

CYTAUXZOOM FELIS:
AN EMERGING FELINE PATHOGEN AND POTENTIAL THERAPY

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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Dedication

To my husband, Kyle

I couldn't have done this without you.

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LIST OF ABBREVIATIONS

AUC - area under the curve

CL - systemic clearance

C_{MAX} - peak plasma concentration

Ct - Cycle threshold

DIC - disseminated intravascular coagulation

FS - female spayed

FI - female intact

HPLC - high pressure liquid chromatography

IM - intramuscular

LOQ - limit of quantification

MC - male castrated

MI - male intact

MRT - mean residence time

PCR - polymerase chain reaction

T_{MAX} - time of peak concentration

Vd - apparent volume of distribution

ABSTRACT

Cytauxzoon felis is a hemoprotezoal parasite that causes substantial morbidity and mortality during the acute phase of infection in domestic cats. It is a tick vectored infection and the bobcat appears to serve as the natural reservoir host. However, domestic cats that survive the acute illness remain persistently infected and may also serve as a reservoir for the tick-transmitted pathogen.

Bobcats are believed to only develop a brief illness prior to entering the persistent carrier state. However, only fatal cytauxzoonosis had been reported in non-native wild felids (captive-reared Asian tigers (*Panthera tigris*) and an African lioness (*Panthera leo*) and her cub). We collected blood from eight tigers, a lion, cougar, bobcat, and six domestic cats living in an area endemic for *Cytauxzoon felis*. Blood smears were reviewed via light microscopy for the presence of intraerythroid organisms consistent with *C. felis*. PCR analysis for *C. felis* was performed, and the 18S rRNA gene sequence from positive samples was characterized. Four clinically normal tigers were found to be infected with *C. felis*. Intraerythrocytic organisms consistent with *C. felis* were identified microscopically in one of the four tigers. Genotyping of the pathogen from all infected tigers revealed all amplicons shared >99.8% identity with previously reported 18S rRNA sequences from *C. felis* infected North American domestic cats, and were identical to amplicons from domestic cats on the premises. Although not native to the USA, tigers may become infected by a North American strain of *C. felis* without observed

clinical illness. PCR assay for *C. felis* was more sensitive and specific than cytologic recognition of piroplasms in tigers.

Perinatal transmission of other hemoprotozoa, including related *Theileria* species, is well documented. If perinatal transmission of *C. felis* were possible, then recovered domestic queens could produce offspring that might serve as reservoirs for infection of other domestic cats via a tick vector. The objective of this study was to determine if perinatal transmission occurs between parasitemic carrier queens to their offspring. Two naturally infected intact female domestic shorthair cats were bred to produce a total of fourteen kittens in three litters. None of these kittens was PCR positive for *C. felis*. This study failed to document perinatal transmission of *C. felis* in domestic cats. If such transmission occurs, it is likely to be uncommon and probably plays little, if any, role in the spread of cytauxzoonosis to domestic cats.

Our next study objective was to characterize the pharmacokinetic profile of the anti-protozoal drug diminazene diaceturate in cats, which has shown promise in treating cats with cytauxzoonosis. Using four healthy purpose bred cats, we determined drug pharmacokinetics in the species. A powdered commercial drug formulation (Veriben®, Ceva Sanet Animale) was reconstituted with sterile water to a concentration of 7 mg/mL prior to intramuscular administration of 3 mg/kg diminazene diaceturate. Heparinized blood samples were collected just before (hour 0) or 0.5, 1, 2, 4, 8, 12, 18, 24, 36, 48, 72, 120, and 168 hours later. Concentrations of diminazene were measured by HPLC analysis using UV absorption and ion-pairing conditions. The pharmacokinetic profile was analyzed using a simple one-compartment model. Diminazene had a mean terminal half life ($T_{1/2}$) of 1.70 (+/- 0.29) hrs and mean peak

plasma concentration (C_{MAX}) 0.51 (+/- 0.11) $\mu\text{g/mL}$. The mean residence time (MRT) of diminazene was 2.45 hrs (+/- 0.42). Systemic clearance (CL/F) was 1.38 (+/- 0.26) L/kg/hr. The volume of distribution per fraction absorbed (VD/F) was 3.36 (+/- 0.72) L/kg. The single intramuscular dose of diminazene diacetate was well tolerated by all 4 cats.

We investigated the ability of the antiprotozoal compound diminazene diacetate to eliminate the pathogen from naturally infected *C. felis* carriers. Seven healthy, chronically infected domestic cats were treated in a masked fashion with diminazene diacetate (3 mg/kg) or placebo intramuscularly in a series of two injections seven days apart. Samples were collected at 0, 3, 6 and 10 weeks. All animals remained positive on PCR and microscopic review of stained blood smears, and there was no significant difference between placebo and treatment groups in degree of parasitemia. Toxicity at this dose was minimal and self-limiting, and included hypersalivation and injection site soreness. Pre-medication with atropine alleviated hypersalivation. At 3 mg/kg administered twice, diminazene diacetate was unable to eliminate the pathogen or significantly reduce parasite burden in healthy, chronically infected cats.

In order to determine if the drug might clear the carrier state at a more dose intense protocol, five naturally infected chronic carrier cats were administered 4 mg/kg of diminazene diacetate intramuscularly for five consecutive days. Clearance of the organism was assessed via semi-quantitative PCR and light microscopy 1, 3, 6, and 10 weeks after starting treatment. Additionally, cats were monitored for adverse drug reactions by daily observation and examination, CBC, biochemical profile, and urinalysis at 1, 3, and 10 weeks. Adverse events were common at this higher dose and included profuse self-limiting salivation and nausea at the

time of injection, monoparesis in the injected leg, potential hepatotoxicity, and proteinuria.

Unfortunately, degree of parasitemia was not reduced. Therefore, the 4 mg/kg dose intense treatment protocol cannot be recommended for elimination of the carrier state.

CHAPTER 1

CYTAUXZOOM FELIS

Etiology

The vector-borne hemoprotozoan parasite *Cytauxzoon felis* is the causative agent for the often fatal disease feline cytauxzoonosis. It is an Apicomplexa parasite within the order Piroplasmida and family of Theileriidae. It affects both domestic and wild Felidae, but is incapable of infecting other mammals outside this realm [1].

Within the United States, the disease cytauxzoonosis occurs in specific geographical regions including the South-central, Mid-central and mid-Atlantic regions of the USA, which correlate to regions where the tick vector is common (Figure 1) [2-4]. *Amblyomma americanum* (the Lone Star tick) is considered to be the primary vector, but *Dermacentor variabilis* (the American dog tick) has also been demonstrated to be a competent tick vector in the laboratory setting [5, 6]. The range of *C. felis* seems to best correlate with that of *Amblyomma*, and outside the laboratory setting, *D. variabilis* has not been described to carry *C. felis* [7]. Since the disease was first reported in the mid-1970s from Missouri, reports have documented the apparent spread of this emerging disease to areas well beyond the Mid-central states. In fact, although domestic cat infection has yet to be recognized, infection has been documented in bobcats as far north as Pennsylvania and North Dakota [8, 9]. Interestingly, there appear to be geographic regions

where the pathogen may be less virulent, as exemplified by a high number of survivor animals within certain geographic locales, such as Oklahoma and northern Arkansas [10].

Not surprisingly, the acute disease cytauxzoonosis demonstrates temporal predilection for warm months [11]. This temporal spacing is thought to coincide with peak activity of the tick vector. Adult and nymphal ticks, both of which can transmit infection, are most active from March to May, with a lesser peak in August and September [11].

Bobcats (*Lynx rufus*) are reported to be the reservoir host for *C. felis*, but infection leads to important disease in domestic cats (*Felis domesticus*) [12]. Although the pathogen is able to infect a wide variety of felids, it cannot infect and produce disease in laboratory species, domestic livestock, or non-felid wildlife [1]. Infection has been reported in lions, tigers, ocelots, pumas, leopards and the Florida panther [13-17]. The infection may vary in virulence between Felidae species. In native wild Felidae including bobcats and panthers, most infections produce only mild illness, although fatal infections have been reported [12, 18-20]. On the other hand, the vast majority of infections reported in domestic cats, lions, and tigers result in fatal disease [4, 13-15, 21].

While *C. felis* is the only species of *Cytauxzoon* known in the USA, additional species are found in other parts of the world. For instance, *Cytauxzoon manul* affects Pallas cats (*Felis manul*) in Asia [22]. This species is capable of infecting domestic cats too, but does not appear to cause substantial clinical illness [23]. Unfortunately, inoculation of domestic cats with *C. manul* does not provide protective immunity against *C. felis* [23]. Highly endangered Iberian lynx in Spain, as well as domestic cats in the same region, are sometimes infected with another species of *Cytauxzoon* that is as yet unnamed [24, 25]. Domestic cats in both France and

northern Italy have also been documented to harbor *Cytauxzoon* parasites [24, 26, 27]. Multiple reports of cytauxzoonosis in feral domestic and in native wild cats in Brazil may be due to either *C. felis*, or another closely related *Cytauxzoon* species [16, 28, 29].

Pathogen Life cycle

In nature, transmission of the pathogen requires an appropriate tick vector (e.g., *A. americanum*). Within its feline host, *C. felis* exists in two forms during a short-lived non-erythrocytic schizogenous phase followed by a chronic erythrocytic piroplasm phase. Typically the naïve tick feeds on a carrier bobcat, ingesting erythrocytes containing piroplasms. The sexual phase of reproduction occurs within the tick gut, after which ookinetes migrate to the tick salivary glands. Once in the salivary glands, the ookinetes undergo merogony to form sporozoites. When the tick then feeds on a second, non-infected felidae, the tick transmits sporozoites. In the host, the sporozoites infect host mononuclear cells. Within the mononuclear cell, the sporozoite undergoes merogony; this phase of infection is known as schizogony. Numerous daughter merozoites are formed within the mononuclear cells, which eventually leads to cell rupture and release of merozoites into the bloodstream. The merozoites are endocytosed by erythrocytes, and are then termed piroplasms. Schizonts are found in mononuclear cells typically 1 to 3 days before piroplasms become identifiable within erythrocytes. Within the erythrocyte, the piroplasms continue to reproduce via asexual binary fission. An infected bobcat will undergo a brief schizogenous phase of illness, and will usually recover to become a lifelong carrier, although the occasional bobcat succumbs to the disease [12, 18]. However, if the infected tick instead feeds on a domestic cat, the cat may experience clinical illness due to

massive schizogenous replication causing obstruction of blood flow, organ damage and disseminated intravascular coagulation (DIC). Some cats will recover from acute illness to become chronic carriers of *C. felis* but do not appear to suffer long term ill effects of their carrier status, much like infected bobcats [10, 30, 31]. Piroplasms have also been identified in cats that have not been reported to show any clinical signs compatible with acute illness [29, 32]. The reason why many domestic cats sicken and die while some do not remains unknown.

Experimentally, *C. felis* has also been transmitted via inoculation with schizont-laden splenic homogenate or transfusion with blood from acutely ill cats, presumably containing schizonts [12, 33]. Transfusion of blood from carrier cats with piroplasms but no schizonts in the blood leads to transfer of the piroplasm stage of infection, but not to clinical illness in the transfused cat [33]. Interestingly, cats infected by transfusion of piroplasms are not immune to infection and illness related to schizonts, but cats that survive the schizogenous phase of infection are immune to recurrent illness [12, 34]. Close cat-to-cat contact alone has not been demonstrated to transmit disease [35]. Although many hematoparasites are capable of perinatal transmission, until our study this route of infection had not been investigated for *C. felis* [36-39].

Pathogenesis

The vast majority of the considerable pathologic damage that occurs during acute illness is due to the schizogenous phase of parasitemia. The schizonts distend the mononuclear cells so substantially that the schizont laden macrophages occlude the small veins and capillaries of

the host's organs, most notably the liver, lung, spleen and lymph nodes [3, 40]. This obstruction then fuels hypoxic tissue damage and release of inflammatory cytokines, which can lead to multi-system organ failure, systemic inflammatory reaction syndrome and disseminated intravascular coagulation (DIC). The degree of schizogony appears to be correlated with severity of disease; bobcats (the natural host) undergo a brief and milder schizogenous phase as compared to domestic cats [20]. Because the schizogenous phase of replication is brief in both bobcats and domestic cats, the illness is typically acute and short lived with most cats either succumbing to disease or improving within only several days [10, 33, 41]. Occasionally, the presence of piroplasms is suspect to trigger hemolysis at the end of acute illness, however, hemolytic anemia has not been noted in chronic carriers [10, 42, 43].

Clinical Presentation

Infected domestic cats share many similar characteristics. They are typically outdoor cats from wooded suburban or rural areas, where they are more likely to encounter a tick that has recently fed on an infected bobcat [11]. There is no breed, sex or age predisposition, and retroviral status has not been proven to predispose cats to infection [11]. Often, the infected cats are young adults and previously quite healthy.

Tentative clinical diagnosis of cytauxzoonosis in domestic cats is often made based on a combination of clinical signs and hematologic abnormalities in a cat with outdoor exposure living in an endemic area. Common findings include an acute onset of lethargy and anorexia, vocalization, high fever, icterus, elevation of the third eyelid, tachypnea, lymphadenopathy,

hepatomegaly, and splenomegaly [41, 42]. Laboratory abnormalities reflect the inflammatory nature of the disease and the DIC that often accompanies it. Abnormalities of the CBC most commonly include pancytopenia and characteristic signet ring intraerythrocytic inclusions that are frequently visualized on blood smear analysis. Various combinations of neutropenia, neutrophilia, thrombocytopenia and non-regenerative anemia may be observed on the hemogram [21]. The most common biochemical abnormalities include hyperbilirubinemia, elevation in liver enzyme activity, pre-renal azotemia, hyperglycemia, electrolyte and acid base disturbances, hypoalbuminemia, and hypocholesterolemia [42]. Urinalysis often demonstrates a bilirubinuria. The above abnormalities are not pathognomonic for cytauxzoonosis, and many other infectious and non-infectious diseases will have a similar presentation.

Diagnosis

Definitive diagnosis has typically been made by direct observation of the organism. Aspiration of an enlarged lymph node, liver or spleen will potentially yield a macrophage containing large numbers of schizonts [21, 42]. The diagnosis is more commonly made by careful examination of a Wright-Giemsa or Diff Quik stained blood smear [44]. Observation of circulating monocytes containing schizonts is possible, but more commonly piroplasms within erythrocytes are noted. Some of these piroplasms have a classic and distinctive “signet-ring” morphology, although additional morphologies are also possible, including the safety pin, tetrad or cocci-like chains (Figure 2) [42]. Although supportive of a diagnosis of cytauxzoonosis, detection of piroplasms is not a highly accurate means of diagnosis as it lacks both sensitivity and specificity. Intraerythrocytic piroplasms can be infrequent or absent since illness

accompanies the schizogenous phase and may precede the appearance of piroplasms by several days. A meticulous scan of the blood smear may be required in order to detect organisms, and even then they may not be found in acutely ill cats [45]. Other hemoprotozoa are indistinguishable on light microscopy (e.g. *Babesia felis*, *Cytauxzoon manul*). As these are not reported in the United States at this time, more common causes of false positive blood smear results include stain precipitant artifact, Howell-Jolly bodies or *Mycoplasma hameofelis* being mistaken as *C. felis* by the microscopist.

Molecular methods offer increased sensitivity and specificity for accurate detection of the *C. felis* organism. A commercially available, highly sensitive polymerase chain reaction (PCR) for whole blood samples (North Carolina State University Vector Borne Disease Diagnostic Laboratory, Raleigh, NC; IDEXX Laboratories, Westbrook, ME) has been developed. The test is able to detect as little as 0.01 gene copies/ μ l, and consistent detection requires only 50 copies of the target per reaction [46]. The disadvantage to this method is that the time course of disease is very rapid (hours to days), and the time for shipment and testing may preclude clinically utility. As it takes days to weeks to develop an appreciable antibody response, serology is not of value in acute cytauxzoonosis, but serology has been used in a research setting and for prevalence studies conducted in wild cat populations [10, 17, 47, 48].

The diagnosis is readily confirmed on necropsy [40]. Gross findings usually include splenic and hepatic enlargement and mottling, mild to moderate lymphadenomegaly, and pulmonary edema [40, 49]. Petechial and ecchymotic hemorrhages are also common. In cats which die in the acute phase of disease, schizonts are readily demonstrated in mononuclear phagocytes within tissues, often occluding the vascular lumen. Occlusion is often severe enough

that venous congestion results. Interestingly, inflammation is not a prominent finding in cats that die of acute disease, but ill cats certainly fulfill the criteria of systemic inflammatory response syndrome. While histopathology is rarely required to provide a diagnosis, it is an effective way to demonstrate the schizont laden macrophages within affected tissues (Figure 3). Indeed, it is very unlikely that the diagnosis of acute cytauxzoonosis would be missed on necropsy with histologic review of tissue.

Treatment

In the early history of the disease, treatment options for cytauxzoonosis were considered ineffective and the disease was regarded as uniformly fatal. However, reports of cats that survived acute infection have emerged during the last decade. Some of these cats likely recovered spontaneously, but in some cases, these survivors may have benefitted from aggressive supportive care, to go on to become apparently healthy carrier cats [10]. Supportive care typically involves crystalloid fluids to address components of pre-renal azotemia, dehydration and improve perfusion of organs. Additionally, some experts advocate administration of heparin due to the common complication of DIC [41]. Whole blood transfusion or blood component therapy may also be used to address anemia caused by hemolysis and coagulopathy triggered by DIC. Some practitioners use non-steroidal anti-inflammatory drugs or glucocorticoids (i.e. prednisolone) for the anti-pyrexia effects as well as their analgesic properties [41]. No controlled studies have examined whether these therapies improve or worsen clinical outcome. There are also numerous reports of antimicrobial drugs used in the therapy of cats afflicted with cytauxzoonosis, but the drugs utilized (enrofloxacin, doxycycline and/or sodium ampicillin)

seem unlikely to have contributed substantially to outcome as they do not possess anti-protozoal activity [21, 50]. Given the multisystemic and inflammatory nature of the disease, it is likely that appropriate supportive care may play a substantial role in recovery independent of anti-protozoal therapy.

Specific antiprotozoal therapies have been investigated. The antiprotozoal drugs parvaquone and buparvaquone were both investigated in the early 1990s and found to be ineffective at treating experimental cytauxzoonosis [34]. Imidocarb dipropionate is a urea derivative that has been used in veterinary medicine for treatment of *Babesia* infections [51]. It is widely available within the United States and represents a cost effective option for treatment of veterinary protozoal disease. Typically it is administered as a series of two intramuscular injections, with variation in published doses varying from 2-5 mg/kg and interdosing intervals that range from four to seven days apart [42]. Side effects include salivation, emesis, nasal drip, diarrhea, and pain at injection site [52]. The cholinergic effects can be overcome with pretreatment with an anticholinergic, such as atropine. Unfortunately, despite its easy accessibility, the drug has not proved to be a particularly effective treatment for cytauxzoonosis, with only 25% of patients treated with the drug surviving [41]. Additionally, imidocarb does not appear effective at eliminating the parasitemia in carrier cats [53].

More recently, a combination therapy of atovaquone and azithromycin, along with supportive care, yielded the most promising results to date with a survival rate of 60%, statistically improved as compared to the imidocarb control [41]. In recovered carrier cats, the combination of atovaquone and azithromycin was unable to consistently clear infection, but in

several treated cats pathogen burden dropped to levels below PCR detection for a period of time, only to recrudesce [53].

A third therapeutic option is diminazene, an anti-protozoal used extensively in Africa, South America and the far East for treatment of other protozoal diseases, primarily *Trypanosoma* and *Babesia* infections [54, 55]. The major drawback to the therapeutic use of this drug is lack of availability within the United States of America as it lacks approval from the Food and Drug Administration. Methods exist by which the drug may be imported for compassionate use, but the time required to fulfill these legal importation requirements for a patient afflicted with acute cytauxzoonosis is often unacceptably long, essentially rendering it unavailable for clinical patients. However, a single retrospective report from 1999 indicated that five of six cats treated with diminazene and supportive care survived, a reported survival of 83% [30].

Prevention

At this time, prevention of disease relies on preventing tick bites. Unfortunately, given domestic cats sensitivities to many commonly used parasiticides, the arsenal of acaricides is limited to fipronil and possibly selamectin, although the latter does not make a label claim of efficacy against ticks in cats [56, 57]. While both of these drugs may kill ticks, neither of these drugs prevent tick bites entirely [58]. Therefore, the disease may still be transmitted even with an effective parasite control plan. The strategy most likely to be effective is a combination of acaricides and confinement indoors in endemic regions, although this does not entirely insure

that the cat is completely protected from the infection as ticks may be carried into the home by other pets or humans.

A potentially helpful tool would be the development of an effective vaccine to prevent infection or illness. The financial requirements for such a vaccine development program would be substantial. Anti-protozoal vaccines often are of questionable efficacy, as seen by the lack of an effective malaria vaccine or, in the veterinary realm, the *Giardia* vaccine [59, 60]. Attempts to use less pathogenic *Cytauxzoon* species, such as inoculating *C. manul* into domestic cats, has failed to produce a reduction in mortality associated with *C. felis* infection in a limited number of experimentally infected cats [23]. Cats that have survived the schizogenous phase of infection appear to be immune to clinical illness upon repeat infection, a finding that offers some hope for vaccine development [34]. Identification of appropriate antigen targets will be a key to vaccine development in the future.

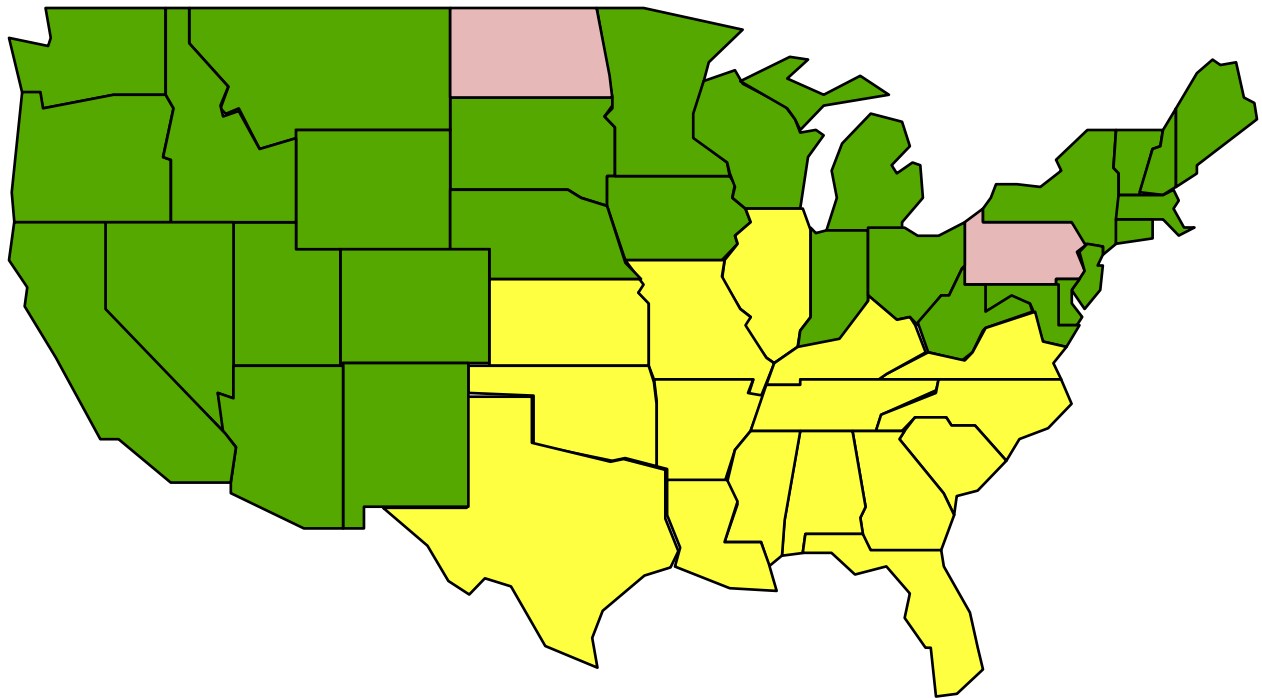


Figure 1.1: States with reported *C. felis* infection in domestic or wild felids. States shaded yellow represent areas where disease has been reported in domestic cats. States in green have not reported the disease in either cats or bobcats. States where *C. felis* has been detected in bobcats but not domestic cats are shaded in pink.

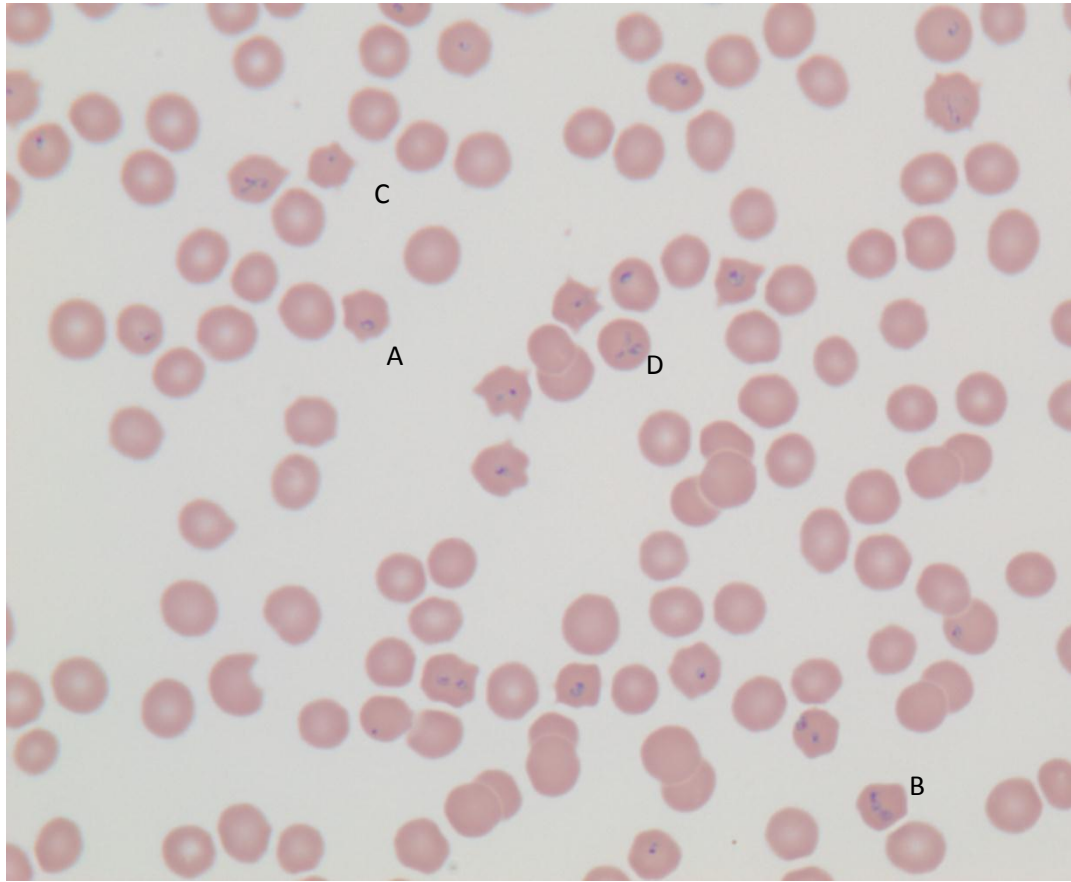


Figure 1.2: Blood smear from infected cat. Various forms of *C. felis* are visible: A) Signet ring B) Safety-pin C) Cocci D) Tetrads. Photomicrograph of a peripheral blood smear at 100 X.
Photo courtesy of Dr. Melanie Spoor.

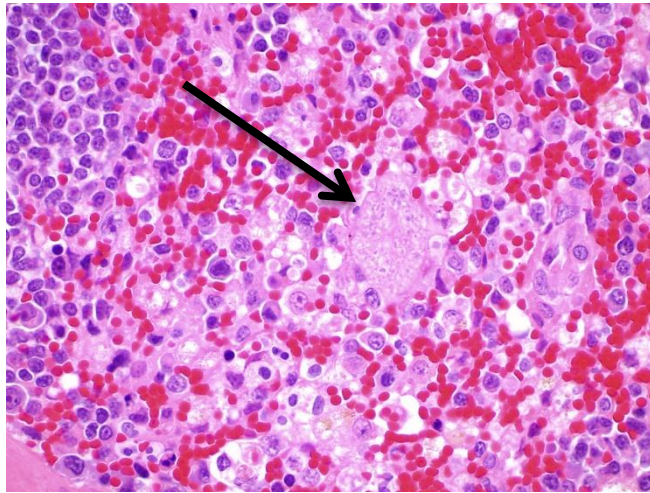


Figure 1.3: Macrophage containing large numbers of schizonts within the splenic vasculature (arrow). Splenic congestion is also present. Photomicrograph at 60X. Photo courtesy of Dr. Kei Kuroki.

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CHAPTER 2

Detection of *Cytauxzoon felis* in apparently healthy captive tigers (*Panthera tigris*)

Title: Detection of *Cytauxzoon felis* in apparently healthy captive tigers (*Panthera tigris*)

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Abstract:

Objective—To determine if captive non-native wild cats living in an area endemic for *Cytauxzoon felis* harbor the protozoal pathogen in a subclinical state.

Design—Observational case series.

Animals— Nine non-native captive-bred large cats and 2 captive-bred large cats native to the USA, all housed in outdoor enclosures at a large cat refuge in Arkansas. Domestic cats on the premises provided additional samples.

Procedure—Blood was collected in EDTA from eight tigers, as well as a lion, cougar, bobcat, and six domestic cats. Blood smears were reviewed via light microscopy for the presence of intraerythroid organisms consistent with *C. felis*. PCR analysis for *C. felis* was performed, and the 18S rRNA gene sequence from positive samples was characterized.

Results— Four clinically normal tigers were found to be infected with *C. felis*. Intraerythrocytic organisms consistent with *C. felis* were identified microscopically in one of the four tigers. In a single tiger, a few intraerythrocytic inclusions were observed, but this tiger was negative for *C. felis* by PCR. Genotyping of the pathogen from all infected tigers revealed all amplicons shared >99.8% identity with previously reported 18S rRNA sequences from *C. felis* infected North American domestic cats, and were identical to amplicons from domestic cats on the premises.

Conclusions and Clinical Relevance— Although not native to the USA, tigers may become infected by a North American strain of *C. felis* without observed clinical illness. PCR assay for *C. felis* was more sensitive and specific than cytologic recognition of piroplasms in tigers.

Abbreviations:

PCR – polymerase chain reaction

FS – female spayed

FI – female intact

MC – male castrated

MI – male intact

Introduction:

Cytauxzoon felis is a hemoprotozoan parasite of wild and domestic felids that is transmitted by the bite of a tick vector.¹ Cytauxzoonosis develops due to the initial schizogenous phase of parasite replication in the mononuclear cells which cause widespread occlusion of blood vessels and a profound systemic inflammatory response.^{2,3} Mononuclear cells eventually become distended with organisms and rupture, releasing merozoites. Merozoites are then taken up by erythrocytes where the erythrocytic parasites are referred to as piroplasms.³ Cats that survive the schizogenous phase of infection generally remain healthy despite persistence of piroplasms for months to years.⁴⁻⁶ Bobcats, the primary reservoir hosts, are believed to only develop a brief illness prior to entering the persistent carrier state. In contrast, many domestic cats develop a profound illness that often results in the cat's death.⁶⁻¹¹ Although related *Cytauxzoon* species have been reported in Asia and Europe, infections with *C. felis* are largely limited to the Americas.¹²⁻¹⁹ In the USA, the disease in domestic cats is recognized predominantly in the south central, south eastern, and Atlantic states, although the pathogen has been documented in bobcats in states outside these regions including North Dakota and

Pennsylvania.^{16,20,21} Native bobcats (*Lynx rufus*) and perhaps cougars (*Puma concolor*) seem to be the predominant reservoir hosts in the USA.^{21,22}

While disease is often mild in native American wild cats, as with domestic cats, severe morbidity and mortality has been reported in felids that are not native to the Americas. Fatal cytauxzoonosis has been reported in a two captive-reared Asian tigers (*Panthera tigris*), and an African lioness (*Panthera leo*) and her cub.²³⁻²⁵

A carnivore preservation sanctuary located in Eureka Springs, Arkansas^a contacted the investigators after several tigers died over a period of years due to presumed or necropsy-confirmed cytauxzoonosis. The sanctuary houses more than one hundred twenty large felids, most of which are tigers, but also includes several lions, leopards, cougars, bobcats, and other wild felidae. All the large cats housed at the refuge were captive-bred animals taken in by the sanctuary after the prior owners became unable or unwilling to care for the cats. The purpose of this study was to determine if subclinical parasitemia could be identified in captive bred large cats that are not native to the Americas, such that these cats might serve as a reservoir of infection for other cats housed on the premises.

Methods and Materials:

All large cats included in the study were housed in groups of two to three in large outdoor enclosures in a rural wooded area of northern Arkansas^a. Staff zoologists design the animal's diet and general husbandry practices, and observe each animal at least several times each day. The premises were regularly treated with carbaryl^b to minimize the animals' ectoparasite exposure.

Blood was collected from a convenience sample of one lion (15 year old FS, residing on premises 14 years), one cougar (15 year old FS, residing on premises four years), one bobcat (four year old MC, residing on premises 3.5 years) and eight tigers (Table 1) that were anesthetized for routine care. Six of the tigers and all of the other large cats were apparently healthy. The healthy tigers had been housed on the premises from 2.8 years to 15 years (mean 11.8 years). Healthy animals were fasted overnight prior to being anesthetized to facilitate safe handling; anesthesia was induced via intramuscular injection of ketamine and xylazine. During the anesthetic episode, each animal underwent a complete physical examination and blood was collected via the medial saphenous vein for CBC, serum biochemical analysis, and PCR analysis for *C. felis*. Blood samples were also obtained from two sick tigers undergoing diagnostic evaluation under the care of the facility's regular veterinarian (R. Eby). Additional blood samples were made available from domestic cats living on the premises. Unstained blood smears were prepared by the staff zoologists from 6 feral domestic cats killed on the premises. Additionally, blood samples were collected in EDTA from one feral cat, and from 5 pet cats that had been housed on the same premises as indoor/outdoor pets for a period of 1 to 3 years.

Blood collected in EDTA was used to prepare blood smears and to perform CBC, while clotted blood was used to harvest serum for biochemical analysis. Smears were stained with Wright-Giemsa stain in a routine fashion and reviewed by a single boarded clinical pathologist (MW) who was unaware of animal species or PCR results. CBC and serum biochemical analysis were performed using in-house laboratory equipment within 12 hours of blood collection. Total deoxyribonucleic acid was extracted from anticoagulated blood and subjected to PCR analysis using a previously published method with minor modifications.²⁶ Briefly, each reaction

consisted of 12.5 µl 2X PCR master mix^d, 7 µl water, 50 pmol of each oligonucleotide primer (5'-GCCAATCGCATTGCTTTATGCT-3' and 5'-CCAATTGATACTCCGGAAAGAG-3') and 5 µl sample. Thermal cycling conditions were: 98°C for 30 seconds followed by 45 cycles 95°C for 5 seconds and 60°C for 5 seconds. The final extension step was 72°C for 5 minutes. Melting curve analysis was initiated at 75°C and data were captured at increasing increments of 0.5°C for 30 time points. For each PCR assay positive (previously characterized *C. felis* samples) and negative (no template) controls were used. Standard precautions were used to prevent amplicon carryover.

In order to further characterize the positive samples, a PCR assay that amplified a near full-length portion of 18S rRNA gene sequence in multiple Apicomplexan species was utilized to characterize the 18S sequences from *C. felis*-infected blood samples. Selected primer sequences were 5'-GTTGATCCTGCCAGTAGT-3' and 5'-AACCTTGTTACGACTTCTC-3'. Each 50 µL reaction contained 1 µL of DNA template, 50 pmol of each primer, 10 nmol dNTPs, 75 nmol of MgCl₂, 3.75 U DNA polymerase and a 1X concentration of PCR buffer^e. Optimized thermal cycling conditions consisted of an initial denaturation at 94 °C for 5 minutes, followed by 50 amplification cycles (94 °C for 20 seconds, 56 °C for 30 seconds, and 68 °C for 3.5 minutes) and a final extension step at 72 °C for 7 minutes^f. Positive control consisted of confirmed *Babesia* infected canine whole blood and negative controls consisted of water (no DNA). Standard precautions were used to prevent amplicon carryover. Amplicons were visualized by ethidium bromide staining^g and ultraviolet light transillumination after electrophoresis in a 1% agarose gel^h. Amplicons were purified using a commercially available kitⁱ and sequenced directly^j. In addition to the primers used to generate the amplicons, two internal primers (5'-

TGCTTTCGCAGTAGTTCGTC-3' and 5'-GCGAATCGCATTGCTTTATGCT-3') were utilized for sequencing. Each chromatogram was carefully inspected for heterogeneity and contigs were assembled using a commercially available software package.^k

Results:

Most cats were believed to be healthy at the time of sampling based on attitude and appetite, but two tigers were known to be ill. Rectal temperature, pulse, and respiratory rate were normal for all of the animals believed to be in good health. The sick tigers, one male and one female, had been anorexic and lethargic several days prior to examination. Both sick tigers were normothermic. The male had no specific abnormal exam findings. The female had tachycardia, tachypnea, and a profuse vaginal discharge. Serum biochemical analysis and CBC were normal in all but one of the presumably healthy tigers and the ill female tiger. An apparently healthy 11.5 year old male tiger was azotemic (creatinine 7.3 mg/dL; reference interval 1.6 - 4.4 mg/dL and blood urea nitrogen 40 mg/dL; reference interval 12 – 44 mg/dL). This animal died several months later due to presumed renal failure; histopathologic examination of tissue was not performed. The anorexic female tiger had a leukocytosis and hypocalcemia. This tiger was diagnosed with pyometra and died 3 months post-hysterectomy and PCR testing. Postmortem histologic evaluation of kidney, heart and spleen revealed no evidence of cytauxzoonosis. The other sick tiger that lacked a specific diagnosis was treated with imidocarb and eventually recovered.

Erythrocytic inclusions were observed on microscopic evaluation of blood smears from two tigers. A signet ring morphology consistent with *C. felis* piroplasms was observed in some

red blood cells of the clinically well but azotemic tiger (approximately 1 organism per ten 1000x oil immersion fields). From the female tiger determined to have pyometra, a few erythrocytic inclusions (less than 1 per twenty 1000x oil immersion fields) were seen but these did not have the classic signet ring morphology and could not convincingly be identified as *C. felis* piroplasms; they may have been staining artifacts. *Cytauxzoon felis* was detected by the initial PCR from the azotemic tiger as well as three additional healthy tigers, but in neither of the ill tigers, nor in the lion, cougar, or bobcat. A nearly full-length 18S rRNA amplicon of the expected 1741 bp size was amplified from all of the positive samples (n=4). The DNA sequences of these 4 amplicons shared >99.8% identity with previously reported 18S rRNA sequences from North American felids infected with *C. felis* (Table 2).

Evidence of *C. felis* infection was found in most of the domestic cats evaluated. Piroplasms were identified on microscopic review from 6 of 7 feral domestic cats, and from 5 of 5 pet cats. Blood was available for PCR assay from the 5 pet cats and one feral (piroplasm +) cat; all samples were positive for *C. felis* by PCR.

The nucleotide positions shown in Table 2 represent the only positions with any variation detected between our sampled population of tigers and the six domestic cats, or between the ten samples (four tigers, six domestic cats) and the four previously published full-length 18S sequences in GenBank. Secondary peaks were detected at position 256 from all four infected tigers and all six infected domestic cats, with both cytosine and thymine recognized in that position.

Discussion:

Cytauxzoon felis has previously been reported in small numbers of captive tigers and

lions that died of the disease.²³⁻²⁵ In this investigation, four of six clinically normal tigers were PCR positive for *C. felis*. This finding indicates that tigers, like felidae species native to the Americas, may harbor subclinical infection.

Until recently, only native American wild cats were believed to be capable of serving as reservoir hosts since *C. felis* infection was fatal in domestic cats and in the rare instances of infection in tigers and lions.^{8,23-25,27,28} Over the last decade it has become clear that some domestic cats survive infection and can be competent pathogen reservoirs, although the importance of domestic cats in the natural transmission of *C. felis* has yet to be determined.^{1,29} While our report does not address the ability of tigers to serve as a competent reservoir host, the finding that 4 of 8 tigers had been infected with no history of an acute febrile illness certainly suggests that this species can survive acute infection and develop a non-clinical carrier state similar to that which occurs in bobcats. Although considered less likely, it is possible that tigers may not have incurred a schizogenous phase of infection and instead may have been infected via direct blood inoculation, perhaps when infected domestic cats were killed by the tigers.

The tigers in this study were housed in a heavily wooded rural area in northern Arkansas. It has been suggested that a strain of *C. felis* less virulent to domestic cats may exist in this region because a number of cats from the geographic region have been recognized as having chronic infection without a history of acute febrile disease.^{4,6} In fact, 5 pet cats housed on the premises and allowed access to the outdoors were positive for *C. felis* by both blood smear examination and PCR without any history of clinical illness. Additionally, blood smears made post-mortem from 5 of 6 feral domestic cats that were killed by the captive large cats after entering enclosures were determined to contain piroplasms. While no history was available for

the feral cats, it is unlikely that they were acutely ill since they were apparently foraging for food when they were killed. An additional feral cat caught in a live trap on the same premises was apparently healthy despite identification of piroplasms on blood smear and a positive *C. felis* PCR.

It cannot be definitively determined if the tigers, which came from various locations throughout the USA, acquired *C. felis* infection at the rescue facility but it seems likely. The sanctuary is located in a heavily wooded area where cytauxzoonosis is considered common in domestic cats, and most of the tigers had been kept there for years prior to testing. Ticks are occasionally noticed on the tigers despite routine application of carbaryl to the enclosures. While ticks were not detected at the time of examination and blood collection, most tigers were sampled during colder weather when the predominant tick vector, *Amblyomma americanum*, is less likely to be active.¹ Additionally, ticks present on the tigers may simply have been missed due to the cat's large size and thick coats.

PCR analysis was more sensitive and specific for detection of *C. felis* in the tigers than routine microscopic examination of stained blood smears. Interestingly, the only tiger in which piroplasms were definitively identified had concurrent chronic kidney disease. It is possible that concurrent illness permitted a greater level of parasitemia, but it is also possible that this was simply coincidence. Rare inclusions identified on blood smears of the tiger with pyometra were seen, but this cat was negative for *C. felis* by PCR. It is more likely that these inclusions represented a stain artifact or a morphologic RBC change rather than that the PCR result was falsely negative. Unlike the tigers, RBC piroplasms were seen on routine blood smear from all 5 infected pet cats as well as 6 of 7 feral cats.

Molecular techniques are necessary to distinguish between closely related *Cytauxzoon* species as morphologic observation of inclusions is inadequate to the task. In South America, native pumas (*Puma concolor*), ocelots (*Leopardus pardalis*), jaguars (*Panthera onca*), oncillas (*Leopardus tigrinus*) and domestic cats have all been documented to be chronic carriers of a Cytauxzoon-like parasite.^{19,30} These pathogens may be distinct from the North American *C. felis* (unpublished data). Further, *C. manul* and a *Cytauxzoon* species isolated from Iberian lynx (*Lynx pardinus*) and domestic cats in Spain appear morphologically identical to *C. felis* on blood smear examination.^{15,31,32} Feline *Babesia* species, found predominantly in Africa, also appear morphologically similar.³³ In our study, PCR analysis of the 18S rRNA gene from parasites obtained from infected tigers confirmed that the infectious agent was indeed North American *C. felis*.

While the *C. felis* 18S gene sequences amplified from the tigers were nearly identical to the only four full-length *C. felis* sequences in GenBank, we did identify secondary nucleotide peaks at one position (corresponding with position 256 on Genbank accession AY679105.1); both cytosine and thymine nucleotides were detected in this position. Besides this region, there was no variation documented between sequences from the 4 tigers. The biologic significance of variation at this position is unknown, but may represent microheterogeneity between rRNA operons in the *C. felis* genome. Alternatively these could represent a polymorphic site and are indicative of simple genetic diversity within the *C. felis* population. The identical sequences of the 18S gene in both the outdoor domestic cats and tigers housed at the sanctuary can be used to support the idea of a common source of *C. felis* infection at the facility.

We have demonstrated that apparently healthy captive tigers can be carriers of *C. felis*. Because only a single lion was tested, we cannot state whether this species too can maintain chronic, non-clinical infection. However, such chronic infections have now been identified in domestic cats, native American wild cats, and cats not native to the Americas.^{5,8,22,25,30} Screening via PCR of any captive wild cats coming from or being moved to an endemic region may be warranted. Identification of carriers would prevent misdiagnosis of subsequent concurrent disease in these cats as cytauxzoonosis, a mistake that might otherwise be made based on recognition of piroplasms on peripheral blood smears or amplification of *C. felis* DNA by PCR. Although Asian and African wild cats would not be important reservoirs of disease for domestic cats in North America, they might serve as reservoirs for other cats housed in zoo or large cat sanctuaries. It seems appropriate that zoos and large cat sanctuaries should exercise precautions including attempts at tick control in an effort to minimize risks to captive cats. Additionally, limiting contact and access of all outside felids, wild, domestic, and feral, seems prudent.

Footnotes:

a – Turpentine Creek Wildlife Refuge, Eureka Springs, AR

b-Sevin Dust, GardenTech, Lexington, KY

c- IdexxVet Test Chemistry Analyzer, Idexx Laboratories, Westbrook, Maine

d- SsoFast™ EvaGreen® Supermix Bio-Rad, Hercules, CA, 94547

e- AmpliTaq Gold DNA and GeneAmp PCR Gold Buffer , Applied Biosystems, Carlsbad, CA

f- Techne Inc., Burlington, NJ

g- Fisher Scientific, Pittsburgh, PA

h- SeaKem GTG agarose, Lonza Rockland, Inc., Rockland, ME

i- QIAquick PCR purification kit, Qiagen Inc., Valencia, CA

j- MCLAB, South San Francisco, CA

k- ClustalW (Full Multiple Alignment Default Settings) BioEdit Sequence Alignment Editor

Table 2.1: Summary of tiger signalment, clinical findings and PCR/light microscopy findings

	Age (yr)	Sex	Time at facility (yr)	Clinical findings	PCR/microscopy
Tiger 1	11.5	MI	9.0	azotemia	+/+
Tiger 2	15.3	MI	15.3	normal	+/-
Tiger 3	14.5	MN	14.5	normal	+/-
Tiger 4	14.5	MN	14.5	normal	+/-
Tiger 5	15.5	MN	15.5	normal	-/-
Tiger 6	15.3	FI	15.3	normal	-/-
Tiger 7	14.3	MN	14.3	anorexia	-/-
Tiger 8	14.8	FI	2.8	pyometra	-/?

+= positive test result, -=negative test result, ?=ambiguous result

Table 2.2: Nucleotide variability between isolates from the tigers and domestic felids of this report, and published full-length *C.felis* 18S sequences isolated from domestic cats. Numbering of nucleotide positions is based on Genbank accession AY679105.

Sample	NP 109	NP 256*	NP 1585	NP 1638	Animal Infected
AY679105 ^a	T	T	T	T	domestic cat
AF399930 ^b	T	T	T	T	domestic cat
AY531524 ^c	T	T	C	T	domestic cat
L19080 ^d	N	T	T	N	domestic cat
Tiger 4	T	T/C	T	T	tiger
Tiger 3	T	T/C	T	T	tiger
Tiger 2	T	C/T	T	T	tiger
Tiger 1	T	T/C	T	T	tiger
Pet cat 1	T	C/T	T	T	domestic cat
Pet cat 2	T	C/T	T	T	domestic cat
Pet cat 3	T	C/T	T	T	domestic cat
Pet cat 4	T	T/C	T	T	domestic cat
Pet cat 5	T	T/C	T	T	domestic cat
Feral cat	T	C/T	T	T	domestic cat

Notes:

Except for those nucleotides shown in the table, sequences were identical

NP= nucleotide position, C=cytosine, T=thymine, N=unresolved nucleotide sequence

*Secondary peaks identified in chromatogram; first nucleotide listed is dominant in chromatogram

^a- AY679105.1, Bendele, 2005 : <http://www.ncbi.nlm.nih.gov/nuccore/AY679105.1>

^b- AF399930.1, Wagner, 2002: <http://www.ncbi.nlm.nih.gov/nuccore/AF399930.1>

^c- AY531524.1, Holman, 2005: <http://www.ncbi.nlm.nih.gov/nuccore/AY531524.1>

^d- L19080.1, Allsopp, 1995: <http://www.ncbi.nlm.nih.gov/nuccore/L19080.1>

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CHAPTER 3

Lack of evidence for perinatal transmission of *Cytauxzoon felis* in domestic cats

Title: Lack of evidence for perinatal transmission of *Cytauxzoon felis* in domestic cats

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Cytauxzoon felis is a hemoprotozoan parasite of cats capable of causing severe, often fatal disease. The parasite initially undergoes a schizogenous phase of replication inside mononuclear cells. During this phase the mononuclear cells cause widespread occlusion of blood vessels with a profound systemic inflammatory response. Mononuclear cells eventually become distended with organisms and rupture, releasing merozoites. Merozoites are then taken up by erythrocytes where the erythrocytic parasites are referred to as piroplasms (Cohn, 2011). The natural lifecycle of the parasite relies on an appropriate tick vector, *Amblyomma americanum* or *Dermacentor variabilis*, feeding on an infected host and later feeding on a second susceptible felid to transmit the pathogen (Blouin et al., 1984; Reichard et al., 2010). The natural host is considered to be the bobcat (*Lynx rufus*), but others felids that survive initial infection may also serve as reservoirs (Birkenheuer et al., 2008; Blouin et al., 1984).

Perinatal transmission of other hemoprotozoa, including related *Theileria* species, is well documented (Allsopp et al., 2007; Melendez et al., 1993). If perinatal transmission of *C. felis* were possible, then recovered domestic queens could produce offspring that might serve as reservoirs for infection of other domestic cats via a tick vector. The objective of this study was to determine if perinatal transmission occurs between parasitemic carrier queens to their offspring.

Two naturally infected intact female domestic shorthair cats (cat A and cat B) were separately donated to the University of Missouri specifically for study of cytauxzoonosis. The donors had lost numerous pet cats to cytauxzoonosis and wished to remove these infected cats from their premises. Both were outdoor, semi-feral cats that had lived on the owners' premises in rural Arkansas for an extended period. The reproductive history of cat A was not known, but

she was clearly pregnant at the time she was confined for removal. She delivered a litter of 7 apparently healthy kittens (litter A1) during her confinement in a fenced outdoor enclosure on the owner's premises before the investigators took possession of the cat. This litter was several weeks old before transfer could be arranged and the investigators took physical possession of the cat and her kittens. The other cat (cat B) had previously delivered several litters of kittens that she raised to weaning. Some of these kittens kept as "barn cats" had died of an acute illness presumed to be cytauxzoonosis as young adults. Microscopic slide review of blood smears from both adult cats made prior to the investigators taking possession of the cats revealed RBC piroplasms consistent with *C. felis*. The use of these cats and all procedures were approved by the University of Missouri Animal Care and Use Committee (Protocol # 6901).

At the time of donation, both cats and the kittens of litter A1 were in apparent good health, and physical examinations were unremarkable. Based on the donor's recollection and examination by the investigators, both queens were mature females approximately five years in age. The only laboratory abnormality identified in the adults by CBC with microscopic slide review, serum biochemical panel, urinalysis and feline retrovirus testing^a was the presence of signet ring shaped parasites in occasional RBC. Both cats were positive by PCR for *C. felis* infection. Adult cats were dewormed with a combination product containing emodepside/praziquantel^b and were treated with fipronil^c for internal and external parasites. The A1 kittens were separated from the queen at 7 weeks of age, vaccinated with FVRCP and dewormed with pyrantel pamoate.

Both queens were bred several months after donation. A specific pathogen free male cat was housed with each female until visual inspection revealed that the queens were obviously pregnant. Both queens remained well throughout pregnancy, and delivered six live-born kittens (Litter A2), or one live kitten (Litter B). The queens were housed with their litters until weaning at approximately 7 weeks of age. Kittens were observed daily for signs of clinical illness (anorexia, lethargy, failure to thrive) but due to the fractious nature of the queens, kittens were not handled until they were old enough to voluntarily approach the investigators at several weeks of age. Kittens remained healthy other than mild conjunctivitis in some of the kittens in litters A1 and A2, which resolved with application of oxytetracycline ophthalmic ointment. All kittens received routine vaccination with FVRCP and were dewormed with pyrantel. Kittens from all three litters were adopted to private homes at approximately 12 weeks of age.

Whole blood was collected in EDTA from the jugular veins of all cats and kittens for blood smear review and PCR testing. Blood smears were stained with Wright-Giemsa stain in a routine fashion for slide review, and a minimum of 500 red blood cells were observed. Total deoxyribonucleic acid was extracted from anticoagulated blood and subjected to PCR analysis using a previously published method with minor modifications.(Birkenheuer et al., 2006; Cohn et al., 2011) Briefly, each reaction consisted of 12.5 µl 2X PCR master mix^d, 7 µl water, 50 pmol of each oligonucleotide primer and 5 µl sample. Thermal cycling conditions were: 98°C for 30sec followed by 45 cycles 95°C for 5 seconds and 60°C for 5 sec. Melting curve analysis was initiated at 75°C and data were captured at increasing increments of 0.5°C for 30 time points. For each PCR assay positive (previously characterized *C. felis* samples) and negative (no template) controls were used. Standard precautions were used to prevent amplicon carryover.

Adult queens were tested at donation, prior to breeding, and at the time of weaning kittens; both cats were positive by both smear review and PCR each time they were tested. Litter A1 was tested at 6, 7, and 8 weeks of age; litter A2 was tested at 6, 8 and 10 weeks of age, and the single kitten in litter B was tested at 8, 10 and 12 weeks of age. All 42 blood samples obtained from 14 kittens in 3 litters were negative for *C. felis* infection by both microscopic review and PCR. The breeding male was also tested after being removed from both queens, and was negative by both smear review and PCR assay.

Although perinatal transmission was not documented in this study, such transmission might occur uncommonly and could represent an important potential reservoir of infection for naïve cats since healthy domestic carriers are competent reservoirs for this tick-transmitted infection (Reichard 2010). Maternal transmission of human hemoprotezoal infections typically spread by arthropod vectors include malaria (*Plasmodium spp*) and Chagas disease (*Trypanosoma cruzi*), both important public health concerns (Petersen, 2007). Perinatal transmission of other protozoal diseases of veterinary importance have also been documented (Petersen, 2007). In a small study of carrier mares infected with *Theileria equi*, perinatal infection rates approached 100% (Allsopp et al., 2007). Transplacental transmission of experimental *Babesia gibsoni* infection has been reported; in a single bitch infected with *Babesia gibsoni*, congenital Babesiosis was demonstrated in five of five puppies (Fukumoto et al., 2005; Itoh and Itoh, 1990). Perinatal transmission is common in cattle infected with *Trypanosoma vivax* and infection during gestation has been demonstrated to cause a substantial perinatal losses (Melendez et al., 1993; Okech et al., 1996). In contrast, *Trypanosoma cruzi* infection rates vary from 2%-12% of children born to infected mothers and congenital malarial infections occur in

only 33% of children born in endemic areas (Petersen, 2007). It is possible that transmission of *C. felis* to kittens born to infected queens might have gone undetected in our study due to sample size. Ideally, a larger number of kittens from multiple queens would be investigated.

Maternal infection by feline hemoparasites, such as *Bartonella henslae*, has previously been reported to lead to decreased fertility and failure to maintain pregnancy (Guptill et al., 1998). In our study, one of the queens produced only a single kitten, which is less than would be anticipated for a multiparous queen. It is possible that chronic *C. felis* infection may lead to decreased breeding efficiency; however the other queen in the study produced two litters of kittens with robust numbers of six and seven kittens each. Additional studies would be necessary to assess the effects of cytauxzoonosis on fertility.

Kittens were tested at three different time points to minimize the likelihood of missing a periodic parasitemia. Due to the feral behavior of the queens, testing prior to weaning was not possible without undue stress to the dam and offspring. We are thus unable to state that the kittens were not congenitally infected, and then later cleared the infection, but this is considered unlikely since parasitemia with the piroplasm seems to be persistent both after recovery from natural infection, and after iatrogenic infection via transfusion of piroplasm containing RBC (Brown et al., 2008; Glenn et al., 1983; Kier et al., 1982; Meinkoth et al., 2000). In conclusion, this study failed to document perinatal transmission of *C. felis* in domestic cats. If such transmission occurs, it is likely to be uncommon and probably plays little if any role in the spread of cytauxzoonosis to domestic cats.

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Footnotes:

a- SNAP FeLV/FIV Combo test, IDEXX Laboratories, Westbrook, Maine, 04092

b-Profendor, Bayer, Shawnee, Kansas, 66201

c- Frontline Spray, Merial, Duluth, GA, 30096

d- SsoFast™ EvaGreen® Supermix Bio-Rad, Hercules, CA, 94547

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CHAPTER 4

DIMINAZENE

Background

Diminazene is an aromatic diamidine (figure 4.1), and is commercially available as one of two diminazene salts: diminazene aceturate and diminazene diaceturate. These two compounds vary in molecular weight, with weights of 515.5 and 587.6, respectively. Although the active compound is the same, it has been suggested that differences in the performance of the two different salts may exist, as suggested by a 2007 study in horses that found that diminazene aceturate was 90% effective in clearing equine babesial infections as opposed to 80% with diminazene diaceturate (statistical analysis to significance was not provided). However, both drugs were dosed at 3.5 mg/kg, leading to a 13% diminished dose of the active base with the diaceturate salt as compared to the aceturate salt due to differences in molecular weight [1]. This difference may explain the difference in outcome noted in the previous study, or there may truly be a biological difference in outcome between the two preparations. The difference between the two preparations was further explored in a pharmacokinetics study in cattle, where diminazene diaceturate was found to be equivalent to diminazene aceturate with respect to the area under the plasma concentration versus time curve, but not in terms of the maximum plasma drug concentration and the time to maximum plasma drug concentration [2].

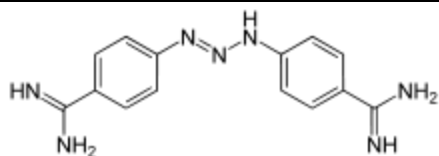


Figure 4.1: Diminazene molecular structure

The drug was first commercially introduced in 1955 as a treatment for trypanosomiasis and babesiosis in livestock, and has obtained widespread use since then [3]. In aqueous solution, diminazene salts are stable for 48-72 hours, and so the commercial preparations also contain a stabilizer, antipyrine (pheyldimethyl pyrzone) [3]. Both salts are commercially available and are marketed under a wide variety of brand names internationally, including Azidine®(diminazene acetate), Berenil®(diminazene acetate), Ganaseg®(diminazene acetate) and Veriben®(diminazene diacetate). However, none of these formulations are approved for use in the United States by the Food and Drug Administration in any species. The drug is typically administered intramuscularly, as it is unstable at acidic conditions, making oral administration unlikely to be effective [4]. Dosing varies depending on the host species and breed as well as the parasite being treated. One of the more common doses for cattle is 3.5 mg/kg for most susceptible protozoa, but treatment of *Trypanosoma brucei* and *T. theileri* may require doses of 7.0 mg/kg [3].

Mechanism of Action

Multiple mechanisms of action may explain the ability of diminazene to eliminate protozoal pathogens. Diminazene binds to kinetoplast (kDNA) within the trypanosomal parasite which

inhibits kDNA replication. Binding takes place via interaction with areas of DNA with high proportions of adenine-thymine (A-T) base pairs and appears to be somewhat sequence specific. This binding is through non-covalent mechanisms, which include Van der Waals forces, hydrogen bonding, and electrostatic forces [5]. The drug also binds double stranded DNA within the minor groove. Another mechanism involves inhibition of mitochondrial type II topoisomerase, which inhibits the normal functions of replication, transcription, recombination and repair [6]. Additionally, diminazene has also been shown to inhibit serine proteases, which impede generation of normal signaling pathways leading to increased intracellular calcium within the mammalian cell [7, 8]. This increase in free intracellular calcium then allows for organization and fusion of the host lysosomes, which are believed to be crucial for host cell invasion by *Trypanosoma*.

Uses

Diminazene is used to treat trypanosomiasis in cattle, pigs, buffalo, gazelle mules and horses [3, 9, 10]. There are literally dozens of known *Trypanosoma* species. These species are similar to *Cytauxzoon* species in that they are vector borne protozoal heterogeneous pathogens. The organisms are present within the bloodstream but many are not intracellular pathogens (*C. felis* is a strictly intracellular pathogen). *Trypanosoma* species can infect a large number of host species (including cats), producing various different disease manifestations. Diminazene shows variable efficacy in trypanosomal treatment. A dose of 3.5 mg/kg was highly effective at clearing *T. evansi* infection in equids with the first dose, as determined by blood smear examination and mouse inoculation. However, when the treated animals relapsed or became reinfected, and

required repeat treatment six weeks later, 25% and 50% of the donkeys and horses, respectively, were positive for the parasite [9]. Treatment of *T. vivax* infected horses also follows an unrewarding path, whereby the animals initially respond, but eventually relapse and many die [11]. However, in *T. congolense*, a single dose of diminazene is curative in both cattle and dogs [12].

Diminazene is an effective treatment for Babesiosis in dogs, horses, donkeys and cattle [1, 3, 13]. Like *C. felis*, *Babesia* are hematoprotzoal parasites, but unlike *C. felis*, *Babesia* infect only the host red blood cells and undergo only a single life cycle phase within the mammalian host. When treating *Babesia* with diminazene, doses range from 2.5 to 10 mg/kg in dogs, with higher doses being more likely to effect a cure for *Babesia canis* [12]. A recent report demonstrated remarkable efficacy for the treatment of *Rangelia vitale*, a parasite closely related to *Babesia* spp., in dogs [14].

Diminazene has also been applied therapeutically to treat leishmaniasis, another protozoal disease, in humans. Efficacy and mechanism of action for the treatment of leishmaniasis has been studied in laboratory mice. Due to its affinity for kDNA, it causes dyskinetoplasty in the parasite and may have future therapeutic advantages for disease treatment when combined with other drugs [15, 16].

Use of diminazene in domestic cats is rare. We are aware of only two published studies prior to the studies reported here. The first, published in 1999, retrospectively described the use of diminazene aceturate (2.0 mg/kg, two doses 7 days apart) in six cats affected by acute cytauxzoonosis [17]. In this report, five out of six cats survived the infection but information

addressing long term parasite clearance was unavailable. In a more recent study, seven cats were experimentally infected with *Trypanosoma evansi* and then treated with diminazene aceturate at a dose of 3.5 mg/kg for five consecutive days. This therapy was 85.7% efficacious in clearing the organism, as determined by PCR analysis, and treatment appeared to be safe [18].

Although its use in veterinary medicine has primarily been as an anti-protozoal medication, many researchers are now looking at this older drug in a new light. The drug has been used to treat bovine francisellosis (aka, tularemia), a disease caused by gram negative bacteria [3]. Perhaps less intuitive uses have also been investigated. Due to the specificity of its DNA binding, researchers in other fields have begun to utilize its binding affinity to AT rich areas of DNA. Emerging uses for this aromatic diminadene include use in plasmid purification, which produces higher yields than other previously utilized methods for purification [19]. This potential for increased efficiency make plasmids more available for genetic engineering or DNA vaccines. Researchers are also looking to expand the therapeutic realm of diminazene by synthesizing of platinum-diminazene compounds as chemotherapy for in vitro breast cancer experiments [20]. Due to the high binding affinity for AT rich regions, these new diminazene compounds bind with more affinity than cisplatin, a commonly used chemotherapeutic. Additionally, the ability to inhibit topoisomerase likely contributes to their cytotoxicity to in vitro neoplastic cell populations.

Pharmacokinetics

The variable pharmacokinetics of diminazene have been investigated in several animal species. Modeling of pharmacokinetic data typically finds the best fit in a two compartment

model, although some studies have found that it is better represented by a one or three compartment model [21-24]. The drug is highly bioavailable and rapidly attained plasma levels after intramuscular injection in most species [21, 25-27]. Several pharmacokinetic parameters in multiple species are summarized in Table 4.1.

Table 4.1: Pharmacokinetic data for diminazene

Animal Species	Dose (mg/kg)	Route	Compartment Model	Elimination half-life (hours)	Vd (L/kg)	Cmax (mcg/mL)	Tmax (min)	ref
Dog	4.2	IM	Two	5.3	24.5	1.85	20	[12]
	3.5	IV	Two	11.6	2.39	n.d.	n.d.	[28]
Cattle	3.5	IM	Three	n.d.	1.37	4.76	36	[25]
	3.5	IM	Two	222.1	0.92	3.24	49.8	[2]
	3.5	IM	Two	2	n.d.	4.65	15-45	[27]
	3.5	IM	One	107.5	0.74	n.d.	n.d.	[23]
Sheep	3.5	IM	Two	18.8	n.d.	8.11	1.12	[21]
Goats	3.5	IM	Two	16.54	n.d.	7	0.92	[21]

In dogs, pharmacokinetic analysis revealed that diminazene is rapidly distributed and sequestered in the liver, and then is redistributed more slowly to the peripheral tissues and/or renally eliminated [22]. However there appears to be wide inter-individual variation for diminazene pharmacokinetics in dogs, which may explain why some dogs have toxic side effects at published therapeutic doses [22, 29]. In all animals where elimination route was characterized, both fecal and urinary excretion are routes of elimination [3]. The drug is also excreted into the milk of mammals, and may be detectable within minutes and persist in measurable levels for days [21].

Published data regarding the pharmacokinetics of diminazene in cats have not been available prior to our studies.

Resistance

Diminazene is widely used in areas with endemic trypanosomiasis, and patterns of resistance in highly endemic areas have emerged leading to reports of decreased treatment efficacy [30]. Trypanosomiasis has a great economic impact on food animal production, thus spawning multiple studies investigating mechanisms behind drug resistance. Characterizations of resistant parasite populations of *T. congolense*, *T. brucei* and *T. evansi* have demonstrated a loss of the purine transporter gene; this gene encodes the main transporter by which the drug is accumulated within the parasite [31-33]. Additionally, upregulation of a novel gene, TeDR40, in *T. evansi* isolates induced significantly reduced sensitivity to the drug [31]. Resistance in Babesial organisms has also received attention. Resistant strains of *Babesia gibsoni* are noted to demonstrate decreased levels of transcription of heat shock protein BgHsp70, although the actual mechanism of resistance has yet to be determined [34].

It has been suggested that drug resistance may also be related to the chronicity of infection. According to this theory, at least some treatment failures with diminazene are not due to innate or acquired resistance to the drug, but instead these failures are due to an extended duration of infection that allows colonization of tissues with poor drug penetration (i.e. the brain) [3]. For example, treatment of mice infected with *T. brucei brucei* at 3 or 7 days post infection will result in a cure, while treatment at 14 days leads to uniform relapse.

Adverse effects and Toxicity

Diminazene may cause serious and even fatal adverse events. Cattle have reported tolerated doses up to 21 mg/kg without adverse effects [3]. It is fatal in mice when administered at dosages varying from 9 to 20 mg/kg in mice [35, 36]. Camels and dogs appear particularly susceptible to adverse effects. Toxicity in camelids has been reported at 7 mg/kg. The toxic dose for dogs is 20-50 mg/kg intramuscularly or 12.5 mg/kg administered intravenously, but doses within the recommended dose range of 4.2 mg/kg have caused neurologic lesions [12]. Toxicity in dogs appears to be related predominately to CNS edema, hemorrhage and malacia in the cerebellum, thalamus and midbrain [12]. Non-lethal side effects include a transient tachycardia, hypotension and collapse (when administered IV), gastrointestinal disturbance, urination, injection site swelling and neurologic symptoms (tremors, ataxia, nystagmus) in dogs, while horses and mules experience salivation, edema, restlessness, recumbence and dyspnea [9, 22, 29, 37]. Hepatotoxicity is also possible and is reported in camelids and dogs [38, 39]. Prior to our studies, the drug had only been used in thirteen cats at doses of 2 mg/kg or 3.5 mg/kg of diminazene aceturate [18, 40]. In these two studies, one animal died after receiving diminazene, but the cause of death was likely due to Cytauxzoonosis. Other toxicities were not reported in treated cats.

Although many of these side effects are often associated with excessive parasympathetic stimulation, a mechanism remains undetermined. Although it has been postulated that diminazene could be inhibiting cholinesterase activity, experimental evidence has not been consistent with this hypothesis [41]. Other researchers have proposed, but have not proven, that diminazene may bind directly to parasympathetic receptors or affect secondary messengers to reach the same effects [12].

Other side effects may not be apparent initially, but can become manifest in economically important ways. In rams, the drug induces substantial decreases in sperm concentration, volume and motility [42]. The persistence of the drug in the milk requires appropriate withdrawal times when administered to milk-producing species. Despite the fact that diminazene interacts substantially with DNA, it has not proven to be tetratogenic [3].

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CHAPTER 5

PHARMACOKINETICS OF DIMINAZENE DIACETURATE IN HEALTHY CATS

Pharmacokinetics of Diminazene Diacetate in Healthy Cats

Short title: Diminazene Pharmacokinetics in Cats

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Abbreviations:

HPLC- high pressure liquid chromatography; IM - intramuscular; AUC- area under the curve;

CL/F - apparent extravascular clearance; C_{MAX} - peak plasma concentration; T_{MAX} - time of peak concentration; V_d/F - apparent volume of distribution; MRT - mean residence time; LOQ - limit of quantification

Survival of five of six cats treated for acute cytauxzoonosis with diminazene aceturate has been reported.(Greene et al., 1999) Diminazene, an aromatic diamidine, has been used to treat babesiosis and trypanosomiasis in dogs, leishmaniasis in humans, and a variety of other protozoal diseases in domestic livestock. It was administered at a dose of 2 mg/kg IM (two doses, one week apart) in the few cats with *C. felis* infection. Diminazene was administered to seven cats experimentally infected with *Trypanosoma evansi* at a dose of 3.5 mg/kg via intramuscular injection on five consecutive days.(Silva et al., 2009) The treatment was 85.7% efficacious in eliminating the parasite, and no adverse effects were observed. Although commercially available in both acetate (MW 515.5) and diacetate (MW 587.6) salts, the drug is not FDA approved for use in any species in the United States of America.

Feline cytauxzoonosis is a disease with high morbidity and mortality. Left untreated, 97% of cats with clinical cytauxzoonosis will die of the disease.(Birkenheuer et al., 2006) Recently, the combination of the anti-malarial drug atovaquone with the antibiotic imidocarb dipropionate produced improved survival, but 40% of treated cats still died of the disease.(Cohn et al., 2011) Additionally, this combination therapy requires thrice-daily oral dosing and can be prohibitively expensive. Diminazene, if found to be effective for the treatment of *C. felis*, would offer advantages both in practicality of dosing and in cost. Before additional clinical studies can be performed, the disposition of diminazene needs investigation. Therefore, our study objective was to characterize the pharmacokinetic profile of diminazene diacetate in cats.

Four adult cats (2 male and 2 female) weighing 3.1 to 5.3 kg were used. The cats were healthy based on physical examination, CBC, biochemical panel and urinalysis. The day prior to study, cats were sedated with propofol (3-5 mg/kg to effect) and indwelling jugular catheters

were placed to facilitate sample collection for the first 48 hours. After that, samples were obtained via jugular or medial saphenous venipuncture. Heparinized blood samples (2.0 ml) were collected just before (hour 0) and 0.5, 1, 2, 4, 8, 12, 18, 24, 36, 48, 72, 120, and 168 hours after intramuscular administration of diminazene diaceturate. Plasma was separated by centrifugation within 30 minutes of collection and frozen (-80°C) until analysis. Cats were observed several times daily to monitor for adverse events such as anorexia, lethargy, injection site pain, abnormal mentation, seizures, vomiting, ptilyism, or diarrhea. Temperature, heart rate, respiratory rate and effort were recorded 0,12, 24, 48, 72, 120 and 168 hours after drug administration. At 168 hours post-injection, CBC, biochemical panel and urinalysis were repeated. All procedures were approved by the University of Missouri Animal Care and Use Committee.

A powdered commercial drug formulation of diminazene diaceturate (Veriben®, CevaSante Animale) was freshly reconstituted with sterile water to a concentration of 7 mg/mL and the solution was filtered (0.2 micron disk filter, B.Braun, Bethlehem, PA) prior to administration. The dose was calculated at 3 mg/kg diminazene diaceturate (equivalent to 1.68 mg/kg base or 2.6 mg/kg of diminazene aceturate). The total volume of reconstituted drug varied from 1.3 to 2.3 mL per cat; all volumes exceeding 2.0 ml were administered in two separate intramuscular (IM) locations.

Concentrations of diminazene were measured by high pressure liquid chromatography (HPLC) analysis using UV absorption and ion-pairing conditions. Because the assay detects the amount of diminazene base, it can be used for detection of either diminazene aceturate or diminazene diaceturate. The reference standard of diminazene (Sigma Aldrich, St Louis, MO,

USA) was dissolved in distilled water to make up a 1 mg/ml stock solution. From this stock solution, further dilutions were made in distilled water in order to generate calibration curves in plasma and to generate quality control samples. The calibration curve included 8 standards, including zero (blank). The calibration curve linear range was 0.01 to 10 µg/mL. The mobile phase consisted of 73% distilled water, 27% acetonitrile and an ion pairing reagent added as a mobile phase modifier. As diminazene is a highly polar molecule, the ion-pairing reagent (S8) was needed at a concentration of 0.1% to optimize column retention time. The HPLC system consisted of a quaternary solvent delivery system (Agilent Technologies, Wilmington, DE, USA), at a flow rate of 1 mL/min, an autosampler (1100 Series Autosampler, Agilent Technologies) set for injection volume of 30 µL, and ultraviolet detector set at a wavelength of 370 nm (1100 Series UV–Visible Detector, Agilent Technologies). The chromatograms were integrated with a computer program (1100 Series Chemstation software, Agilent Technologies). A C18 reverse-phase column (ACE 5 micron C18-AR) kept at column temperature of 40° C was used for separation. Calibration samples, quality control samples, and incurred samples were all extracted using an identical procedure. Extraction of diminazene was accomplished by adding 400 µL of acetonitrile to an equal volume of sample. The mixture was vortexed and centrifuged at 1000g for 10 minutes. A volume of 500 µL of the supernatant was evaporated to a dry residue under a stream of air at a temperature of 40° C for 20 minutes. Samples were then reconstituted with 200 µL of mobile phase and vortexed. The resulting solution was transferred to HPLC vials and injected. Retention time for peak of interest was 8 to 9 minutes. Limit of quantification for diminazene in feline plasma was 0.01 µg/mL, which was determined from the lowest point on a linear calibration curve that produced a signal-to-noise ratio above 10:1. Feline plasma fortified

with standard of diminazene were used to validate the assay. The precision was with (CV) of +/- 15% and accuracy was within +/- 15% of the true value.

Mean plasma drug concentrations of diminazene were plotted on linear and semi-logarithmic graphs for analysis. Non-compartmental pharmacokinetic analysis was conducted using a commercially available pharmacokinetic program (Phoenix WinNonlin, version 6, Pharsight Corporation, Mountain View California). The area under the curve (AUC) from zero to infinity was calculated using the log-linear trapezoid method. The terminal half-life was estimated from the slope after the peak concentration. Secondary parameters of importance were systemic clearance per fraction absorbed (CL/F), peak plasma concentration (C_{MAX}), time of peak concentration (T_{MAX}), apparent volume of distribution per fraction absorbed (Vd/F), and mean residence time (MRT). Bioavailability could not be assessed because there was not an accompanying intravenous dose.

A single intramuscular dose of diminazene diacetate was well tolerated by all cats. No salivation, vomiting, diarrhea, behavior or mentation changes were noted. Vital parameters remained normal with the exception of mild tachypnea at 48 and 72 hours post injection in three cats. Thoracic auscultation and respiratory effort remained normal despite respiratory rates from 48 to 60 breaths per minute, suggesting that tachypnea was a behavioral response to handling. Only clinically irrelevant laboratory alterations were noted. In two cats, mild eosinophilia was detected (1.76 and $1.61 \times 10^3 /\mu\text{L}$; reference interval $0-1.5 \times 10^3 /\mu\text{L}$), and a minor increase in ALT was noted in one cat (83 U/L ; reference interval $18-77 \text{ U/L}$).

Mean plasma diminazene concentrations at each time point are reported in Table 5.1, with accompanying linear graphs of plasma concentration vs. time shown in Fig 5.1. Diminazene was not detected in any of the time 0 samples.

Inter-individual variation in measured parameters was relatively minimal, but there were only four animals in the study; therefore, population variability is difficult to assess from this study. Absorption of the drug after intramuscular administration produced a peak at 30 minutes, but there were not enough early samples to assess the rate of absorption and whether or not a higher peak may have appeared earlier (Table 5.2). The half life of the drug in health cats is quite short at 1.7 hours, as compared to an elimination half life of 5.31 hours in dogs administered intramuscular diminazene. (Miller et al, 2005) Although it may be tempting to try to extrapolate this data to clinical dosing schedules, it is important to recognize that without knowing the drug concentration required to inhibit or kill a particular pathogen, it is not yet possible to make suggestions regarding optimum dosing schedules. Clearly, the short half-life in cats and the briefly sustained plasma concentrations from IM injection of this formulation could pose a challenge if high sustained concentrations are needed for therapeutic efficacy.

In this study, diminazene diacetate was well tolerated in healthy cats at a dose of 3 mg/kg administered intramuscularly once. Very limited information has been published regarding diminazene toxicity in cats. In the two published studies of diminazene use in cats, 12 of 13 cats appeared to tolerate the drug without adverse effects. (Greene et al, 1999; Silva et al, 2009) The remaining cat died within hours of drug administration, but the cat's death was more likely related to cytauxzoonosis than to drug toxicity. Additional data to determine maximum

tolerated dose and to look for evidence of biochemical or hematological toxicity is required before routine clinical use of diminazene in cats can be considered. Without any information regarding the concentration of drug required to inhibit or kill a given protozoal agent, predictions cannot be made regarding optimum dose or dose frequency for the treatment of infection. However, this pharmacokinetic study provides some foundation from which other studies can be designed. Further studies are warranted to determine efficacy of the drug in feline protozoal diseases, including *Cytauxzoon felis*.

Table 5.1: Mean Plasma Diminazene Concentration (n=4)

TIME (hour)	Mean* +/-Std. Dev (µg/ml)
0	0 +/- 0
0.5	0.46 +/-0.14
1	0.31 +/-0.06
2	0.20 +/-0.04
4	0.09 +/-0.03
8	0.02 +/-0.005
12	0.01 +/-0.0
18	<0.01
24	<0.01

*Limit of quantification (LOQ): 0.01 µg/mL. Results for all cats at times (36, 48, 72, 120, 168)

were not detectable, or below LOQ.

Table 5.2: Pharmacokinetic Parameters

	Mean	(+/-) Std. Dev.
Volume of distribution per fraction absorbed (VD/F)	3.36 L/kg	+/-0.72
Elimination rate constant	0.42 hr ⁻¹	+/-0.08
Area under the curve (AUC)	1.22hr×µg/mL	+/-0.26
Half-life (T1/2)	1.70 hr	+/-0.29
Peak plasma concentration (Cmax)	0.51 µg/mL	+/-0.11
Systemic clearance (CL/F)	1.38 L/hr/kg	+/-0.26
Mean residence time (MRT)	2.45 hr	+/-0.42

Figure 5.1: Mean Plasma Diminazene Concentrations

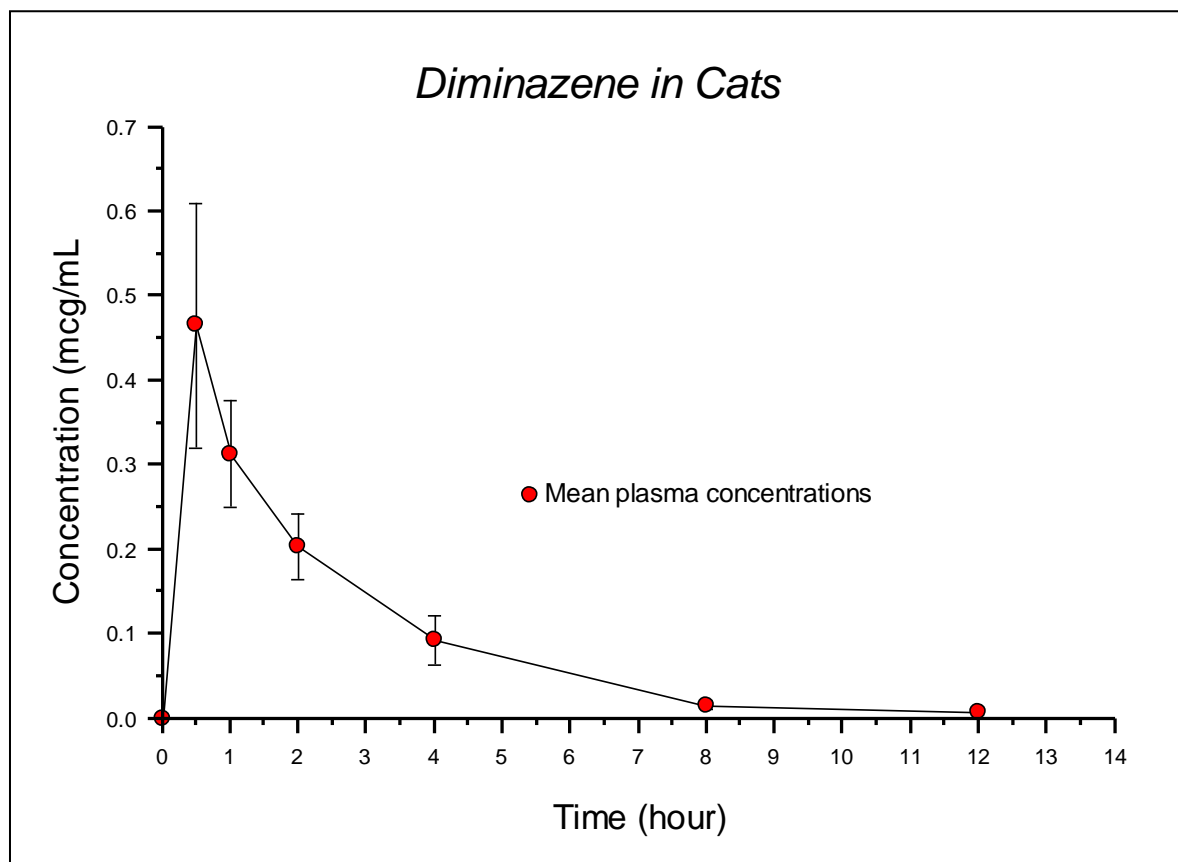


Fig. 5.1. Mean plasma diminazene concentrations (+/- Standard Deviation) at time points 0 through 12 hours. Hours 18, 36, 48, 72, 120, 168 are not shown as concentrations were below LOQ ($< 0.01 \mu\text{g/mL}$).

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CHAPTER 6

DIMINAZENE DIACETURATE FOR THE TREATMENT OF CHRONIC *CYTAUXZOOM FELIS* INFECTION IN NATURALLY INFECTED CATS

Title: Diminazene diacetate for the treatment of chronic *Cytauxzoon felis* infection in naturally infected cats

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Key Words: Cytauxzoonosis, piroplasm, anti-protozoal, carrier

Abstract

Cytauxzoon felis is a hemoprotozoal parasite that causes substantial morbidity and mortality during the acute phase of infection in cats. However, cats that survive the acute illness remain persistently infected and may serve as a reservoir for the tick-transmitted pathogen. We investigated the ability of the antiprotozoal compound diminazene diaceturate to eliminate the pathogen from naturally infected *C. felis* carriers. Seven healthy, chronically infected domestic cats were treated in a masked fashion with diminazene diaceturate (3 mg/kg) or placebo intramuscularly in a series of two injections seven days apart. Clearance of the organism was assessed via light microscopy and semi-quantitative PCR at 0, 3, 6 and 10 weeks. Additionally, cats were monitored for behavioral changes or for changes on physical examination, CBC, plasma biochemical profile, and urinalysis periodically. Those cats which remained parasitemic at the end of 10 weeks were switched to the alternative treatment and similarly monitored for an additional 10 weeks. Adverse events associated with treatment were limited to self-resolving hypersalivation and injection site soreness; the former was ameliorated by premedication with atropine. Parasite burden, as assayed by both light microscopy and semi-quantitative PCR, did not differ between diminazene and placebo treated cats at any time point. Diminazene diaceturate was unable to eliminate the pathogen or significantly reduce parasite burden in healthy, chronically infected cats.

Introduction:

Cytauxzoon felis is a hemoprotozoan parasite of that causes substantial morbidity and mortality in cats. The natural lifecycle of the parasite relies on an appropriate tick vector feeding on an infected host and later feeding on a second, susceptible felid to transmit the pathogen.^{1,2} The acute phase of infection begins with schizogenous replication within the host mononuclear cells. Schizont distended macrophages can lead to widespread obstruction of the microvasculature with subsequent profound systemic inflammatory response and multisystem organ failure. Mononuclear cells eventually rupture, releasing merozoites that are then taken up by erythrocytes as piroplasms.³⁻⁵ Cats that survive initial infection will remain persistently parasitemic without displaying signs of illness.⁶⁻⁸

Although once considered a uniformly fatal disease, it has become apparent that some cats survive acute infection.⁶⁻⁸ In fact, the combination of atovaquone and azithromycin along with supportive care led to a survival rate of 60%, but treatment did not eliminate the carrier state.^{9,10} These chronic carriers are capable of transmitting infection through the appropriate *Amblyomma americanum* tick vector.¹

Diminazene has been used to treat a variety of protozoal diseases in domestic livestock throughout the world. The drug is produced as either of two salts, diminazene aceturate and diminazene diacetate, both of which contain the same active compound but differ in molecular weight. In a case report of six cats with acute cytauxzoonosis treated with diminazene aceturate at 2 mg/kg intramuscularly, five cats survived.¹¹ Although the drug is not FDA approved for use in any species in the USA, it may be possible to obtain the drug for a specific animal patient

under Compassionate Use Protocols. While such importation is impractical for cats suffering acute cytauxzoonosis, it should be possible for the treatment of chronic carriers were it to be demonstrated to be efficacious in eliminating the pathogen.

Despite improved treatments, feline cytauxzoonosis remains a disease with high morbidity and mortality. We hypothesized that diminazene, administered in a two dose protocol equivalent to that used for the treatment of acute cytauxzoonosis, could eliminate *C. felis* infection from naturally infected carrier cats as assessed by light microscopy and PCR with few adverse effects. Were this treatment to prove effective, it could not only be used to reduce the chance that recovered cats could serve as an indirect source of infection for others, but would provide impetus for further study of the drug for the treatment of acute cytauxzoonosis.

Methods and Materials

Study Design

This was a prospective, crossover study with a block design. Two cats were knowingly assigned to receive placebo during the first phase, while the other cats were arbitrarily assigned to receive either placebo or drug. Only a single investigator (LAC) was aware of the treatment assigned to each cat; this investigator did not participate in microscopic slide review or PCR analysis. All cats that remained infected after completion of phase one were then assigned to the alternative treatment/placebo in phase two of the study.

Animals

Seven adult domestic cats (2 female intact, 2 female spayed, 3 male castrated) were donated for the study of cytauxzoonosis by their owners. All cats were from *C. felis* endemic areas of northern or central Arkansas, had extensive outdoor exposure, and all were suspected or confirmed to harbor the pathogen prior to donation. One cat had been owned for only 2 months, but the others had been owned for several (up to 5) years. None of the cats had a known history of illness. All cats appeared to be young to middle aged adults, and weights ranged from 3.2 to 5.8 kg (mean 4.8 kg +/- 0.95 kg).

At the time of donation, each cat underwent a complete physical examination along with feline leukemia (FeLV) and feline immunodeficiency virus (FIV) antigen and antibody testing^a, CBC, biochemical profile, urinalysis, and PCR testing for *C. felis*. No important abnormalities were identified on physical examination, biochemical profile, or urinalysis, and all cats were negative for retroviral infection. All cats were positive for *C. felis* by both PCR and microscopic review of stained blood smears. None of the cats was anemic, but a single cat had persistent neutropenia (segmented neutrophils $1.73 \times 10^3/\text{ul}$ to $2.38 \times 10^3/\text{ul}$; reference range $2.5\text{-}12.5 \times 10^3/\text{ul}$). Before study entry the neutropenic cat was evaluated with *Ehrlichia* spp. PCR, bone marrow aspirate evaluated microscopically and by immunofluorescent antibody (IFA) testing for FeLV, and given a trial course of doxycycline. All cats were vaccinated with FVRCP and rabies vaccines, administered an anti-helminthic (fenbendazole 50 mg/kg PO or emodepside/praziquantel^b topically 15 mg/60 mg per cat) and treated with topical anti-parasitic (fipronil^c). Animals were cared for according to the principles outline in the National Institutes of Health

Guide for the Care and Use of Laboratory Animals, and were acclimated to housing in an accredited facility for a minimum of 30 days prior to initiation of the study. All animal use was approved by the University of Missouri Animal Use and Care Committee (protocol number 6665).

Treatment

A commercially prepared product of diminazene diaceturate^d (70 mg/mL) and phenazone (375 mg/mL) in sterile water was stored as per manufacturer directions. Cats were given either 3 mg/kg of diminazene diaceturate or an equivalent volume of sterile saline in the epaxial muscles or the biceps femoris on day 1 and 8 of each treatment phase. Due to their fractious nature, 2 cats required sedation with ketamine (10-15 mg/kg) administered IM prior to treatment or blood sample collection. Because excessive ptialism was noted in several cats immediately after the first treatment, all animals were subsequently pretreated with atropine (0.03 mg/kg) subcutaneously 15 minutes prior to drug/placebo administration.

Monitoring

Cats were monitored for 10 weeks after each phase of the study. Physical examination, CBC with careful blood smear review, biochemistry profile, urinalysis, and *C. felis* PCR were performed prior to the first treatment, then again at weeks 3 and 10; at week 6, the same tests were performed with the omission of biochemistry profile and urinalysis. For the first two hours after each injection, cats were monitored for changes in heart or respiratory rates, and the injection site was palpated. The attitude and appetite of all cats was observed twice daily for one

week after each injection, and once daily for the study duration. At study completion, the two fractious intact female cats were euthanized and necropsy performed.

Quantitative CBC, plasma biochemistry profile, and urinalysis were performed in routine fashion by the University of Missouri Veterinary Medical Diagnostic Laboratory. In addition, a trained technician thoroughly reviewed Wright-Giemsa stained blood smears for piroplasms and counted the number of piroplasms per 1500 erythrocytes at 40X.

Polymerase Chain Reaction

Anticoagulated blood was analyzed using a previously published technique.¹² Briefly, each reaction consisted of 12.5 µl 2X PCR master mix^e, 7 µl water, 50 pmol of each oligonucleotide primer and 5 µl sample. Thermal cycling conditions were: 98°C for 30sec followed by 45 cycles 95°C for 5 seconds and 60°C for 5 sec. Melting curve analysis was initiated at 75°C and data were captured at increasing increments of 0.5°C for 30 time points. For each PCR assay positive (previously characterized *C. felis* samples) and negative (no template) controls were used. Cycle threshold (Ct) determined from real time PCR was used as an estimate of parasitemia. Typical precautions (i.e. disposable gloves, clean to dirty workflow and physical separation of pre and post PCR samples) were used to prevent amplicon carryover.

Statistical Analysis

A Two Way Repeated Measures ANOVA was used to compare the number of piroplasm-containing erythrocytes and the Ct for each positive PCR. Pairwise multiple comparisons of

means (Holm-Sidak method) carried out after the two way ANOVA. Calculations were performed by standard statistical software.^f Significance was considered as a p value of <0.05.

Results

Observed adverse events were minor and self-limiting. Immediately after the first set of injections, several cats salivated profusely for more than an hour. For all subsequent injections, each cat was pre-treated with atropine; thereafter hypersalivation was absent or minimal. No changes were noted in heart rate within the first few hours of injection, but transient tachypnea without respiratory distress developed in one cat after both drug and placebo injection, and in two different cats after drug or placebo injection. Subjectively assessed injection site soreness was noted on palpation of the injection site in only a single cat after diminazene administration, but this resolved by the following day. Vomiting was not seen within hours of injection, but during the entire 10 weeks both cats treated with diminazene (n=3) and placebo (n=3) vomited on occasion. All cats retained normal appetite and attitude through the entire study course.

Hematologic abnormalities were limited to the presence of piroplasms at low numbers and neutropenia. A single sample from week 10 was not available for CBC due to technical issues. None of the cats was ever anemic. Moderate neutropenia was noted in one cat before study entry (nadir 1.55; day of study entry 1.73; reference range $2.5-12.5 \times 10^3/\mu\text{l}$). Bone marrow aspirate obtained prior to entry confirmed marked myeloid hypoplasia, and although occasional RBC containing piroplasms were identified in the marrow, schizonts were never seen. In addition to the negative FeLV antigen ELISA on whole blood, the marrow was negative by IFA for FeLV. Although the cat was negative by PCR for *Ehrlichia*, a trial course of doxycycline (10

mg/kg/day) was administered for three weeks without improvement in neutrophil count. This cat continued to have neutrophil counts just above or below the lower reference range for the duration of the study (Figure 6.1). Mild neutropenia (2.07 to $2.48 \times 10^3/\mu\text{l}$) was detected in two other cats at three separate time points, 3 weeks after diminazene treatment ($n=2$) or 3 weeks after placebo ($n=1$).

Biochemical panel and urinalysis were unremarkable from most cats at most time points. Mild hyperglycemia was detected from both semi-feral cats after both treatment and placebo treatments. In a single cat, mild hypokalemia (2.8 ; reference range 3.0 - 4.7 mEq/L) was noted at several time points after both diminazene and placebo. Plasma alanine transaminase (ALT) was increased (110 ; reference range 18 - 77 U/L) in one cat 3 weeks after diminazene injection. Proteinuria ($2+$ dipstick, 100 mg/dL sulfosalicylic acid) was detected in a single concentrated urine sample but the sediment in that sample was active and *Capillaria* eggs were identified as well.

No statistically significant difference was noted on the number of piroplasm infected erythrocytes seen on light microscopy between treatment and placebo treatments ($p=0.85$). Piroplasms were identified in low numbers on every blood smear from every cat at every time point, with a maximum of 3 piroplasms per 1,500 erythrocytes.

All cats remained *C. felis* positive by PCR at all time points of the trial (Figure 6.2). Due to tube mislabeling, samples for analysis of Ct PCR were unavailable for two cats at week 10 after diminazene administration. For statistical calculations, the values used for these two cats were recorded from the next closest sample to week 10, which was the week 6 sample. Cycle

threshold values were not significantly different in the treatment group as compared to the placebo group at any time point ($p=0.70$).

Gross lesions were not identified on necropsy of the two euthanized cats. Histologic evidence of mild focal lymphocytic pancreatitis and hypertrophic cardiomyopathy was observed in one cat, mild pancreatic amyloidosis was noted in the other. No evidence of *C. felis* schizonts were noted in the tissues from either cat.

Discussion

We demonstrated that 3 mg/kg diminazene diacetate given intramuscularly in two doses one week apart was unable to eliminate parasitemia in chronic *C. felis* carrier cats. The dose chosen was essentially equivalent to the dose of diminazene used in a series of six cats, five of whom survived acute cytauxzoonosis.¹¹ In that study, two doses of 2 mg/kg diminazene acetate were administered 7 days apart. Correcting for difference in molecular weight between the two diminazene salts, our dose of diminazene diacetate was equivalent to 2.6 mg/kg diminazene acetate.

Although cytauxzoonosis was once believed to be nearly uniformly fatal in domestic cats, it is now obvious that some portion of domestic cats survive acute infection to become chronic carriers. As with the cats donated for this study, there may be no known antecedent illness suggesting that these cats, like the natural reservoir host the bobcat, experienced a limited schizogenous phase of infection with only mild illness.^{13,14} Additionally, newer treatment

regimens are associated with survival of as many as 60% of treated cats but these recovered cats can continue to carry piroplasms in the RBC.⁹(Cohn, personal communication) Much less is known about the chronic stage of infection than the acute. Animals with chronic infection appear well, but it is possible that there may be important sequela to such infections.^{8,15} Although anemia never occurred in the cats studied here, it is possible that parasitized RBC might predispose to hemolytic anemia.

Neutropenia was a frequent finding in a single cat, and an occasional finding in two others. Neutropenia in one cat was identified at the time of his donation and before he was entered into this study. Despite a search, no cause for the neutropenia was identified. Neutropenia has not been previously identified as a consequence of chronic *C. felis* infection, and was not present in all of the cats included in this study. While it is possible that chronic *C. felis* infection may cause neutropenia in some cats, it is also possible that we either missed another co-morbid condition, or that for this cat, a low neutrophil count is simply normal. Despite demonstrated moderate neutropenia, this cat has remained well for 12 months.

Evidence of hypertrophic cardiomyopathy was noted in one of the euthanized cats. It is well established that certain drugs may induce cardiomyopathy, but these are more commonly dilated cardiomyopathy.¹⁶⁻¹⁹ Although it is possible that either diminazene diaceturate may have induced cardiomyopathy or that the finding was somehow related to *C. felis* infection, it is more likely an unrelated finding as hypertrophic cardiomyopathy has been reported in 15% of apparently normal cats.²⁰ Similarly, pancreatic amyloidosis and lymphocytic pancreatitis

identified at necropsy were most likely incidental findings but a relationship to either drug administration or chronic *C. felis* infection cannot be definitively ruled out.

Even if chronic *C. felis* infection does not lead to disease sequela for the individual cat, it might be of importance to the spread of disease. Once they are infected, domestic cats remain persistently parasitemic.^{6,7,15} These parasitemic cats might pose an indirect threat to other cats in the area. We now know that domestic cats are capable of transmitting the pathogen to a vector tick that can in turn transmit infection to another susceptible cat.²¹ Cytauxzoonosis is an emerging disease with an expanding geographic range.^{3,22-24} Although the bobcat (*Lynx rufus*) is the natural reservoir host, it seems plausible that chronically parasitemic domestic cats might have a role to play in this geographic expansion.^{1,14} In fact, the cats used in this study were donated by owners who did not want chronic carriers on their property for the fear that they increased the risk of infection for other cats in the area; had they not been donated for our study these cats were to have been euthanized. Effective treatment would potentially enable these cats to have remained with their owners.

Diminazene diacetate used at 3mg/kg produced only mild, transient adverse effects. Ptyalism, which was not reported in any of three previous studies of diminazene use in cats, was ameliorated with the administration of atropine sulfate prior to injection.^{11,25,26} This suggests that like the anti-protozoal compound imidocarb dipropionate, parasympathomimetic effects may be responsible.^{27,28} Injection site pain was only identified in one cat, and resolved quickly. Hyperglycemia seen in the two fractious cats after administration of both diminazene and placebo was likely a stress response and not mediated directly by diminazene. In one cat, a

transient increase in ALT was noted. Hepatotoxicity of diminazene has been reported dogs and camelids, but was not been previously reported in cats.²⁹⁻³¹ Histologic evaluation of two cats necropsied 10 weeks after diminazene administration, including the cat that had an increased ALT, did not reveal any pathologic changes in the liver. Although proteinuria was noted in one cat at one time point after diminazene administration, this same urine sample had both *Capillaria* parasites and an active sediment that may well have accounted for this finding. Additionally, this sample was from one of the fractious animals that required intramuscular administration of ketamine to facilitate safe handling; proteinuria has been associated with stress and exertion.^{32,33}

Piroplasms were consistently identified at low numbers in all blood samples. Because feline erythrocytes can remain in circulation for approximately 70 days, the study was continued for 10 weeks, enough time that any RBC containing parasites that might have been killed by treatment should have been cleared from circulation.³⁴ No difference was detected on semi-quantitative light microscopic review of blood smears between the treatment and placebo group.

Polymerase chain reaction is a more sensitive means of detecting the pathogen than blood smear review, and the Ct from real time PCR offers a semi-quantitative measure of the degree of parasitemia. All samples were PCR positive for *C. felis*. Additionally, no difference in parasite burden was evident by documenting Ct.

Although diminazene diacetate was ineffective at eliminating *C. felis* from chronic carriers, it may still be efficacious in the treatment of acute cytauxzoonosis. The acute stage is characterized by schizogenous replication that can lead to substantial organ dysfunction with substantial morbidity and mortality. At a dosage of 3 mg/ kg administered intramuscularly

twice seven days apart, most side effects were transient and mild, and premedication with atropine prior to injection is eliminated excessive salivation. Future studies using diminazene to treat cats with acute cytauxzoonosis infections are warranted.

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a-SNAP FeLV/FIV Combo test, IDEXX Laboratories, Westbrook, Maine, 04092

b-Profendor, Bayer, Shawnee, Kansas, 66201

c- Frontline Spray, Merial, Duluth, GA, 30096

d- Veriben RTU, Ceva Sante Animale, South Africa

e- SsoFast™ EvaGreen® Supermix Bio-Rad, Hercules, CA, 94547

f- Sigma Plot v11; Systat Software Inc San Jose, CA 95110

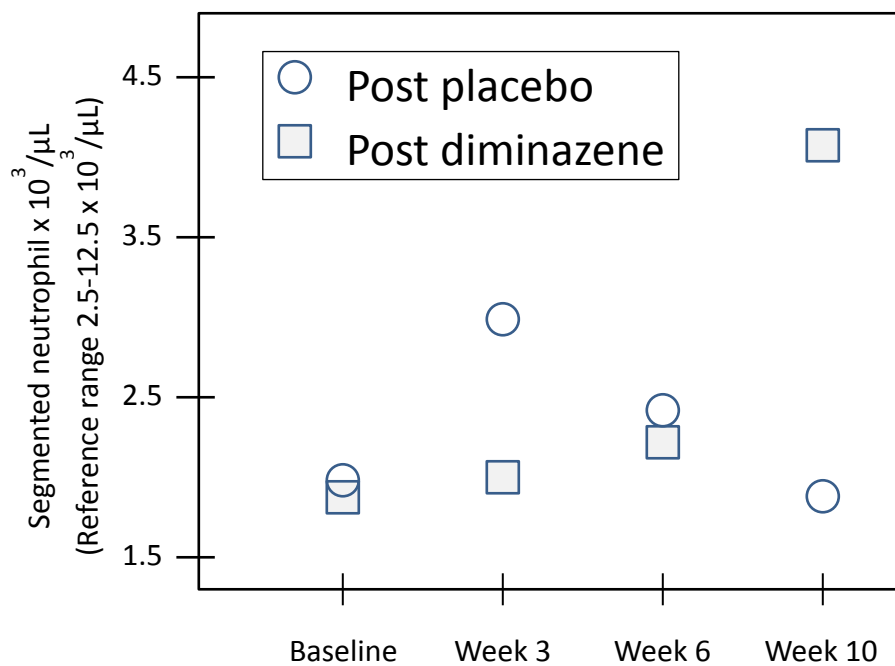


Figure 6.1. Neutrophil counts of the single cat that documented neutropenia before study entry.

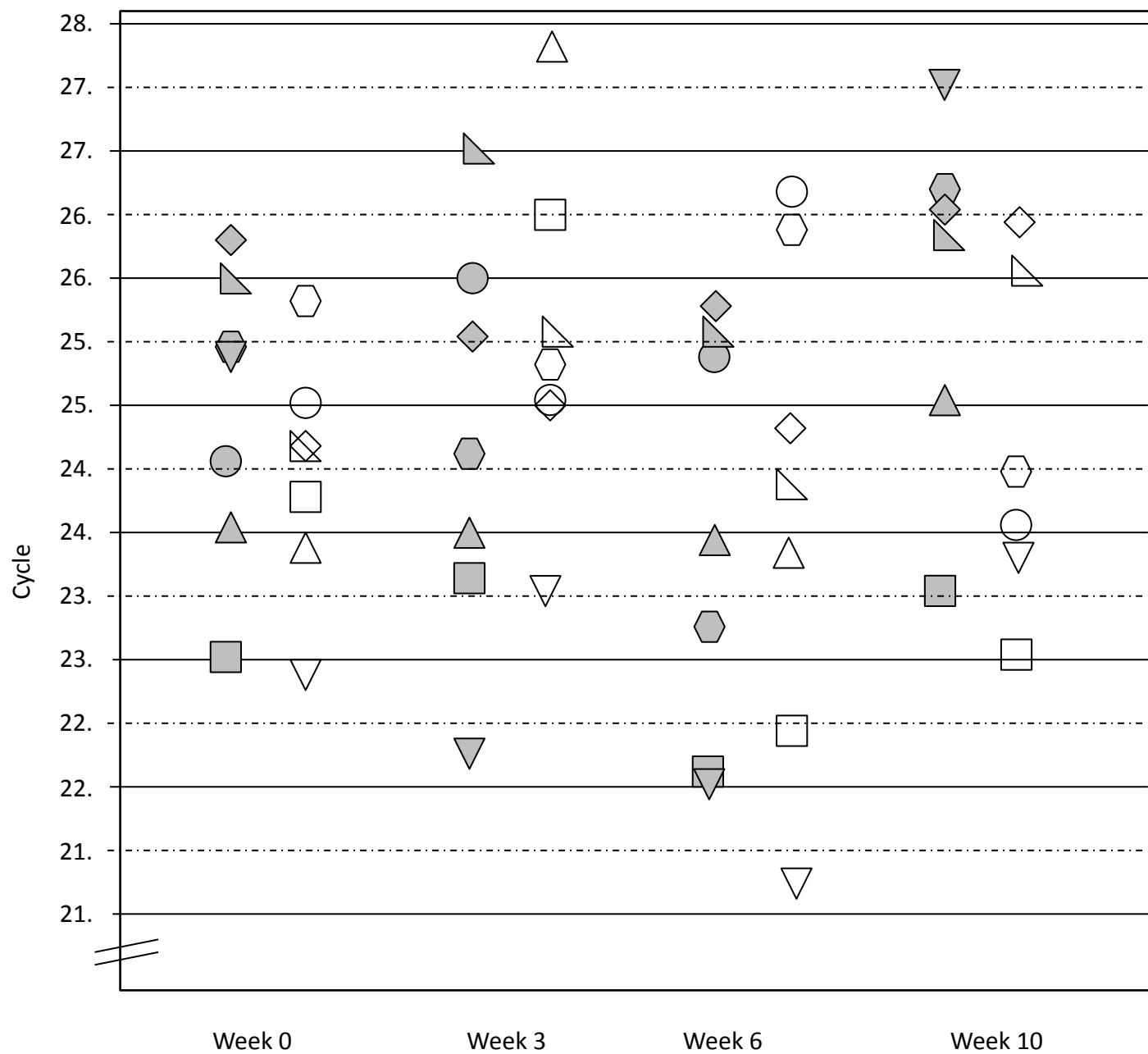


Figure 6.2: Cycle threshold (Ct) from real time PCR for all cats at 0, 3, 6, and 10 weeks after injection with 3 mg/kg diminazene diaceturate IM. Each shape represents the Ct count from an

individual cat. Open shapes are Ct values after placebo while shaded shapes represent values after diminazene treatment. Datum from two individual cats are missing at week 10 (one each placebo and drug) due to a labeling error. Lower Ct values roughly correspond with higher numbers of parasites. There was no statistic difference in parasite burden after treatment.

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CHAPTER 7

DOSE INTENSE DIMINAZENE DIACETURATE PROTOCOL FOR THE TREATMENT OF CHRONIC *CYTAUXZOOM FELIS* INFECTION IN NATURALLY INFECTED CATS

Title: Dose intense diminazene diacetate protocol for the treatment of chronic
Cytauxzoon felis infection in naturally infected cats

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Key Words: cytauxzoonosis, anti-protozoal, piroplasm, clearance, therapy

Abstract

Cytauxzoon felis is a hemoprotozoan parasite of cats. While many infected cats die of acute illness, those that recover enter a chronic carrier state. To date, no treatment has been documented to clear the chronic carrier state leaving recovered cats to act as a potential indirect source of infection via a tick vector. Diminazene diacetate is an anti-protozoal therapy that has been suggested to be efficacious for the treatment of acute cytauxzoonosis. In order to determine if the drug might clear the carrier state, five naturally infected chronic carrier cats were administered 4 mg/kg of diminazene diacetate intramuscularly for five consecutive days. Clearance of the organism was assessed via semi-quantitative PCR and light microscopy 1, 3, 6, and 10 weeks after starting treatment. Additionally, cats were monitored for adverse drug reactions by daily observation and examination, CBC, biochemical profile, and urinalysis at 1, 3, and 10 weeks. Adverse events were common and included profuse self-limiting salivation and nausea at the time of injection, monoparesis in the injected leg, potential hepatotoxicity, and proteinuria. Unfortunately, degree of parasitemia was not reduced. Therefore, this treatment protocol cannot be recommended for elimination of the carrier state.

Introduction:

Cytauxzoon felis is a hemoprotozoan parasite of felids that induces considerable morbidity and mortality. The acute phase of infection begins with schizogenous replication within the host mononuclear cells. Schizont distended macrophages can lead to widespread obstruction of the microvasculature with subsequent profound systemic inflammatory response and multisystem organ failure. Mononuclear cells eventually rupture, releasing merozoites that are then taken up by erythrocytes as piroplasms.(Cohn, 2011) If a cat survives initial infection, piroplasms may persist within the erythrocytes for the life of the cat. (Brown et al., 2008; Haber et al., 2007; Meinkoth et al., 2000) These chronic carriers are capable of transmitting infection through the appropriate *Amblyomma americanum* tick vector. (Reichard et al., 2010)

Diminazene, an aromatic diamidine produced as either of two salts, has been used to treat a variety of protozoal diseases in domestic livestock worldwide. In a case report of six cats with acute cytauxzoonosis treated with diminazene aceturate at 2 mg/kg intramuscularly, five cats survived.(Greene et al., 1999) Although the drug is not FDA approved for use in any species in the USA, it may be possible to obtain the drug for a specific animal patient under Compassionate Use Protocols. While such importation is impractical for cats suffering acute cytauxzoonosis, it should be possible for the treatment of chronic carriers were it to be demonstrated to be efficacious.

Diminazene aceturate was able to eliminate *Trypanosoma evansi* from the majority of experimentally infected cats at a dose of 3.5 mg/kg (equivalent to 4.1 mg/kg of diminazene diacetate) given intramuscularly for five consecutive days, without adverse effects.(Da Silva et

al, 2009) Recently, we investigated the ability of a lower, less intense dosing protocol, similar to that used in the single case series for the treatment of acute cytauxzoonosis, to reduce parasite burden from carrier cats without success.(Lewis et al.,unpublished data)^a We hypothesized that a dose intense protocol would be able to reduce or eliminate the parasite burden in naturally infected cats with chronic *C. felis* infection, thus potentially reducing their ability to act as a reservoir of infection.

Methods and Materials

Animals

The study was approved by the University of Missouri Animal Use and Care Committee (protocol number 7174), and cats were group housed in an accredited facility and cared for according to the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Five adult cats (2 female spayed, 3 male castrated), all from the same region of northern Arkansas, were donated for the study of cytauxzoonosis by their owner after they were confirmed to be *C. felis* carriers. The middle-aged cats ranged from 5.1 to 7.3 kg (median 5.7 kg +/- 0.96 kg) and had been owned for two to five years. All had been vaccinated for FVRCP and rabies, and none had a known history of illness. At the time of donation, each cat underwent a complete physical examination, CBC, biochemical panel, *C. Felis* PCR, and feline leukemia and feline immunodeficiency virus antigen and antibody (respectively) testing. Cats were administered an anti-helminthic (fenbendazole 50 mg/kg PO or emodepside/praziquantel topically 15 mg/60 mg per cat) and topical anti-parasitic (fipronil) at donation. Before use in this dose intense study, these cats had been previously treated with 2 injections of 3

mg/kg diminazene given one week apart. However, the treatment failed to reduce parasitemia as determined by both PCR and light microscopic review of stained blood smears.(Lewis et al.,unpublished data)^a The last dose of diminazene had been administered 3.5 months prior to enrollment in this dose-intense study.

Treatment

A commercial preparation of diminazene diacetate (70 mg/mL) and phenazone (375 mg/mL) in sterile water^b was obtained and stored as per manufacturer directions. Cats were premedicated with 0.03 mg/kg atropine 15 minutes prior to injection of a 4 mg/kg intramuscular dose of diminazene diacetate q 24 hours for 5 total doses. On the first day of treatment, the entire volume (0.29 to 0.42 ml) was administered in the biceps femoris, but after two cats developed monoparesis in the injected limb, injections were divided equally between the epaxial muscles and the quadriceps on days 2 through 5 of treatment. Due to excessive vomiting and ptilyism despite atropine premedication, 1 mg/kg of maropitant^c was administered subcutaneously 30 minutes prior to diminazene injection on days 3 through 5 of the study.

Clinical and laboratory evaluation

A baseline CBC with quantification of erythrocyte piroplasms, plasma biochemical panel, urinalysis, and semi-quantitative PCR of blood for *C. felis* were obtained just prior to study entry. Respiratory rate, respiratory effort and heart rate were recorded every 15 minutes for 2 hours immediately post injection. Cats were monitored for attitude as well as injection site

reactions twice daily for six days, and then daily for the following 9 weeks. CBC with quantification of erythrocyte piroplasms, biochemical panel, urinalysis, and PCR of blood for *C. felis* were repeated at weeks 1, 3 and 10, while CBC with piroplasm quantification alone was performed at week 6. All cats had echocardiograms performed by a boarded cardiologist after the study was completed.

CBC, biochemical panel, and urinalysis were performed in the University of Missouri Veterinary Medical Diagnostic Laboratory in a routine fashion. Additionally, a trained technician reviewed Wright-Giemsa stained blood smears. After thorough slide review, the number of piroplasm infected erythrocytes per 1500 erythrocytes were counted.

Polymerase chain reaction on anticoagulated blood was analyzed using a previously published technique.(Birkenheuer et al., 2006) Briefly, each reaction consisted of 12.5 µl 2X PCR master mix^d, 7 ul water, 50 pmol of each oligonucleotide primer and 5 µl sample. Thermal cycling conditions were: 98°C for 30sec followed by 45 cycles 95°C for 5 seconds and 60°C for 5 sec. Melting curve analysis was initiated at 75°C and data were captured at increasing increments of 0.5°C for 30 time points. For each PCR assay positive (previously characterized *C. felis* samples) and negative (no template) controls were used. Cycle threshold (Ct) determined from real time PCR was used as an estimate of parasitemia. Typical precautions (i.e. disposable gloves, clean to dirty workflow and physical separation of pre and post PCR samples) were used to prevent amplicon carryover.

Statistical analysis

A One Way Repeated Measures ANOVA was used to look for changes in both the number of infected erythrocytes observed microscopically as well as Ct PCR after treatment. Data met criteria for normalcy and equal variance. Calculations were performed by standard statistical software.^e A *p-value* of less than 0.05 was considered statistically significant.

Results

Adverse reactions and toxicity

Adverse reactions were common. Despite the use of atropine premedication, 3 cats demonstrated severe ptialism soon after injection and 4 cats experienced repeated vomiting in the hours following injection. All the cats appeared nauseous and lethargic for several hours after injection, but the addition of maropitant pre-treatment on day 3 through 4 resulted in dramatically less salivation and eliminated vomiting after injection. Additionally, during the 10 weeks of observation a small amount of vomitus was found in the cats' housing on an occasional basis. Three cats were quite sore near the sites of injection for 1 to 2 days afterwards. Two cats developed monoparesis in the injected leg on Day 1. In one cat, paresis resolved within 24 hours, but in the other cat the monoparesis persisted and improved gradually over months.

Hematologic values changed little during treatment. Neutropenia (segmented neutrophils $1.55 \times 10^3/\text{ul}$; reference range $2.5\text{-}12.5 \times 10^3/\text{ul}$) was noted in one cat 10 weeks after treatment; this same cat had demonstrated neutrophil counts either below or at the bottom of the reference range since acquisition 10 months prior to study completion. Anemia was not identified in any cat at any time point (mean hematocrit at baseline $36.8\% \pm 4.5$; mean hematocrit from all time points $37.4\% \pm 4.6$; reference range 24-45%). Platelet clumping prevented accurate enumeration

of platelet counts on several samples, but estimated platelet numbers were always considered appropriate.

Serum alanine aminotransferase (ALT) increased in 4 of 5 cats after treatment (Figure 7.1). Two cats were noted to have diminished appetite and to have lost body weight at weeks five to six post treatment. It was discovered that they had been transitioned to a new brand of commercial dry food abruptly during week two of the study. At week six, serum ALT in the two cats with weight loss were 88 U/L and 238 U/L (reference range 18-77), serum alkaline phosphatase (ALKP) were 75 U/L and 193 U/L (reference range 5-55), and bilirubin 0.2 and 2.0 mg/dL (reference range 0-0.3). All five cats were transitioned back to the original diet; the cat with normal serum bilirubin gained weight rapidly, but the cat with hyperbilirubinemia failed to improve and became completely anorexic. Biochemical abnormalities, abdominal ultrasound findings, and hepatic cytology all supported the clinical syndrome of hepatic lipidosis in this cat. The cat improved rapidly after an esophagostomy tube was placed to facilitate feeding on a high calorie prescription diet (Hill's a/d); the feeding tube was removed after 10 days.

Every cat had well concentrated urine at every time point (mean urine SG 1.068 ± 0.009). Despite benign urine sediment, moderate proteinuria as indicated by both 2+ dipstick and sulfosalicylic acid of 50 mg/dL was detected in two cats during week three, but resolved thereafter. In a single cat, an unusual rhomboidal crystal was identified in the urine at week 10.

Echocardiography revealed one cat with each of the following findings: focal septal hypertrophy, tricuspid regurgitation, an enlarged left atrium and a structurally normal heart. Two cats had increased moderator bands and a left anterior fascicular block.

Parasitemia

Parasitized erythrocytes were evident microscopically from every cat just prior to study entry, but only at very low numbers (0 to 2 per 1500 erythrocytes). Parasites could not be identified after thorough slide review from two cats at week 3 after treatment, but were again identified from these same cats at weeks 6 and 10. Parasites were observed on all other blood smear samples, always at low numbers. As compared to baseline, the average number of piroplasms noted per 1500 erythrocytes was not significantly different at one, three, six or ten weeks, with a range of 0 to 2 infected erythrocytes per 1500 erythrocytes ($p=0.29$).

All cats were PCR positive for *C. felis* at every time point. Cycle threshold values, which inversely correspond with parasite burden, did not statistically differ from baseline (Figure 7.2) ($p=0.05$).

Discussion

A high dose, dose intense protocol of diminazene administration was unable to reduce the parasite burden in cats that were chronic *C. felis* carriers. Because the cats were followed for 70 days, and the life-span of feline erythrocytes is approximately 70 days, we eliminated the possibility that either microscopy or PCR would detect non-viable parasites in erythrocytes that had simply not yet been removed from circulation.(Kaneko et al., 1966) The degree of parasitemia did not appear to vary much over time. Other hemoprotozoan infections demonstrate marked variations in parasite numbers in an individual even over short periods of time. An example of this phenomenon is seen in malaria (*Plasmodium falciparum*), where 100-fold or greater variations in parasite density can be noted within a 6-hour period.(Delley et al., 2000) In

cats, a similar phenomenon has been recognized in *Mycoplasma haemofelis* when parasites may be abundant on one sample and undetectable on another sample obtained the same day. (Alleman et al., 1999) Our study data would tend to indicate relatively constant low levels of parasitemia are present in cats with chronic *C. felis* infection.

As compared to the few prior published studies of diminazene use in cats, we noted far more adverse events.(Da Silva et al., 2009; Greene et al., 1999; Lewis et al., 2012, Lewis et al.,unpublished data)^a Two of the three previously published studies used a lower dose administered less often than was done in our study.(Greene et al., 1999; Lewis et al., 2012, Lewis et al.,unpublished data)^a However, the study reported here used an equivalent dose of diminazene base as was used for the treatment of trypanosomiasis.(Da Silva et al., 2009) Da Silva et al reported no adverse effects in 7 treated cats; hepatic and renal parameters remained normal when tested every 14 days for 49 days after treatment but urinalysis were not performed. Perhaps the greater number of adverse events was related to our use of diminazene diacetate at an equivalent to the dose of diminazene acetate used in the prior study, or to a difference in the inactive components of the drug preparations used. Alternatively, there may be differences inherent to the cats treated (e.g., young versus middle aged, infection with *Trypanosoma* versus *Cytauxzoon* spp) that might explain the greater incidence of adverse events seen in our study.

At the 4 mg/kg dose, many cats developed significant ptialism and emesis that were ameliorated with the administration of atropine and maropitant prior to injection. These signs were not reported in a study using an equivalent diminazene dose in cats, but have been reported in other species.(Da Silva et al., 2009; Homeida et al., 1981; Kettner, 2007; Miller et al., 2005)

In our previous study using a lower drug dosage, hypersalivation occurred but not vomiting. Because ptyalism and vomiting were not ameliorated by pre-treatment with atropine alone, parasympathomimetic effects are unlikely to be completely responsible for these effects. The drug itself may act at the chemoreceptor trigger zone to induce vomiting.

The observed monoparesis in two cats was thought to be precipitated by inadvertent injection of the drug into the sciatic nerve. Because it involved only the injected leg and occurred very soon after injection a peripheral neurotoxicity seemed far less likely, although nervous system toxicity has been reported in dogs treated with diminazene.(Naude et al., 1970) It is possible that the drug caused direct damage to the skeletal muscle. Diminazene has been reported to be toxic in-vitro to cultured rat myocyte cells, skeletal muscle edema has been noted in dogs administered the drug, and muscle necrosis has been identified at the site of IM injection in cattle.(Fairclough, 1963; Gillingwater et al., 2010; Losos and Crockett, 1969) No additional paresis was observed after dosing was altered to be given in two equivalent volumes in the quadriceps and epaxial musculature.

Hepatic enzymes were increased in several cats after diminazene administration. In two cats, hepatic lipidosis was either confirmed or suspected based on clinical findings and the resolution of weight loss and improvement in hepatic enzymes upon return to feeding. It was unfortunate that the cats' diet was changed during the study period, which makes it difficult to know if anorexia, weight loss, and hepatopathy were drug related or not. Hepatic lipidosis in cats may be related to abrupt diet change, a diet that is unacceptable to a cat, or secondary to other underlying disease such as cholangitis or neoplasia.(Armstrong and Blanchard, 2009; Willard et

al., 1999) In other species, xenobiotic toxicosis has also been reported to cause histologic evidence of hepatic lipid accumulation, although to the authors' knowledge this has not been reported in cats.(Amacher, 2011) Because serum ALT was increased in 4 of 5 cats, including two with no evidence of diminished appetite or weight loss, it seems likely that the drug itself was related at least to the increased cytosolic leakage enzyme ALT. Although no increase in hepatic enzymes were reported in by Da Silva et al in 7 cats treated with the dose equivalent of diminazene used here, diminazene administration in dogs can cause increases in liver enzyme activity. (Akpa et al., 2008; Da Silva et al., 2009; Miller et al., 2005) The drug is sequestered in the liver and accumulates there for prolonged periods in dogs, rabbits, and cattle. (Gilbert, 1983; Kellner et al., 1985; Miller et al., 2005)

Two cats demonstrated a transient proteinuria while serum albumin remained normal. Other markers of renal injury, such as tubular casts, glucosuria, pyuria or loss of concentrating ability were not noted. The significance of the described rhomboid crystal is unclear. Abnormal urinary crystals have been reported with toxins such as ethylene glycol and the combination of melamine and cyanuric acid.(Thompson et al., 2008) Additional doses would have to be given in a larger population to see if crystalluria is indeed a side effect of diminazene diacetate administration.

The only hematological abnormality was neutropenia in a single cat that had consistently demonstrated neutrophil counts at or below the lower reference range during the 10 months since his acquisition and before ever receiving diminazene diacetate. Prior to entry into this study, the cat had tested negative for feline leukemia virus by ELISA and IFA of bone marrow,

negative for feline immunodeficiency virus antibody by ELISA, and negative for *Ehrlichia spp* by PCR. A bone marrow examination revealed marked myeloid hypoplasia without an apparent cause. Despite periodic neutropenia, the cat has remained healthy. Diminazene has not been reported to cause hematologic abnormalities. (Da Silva et al., 2009; Homeida et al., 1981; Peregrine and Mamman, 1993; Tuntasuvan et al., 2003) The neutropenia observed in this single cat is unlikely to be related to diminazene administration. It is possible neutropenia relates to chronic cytauxzoonosis; although none of the other cats in this study were neutropenic, two others from this group have consistently maintained neutrophil counts near the bottom or just under the reference range during the 12 months they have been monitored.

Because we had identified cardiomyopathy in a cat treated with two injections of 3.0 mg/kg diminazene diacetate, all cats in the current study were examined after study completion by echocardiography. (Lewis et al., unpublished data)^e Unfortunately, baseline cardiac investigation was not performed as cardiotoxicity has not been previously reported in association with diminazene use. Echocardiography was performed one month after completion of the study reported here, and six months after the same cats had received two injections of a lower dose of diminazene. Minor echocardiographic abnormalities were found in four of five cats. Given the heterogeneity of the abnormalities, it is likely that these simply represent a spectrum of spontaneous (naturally) acquired disease, and are unlikely to represent a drug induced cardiomyopathy.

This study included a small number of infected cats. It is possible that effect of diminazene treatment on parasite levels might have reached statistical significance if a larger

sample were used. However, since the most biologically relevant endpoint is complete clearance of the organism, this would not change the clinical application of our results.

Infection with *C. felis* causes acute disease characterized by substantial morbidity and mortality as well as chronic infection in clinically normal cats. Although diminazene failed to clear infection of chronic carriers, it may still be effective for the treatment of acute disease. Indeed, Greene et al reported that five of six cats treated with diminazene aceturate for acute cytauxzoonosis survived the disease.(Greene et al., 1999) The most effective treatment to date for acute infection, azithromycin and atovaquone, is associated with a 60% survival, but is ineffective at clearing the chronic infection.(Cohn et al., 2011; Cohn et al., 2008) Therefore, elimination of chronic parasitemia may not be necessary for a drug to produce improved survival from acute cytauxzoonosis.

In summary, diminazene diacetate failed to eliminate *C. felis* from chronically infected carrier cats. At a dose of 4 mg/kg given intramuscularly for five consecutive days, cats experienced multiple adverse effects, including vomiting, salivation, injection site soreness, monoparesis, hepatotoxicity and proteinuria. Additional studies are warranted to investigate the use of diminazene diacetate for the treatment of acute cytauxzoonosis, but treatment of chronic carrier cats with diminazene cannot be advocated.

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a- Lewis, K., Cohn, L., Marr, H., Birkenheuer, A.J. Diminazene diacetate for the treatment of chronic *Cytauxzoon felis* infection in naturally infected cats (University of Missouri).

b- Veriben RTU, Ceva Sante Animale, South Africa

c-Cerenia, Pfizer Animal Health, New York, NY

d- SsoFast™ EvaGreen® Supermix Bio-Rad, Hercules, CA, 94547

e- SAS v9; SAS Institute Inc, Cary, NC

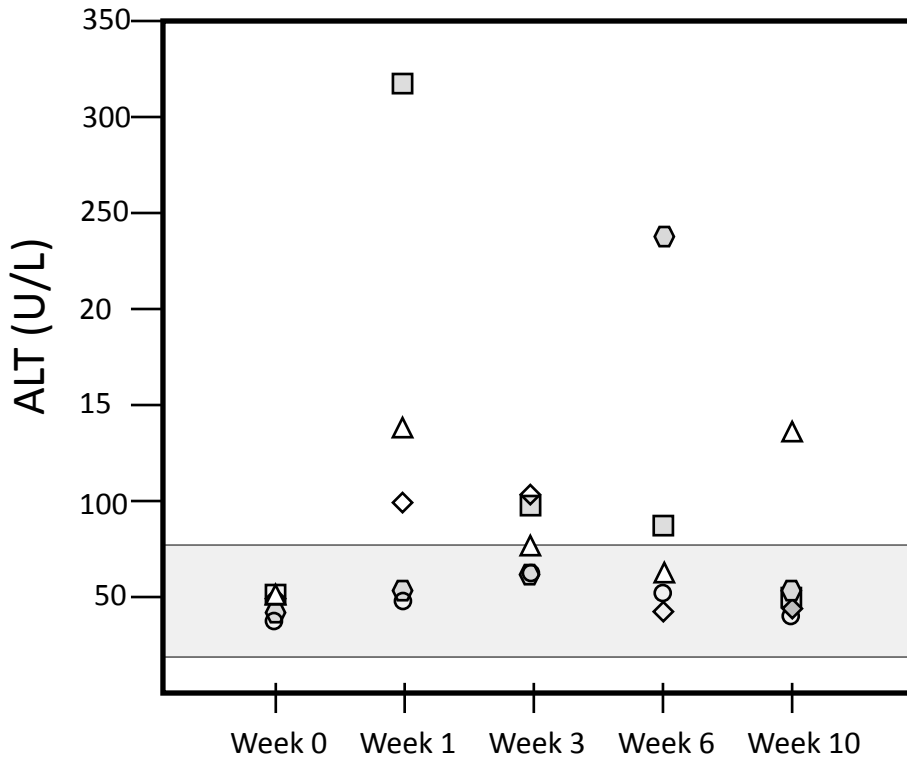


Figure 7.1: Plasma ALT of each cat at 0, 1, 3, 6, and 10 weeks after injection of 4 mg/kg diminazene diaceturate IM. The area in light grey rectangle represents the reference range for ALT. The cat represented by the colored square and hexagon are the two diagnosed with suspected mild or severe hepatic lipidosis, respectively.

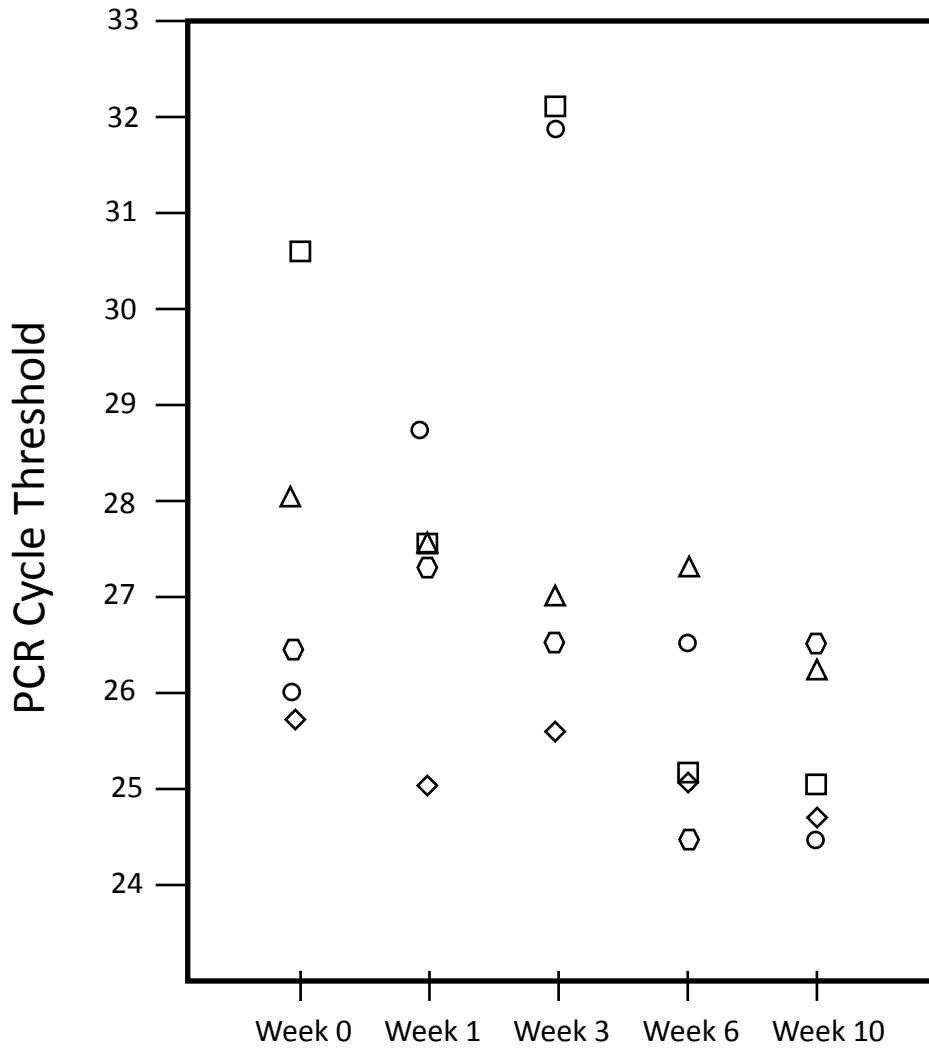


Figure 7.2: Mean cycle threshold (Ct) PCR for all cats at 0, 1, 3, 6, and 10 weeks after injection of 4 mg/kg diminazene diaceturate IM. Lower Ct values roughly correspond with higher numbers of parasites. There was no statistic difference in parasite burden after treatment.

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CHAPTER 8

CONCLUSIONS AND SUMMARY

Our knowledge of *Cytauxzoon felis* is rapidly evolving. Just a decade ago, it was believed that although felids other than bobcats could become infected, they served as dead end hosts and the disease was ultimately fatal in these accidental hosts. This understanding has changed as reports of domestic cats surviving the infection began to emerge. In some cases, domestic cats were reported to survive a severe clinical illness. In others, apparently healthy cats with no known illness were found to be chronic carriers of the protozoan, demonstrating that infection with *C. felis* and the disease cytauxzoonosis were not always synonymous. Likewise, our previous understanding of the infection in exotic, non-native felids such as lions and tigers was that they uniformly succumbed to the disease. We documented chronic infection in tigers without recognized clinical illness, altering what was understood about the infection in these non-native species.

It is not surprising that severely ill animals comprise the bulk of the initial reports of a disease process. Severe morbidity and mortality triggers veterinarians and researchers to search for a cause of illness. In contrast, apparently healthy carrier animals with minimal parasite burdens can easily be overlooked. The development of molecular techniques has further

expanded our knowledge of chronic carrier animals, by allowing the detection of animals with low levels of parasitemia, as appears to be typical of chronic *C. felis* carriers. Perhaps, we will come to understand that the clinically ill animals are only a relatively small portion of the animals infected by the disease. While we cannot ignore the fact that extensive studies of experimental infection in the 1980's led to near uniform mortality, there are reports of what might be a less virulent strain of pathogen in areas of Arkansas. (Brown et al., 2010; Meinkoth et al., 2000) Indeed, we reported not only healthy but chronically infected tigers from this same region, but also reported healthy but chronically infected domestic cats. Natural selection often favors pathogens that are not highly virulent and do not rapidly kill their hosts. Future studies of pathogen prevalence in healthy domestic and exotic felidae in the United States are warranted. If we find that infections are common in healthy animals from given regions, alterations in animal husbandry such as regular testing for the pathogen or altered ectoparasite control regimens may be useful to help control disease.

The life cycle of this Apicomplexan parasite still remains somewhat nebulous. Although the initial model focused on the tick vector's role in transmission from the natural reservoir host, the bobcat, to an aberrant host, in the domestic cat other modes of transmission have been experimentally demonstrated. Importantly, close contact (e.g., mutual grooming) has not been shown to cause disease without a tick vector present. Alternate routes of infection include inoculation with schizont-laden splenic homogenate or transfusion with blood from acutely ill cats.(Glenn et al., 1983; Kier et al., 1982; Wagner et al., 1980) In other protozoal diseases, vertical transmission may play a significant role in the parasitic lifecycle. The role of the chronic carrier queen in transmitting *C. felis* to her kittens was completely unknown prior to our studies.

We failed to document perinatal transmission of *C. felis* in chronically infected cats. Larger studies would be required to definitively exclude this as a mode of transmission, but our initial study indicates that this is unlikely to be a major route of natural infection.

At present, cats that are infected and develop clinical cytauxzoonosis have substantial morbidity and mortality even when provided with aggressive medical care. (Cohn, 2011) Novel therapeutic options should be explored for treatment of this disease. One such potential novel therapy is diminazene. If the antiprotozoal diminazene were proven even only equally effective as the current standard therapy, it would offer significant advantages. The combination of the antiprotozoal drug atovaquone combined with the antibiotic azithromycin is the current standard therapy for cytauxzoonosis. However, this regimen is very expensive and must be administered orally multiple times daily for 10 days. On the other hand, diminazene is very inexpensive and is given by a single intramuscular injection that may be repeated.

Our first step to investigate this drug for use in the treatment of cytauxzoonosis was to characterize the drug pharmacokinetics in healthy young adult cats. We found that a high V_d/F suggests that this drug is likely widely distributed through the body, something that would be necessary to treat a systemic protozoal infection. We also found that the plasma half-life of diminazene when administered intramuscularly in cats is quite short at 1.7 hours. Although at first glance this might appear problematic, the drug is used often as a single injection in many other species, or when given repeatedly, the repletion is often delayed a week or more even while the elimination half-life of the drug is consistently less than 6 hours. (Miller et al., 2005) Although our studies were not designed to identify drug toxicity, no adverse reactions were

identified in these healthy cats given 3 mg/kg, setting the stage for further investigation. Further work looking at the pharmacodynamics of this drug, such as sites of accumulation and biologic half-life, are also needed to provide the clinician important information needed to formulate dosing protocols. Ideally, pharmacokinetic and pharmacodynamic information for antimicrobial drugs are combined with data on the minimum inhibitory concentration for a given microbe. Unfortunately, this has yet to be determined for cytauxzoonosis. The lack of a long term culture system for the organism currently hinders this work, which would then allow us to create guidelines for clinical use.

Diminazene might be used in either of two populations of infected cats; cats acutely ill with cytauxzoonosis, or chronic carrier cats which pose a risk for indirect transmission (via a tick vector) to other cats. We began our studies on the latter group. Success in eliminating infection could reduce indirect transmission, impact geographic spread of this disease, and might even protect the treated cat from (as of yet unreported) consequences of chronic infection. We used a group of chronic carrier cats donated for these studies, a group distinct from the typical clinically ill patient presented to the veterinary practitioner during the schizogenous phase of infection.

Our first therapeutic trial used a low dose, low intensity of diminazene, based on the single previous report in the literature of treatment of feline cytauxzoonosis. (Greene et al., 1999) At a dose of 3 mg/kg administered intramuscularly twice at a seven day interval, we were unable to demonstrate clearance of the *C. felis* organism via PCR or light microscopy. Additionally, the treatment failed to significantly decrease the level of parasitemia as assessed by semi-quantitative methods (cycle threshold PCR) as compared to placebo. While there were no

serious adverse events in the treated cats, profuse salivation and occasional vomiting were noted in these cats but not in the healthy cats used in our pharmacokinetic study. The reason for this is unclear, but may be related to animal age since the carrier cats were middle aged adult cats as compared to the young adult cats used for the pharmacokinetics study. Potentially infection itself might impact the recognized adverse events associated with diminazene administration. Because premedication with atropine resulted in diminished side effects, these effects can be reasonably assumed to be parasympathetically mediated.

After determining that the low dose, low intensity drug protocol was ineffective at parasite clearance, we tried a different dose and dose regimen in the same previously treated cats. A recent study was able to eliminate another hemoprotozoa, *Trypanosoma*, in cats with a higher dose and more intense dosing protocol.(Da Silva et al., 2009) Following this paper, we elected to administer 4 mg/kg diminazene diacetate (equivalent to the 3.5 mg/kg diminazene acetate used in the *Trypanosoma* study) intramuscularly daily for five consecutive treatments. Despite the dose intensification and escalation, we were again unable to clear the chronic carrier state. However, at the higher dose, the cats demonstrated more severe side effects than during the lower dose study. Nausea and emesis were much more severe, and necessitated adding an anti-emetic drug as part of a pre-treatment. Two cat developed monoparesis, and in one cat this persisted for months, believed to be secondary to damage to the sciatic nerve or biceps femoris muscle. Our conclusions at this time are that diminazene diacetate does not appear to be an effective treatment for the clearance of cats with chronic *C. felis* erythroparasitemia at either a 3 mg/kg dose administered IM twice one week apart or at 4 mg/kg administered IM for 5 days.

Although the above results could be viewed as discouraging, it is worthwhile to point out that our test population of chronic carrier cats is distinct from the acutely infected clinically ill cat. During the acute phase, the morbidity is caused by infection of the monocytes with occlusion of the vascular tissue supply and a massive inflammatory response of the host. In the chronic phase, the erythrocytes are the focus of the infection, and the substantial systemic inflammatory response is absent. The previously report in which five of six cats treated with diminazene survived infection involved acutely ill cats. Therefore, the next step in this line of research would be to treat cats with acute cytauxzoonosis with diminazene as compared to the current best line of therapy, azithromycin and atovaquone. Because diminazene is not approved by the FDA for any species in the United States, such a trial could not be performed in client owned, naturally infected animals.

Future work in cytauxzoonosis should focus on development of more effective treatment and prevention strategies. It may be that by the time acute disease is recognized, the damage has been done and even elimination of the parasite may not prevent a fatal outcome. The complex interactions between host and parasite are integral into expanding our understanding of how the disease progresses from acute illness to chronic carrier state, and why some animals will succumb to the disease while others will progress to a chronic carrier state. It is my sincere hope that future research is able to elucidate these as well as provide additional treatment and prevention strategies for a serious illness of feline patients.

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