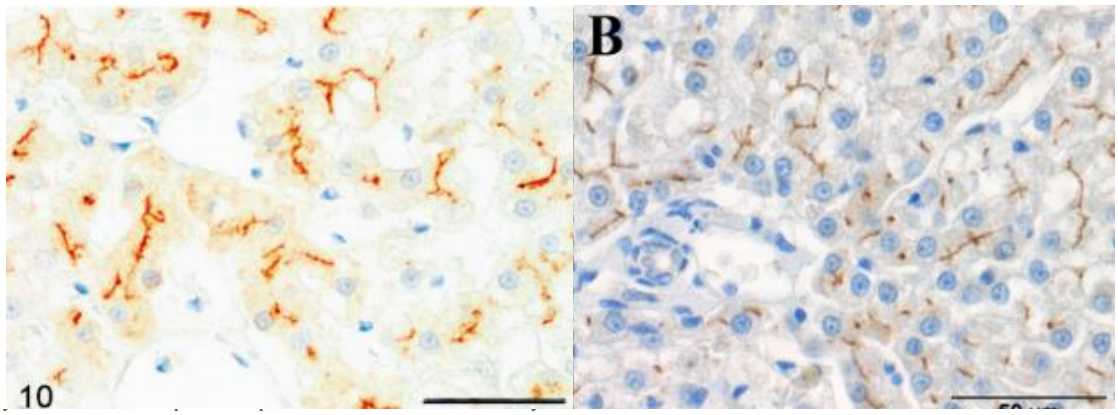
Primary antibody	Dilution	Final concentration (μg/mL)	Antigen retrieval method	Detection method
TNF-α	1:100	N/A	L.A.B	Polymer
IL-1β	1:10	10	Citrate	ABC
IL-6	1:10	50	N/A	ABC

Immunohistochemical hepatic expression of MRP2 and BSEP in feline cytauxzoonosis

In conjunction with increased hepatic immunoreactivity for IL-6, TNF-α, and IL-1β, functional cholestasis should prompt down regulation of the MRP2 and BSEP transport proteins on the biliary canalicular membrane surface of Zone 3 (centrolobular) hepatocytes.[7] This should result in reduced immunolabeling intensity for MRP2 and BSEP on the hepatocellular canalicular membrane surface relative to normal feline hepatocytes. What follows is a truncated literature review, plan for IHC protocols, and anticipated pitfalls for immunohistochemical evaluation of MRP2 and BSEP in feline FFPE liver sections.

Several publications describing the immunohistochemical microanatomic localization of MRP2 in FFPE sections of normal and diseased feline liver sections are available as starting

points for this study.[7,132] Ijzer et al. described the presence of MRP2 on the biliary canalicular surface of normal feline hepatocellular membranes (Figure 22, upper left image). Subjectively, the immunolabeling intensity was greatest in centrolobular (i.e. Zone 3) hepatocytes. Van Sprundel et al. evaluated MRP2 expression in a series of feline hepatic neoplasms with comparison to normal feline hepatic tissue (Figure 22, upper right image). These findings corroborate the expression and appropriate tissue localization of MRP2 in feline hepatocytes indicating that appropriate cellular machinery is present for a functional cholestasis. Both publications used the same anti-MRP2 antibody and immunohistochemical protocol. Full validation of the anti-MRP2 antibody via a Western Blot was not performed in either case. Instead immunohistochemical localization to the appropriate anatomic region was cited as evidence for specificity of the antibody. Complete validation would be needed.



Antibody	Manufacturer	Type	Clone	Antigen retrieval	Dilution	Wash buffer	Incubation
MRP2	Monosan	Mouse mAb	M_2III-6	Citrate	1:300	PBS	O/N 4 °C

Figure 22: Membranous anti-MRP2 immunolabeling in normal feline hepatocytes as published by Ijzer et al (left, image and table reproduced with open access permission from jzer J, Kisjes J, Penning L, et al. The progenitor cell compartment in the feline liver: An (immuno)histochemical investigation. *Vet Pathol* 2009;46:614-621.)[7] and van Sprundel et al. (right, image and table reproduced with permission from Science Direct.)[9] The same anti-MRP2 antibody & protocol were used in both publications.

No prior publications on immunohistochemical evaluations of feline hepatic tissue for BSEP were identified in a literature search. However, BSEP mRNA has been isolated from normal feline hepatic tissues with subsequent sequencing and confirmation of protein expression in cultured cells identified via western blot[133] The feline cDNA sequence for BSEP is highly

conserved with that of dogs and humans and functional studies using BSEP in cell culture membrane vesicles suggest that feline BSEP has a similar structure and function to that of dogs and humans.[133] The presence of BSEP in cell membrane vesicles of cultured hepatocytes is further supporting evidence that feline hepatocytes are capable of developing a functional cholestasis.

Identification of a suitable anti-BSEP antibody will be necessary for this portion of the experiment. The antibody used in the western blot performed above by van Beusekom et al. was a gifted polyclonal anti-rat BESP antibody which is not commercially available. However, the homology between the human and cat proteins indicates that commercially available anti-human BESP antibodies are likely to work. Optimization of antibody selection and immunohistochemistry protocol development would be necessary.

Comparison of serum biochemical hepatic enzyme patterns in other forms of feline sepsis and non-infectious systemic inflammatory response syndrome (SIRS)

In Chapter 2, similarities between serum hepatic enzyme profiles in domestic cats with feline cytauxzoonosis and those reported in domestic cats with sepsis and SIRS were discussed. These prior publications on sepsis and SIRS did not differentiate between inciting causes of inflammation or infection. Thus, it is unclear if the patterns described in protozoal and bacterial sepsis would also be expected in fungal or viral sepsis or noninfectious forms of SIRS. Furthermore, our retrospective analysis of feline cytauxzoonosis was hindered by the availability of only two serum hepatic enzymes. To better characterize expected hepatocellular enzyme patterns in cases of systemic inflammation in feline patients, I propose a prospective evaluation of cats with various forms of bacterial, protozoal, viral, and fungal sepsis and non-infectious SIRS to: 1) confirm the repeatability of a low/low normal ALP and ALT serum concentrations in sepsis from other etiologies; 2) determine if changes in other other hepatic enzymes (GGT, AST, and GLDH) provide additional insight into the pathophysiologic mechanisms of hepatocellular injury and cholestasis in feline sepsis/SIRS, and 3) potentially evaluate the diagnostic sensitivity of hepatic enzyme activity patterns between septic and nonseptic cats or between inciting causes of feline sepsis.

Cases would be prospectively enrolled based on the presence of two or more of the following criteria: pyrexia (>/= 103.5◦F) or hypothermia (</= 100.0◦F); tachycardia (>/=225 bpm) or bradycardia (</= 144 bpm); tachypnea (>/= 40 bpm); and leukocytosis (>/= 19,500 WBC/μL) or leukopenia (</= 5,000 WBC/μL) or >/= 5% band neutrophils.[78] Selecting cases prospectively would allow for collection of more complete clinical histories and their comparison with histologic sections of any cats that subsequently died and were necropsied. In addition, full serum biochemical profiles with the addition of GGT, AST, and GLDH, which are not part of a standard feline serum biochemical profile would be possible. Evaluation of and comparison to healthy control cats in the same time frame would allow for statistical evaluation of any potential diagnostically significant cut-off values via ROC analysis. Complete blood counts and urinalyses may also be collected to look for differences in leukocyte and erythrocyte responses and better characterize expected urinalysis results, such as urine specific gravity, proteinuria, and bilirubinuria. In addition, serum samples may be preserved via deep freezing at -20ºC for future evaluation of potentially prognostic analytes in septic patients such as total thyroxine levels or serum amyloid A.[134]

Prospective analysis is limited by the relatively few cases of systemic inflammation identified in a given year, even at tertiary care facilities. Extending the enrollment period and creating collaborative projects between multiple tertiary care facilities would help alleviate the potential for low case numbers. However, the benefits over retrospective analysis are clear. Retrospective analysis would limit the availability and quality of serum biochemical and clinical data and fail to overcome the interpretation challenges already present in the literature. Identifying cases would prove difficult as searching case records for clinical data, which may be absent or mis-recorded, in a paper-based medical records database is time-consuming and unrewarding. Furthermore, identifying cases for potential histologic correlation of lesions would be challenging as clinical histories are insufficient or absent and final diagnosis of “sepsis” is reserved as a clinical syndrome with no matching morphologic entity in histopathologuy reports. Retrospective identification of specific etiologies in histologic specimens that have clinical presentations compatible with sepsis, such as *C. felis*, *Francisella tularemia*, feline coronavirus (i.e. feline infectious peritonitis), and *Histoplasma capsulatum*, might be done for comparison studies. However, this type of retrospective data analysis will exclude miscellaneous causes of

bacterial sepsis and non-infectious SIRS, such as intestinal obstruction/rupture, post-surgical complications, peritonitis, pyothorax, pyometra, pyelonephritis, etc.[69]

Association between *C. felis* strain and clinical outcome in naturally infected cats in Kansas

Questions remain regarding the effect of *C. felis* strains as characterized by IST1/IST2 genotype on the clinical outcome of infected cats. A positive association between survival and the *C. felis* ITSa genotype was demonstrated in naturally infected cats from northwest Arkansas, USA.[6] However, this same strain is known to cause fatal infections in domestic cats in Arkansas and surrounding states.[6,15,16] In addition, ITSa is the primary *C. felis* genotype circulating in wild bobcats (*Lynx rufus*) throughout the endemic United States, involving areas without natural infection survival rates as high as those reported in northwest Arkansas.[49] Retrospective sequencing of *C. felis* ITS1/ITS2 sequences in formalin-fixed paraffin-embedded tissues of infected cats in Georgia indicated a predominance of the ITSa sequence in deceased cats (Figure 10).[6] In addition, bobcats in Georgia have been demonstrated to be carriers of the ITSa strain.[13] However, no retrospective analysis of changing strains in Arkansas or identification of reservoir host strains has been published. The disconnect between *C. felis* genotype and domestic cat survival rates, both between and within geographic regions, may indicate a unique host-organism interaction with the at-risk domestic cat population in northwest Arkansas and nearby regions. This question of whether the geographical increase in survival rate is a primary function of the infecting *C. felis* strain or the immune responses within a localized at-risk domestic cat population should be addressed before conducting large-scale evaluations of pathophysiologic mechanisms with results that may not be applicable to all cats in all *C. felis* enzootic regions.

I hypothesize geographically confined increases in survival rates in at-risk domestic cat populations are not associated the infecting *C. felis* strain. Kansas may be an ideal location for this proposed retrospective study for several reasons: 1) relatively high prevalence of carrier cats[2], 2) access to histologic sections of fatal feline cytauxzoonosis, and 3) prior identification of the *C. felis* ITSa genotype in Kansas's bobcat population[13]. Furthermore, sequence analysis of the *C. felis* ITS1/ITS2 genotype has only been performed on a small handful of samples from Kansas, leaving much unknown about *C. felis* strains in our geographic area.[16] A single study

evaluating associations between strain and survival in a single geographic region with a high prevalence of survivors, frequent fatal cases, and known sequences in reservoir hosts is needed. To begin addressing this question, *C. felis* genotype strains in both fatal and nonfatal infections of domestic cats must be characterized. I propose a retrospective evaluation and comparison of *C. felis* ITS1-ITS2 gene sequences in formalin-fixed paraffin-embedded (FFPE) histologic sections of deceased cats with those in identified *C. felis* carrier populations in Kansas. This retrospective sequencing proposal will serve two purposes: 1) determine if the *C. felis* genotype in a large population of surviving domestic cats is the same as that reported in the local reservoir host population, and 2) determine if there as an association between survival and *C. felis* genotype outside of Arkansas.

Recent molecular evaluation of blood samples for *C. felis* Cox3 mitochondrial DNA from 1,104 asymptomatic owned, rescued, and feral cats in eastern Kansas indicated a *C. felis* carrier prevalence of 25.8%.[2] *C. felis* DNA extracted during this study may still be available for sequencing. This 25.8% prevalence in asymptomatic carriers is higher than the reported 15.5% in Arkansas and 12.9% in nearby Missouri targeting the *C. felis* 18s rRNA gene.[51] Although different molecular targets were utilized in these studies, findings indicate Kansas has a significant population of persistently infected domestic cats similar to Arkansas allowing for a statistically powerful number of carrier cats for comparison with fatal cases.

A cursory search of the Kansas State Veterinary Diagnostic Laboratory for histologic and necropsy cases with “cytauxzoon” in the diagnosis and comment fields between 2018 and 2021 yielded only 7 potential cases for retrospective analysis. This relatively low number of histologic sections is likely due to strict inclusion criteria for the initial retrospective search. The 2018-2021 time frame for retrospective analysis of FFPE sections was chosen as these samples are more likely to reflect fatal strain types in the domestic cat population during determination of *C. felis* carrier prevalence by Wikander et al.[2] Extending the retrospective database search to 2000 would encompass the complete time frame of the data collected in Chapter 2 and allow for detection of changing strain types over the last two decades, beginning round the time carrier cats were first being described in the literature. Data on the number of histologically diagnosed cases feline cytauxzoonosis before 2018 are not available to the author.

Formalin-fixation can induce DNA fragmentation limiting their utility in DNA sequencing studies. However, successful *C. felis* DNA extraction from FFPE samples follow by

amplification and sequencing of both the *C. felis* ITS1 and ITS2 genes has been previously described by Brown et al. and similar protocols would be followed here (Figure 25).[48] Primers used by Brown et al. targeted a 651 bp sequence incorporating a 458 bp sequence of the ITS1 region and a 431bp sequence incorporating a 265 bp sequence of the ITS2 region. These targets were small enough to avoid the effects of formalin-induced fragmentation while long enough to ensure appropriate identification of strains. FFPE dehydration and DNA extraction methods were adjusted to accommodate a 15 mg tissue specimen. Adjustments included prolonged deparaffinization and tissue digestion times.

Table 4: ITS1/ITS2 *C. felis* sequences and associated strains identified in FFPE tissue sections from *C. felis* infected cats in Georgia[6] (Table reproduced with open access permission from Brown H, Berghaus R, Latimer K, et al. Genetic variability of Cytauxzoon felis from 88 infected domestic cats in Arkansas and Georgia. *J Vet Diagn Invest* 2009;21:59-63.)

Table 1. Nucleotide variation and frequency of *Cytauxzoon felis* internal transcribed spacer region (ITS1 and ITS2) sequences.

	ITS1 nucleotide site						ITS2 nucleotide site						
Sequence	338	349	375	397	409	415	117	180	232	243	257	*n*	GenBank accession no. (ITS1, ITS2)
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.

Several limitations for this proposed study exist. Resulting data would be reflective of *C. felis* infections in Kansas and possibly nearby surrounding areas but may not be reflective of findings in other enzootic regions. Direct comparison between *C. felis* strains in fatal and surviving cases in multiple geographic locations would provide additional insight into the complex associations between this organism and the localized host population immunologic responses. In the published literature, the highest number of asymptomatic *C. felis* carriers have been identified in Arkansas, Missouri, Oklahoma, and Kansas. Comparing data from Kansas with a significant number of surviving cats from the Atlantic or Gulf Coast region may prove difficult. Finally, sequencing large numbers of samples, as would occur here, is expensive, time

consuming, and outside my primary area of expertise. Collaboration with other researchers for samples collection, sample analysis, and grant funding would be required.

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