CYTAUXZOONOSIS:

NOVEL DIAGNOSTICS AND THERAPEUTICS

By

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Abstract:

Cytauxzoonosis is a tick-borne disease in the domestic cats with high mortality and a narrow therapeutic window for initiation of treatment. It is caused by the apicomplexan protozoal parasite *Cytauxzoon felis* and transmitted primarily by *Amblyomma americanum* ticks in the North America. To date, there is no effective treatment and no vaccine available for prevention, and the infected cats usually have rapid disease progression and succumb to infection. Thus, the early diagnosis and effective therapy is crucial for the survival of these cats.

In this thesis, two novel diagnostics including a *C. felis* probe-based droplet digital PCR (ddPCR) and a *C. felis* IgM ELISA were developed and validated for early detection of feline acute cytauxzoonosis. The probe-based ddPCR was designed using the hydrolysis probe and droplet digital technology. The assay is highly sensitive and specific that can detect infection in all clinical cats presented with acute cytauxzoonosis and had 100% agreement with blood smear evaluation. Infections were also detected in experimentally infected cats as early as one day prior to developing clinical signs. Additionally, it can monitor parasite loads over time to assess therapeutic efficacy. An indirect ELISA was developed utilizing the recombinant schizogenous antigen contig88 as coating protein to capture *C. felis*-specific IgM in infected cat plasma samples. IgM was detected by goat-anti-cat IgM antibody-HRP. The absorbance was measured at 450 nm. The IgM ELISA had 100% specificity and 86.21% sensitivity for strong positive samples and 75.86% specificity and 100% sensitivity in weak positive samples.

Lastly, a clinical study was conducted evaluating the efficacy of combined immunomodulatory therapy (atovaquone + azithromycin + dexamethasone) in treating cats with acute cytauxzoonosis. Although there was no significant difference in survival (p=0.8343), increased expression of pro-inflammatory cytokines (IFNγ, IL-1β, IL-8, IL-12, RANTES and Flt-3L) were detected in cats with acute cytauxzoonosis compared to healthy cats (p<0.05), while expression of PDGF-BB, SDF-1, MCP-1 and IL-18 was decreased in *C. felis* infected cats (p<0.05).

Collectively, these studies developed novel, applicable diagnostics for cytauxzoonosis and insight on the immunologic alteration during acute *C. felis* infection, which has the potential to become therapeutic targets in the future.

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CHAPTER 1

INTRODUCTION

Life cycle and Epidemiology of *Cytauxzoon felis*

Cytauxzoonosis is an emerging tick-borne infectious disease affecting the domestic cats and wild felids. The etiology, *Cytauxzoon felis*, is an apicomplexan protozoal parasite that belongs to the order Piroplasmida and the family Theileriidae. [1-3] The organism exists in two distinct forms (intra-erythrocytic and extra-erythrocytic) in its mammalian host, just like other members of Theileriidae. After its first discovery in Missouri in 1976, [1] it has been a disease of concern almost exclusively in the southcentral and southeastern United States. [2-4] However, it has recently been reported in the South America[5-7] and also a few other *Cytauxzoon* species have been identified in Europe,[8] Mongolia,[9] Iran,[10] and Zimbabwe. [11]

Cytauxzoon felis is transmitted to domestic cats primarily by *Amblyomma americanum* ticks in the North America. In the life cycle of *C. felis*, domestic cats acquire sporozoites during tick feeding. The sporozoites enter peripheral circulation, where they get phagocytized by mononuclear cells. Intracellular sporozoites mature into schizonts, which undergo schizogony and binary fission within the macrophages. Due to the multiplication of intra-cellular schizonts, the macrophages increase significantly in size.

It is usually at this stage that mammalian hosts develop clinical signs due to the protozoal thrombi (large schizont-laden macrophages) deposited within the microvasculature of vital organs. [2-4,12,13] During later stage of the disease, the intracellular schizonts mature into numerous merozoites. The macrophages eventually rupture, and merozoites are released into circulation or tissue fluid. The merozoites then penetrate erythrocytes, resulting in the classic 1 – 2 μm, "signet ring" shaped parasites observed cytologically. [2-4,12] The merozoites are capable of undergoing asexual reproduction and infecting other erythrocytes, causing parasitemia of its mammalian host. After another tick feeding, the infected erythrocytes are digested in the hindgut of the tick. After digestion, the merozoites mature into male and female gametes and undergo sexual reproduction. Thereafter, the zygotes mature into motile ookinetes, invading the salivary glands of the tick. The sporogony occurs and infective sporozoites are delivered into another mammalian host during tick feeding. [3,4,12]

Although transmission of *C. felis* have been demonstrated experimentally in both *Amblyomma americanum* (lone star tick) [14,15] and *Dermacentor variabilis* (American dog tick), [16] the occurrence of disease in cats coincides with the overlapping distribution of the bobcat population and *A. americanum* in the North America. [14] Thus, it is convincing that *A. americanum* serves as the primarily definitive host to complete the *C. felis* life cycle in most natural environments in the North America.

Pathogenesis of Cytauxzoonosis in Domestic Cats

As outlined above, the schizogenous phase of parasite replication is responsible for the clinical illness in its mammalian hosts. However, differences of disease severity exist among different mammalian hosts, [17-19] and domestic cats from different enzootic regions exhibit a distinct difference in mortality rates. [20] Bobcats are known to be the reservoir host of

cytauxzoonosis in the North America because they mostly develop mild clinical symptoms and recover subsequently even with persistent parasitemia. [17-19] This is due to a limited schizogony that occurs in bobcats as compared to domestic cats. [21] In domestic cats infected with *C. felis*, asexual schizogenous reproduction usually causes severe clinical illnesses due to the occlusion of vascular lumina by numerous large schizont-laden macrophages, [2-4,12,13] and is most evident in organs such as lungs, livers, spleen and lymphoid tissues. [3,12] The protozoal parasites also cause vascular endothelial disruption, resulting in activation of clotting cascade, eventually resulting in disseminated intravascular coagulation (DIC). [12,13,22] A severe inflammatory response against parasite invasion and cellular by-products released during cell death likely contributes to the development of multiple organ failure syndrome and result in death of the infected cats. [12,13,23] Although intraerythrocytic merozoites are not a major contributor of clinical disease, hemolysis has been reported to be associated with the presence of piroplasms. [12]

Clinical Findings in Infected Cats

After infestation by infected ticks, domestic cats usually develop clinical signs in 5 – 14 days post-infestation. [4,12,14,24] The initial clinical signs are vague and non-specific, which typically include anorexia and lethargy. Within hours to days, clinical abnormalities are usually more noticeable to owners and may include fever, jaundice, yellow urine, generalized muscle pain, vocalization, dyspnea, abnormal mentation or even seizures. [2-4] The most consistent physical exam findings are fever with marked temperature elevation (103 – 107 °F or 39.4 – 41.7 °C) and icterus. [3] However, hypothermia is common in moribund cats. [3] Others common findings include dehydration, pale mucous membranes, pain on palpation, dyspnea, tachycardia, lymph node enlargement and organomegaly (especially liver and spleen). The progression is extremely rapid and infected cats usually succumb within 24-48 hours of presenting to veterinary hospitals despite

aggressive medical intervention. [2,3,12] If the cat survives infection, it will carry intra-erythrocytic piroplasms for years while being clinically asymptomatic. [25] However, there is no evidence to suggest that these cats acquire protective immunity against *C. felis*, and relapse/re-infection is reported. [26]

Diagnosis of Cytauxzoonosis

Although laboratory findings in cats with acute cytauxzoonosis may include pancytopenia, lymphopenia, thrombocytopenia, hemolytic anemia (occur in later stage of infection), elevated liver enzymes, hyperbilirubinemia and electrolyte disturbances, [2,3,12] these characteristics are not specific for cytauxzoonosis. To date, a definitive diagnosis of cytauxzoonosis can be reached by the following three methods: (i) Blood smear evaluation for the presence of intra-erythrocytic piroplasms or schizont-laden monocytes; (ii) Fine needle aspiration of organs that are likely to harbor schizont-laden macrophages and demonstration of their presence via cytologic evaluation; (iii) PCR of peripheral blood for amplification of target *C. felis* DNA.

Blood smear evaluation is by far the most commonly used method to confirm cytauxzoonosis in a clinical setting. [2,3] Piroplasms are seen on light microscopy with Wright-Giemsa staining techniques as a 1 – 2 μm diameter ring with a thick round nuclear chromatin located at one point of the ring. [3] Other shapes including a safety pin, less than 0.5 μm round dot shaped, and less commonly comma-shaped, linear, and tetrad forms may be seen. [4] Care must be taken to not mistake other structures as piroplasms, such as Howell-Jolly bodies, water artifact from slide preparation, or other red blood cell parasites. However, since *C. felis* survivors may have identifiable circulating intraerythrocytic piroplasms, interpretation must be taken under appropriate clinical context to diagnose acute cytauxzoonosis. On the other hand, identification of

schizont-laden monocytes is pathognomonic for acute cytauxzoonosis as schizonts only appear at the acute stage of infection. Schizonts are variably sized basophilic, foamy, amorphous protozoal bodies in the cytoplasm of an infected macrophage. [24] The schizont-laden macrophages are generally very large (15–250 μm diameter) and oftentimes located at the feathered edge of the blood smear due to their size. [24] However, identification by blood smear technique has a major limitation in that early infection might be missed when piroplasms are in low numbers or not evident in early stages of disease, and the schizont-laden macrophages are only occasionally detected on peripheral blood smears.

Because schizont-laden macrophages are lodged within vascular lumina of vital organs mentioned above, fine needle aspiration and cytologic examination of the peripheral lymph nodes, spleen, liver and sometimes lungs could be performed to obtain the diagnosis. However, this is a technique is slightly more invasive and riskier because it oftentimes requires moderate sedation of a critically ill patient. Moreover, advanced equipment and training are necessarily to perform this procedure. Thus, this technique is not routinely performed in general practice settings where the vast majority of the *C. felis* cases are seen.

Various PCR assays are available for detecting *C. felis* infection with great sensitivity for individuals with early disease as compared to blood smear/cytologic evaluations. Historically, 18S rRNA gene, ITS1, ITS2, and more recently mitochondrial gene *cox 3* have been utilized as target for PCR amplification in different PCR platforms including nested PCR and real-time PCR, with overall great success. [14,15,24,27,28] Yet compared to other diagnostics mentioned above, the major limitation of PCR is its turnaround time and availability to general practitioner.

Treatment of Cytauxzoonosis

Several anti-protozoal drugs have been proposed to treat cytauxzoonosis with various success. Parvaquone and buparvaquone are effective against bovine theileriosis but has been proven to be ineffective to treat cats with cytauxzoonosis acquired by experimental infection. [29] While diminazene aceturate is currently not approved by the Food and Drug Administration in the United States, intramuscular injection at 2 mg/kg with aggressive supportive care resulted in survival in 6 cats with naturally acquired acute cytauxzoonosis. [30] A more recent open-label clinical study compared the treatment efficacy of imidocarb to the combination use of atovaquone and azithromycin in treating cats with naturally occurring cytauxzoonosis, which resulted in better and fair success (60% survival) when the latter therapy was used. [31] Since then, the combination use of atovaquone and azithromycin has been considered the standard antiprotozoal therapy to treat feline acute cytauxzoonosis in the North America.

In addition to anti-protozoal therapy, aggressive supportive care is necessary and beneficial for infected cats. [2,3,12] This includes but is not limited to: fluid therapy, antiemetic therapy, oxygen supplementation, analgesia, and nutritional support. While disseminated intravascular coagulation is a common complication associated with cytauxzoonosis, the use of antithrombotic drugs in preventing this event is still under debate. [3,22]

Although elevation and increased expression of many pro-inflammatory cytokines have been demonstrated in peripheral blood samples and on histopathologic samples in deceased cats with acute cytauxzoonosis, [13,23] there is no evidence that the use of corticosteroids, non-steroidal anti-inflammatory medications or even immunosuppressants, are beneficial to acutely infected cats. Moreover, very little is known regarding the host immunologic alteration occurred during acute cytauxzoonosis.

Study Objectives

As outlined above, cytauxzoonosis is a highly fatal disease in domestic cats in many enzootic regions in the United States, especially in Oklahoma. With limited treatment choices and an extremely narrow therapeutic window for initiation of therapy, the scope of this thesis is focused on (i) developing novel diagnostics for early disease detection, and (ii) exploring more effective therapies or combination therapies with increased treatment efficacy in order to improve the overall survival of cats with acute cytauxzoonosis. Thus, the objectives of this thesis are as follows:

1. To develop a novel PCR assay using traditional hydrolysis probes and emerging droplet digital technology to diagnose early acute cytauxzoonosis (Chapter II)
2. To evaluate the combination administration of anti-inflammatory doses of dexamethasone and standard anti-protozoal therapy in treating cats with acute cytauxzoonosis (Chapter III)
3. To develop an anti-*C. felis* IgM ELISA and assess its performance in the early detection of feline acute cytauxzoonosis (Chapter IV)

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CHAPTER II

A PROBE-BASED DROPLET DIGITAL POLYMERASE CHAIN REACTION ASSAY FOR EARLY DETECTION OF FELINE ACUTE CYTAUXZOONOSIS [a]

[a] Kao YF, Peake B, Madden R, Robin M, Cowan SR, Scimeca RC, Thomas JE, Reichard MV, Ramachandran A and Miller CA. A probe-based droplet digital polymerase chain reaction assay for early detection of feline acute cytauxzoonosis. Journal of veterinary parasitology 2021;292:109413

A corrigendum was published in the Journal of Veterinary Parasitology in April, 2021 to update the sequences of the reverse primer and probe listed on page 20. The updated sequences are as follows:

Reverse primer: 5' - CGAAATGCCAGTATACTCCT - 3'

Probe: 5' - TGAGTTTGCAAGGGCCATTATAACACC -3'

Introduction

Cytauxzoonosis is a tick-borne disease that affects wild and domestic felids and have been reported in the North America, South America, Europe, South Africa and Asia. [1] The causative agent, *Cytauxzoon felis,* is an apicomplexan protozoal parasite that is primarily transmitted by *Amblyomma americanum* (lone star tick) in the United States. [2-6] While mostly subclinical infections are documented in wild felids, *C. felis* causes severe disease with high mortality in domestic cats, [1,3,4] especially in enzootic areas of the United States such as Oklahoma, Missouri, and Arkansas. [7] During acute infection, domestic cats develop severe clinical signs with rapid disease progression associated with the schizogenous phase of parasite replication, in which large schizont-laden monocytes occlude vascular lumina in vital organs. [2,8,9] Due to the rapid disease progression and relatively non-specific symptoms during early infection, many cats die or are euthanized within 24 hours of clinical presentation despite aggressive treatments. [3,10] To date, there is no vaccine available to prevent cytauxzoonosis, and the current standard-of-care using anti-protozoal therapy (atovaquone + azithromycin) is only moderately effective at reducing mortality and is hindered by a narrow window for initiating therapy. [10]

Early detection and therapeutic implementation are crucial for effective treatment during *C. felis* infection in domestic cats. Cytauxzoonosis can be diagnosed clinically via observation of piroplasm-laden erythrocytes in peripheral blood smears or schizont-laden macrophages in tissue aspirates (i.e. spleen, liver and lung); [3] however, accurate clinical diagnosis can be challenging due to nonspecific clinical signs, inherent difficulties in sample preparation, insufficient clinical expertise, and diffuse lack of protozoal organisms in cytologic specimens that are not present until advanced stages of infection. Polymerase Chain Reaction (PCR) is a more sensitive diagnostic assay compared to cytologic examination and can detect early infection while parasitemia remains low. [11] Various real-time PCR or nested PCR assays targeting the *C. felis* 18S ribosomal RNA gene or internal transcribed spacer region 2 (ITS2) have been developed and

are currently used to diagnose cytauxzoonosis. [2,11-13] A recently-developed real-time PCR assay targeting the *C. felis* mitochondrial gene *cox3* has shown increased diagnostic sensitivity. [14] However, this assay utilizes double-stranded DNA intercalating molecules (SYBR Green I or EvaGreen), which may lead to the detection of both specific and non-specific amplification products during the PCR reaction; thus, increasing the propensity of false positives. [15,16] In the present study, we attempted to improve on currently existing *C. felis* PCR assays by introducing a probe-based assay and ddPCR methodology.

Probe-based PCR assays offer increased specificity and sensitivity over traditional primer-based qPCR assays. Instead of indiscriminately binding all double-stranded DNA in a reaction, probe-based PCR utilizes a fluorescent-labeled, target-specific probe to recognize only a specific sequence within a desired PCR amplicon, [16] thereby improving assay specificity. Droplet digital PCR (ddPCR) offers many advantages over other quantitative PCR methods such as increased sensitivity, increased reproducibility, absolute quantification, faster turnaround time, and multiplexing. [17-21] To date, neither probe-based PCR assays nor ddPCR techniques have been implemented as routine diagnostic methods to detect *C. felis* infection. The objective of the current study was to design a probe-based assay for the detection of *C. felis* infection in domestic feline blood samples and implement this assay using modern ddPCR techniques in order to improve specificity and diagnostic turnaround.

Materials and Methods

Reference blood samples. All reference samples were collected fresh, in EDTA tubes. An "uninfected control sample" was obtained from a healthy cat housed in the Boren Veterinary Medical Teaching Hospital at Oklahoma State University (OSU-VMH). This sample was also used to test primer/probe design and for assay optimization and internal controls. A "chronic *C.*

felis infection sample" was obtained from a previously *C. felis* infected cat, housed at Oklahoma State University Animal Resources. The infection was originally confirmed by nested PCR as previously described. [8] An "acute *C. felis* infection sample" was obtained from a febrile cat with icterus (5 days duration) and cytologic evidence of *C. felis* piroplasms as found upon presentation to the OSU-VMH.

Clinical blood samples. Clinical blood samples used to test and compare *C. felis* PCR methods were acquired as follows. Fresh EDTA anticoagulated blood samples from domestic cats (n=37) with clinical signs suspicious of acute cytauxzoonosis were submitted to the Oklahoma State University Companion Animal and Translational Science Laboratory (OSU-CATS Lab) from clinics in Arkansas, Oklahoma, and Missouri (Supplementary table 1). Serial blood samples acquired after 10 days of treatment with atovaquone and azithromycin, were available in 7 cats. The presence of intra-erythrocytic piroplasms and/or schizont-laden monocytes were confirmed via blood smear evaluation in all cats before receiving treatment for cytauxzoonosis.

Experimental blood samples. Stored EDTA anticoagulated blood samples were obtained from specific pathogen-free cats (n=4) at various stages of experimental *C. felis* infection (up to 19 days duration). Experimental *C. felis* infection methods were as previously described. [8] Briefly, *A. americanum* nymphs were acquisition fed on a donor cat subclinically infected with *C. felis*. [8] Engorged nymphs were collected and allowed to molt to adults. Adult ticks were then transmission fed on *C. felis* naïve cats (principal cats, n=4) until female ticks had fed to repletion (approximately 7 days). Principal cats were monitored daily for clinical signs of cytauxzoonosis as previously described (Reichard et al., 2009). Blood samples (2-3 ml) were obtained from all cats via cephalic venipuncture on days 0, 7, 10, 12, 15, and 19 post-infestation and stored for DNA extraction and PCR analysis as previously described. [8] Blood samples from these cats served as experimental infection reference samples (experimental infection positive controls) for

this study. *C. felis* infection was confirmed in all cats by 18S nested PCR (described below) prior to analysis.

Samples used to assess specificity for the probe-based *C. felis* ddPCR. DNA extracts from ticks infected with various tick-borne pathogens including *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Babesia conradae*, *Hepatozoon sp*., *Borrelia burgdorferi* and commercial pathogen DNA from other blood-borne pathogens including *Toxoplasma gondii* (ATCC, Manassas, VA) and *Leishmania infantum* (ATCC, Manassas, VA), were available and utilized to assess specificity of the probe-based *C. felis* ddPCR. The presence of pathogen in the DNA extracts were confirmed by PCR as previously published methods. [22-27] Additionally, stored EDTA-anticoagulated whole blood samples previously obtained from 18 specific pathogen-free cats housed at OSU Animal Resources were utilized as healthy, uninfected internal controls.

DNA extraction. Total DNA was isolated from all EDTA-anticoagulated samples using a commercially available kit according to manufacturer's instructions (Qiagen DNeasy Blood & Tissue Kits, Qiagen Inc., Valencia, CA). DNA concentration was determined by Thermo Scientific Spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific., Waltham, MA) and the eluted solution was stored in -80°C for subsequent PCR reactions. The same extraction product from each sample was used across all PCR assays described below.

***C. felis* probe-based PCR assay design – probe and primers.** Primers/probe mixture targeting *C. felis* cytochrome c gene *cox3* was designed following the QX200^{TM} Droplet Digital PCR System manufacturer recommendations (Bio-Rad Laboratories, Inc. Hercules, California, USA) and synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA). Optimal primers/probe was selected based on GC content (50-60% for primers and 30-80% for probes), melting temperature (50 - 65°C for primers and 3 - 10°C higher for probe) and concentrations (50 mM salt concentration and 300 nM oligonucleotide concentration). The selected primers/probe sequences are as follows: Forward: 5'-CTACACTCTTTACACGTTTGTG -3', Reverse: 5' - CGAAATGCCAGTATACTCCT - 3', probe: 5' - TGAGTTTGCAAGGGCCATTATAACACC - 3'. BLAST [28] analysis of the primer and probe sequences against the entire database including the domestic cat (*Felis catus*) and the lone-star tick (*Amblyomma americanum)* genomes was performed to ensure no other similar sequences could be amplified. The analyses resulted in E-

values of 0.040 for the forward primer, 0.0001 for the probe and 0.63 for the reverse primer, when compared to the *C. felis* genome to ensure specificity.

***C. felis* probe-based PCR assay design – probe-based ddPCR.** ddPCR was performed using the QX200™ Droplet Digital PCR System and analyzed with QuantaSoft software version 1.7 (Bio-Rad Laboratories, Inc., Hercules, CA). ddPCR master mix was prepared by adding 12 µl (10 µl + 20%) super mix for probes (no dUTP) (Bio-Rad Laboratories, Inc. Hercules, California, USA), 1.2 µl of primer/probe mix (1 µl + 20%), 1.2 µl of DNase free water (1 µl + 20%). 13.3 µl (12 µl + 10%) of master mix was then mixed with 8.8 µl (8 µl + 10%) aliquot of DNA template (each aliquot containing 50 ng DNA) to generate the reaction mixes. Each reaction had a final concentration of 500 nM primers and 250 nM probe. 20 µl reaction mixes were transferred to DG8™ Cartridges (Bio-Rad Laboratories, Inc. Hercules, California, USA) with 70 µl of Droplet Generation Oil for Probes (no dUTP) (Bio-Rad Laboratories, Inc. Hercules, California, USA) added in each well subsequently. The DG8™ Cartridges were inserted into the QX200™ Droplet Generator (Bio-Rad Laboratories, Inc. Hercules, California, USA) to generate 40 µl droplet suspension that contained 20 µl reaction mixes per reaction. The droplet suspensions were then transferred to 96-Deep Well Reaction Modules (Bio-Rad Laboratories, Inc. Hercules, California, USA) and PCR was carried out by C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, California, USA). The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 10 minutes followed by 49 amplification cycles (95 °C for 30 seconds and 59.4 °C for 60 seconds). The annealing temperature of 59.4 °C was determined by gradient PCR. After thermocycling, the modules were cooled down to 4°C and transferred to QX200™ Droplet Reader (Bio-Rad Laboratories, Inc. Hercules, California, USA) for absolute quantification of the target DNA. All samples were run in duplicate, along with reference sample DNA from (i) a cat with confirmed cytauxzoonosis as positive control, (ii) an OSU-VMH blood donor cat as negative control, and (ii) DNase free water as no template control. The results of the *C. felis* probe-based ddPCR were analyzed using QuantaSoft software version 1.7, and thresholds were set between

the droplet cluster with low relative fluorescence value (<1500) and the droplet cluster with relative fluorescence values ranging from 2000-3000. Droplets below thresholds were read as negative droplets whereas droplets above the thresholds were read as positive droplets. Samples were determined as positive when at least one droplet was classified as positive in each duplicate. The absolute copy numbers per reaction was calculated by the software based on the fraction of positive to negative droplets utilizing the Poisson law of small numbers. [29]

Probe-based real-time quantitative PCR. Real-time quantitative PCR (qPCR) amplification was performed using IDT PrimeTime® Gene Expression Master Mix (Coralville, IA). Amplification was performed using identical primers/probe as described in 2.3.1. Real-Time PCR was performed in 20µl volumes containing 10µl of 2X PrimeTime ® Gene Expression Master Mix (IDT, Coralville, IA), 500nM of each primer and 250nM probe, 5.5µl of water, and 2µl of DNA template. Cycling conditions included a polymerase activation step at 95°C for 3 min., followed by 40 cycles of a 30 sec. denaturation step at 95°C and a 1 min. annealing/extension step at 59.4°C. PCR was run on both Applied Biosystems® 7500 Fast Real-Time PCR System (ThermoFisher Scientific, Foster City, CA) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Ct values less than 37 were considered positive.

18S nested PCR. PCR amplification was performed using Qiagen HotStarTaq DNA Polymerase (Qiagen Inc, Germantown, MD). A nested PCR protocol was followed. Primary amplification was performed using previously published primers: C. felis I/II forward (5'-AACCTGGTTGATCCTGCCAGTAGTCATATGCTTG-3'), and C. felis II reverse (5'-TCACCAGAAAAAGCCACAAC-3'). [12] For secondary PCR amplification, Cfnest forward (5'-TCGCATTGCTTTATGCTGGCGATG-3'), Cfnest reverse (5'-GCCCTCCAATTGATACTCCGGAAA-3') primers were used as previously described. [8] Primary and secondary PCR amplicons were detected by agarose gel electrophoresis (2%

agarose) using UView dye (Bio-Rad Laboratories, Hercules, California) on an ultraviolet light platform (UVP BioDoc-It® Imaging System, Wayne, PA).

Blood smear evaluation. For each clinical blood sample, duplicate blood smears were made using the wedge technique as previously described when available. [30] Diff-Quik stain (EKI, Joliet, IL) was used for blood smear staining. Each blood smear was evaluated microscopically under high power field (100x) by one study investigator (YK) for presence of any piroplasm and/or schizont. The total number of piroplasms were counted in 10 high power fields per sample at the monolayer and recorded. The number of schizont-laden macrophages was evaluated at the feathered edge of each blood smear and the total number per sample was recorded. If no piroplasm/schizont were noted on the first 10 high power fields and feathered edges viewed, the blood smears would be continued to be evaluated for 5 minutes. If more than 2 piroplasms were noted during the period of 5 minutes, the sample was considered positive. (Supplementary table 2)

Determination of the efficacy of the C. felis probe-based ddPCR assay. To determine the efficacy of the primers/probe design and the suitability of reference samples, DNA was extracted using the methods described above. from the three reference blood samples: acute *C. felis* infection sample, chronic *C. felis* infection sample and uninfected control sample. The confirmation of infection was determined by either blood smear evaluation or nested PCR. [8] DNA from the three reference samples was then analyzed by ddPCR using the primers/probe and cycling parameters outlined above.

The probe-based ddPCR assay was then used to analyze the clinical feline blood samples to further determine the performance of the assay in testing clinical blood samples. Briefly, DNA was extracted from fresh whole blood of 37 cats with clinical signs suspicious of acute

cytauxzoonosis using the method described above. ddPCR was performed as outlined above. Blood smears for all 37 samples were evaluated by the method described in above.

Determination of the probe-based C. felis ddPCR sensitivity. To determine the probe-based *C. felis* ddPCR sensitivity in samples with low volume and/or DNA concentration, the assay was tested on 1) serial dilution on the positive reference samples and 2) samples from experimentally infected cats.

Serial dilution of the positive reference samples was performed and samples were tested by the ddPCR assay. Acute and chronic positive reference samples were diluted up to 10^7 times to determine the minimum DNA concentration required for target DNA detection using the primers/probe and cycling parameters outlined above.

To determine the earliest extent of *C. felis* probe-based ddPCR performance during acute *C. felis* infection, the assay was tested on serial blood samples from cats with experimental *C. felis* infection (n=4) using the primers/probe and cycling parameters outlined above.

Comparison between different PCR methods. To compare the performance of the *C. felis* probe-based ddPCR to other PCR applications, a subset of clinical samples from cats with acute cytauxzoonosis (n=26) were assayed by probe-based real-time qPCR (outlined above) before (n=19) and after treatment (n=7) with atovaquone and azithromycin. A subset of these samples (n=2 before treatment and n=7 after treatment) were then assayed by 18S nested PCR (outlined above).

Statistical Analysis. All analyses were conducted using GraphPad Prism 8.0 software (La Jolla, California, USA). P-values < 0.05 were considered significant. Descriptive statistics were used to display the average parasite load. Kappa statistic was used to assess the agreement between the result of ddPCR and blood smear evaluation in diagnosing *C. felis* infection. Kruskal-Wallis test by rank was utilized to compare the performance between different PCR methods in detection of

C. felis in feline blood samples. Wilcoxon matched-pairs signed rank test was used to compare the *C. felis* parasite load in selected clinical cat samples before (day 0) and after (day 10) treatment.

Results

Efficacy of the C. felis probe-based ddPCR assay. When testing the reference samples, the *C. felis* probe-based ddPCR detected greater than 2.0 x 10^7 copies of *C. felis* DNA per 20 µl of reaction in both the acute and chronic infection reference samples. Total DNA concentration extracted from the acute infected sample was 23.1 ng/µl and 64.1 ng/µl from the chronic infected sample. No DNA amplification was noted in the sample from the healthy, uninfected cat. Total DNA extracted from this sample was 50.2 ng/µl.

When testing the clinical feline blood samples from cats presented with symptoms suspicious of acute cytauxzoonosis, 31 out of 37 samples (83.7%) were positive for *C. felis* by ddPCR, with average parasite load of $1.30 \times 10^7 \pm 1.59 \times 10^6$ copies/20µl reaction (Figure 2.1.). All samples with positive blood smear results (n=30, blood smear was not available in one sample) also tested positive on ddPCR, and all samples that tested negative (n=6) by ddPCR had negative blood smear results. There was a 100% agreement (Kappa = 1.00, SE of Kappa = 0.00) between the results of ddPCR and blood smear evaluation. (Table 2.1.)

Probe-based ddPCR sensitivity. When subjected to the *C. felis* probe-based ddPCR, the dilution series of the acute/chronic positive reference samples revealed that the target DNA was reliably and successfully amplified at the lowest dilution, followed by a gradual decrease of the parasite load with increased dilutions. The highest dilution that resulted in positive target DNA amplification was 10^6 times dilution for the acute reference sample and 10^4 times dilution for the

chronic reference sample respectively. (Figure 2.2.) While considering the neat DNA sample concentrations, this translated into 0.0000231 ng DNA/reaction for the acute reference sample and 0.00232 ng DNA/reaction for the chronic reference sample.

For the experimentally infected cat blood samples, cats were infested with *C. felis* infected ticks for 7 days (day 0 to day 7). Blood samples were available at days 0, 7, 10, 12, 15 and 19 post-infestation. Circulating *C. felis* DNA was detected in 3/4 of the cats (75%) at day 10 post-infestation (3.500 ±2.539 copies/20µl reaction) with increasing target DNA copies noted throughout the study period. *C. felis* DNA was detected in all 4 cats (100%) by day 12 post-infestation (Figure 2.3.). All cats developed clinical signs of acute cytauxzoonosis at day 11. These results show that the probe-based *C. felis* ddPCR can detect infection up to 1 day prior to the development of clinical signs in experimentally infected cats.

C. felis probe-based ddPCR specifically amplifies C. felis DNA. No amplification of target DNA was observed in DNA samples that contain *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Babesia conradae*, *Hepatozoon sp.*, *Toxoplasma gondii* and *Leishmania infantum.* Testing DNA extracted from whole blood samples of 18 specific pathogen-free cats also resulted in no amplicon identification.

Comparison between different PCR methods. A subset of clinical blood samples before and after treatment were utilized to compared three different *C. felis* PCR assays: the probe-based ddPCR, the probe-based real-time PCR and the 18S nested PCR. As shown in Table 2.2., ddPCR and real-time qPCR assays, which both utilize the probe-based PCR design, can detect *C. felis* infection in all samples assayed before (day 0) and after (day 10) antiprotozoal treatment. 18S nested PCR can detect infection in all day 0 samples tested, but only 71% (5/7) of the day 10 samples. However, the differences in detection rate between the 18S nested PCR versus the probe-based ddPCR or real-time qPCR was not significant ($p = 0.300$, Kruskal-Wallis test).

Probe-based ddPCR also demonstrated capability of monitoring parasite load over the course of treatment via absolute quantification of target DNA copies in samples acquired pre- and post-treatment. As shown in Figure 2.4., parasite load was significantly lower after treatment (day 10) as compared to before treatment (day 0) ($p=0.0156$, Wilcoxon test).

Discussion

Several antiprotozoal therapies have been implemented in the past to mitigate symptoms of feline cytauxzoonosis with limited success. [10,31-33] To date, the combination therapy with atovaquone and azithromycin carries the best success rate (up to 60% survival), but these anti-protozoal treatments are often ineffective if not initiated soon after presentation, or are hindered by resistant pathogen genotypes that reduce survival rates despite treatment (i.e. cytochrome b genotypes conferring atovaquone resistance.[10,34] In fact, clinical progression of cytauxzoonosis in domestic cats is so rapid, the majority of affected cats die or are euthanized within 24 hours of presentation to a veterinary hospital. [4] As such, accurate detection of the earliest stages C. *felis* infection is crucial to the initiation of treatment and potential for survival in infected cats.

Collectively, our probe-based ddPCR design offers several unique advantages over currently used diagnostic assays. The *C. felis* ddPCR assay successfully detected early stages of acute cytauxzoonosis in domestic cats from small sample volumes with low DNA concentrations. Infection was detected up to 1 day prior to the development of clinical signs in experimentally infected cats, and the ddPCR assay also provides absolute quantification of *C. felis* copies/µl of blood, which can be used to track parasite load over the course of treatment. We also demonstrate that this probe-based design can be utilized in traditional real-time PCR systems, with similar detection capabilities as compared to ddPCR.

In general, probe-based PCR assays offer increased specificity and sensitivity over traditional PCR assays. Instead of using intercalating dyes (i.e. SYBR) to bind all double-stranded DNA in a reaction, probe-based PCR utilizes a fluorescent-labeled, target-specific probe to recognize a specific sequence within a desired PCR product. The same probe sequence designed for the ddPCR assay was easily incorporated into a traditional real-time PCR system (as documented in Materials and Methods above), which is more widely available to most diagnostic facilities.

ddPCR utilizes oil-in-water emulsion technology to distribute the sample solution into 20,000 partitions, thereby improving detection sensitivity and allowing absolute target quantification. [17-21] After PCR amplification to endpoint, droplets that contain one or more target copies of DNA are counted, and the absolute copy number of the target DNA molecules present in a reaction can be calculated from the fraction of positive to negative droplets using the Poisson law of small numbers. [29] ddPCR offers improved reproducibility between experiments and multiplexing capability with no need of melting curve analysis. [19,20,29,35]

When comparing the efficacy of probe-based ddPCR, probe-based real-time PCR, and 18S nested PCR in detecting *C. felis* infection in cats, it is noted that all methods are efficient in cases of severe cytauxzoonosis. This is likely due to the high circulating parasite load in blood before initiation of treatment. However, for blood samples collected 10 days after starting antiprotozoal therapy, both probe-based ddPCR and probe-based real-time PCR were able to detect infection in all 7 samples, whereas 18S nested PCR failed to detect 2 of the 7 blood samples tested. Although these results are not significant (likely due to small sample size), it is possible that probe-based ddPCR is more sensitive in detecting *C. felis* DNA in samples with low parasite load as compared to the nested PCR and potentially real-time PCR especially considering the starting DNA quantity/volume used in each PCR assays. Future studies will employ a higher number of samples and an extended time course to investigate the impact of treatment and

decreasing parasite loads on the detection limits of these different diagnostic PCR methods. ddPCR and real-time PCR have significantly faster turnaround times compared to the nested PCR, which is very valuable to provide an accurate diagnosis to veterinary practitioners in a timely fashion.

Although this novel *C. felis* PCR is promising in providing accurate, early diagnosis of acute cytauxzoonosis in cats, ddPCR is not yet widely available to many veterinary diagnostic laboratories and is expensive as compared to other PCR assays or cytologic evaluations. However, the result of this study shows that the *C. felis* probe-based ddPCR is an improved method in diagnosing cytauxzoonosis due to its many advantages over other PCR methods such as absolute quantification, rapid turnaround time, and multiplexing capabilities.

In summary, we report the first probe-based ddPCR assay in detecting *Cytauxzoon felis*. This is an improved assay compared to the existing published *C. felis* PCR assays. Additionally, the capability of ddPCR to provide absolute parasite quantification could be useful in monitoring treatment efficacy and detecting relapse in both research and clinical purposes.

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Animal Welfare Statement

All animal related procedures were approved by the Oklahoma State University Animal Care and Use Committee (Protocol #VM-19-2, Combined host-pathogen transcriptomics to reveal therapeutic targets of Cytauxzoon felis infection, 02/1/2019) and conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Table 2.1. Comparison Between the Results of *C. felis* Probe-based ddPCR and Blood Smear Evaluation in Clinical Cat Blood Samples [b]

		Probe-based ddPCR	
		Positive	Negative
Blood smear evaluation	Positive	30	0
	Negative	0	6

[b] The probe-based ddPCR and blood smear evaluation were performed on blood samples from clinical cats with suspected acute cytauxzoonosis. The results were recorded as positive/negative in each blood sample for both methods as shown in the table.

Table 2.2. Comparison of different PCR assays in detecting *C. felis* DNA in clinical cat blood samples before and after treatment [c]

Cat ID	Days after treatment	Probe-based ddPCR	Real-time PCR	Nested PCR
6	0	+	+	+
7	0	+	+	
10	0	+	+	
11	0	+	+	
12	0	+	+	
15	0	+	+	
16	0	+	+	
17	0	+	+	
18	0	+	+	+
21	0	+	+	
22	0	+	+	
25	0	+	+	
26	0	+	+	
27	0	+	+	
28	0	+	+	
29	0	+	+	
31	0	+	+	
32	0	+	+	
34	0	+	+	
6	10	+	+	-
11	10	+	+	+
12	10	+	+	+
21	10	+	+	+
22	10	+	+	-
25	10	+	+	+
29	10	+	+	+

[c] Clinical blood samples were utilized to compare detectability of *C. felis* infection between probe-based ddPCR, real-time PCR and 18S nested PCR. Probe-based ddPCR and real-time PCR assays detected parasitic DNA in all selected blood samples (all day 0 and day 10 samples). 18S nested PCR successfully detected all day 0 blood samples tested (n=2), but only detected 5 of the 7 day 10 blood samples (71.4%).

Supplementary Table 1. Geographic Information of Clinical Cat Blood Samples [d]

Cat ID	City	State
1	Oklahoma City	Oklahoma
2	Stillwater	Oklahoma
3	Stillwater	Oklahoma
4	Stillwater	Oklahoma
5	Stillwater	Oklahoma
6*	Fayetteville	Arkansas
7	Western Grove	Arkansas
8	Rogers	Arkansas
9	Oklahoma City	Oklahoma
10	Berryville	Arkansas
11*	Berryville	Arkansas
12*	Berryville	Arkansas
13	Berryville	Arkansas
14	Oklahoma City	Oklahoma
15	Berryville	Arkansas
16*	Berryville	Arkansas
17	Berryville	Arkansas
18	Berryville	Arkansas
19	Berryville	Arkansas
20	Elmore City	Oklahoma
21*	Berryville	Arkansas
22*	Berryville	Arkansas
23	Forsyth	Missouri
24	Forsyth	Missouri
25*	Forsyth	Missouri
26	Oklahoma City	Oklahoma
27	Berryville	Arkansas
28	Lincoln	Arkansas
29*	Berryville	Arkansas
30	Berryville	Arkansas
31	Oklahoma City	Oklahoma
32	Forsyth	Missouri
33	Berryville	Arkansas
34	Forsyth	Missouri
35	Forsyth	Missouri
36	Springdale	Arkansas
37	Berryville	Arkansas

[d] Fresh EDTA anticoagulated cat blood samples were acquired from 37 domestic cats with clinical signs suspicious of acute cytauxzoonosis. Samples are from veterinary clinics in different areas of Arkansas, Oklahoma, and Missouri. Cats which serial blood samples were available were marked with *.

Supplementary Table 2. Result of Blood Smear Evaluation of Clinical Cat Blood Samples [e]

Cat ID	Total number of piroplasms in 10 HPF	Total number of schizont-laden monocytes in 2 blood smears	Determination of *C. felis* infection
1	142	8	Positive
2	344	0	Positive
3	346	0	Positive
4	0	0	Positive
5	4	0	Positive
6	0	0	Positive
7	26	2	Positive
8	61	4	Positive
9	7	0	Positive
10	2	0	Positive
11	2	0	Positive
12	33	0	Positive
13	0	0	Negative
14	0	0	Negative
15	1	0	Positive
16	0	0	Positive
17	14	1	Positive
18	3	0	Positive
19	0	0	Negative
20	0	0	Negative
21	208	0	Positive
22	11	0	Positive
23	11	0	Positive
24	0	0	Negative
25	542	0	Positive
26	10	11	Positive
27	2	0	Positive
28	8	0	Positive
29	7	0	Positive
30	28	0	Positive
31	10	2	Positive
32	N/A	N/A	N/A
33	7	0	Positive
34	21	0	Positive
35	21	0	Positive
36	0	2	Positive
37	0	0	Negative

[e] Duplicate blood smears were evaluated for each sample. Total number of piroplasms in 10 high power fields, or HPF (5 fields per each smear), and total number of schizont-laden monocytes in 2 duplicate blood smears were recorded. A sample was determined to be positive when one of the

following three criteria were met: 1) > 1 piroplasm noted in 10 HPF; 2) > 1 schizont-laden monocytes noted in 2 blood smears; or 3) > 2 piroplasms noted after examining each duplicate blood smear for 5 minutes.

Figure 2.1. Detection and quantification of circulating parasite load in cats with suspected *C. felis* infection. Absolute parasitic loads were obtained by probe-based ddPCR in clinical cat blood samples described in 2.1.2. The *x*-axis represents each sample (individual cat blood samples) and the *y*-axis represents absolute parasitic load (copies per 20 microliter reaction). Copies ranging from $5.2 \times 10^3 - 2.0 \times 10^7$ per 20 microliter reaction were noted in the samples tested positive for *C. felis*. Cats 13, 14, 19, 20, 24 and 37 had no amplification of target DNA, and these samples also had negative blood smear results. Pos = positive control; Neg = negative control, and NTC = no template control.

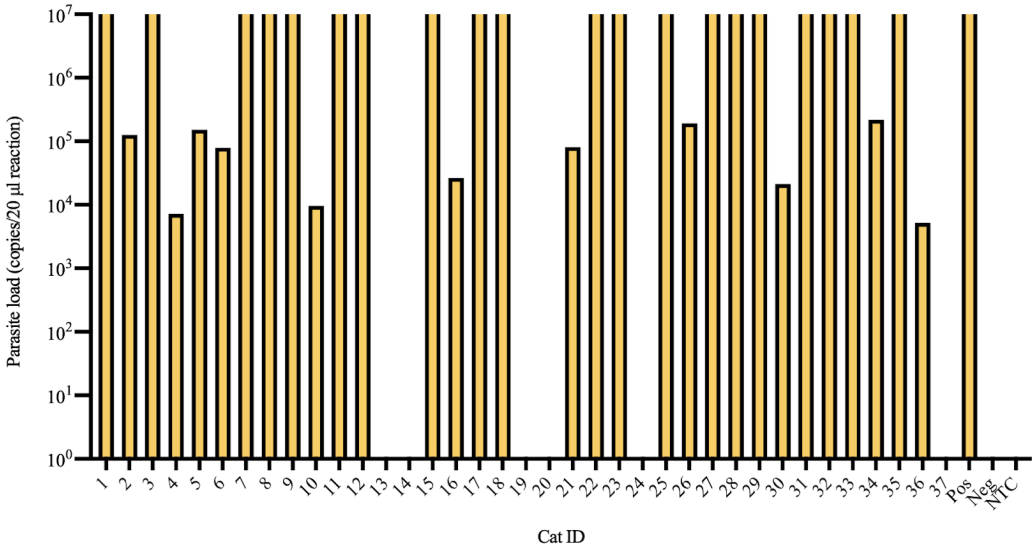


Figure 2.2. Probe-based *C. felis* ddPCR sensitivity. Serial dilutions were performed on acute and chronic positive controls to determine performance and sensitivity of the probe-based *C. felis* ddPCR assay. Target DNA was reliably and successfully amplified in all samples and the assay was able to detect parasitic DNA in up to 512 times dilution in both acute and chronic positive controls.

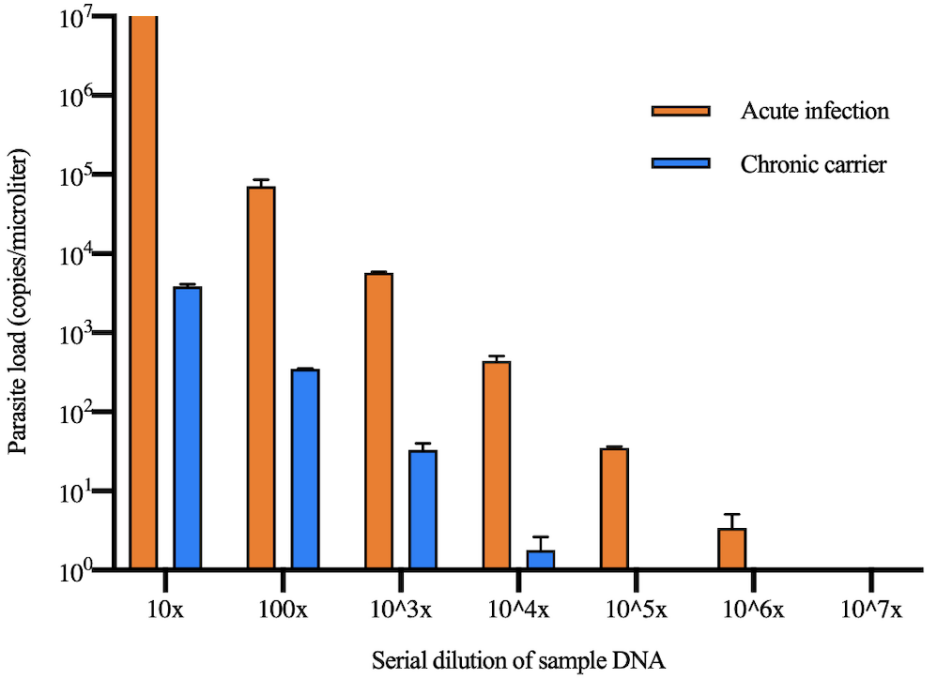


Figure 2.3. Chronological detection of *C. felis* infection by probe-based ddPCR during experimental infection. Absolute parasite loads were quantified by probe-based *C. felis* ddPCR in serial blood samples (day 0, 7, 10, 12, 15 and 19 post-tick infestation) from 4 cats (zag1, mdo6, zae1 and meb3) with *C. felis* experimental infection. Target DNA was detected in 3 of the 4 experimental cats (zag1, mdo6 and zae1) as early as day 10 post tick infestation.

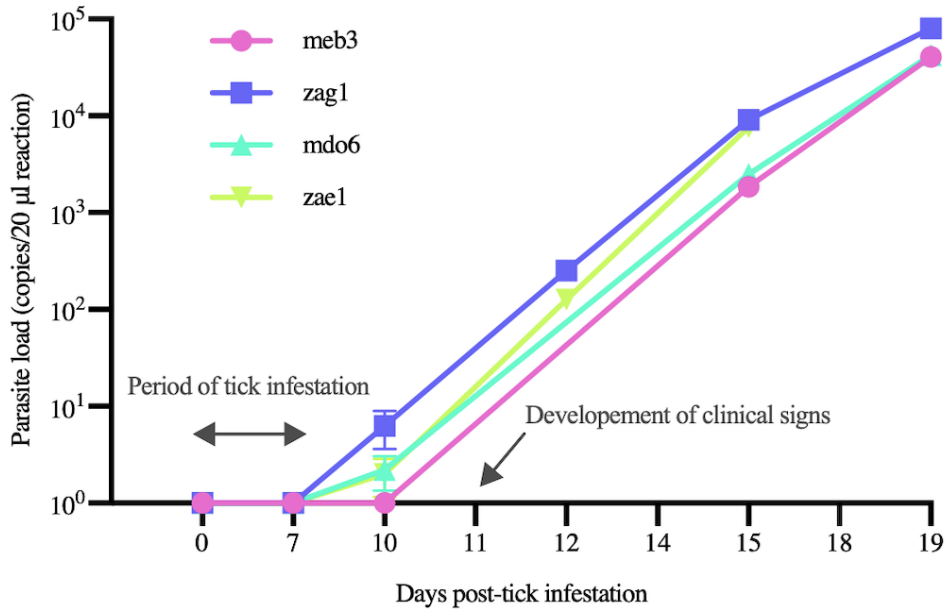
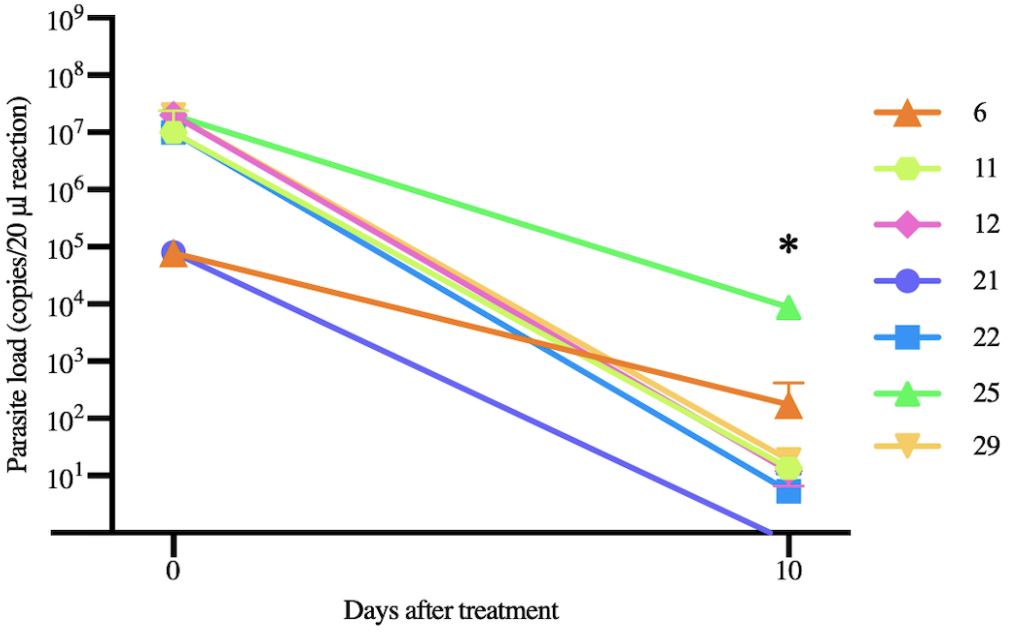


Figure 2.4. Peripheral *C. felis* burden before and after treatment in cats diagnosed with acute cytauxzoonosis. Absolute parasite loads were quantified by probe-based *C. felis* ddPCR in blood samples from cats with acute cytauxzoonosis before and after anti-protozoal therapy. A decreasing trend in parasite load was observed in all cats after treatment. The absolute parasite load was significantly lower after treatment (day 10) as compared to before treatment (day 0). ("*", p=0.0156, Wilcoxon test)



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CHAPTER III

COMBINED IMMUNOMODULATORY THERAPY TO IMPROVE THE SURVIVAL OF CATS WITH ACUTE CYTAUXZOONOSIS

Introduction

Recent studies show that aberrant immune activation may play a significant role in disease progression during acute cytauxzoonosis through increased release of pro-inflammatory mediators and triggering of an acute-phase response. [1,2] Enhanced expression of pro-inflammatory cytokines (TNFα, IL-1β) has been correlated with increased mortality in cats infected with *C. felis*, [1,2] and these findings are paralleled by increased pro-inflammatory cytokine levels in pulmonary tissues during acute infection. [2] Preliminary studies in our lab indicate that enhanced expression of pro-inflammatory cytokines is significantly correlated with increased parasite replication, and that parasite-induced alterations in chemokine signaling may promote defective migration and infiltration of inflammatory cells during acute infection (unpublished data). Thus, there is a critical need to recalibrate modern treatment strategies in order to target the inflammatory drivers of cytauxzoonosis and improve therapeutic efficacy. Dexamethasone is a glucocorticoid agonist with potent anti-inflammatory activity achieved through binding of inflammatory response elements and suppression of pro-inflammatory cytokines. [3-6] Since morbidity and mortality during *C. felis* infection are strongly associated with immune activation and expression of pro-inflammatory mediators [1,2], down- regulation of the inflammatory response with this immunomodulating steroid may drastically improve response to therapy.

Thus, the hypothesis of this study is that using combined immunomodulatory therapy (atovaquone + azithromycin + dexamethasone) will potentiate anti-protozoal treatment protocols (atovaquone + azithromycin) and improve survival in *C. felis*-infected cats by counteracting systemic immune activation. The objectives include 1) characterizing features of systemic immune dysfunction during acute cytauxzoonosis and 2) testing the capacity of immunomodulatory therapy (dexamethasone) to decrease immune activation and potentiate anti-protozoal therapy to improve clinical outcome.

Materials and Methods

Study population. Veterinary clinics from three endemic areas of the United States including Arkansas, Oklahoma and Missouri were recruited for the study. Client-owned cats with one of the following three criteria of eligibility were enrolled in the study. (i) history (have access to outdoor environment, not receiving tick preventives) and clinical signs (i.e. fever, lethargy, hyporexia, icterus, tachycardia, tachypnea, and others) suspicious for acute cytauxzoonosis; (ii) cytologic evidence of *Cytauxzoon felis* organisms on peripheral blood smears and/or tissue aspirates; (iii) positive *C. felis* PCR. Cats met with one of the following criteria precluded eligibility. (i) Body temperature below 100 °F upon presentation; (ii) Attending veterinarian does not expect the cat to survive within the next few hours; (iii) NSAID or glucocorticoid therapy in the past 14 days; (iv) negative *C. felis* PCR result.

Study Design. This was a prospective, randomized, open-label study on client owned cats diagnosed with acute cytauxzoonosis. The study design is shown in Figure 3.1. When a participating veterinarian made a diagnosis of cytauxzoonosis, the owner was offered the chance to enroll in the study and was required to sign an informed consent if they wished to participate. After the consent form was signed, the cat was randomly assigned into one of the two treatment

groups according to the sealed envelopes provided by the study investigator (YK) and treatment began immediately. The treatment drugs were provided by the study investigators (YK, LN, CM). Other than the study drugs, all enrolled cats were required to receive crystalloid fluid therapy (Normosol®, Plasmalyte®, lactated Ringer's solution, or 0.9% NaCl) during hospitalization. Other supportive care (i.e. pain management, antiemetics, appetite stimulants, feeding tubes), additional therapies (whole blood or plasma transfusion, antibiotic therapies or unfractionated heparin), and any other diagnostics were left to the discretion of the treating veterinarian. No glucocorticoids or NSAIDs were allowed other than cats in the group receiving dexamethasone as a component of the study. Cats were required to be hospitalized for 3 days and have a recheck appointment at day 10. If the cat was discharged before finishing the 3-day course of dexamethasone, the owner was required to bring the cat back to the clinic for dexamethasone injections.

Study samples. 1-2 ml of EDTA anticoagulated whole blood samples for each enrolled cat were collected by the participating clinic via venipuncture at three timepoints: day 0 (before starting treatment), day 3 (after last dose of dexamethasone) and day 10 (at discharge). The samples were mailed overnighted with ice-packs to Oklahoma State University (OSU) whenever feasible.

For each sample, at least 100 µl was used for DNA extraction using the method described below. At least 150 µl was kept fresh and underwent antibody staining for flow cytometry analysis using the method described below. At least 300 µl was centrifuged at 2000g for 10 minutes to harvest plasma. The harvested plasma was stored in two aliquots with each >50 µl. The rest of the whole blood was stored in -80 °C.

Confirmatory *C. felis* PCR was performed for day 0 blood samples and cats with negative PCR were excluded from the study. PCR, multiplex immunoassay, and flow cytometry analyses were performed for all cats in each group and at all three timepoints.

Control samples for cytokine analysis. Stored EDTA-anticoagulated plasma samples from 18 healthy specific-pathogen free cats housed at Oklahoma State University Animal Resources were available from another study for cytokine levels comparison to study cats. [7]

Collection of medical record. Participating clinics were required to complete a sample submission form (Appendix 1) that contains basic information and history upon submission of each blood sample. Participating clinics were encouraged to provide results of any laboratory assessments and hospitalization records when possible.

DNA extraction. Total DNA was isolated from all EDTA-anticoagulated samples using a commercially available kit according to manufacturer's instructions (Qiagen DNeasy Blood & Tissue Kits, Qiagen Inc., Valencia, CA). DNA concentration was determined by Thermo Scientific Spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific., Waltham, MA) and the eluted solution was stored in -80°C for subsequent PCR reactions.

PCR amplification. Droplet digital PCR (ddPCR) was performed using the QX200^{TM} Droplet Digital PCR System and analyzed with QuantaSoft software version 1.7 (Bio-Rad Laboratories, Inc., Hercules, CA). The assay was performed as previously described. [7]

Blood smear analysis. For each blood sample, duplicate blood smears were made using the wedge technique as previously described [8] when available. Diff-Quik stain (EKI, Joliet, IL) was used for blood smear staining. Each blood smear was evaluated using the method previously described. [7]

Multiplex immunoassay (MIA) analysis of systemic cytokine secretion. EDTA anticoagulated plasma samples were utilized to measure cytokine concentration in blood using a commercial MILLIPLEX® MAP Feline Cytokine/Chemokine Magnetic Bead Panel. Soluble cytokines (KC, RANTES, Flt-3L, Fas, GM-CSF, IL-2, IL-8, IL-12p40, IL-13, TNFα, IL-1β, INFγ, IL-4, IL-6,

SCF, SDF-1, IL-18, MCP-1 and PDGF-BB) were quantified using a composite panel comprised of microspheres coupled with capture antibodies as described [9] with the following modifications: 25 µl of neat plasma sample was added in the sample well instead of 10 µl neat plasma sample and 15 µl Serum Matrix. Samples were incubated with conjugated microspheres, washed and incubated with biotinylated secondary antibodies and streptavidin-PE. Immune complexes were detected using the Bio-Plex® MAGPIXTM multiplex detection system, and analyte concentration was calculated using a standard curve (Bio-Plex® ManagerTM 6.1).

Flow cytometric analyses. Fresh EDTA anticoagulated whole blood samples were processed for flow cytometry analysis using previously described antibodies and methods. [10,11] with modifications. Briefly, a total 120 µl EDTA-anticoagulated whole blood samples were evenly distributed in to three round-bottom polystyrene tubes. Antibodies and flow cytometry buffer (PBS +1% FBS) were added. The mixture was incubated in 4 °C in dark for 30 minutes. After incubation, the mixture in each tube was centrifuged at 1500 rpm for 2 minutes in 4 °C. The supernatant was removed and 150 µl of flow cytometry buffer was added into each tube and mixed well. The mixture was centrifuged again at the same condition and the supernatant was discarded. 150 µl of flow cytometry buffer was added into each tube and mixed well. IMMUNOPREP® (Beckman Coulter, Inc., Brea, CA) Reagent System was used for red blood cell lysis following manufacturer's instruction with volume adjusted proportionally to the blood sample volume. Sample were kept on ice in all steps above.

For all samples, B-cell (B220+) and T-cell subsets (CD4+, CD8+, CD25+) were quantitated and the proportion of these subsets were used to evaluate changes in circulating immunophenotype. Analyses were performed using BD FACSDiva^{TM} software and previously described antibodies and methods.[10-13]

Statistical Analyses: Descriptive statistics were used to present the signalment, history, physical examination findings and laboratory parameters (including complete blood count and biochemistry values) of the study population gleaned from the medical records and sample submission forms. The survival rate between treatment groups was compared using Kaplan-Meier survival analysis with the primary endpoint with respect to successful treatment efficacy set as survival beyond 10 days. Death, including euthanasia, was considered a treatment failure. Differences in parasite load, cytokine concentration, cellular immunophenotyped were assessed and compared over time and treatment by repeated measures ANOVA or 2-way ANOVA. Values were set as the highest limit of detection when higher than the detection limit. Values lower than the lowest detection limit were set at the lowest detection limit. Values resulting from samples with prolonged sample transit time and inferior quality were excluded from analysis. Simple logistic regression was used to evaluate correlation of survival, parasitic load, clinical history, immunologic function, and treatment efficacy to identify factors affecting survival. All analyses were conducted using GraphPad Prism 8.0 software (La Jolla, California, USA). P-values < 0.05 were considered significant.

Results

Patient demographics. 14 veterinary clinics including one university associated veterinary teaching hospital, one private referral center, and 12 private practices, participated in recruitment of 37 cats with suspected acute cytauxzoonosis from Oklahoma (n = 10), Missouri (n = 6) and Arkansas (n = 21) from May to October 2020. 37 cats were initially enrolled in the study with an average of 2.6 cats enrolled per clinic. 83.7% (31/37) of the cats had positive *C. felis* ddPCR, whereas 6 cats had negative ddPCR and blood smear result and were excluded. In addition, 8 of the 31 cats testing positive on ddPCR either did not meet at least one of the inclusion criteria

upon further assessment or did not adhere to the study requirement and were excluded from analyses except characterizing immunologic parameters during acute infection.

Clinical history. Descriptive statistics were performed on the 23 cats that met the final inclusion. The breeds were mostly domestic short hairs (n = 19) followed by domestic long hairs (n = 2), Siamese (n = 1), and Manx (n = 1). There were 16 males (15 castrated and 1 intact) and 7 females (7 spayed and 0 intact). The mean age of cats was 3.3 ± 2.7 years old, ranging from 4 months to 11 years old. The majority of cats (n = 19) had access to outdoor environment with 2 indoor only cats and 2 cats that with no information provided. For the cats with information available, all except one was not on routine tick preventatives. Cats had illness perceived by owners for 2.6 ± 1.3 (1 - 6) days prior to seeking veterinary care. The cats weighed 10.08 ± 2.57 $(4.25 - 15.4)$ pounds (4.57 ± 1.17 kilograms) upon presentation. Heart rate was variable with some cats presented with tachycardia (>200 beats per minutes; n = 4), bradycardia (<150 beats per minutes; n = 7) and normal (150 – 200 beats per minute; n = 11). 7 cats were tachypneic (< 45 breaths per minutes) and 16 cats had normal respiratory rate. All but one cat had elevated body temperature upon presentation at 104.9 ± 0.8286 $(103.0 - 106.1)$ °C. There was no significant difference in the demography, signalment, history and vital parameters between cats in the two treatment groups (Table 3.1). For the information available, the most common physical examination abnormalities included fever (95.6%), lethargy (93%) and depression (75%). The most common laboratory abnormalities included thrombocytopenia (100%), lymphopenia (93%) and hyperbilirubinemia (88.9%). Table 3.2. summarize the common physical exam and laboratory abnormalities in the study cats.

Confirmation of *Cytauxzoon felis* infection. ddPCR was performed on DNA extracts from each day 0 blood sample to confirm if the enrolled cat had acute cytauxzoonosis. The absolute *C. felis* parasite load in each sample was shown in Figure 2.1. The positive samples had an average

parasite load of $11336482 \pm 9176928\ (5180 - 20000000)$ copies/reaction. Blood smears were made from these samples and evaluated by the method described in Chapter 2. Blood smear was not available in one sample. All other samples testing positive on ddPCR had positive blood smear results, whereas all samples testing negative on ddPCR had negative blood smear results. In average, there were $62 \pm 130\ (0 - 542)$ piroplasms per 10 HPF and the $0.53 \pm 1.3\ (0 - 5.5)$ schizont-laden macrophage per blood smear.

Immunologic characteristics during acute cytauxzoonosis. Blood samples from included cats were processed for flow cytometry analysis and MIA to evaluate the alteration of immunologic parameters in cats with acute cytauxzoonosis. Samples from 18 healthy control cats described above were also processed for MIA analysis to compare to the study cats. 58 samples from 23 cats underwent staining for subsequent flow cytometry analysis. Due to sample deterioration during transportation and the nature of the disease, 22 samples were of poor quality and flow cytometry analyses were not available. 36 samples from 22 cats at day 0, 3, and 10 days post-treatment were included for analysis. Figure 3.2 shows the peripheral immunophenotypic profile in cats presented with acute cytauxzoonosis. Among all the lymphocytes, there were $19.3\% \pm 14.5\%\ (2.10\% - 52.6\%)$ B220+ cells, $20.8\% \pm 15.7\%\ (0.6\% - 60.6\%)$ CD4+ cells, $10.5\% \pm 11.0\%\ (0\% - 30.8\%)$ CD8+ cells, and $1.78\% \pm 6.15\%\ (0\% - 21.3\%)$ CD25+ cells. MIA revealed significant increase in pro-inflammatory cytokines in cats with acute cytauxzoonosis including IL-1β ($p < 0.0001$), INFγ ($p < 0.0001$), IL-12 ($p < 0.0001$), IL-8 ($p = 0.0007$), Flt-3L ($p = 0.0002$) and RANTES ($p < 0.0001$) as compared to healthy control cats, as well as significant decreases in PDGF-BB ($p < 0.0001$), SDF-1 ($p < 0.0001$), MCP-1 ($p = 0.0009$) and IL-18 ($p = 0.0007$) (Figure 3.4).

Effect of treatment on immunologic parameters and parasite load. Alterations in blood parasite load, peripheral immunophenotypes, and cytokine levels were evaluated over time to

assess the effects of the two treatment therapies. A significant decrease in parasite load over time was noted in both treatment groups (Figure 3.5), while no significant difference was noted between the two groups over time. Analysis of peripheral cytokine levels revealed that Flt-3L and IL-12 decreased over time, and PDGF-BB increased over time in cats with acute cytauxzoonosis that received either treatment therapy (Figure 3.6). Dexamethasone showed significant effect in suppressing PDGF-BB elevation over time (Figure 3.7). The proportion of B220+ cells was significantly higher at day 3 in cats treated with A&A plus dexamethasone compared to cats receiving A&A alone (Figure 3.3b). The percentage of CD8+ cells increased significantly in day 10 compared to day 0 and day 3 in cats treated with A&A therapy (both with and without dexamethasone) (Figure 3.3c). Alterations in other immunophenotypic profiles were not significant compared to baseline.

Survival between treatment group and factors affecting survival. For the 11 cats that received A & A and dexamethasone (group A), 6 died/were euthanized and 5 survived to hospital discharge; for the 12 cats that received A & A (group B), 6 died/were euthanized and 6 survived to hospital discharge. The survival rate of group A cats was 45.4% and 50% for group B. The overall survival rate was 47.8%. Kaplan–Meier curve was generated (Figure 3.8.) and survival curves were compared between groups by log-rank test. No significant difference was noted in survival at the time of discharge between groups ($p = 0.8343$). Simple logistic regression was performed to identify factor affecting survival; however, no factor was found to affect survival in the current study set.

Discussion

Although the current study did not identify a significant difference in survival of cats with acute cytauxzoonosis administered standard anti-protozoal therapy with or without anti-inflammatory doses of dexamethasone, our results highlight important clinical and immunopathologic characteristics in cats during acute infection of *C. felis*.

Clinical history, physical examination findings and laboratory assessments extrapolated from study sample submission form and available medical records showed that cytauxzoonosis can affect cats of any breed, age and sex, with a predominance in young, male cats. While other studies did not find a sex predilection, [14-21] the male predominance observed in the current study could be resulting from the bias caused by a relatively small sample size. The study also revealed similar environmental history as previous studies [14-21]; that the majority of cats had exposure to outdoor environment and are not routinely on tick preventatives. However, two cats in the current study were housed strictly indoors with no other housemates, which indicated that other modes of intra-domicile transportation of ticks are possible. Previous studies have also demonstrated indoor cats being infested with ticks. [22, 23] These further substantiate the importance of administering regular tick preventatives regardless of cats' living style (indoor/outdoor). The top three most common physical examination abnormalities are fever (95.6%), lethargy (93%) and depression (75%), which are similar to previous findings. [16,18] Previous studies also documented that some cats can be hypothermic. [18,19] However, hypothermia was not documented in any of the cat in the current study population. This is likely due to exclusion criteria (cats with body temperature <100 °F upon presentation, the treating veterinarian does not expect the cat to live within the next 24 hours) we set for the study since hypothermia was previously noted to be associated with moribund condition and negative outcome. [17,18]

The top three most common laboratory abnormalities in this study (including CBC and biochemistry profile) included thrombocytopenia (100%), lymphopenia (93%) and hyperbilirubinemia (88.9%). While a previous cross-sectional study [24] on characterizing hemostasis in five cats with acute cytauxzoonosis revealed thrombocytopenia, low protein C level, and prolonged prothrombin time, platelet clumping was not evaluated during blood smear evaluation in the current study thus the prevalence of thrombocytopenia might be falsely elevated. Leukopenia has been previously documented to be a common finding and potentially associated with a worse prognosis. [14,18,19] Within leukopenia, neutropenia with a depletion of bone marrow response (decreased myeloid progenitors) and lymphopenia had been documented. [14] Lymphopenia is believed to be part of the stress leukogram during acute infection and the peripheral lymphocytes during the recovering phase of *C. felis* infection might have a large granular morphology [19,25], indicating a non-specific response to an infection. The presence of lymphopenia had made subsequent flow cytometry analyses challenging since well-defined lymphocyte population were oftentimes not identifiable. Hyperbilirubinemia has also been well documented in cats suffering from acute cytauxzoonosis and is believed to resulting from a combination of hepatic infiltration of schizont-laden macrophages and red blood cell hemolysis caused by invasion of merozoites. [17-20] While previous study [16] has identified that total bilirubin concentration is a factor associated with survival to hospital discharge in cats with acute cytauxzoonosis, this was not identified in the cats in our study. This might be due to small sample size and incomplete records regarding laboratory parameters.

Very limited information is available in regard to feline immunologic alterations in response to *C. felis* infection. [1,2] Previous studies demonstrated that pro-inflammatory cytokine levels are increased systemically (TNFα and IL-1β) [1] and in lung tissues (TNFα, IL-1β, IL-6 iNOS and MHC II) [2] in cats that died from acute cytauxzoonosis. In addition, the leukocyte adhesion molecule CD18 that triggers inflammatory response was upregulated in cats with acute

cytauxzoonosis. [1] Although CD18 expression was not evaluated in the current study, we quantified the expression of 19 different cytokines in peripheral blood samples from cats with acute infection to reveal significant elevation of systemic IL-1β, INFγ, IL-12, IL-8, Flt-3L and RANTES, as well as significant decreases in PDGF-BB, SDF-1, MCP-1 and IL-18 (Figure 3.4).

IL-12 is mainly produced by the phagocytes (macrophages and neutrophils) and dendritic cells in response to extracellular or intracellular pathogens. It is a heterodimeric pro-inflammatory cytokine that promotes the T lymphocyte proliferation and differentiation into T_H1 cells, and induces the production of INFγ, the chief pro-inflammatory cytokine responsible for type 1 immunity (T_H1 response). [26,27] As compared to a type 2 immunity (T_H2 response) which is characterized by high antibody titers, a T_H1 response emphasizes on intense phagocytic activity. IFNγ stimulates phagocytosis, oxidative bursts, and killing of intracellular parasites. IFNγ also stimulates antigen presentation to T cells by upregulation of class I and class II MHC molecules on many types of cells. [26] Therefore, elevation in systemic IL-12 and IFNγ levels noted in cats with acute cytauxzoonosis in the current study is supportive of a shift towards T_H1 response, which was previously noted in the hosts during acute infection with other apicomplexan protozoa including *Cryptosporidium*, *Eimeria*, *Neospora*, *Plasmodia* and *Toxoplasma* species. [28]

Increased level of Flt-3L, or FMS-like tyrosine kinase 3 ligand, was observed in cats in this study with acute cytauxzoonosis. Flt-3L plays a crucial role in hematopoiesis by acting like a growth factor for early hematopoietic progenitors. [29,30] It promotes proliferation and differentiation in both myeloid and lymphoid progenitor cells, and Flt-3L is elevated during inflammation with the consequent expansion of hematopoietic cells. [29] As described above, cats with acute cytauxzoonosis commonly exhibit leukopenia and depletion of bone marrow myeloid progenitor cells had been noted. The elevation in Flt-3L in acute *C. felis* infected cats may represent a host response to stimulate bone marrow production and restore the peripheral

leukocyte numbers. Other than its effect on hematopoiesis, increased Flt-3L level has been reported in malarial (*Plasmodium chabaudi*, also belongs to the Apicomplexa phylum) infection in a mice model.[31] In that study, *Plasmodium* sp. induced soluble Flt-3L release by mice mast cells from pre-synthesized membrane-associated precursors, and preferentially stimulated CD8-a+ dendritic cells resulting in increased magnitude of CD8+ T cell activation. The elevation of Flt-3L in *C. felis* infection could share similar mechanisms as *Plasmodium* sp., although increase in the percentage of the CD8+ cells during acute infection was not observed.

Similar to previous cytauxzoonosis studies, increased levels of systemic IL-1β was noted in the current study, and may indicate the activation of the innate immune system in response to *C. felis* parasite invasion. [1,2] IL-1β is a pro-inflammatory cytokine that is secreted by a variety of cell types, particularly monocytes and macrophages, in response to infection and injury. IL-1β is produced as an inactive precursor (pro-IL-1β) in response to pathogen associated molecular patterns (PAMPs) and acts through pattern recognition receptors (PRRs) on macrophages. [32] Mild elevation of IL-8 was also noted in this study, which is also a marker of acute inflammation. IL-8 (CXCL8), is a chemokine responsible for inflammatory response and is produced by sentinel cells including macrophages and mast cells. IL-8 causes activation of neutrophils by attracting them towards sites of parasitic invasion, stimulating the release of neutrophil granules and promoting respiratory bursts. [28,33] Another chemokine, RANTES (more currently known as CCL5), was also observed to be elevated in cats from this study with acute cytauxzoonosis. RANTES is a product of both T lymphocytes and macrophages. It attracts monocytes, eosinophils, and some T lymphocytes to the site of infection and activates both eosinophils and basophils. [28] The elevated RANTES observed in the study cats likely represents consequence of activation of both T lymphocytes and macrophages.

MCP-1 (monocyte chemotactic protein-1) is a chemokine that plays important role in the migration of monocytes, memory T cells and activated natural killer cells. Several studies have documented that MCP-1 resulted in suppression in T_H1 response and T_H2 polarization in viral infections caused by herpesvirus-induced encephalomyelitis and coxsackievirus B3 induced myocarditis in humans. [34,35] No study has evaluated the role of MCP-1 in protozoal infection, but it may indicate that the decrease in MCP-1 noted in the current study in part plays a role in the generation of T_H1 predominant immune response.

SDF-1 is a lymphoid chemokine responsible for lymphocytes interactions in the lymphoid tissue like naïve T lymphocyte homing and B lymphocyte localization in the lymphoid follicles. [36] Deficiency in SDF-1 results in disruption of lymphoid tissue architecture and its primary responses. [36] Peripheral lymph nodes are known to be a location for schizont-laden macrophages accumulation and peripheral lymphopenia is readily noted in cats with acute cytauxzoonosis. Thus, *C. felis* infection might have played a role in the decreased SDF-1 concentrations and resulted in the alterations seen in both lymphocytes and lymphoid tissue. Additionally, SDF-1α level is reported to be inversely correlated with parasite load during *Plasmodium* infection in mice spleen, [37] which also indicates protozoal infection might play a role in negatively regulating SDF-1 concentrations during *C. felis* infection.

PDGF-B (Platelet-derived growth factor B) is a growth factor belongs to the PDGF family and must form homo- or heterodimers (i.e. PDGF-BB) to exhibit its activity. It is known to have important role in wound healing, tumor angiogenesis and regulation in osteogenesis. [38,39] Recent studies have indicated its protective role in preventing lethal sepsis by decreasing the production of pro-inflammatory cytokines and chemokines. [38] Although a decrease rather than increase in PDGF-BB concentration was noted in cats with protozoal infection in the current study, an overall significant increase in PDGF-BB concentration was noted over time in cats that

underwent treatment/survived. This finding coincides with the previous finding that PDGF-BB level was higher in human patients that survived severe sepsis. [40]

Interestingly, a decrease in IL-18 was noted in cats with acute infection of *C. felis* in the current study. IL-18 is a pro-inflammatory cytokine that regulates both T_H1 and T_H2 response. A previous study had shown that IL-18 was increased during *Toxoplasma gondii* infection in mice [41] , and more studies have pointed out a link between IL-18 and immune overactivation in infection caused by various pathogens due to either IL-18 overproduction or over responsiveness of the host response to IL-18. [42] As previously mentioned, increased pro-inflammatory cytokines were noted systemically and in lung tissue from cats that succumbed to cytauxzoonosis. Thus, it is reasonable to think that an increase in IL-18 should be present in cats with acute cytauxzoonosis. However, results from the current study did not support this assumption and the cause of this finding is undetermined.

Analyses from flow cytometry data revealed a predominant proportion (19.3 – 20.8%) of B220+ and CD4+ lymphocytes, a lower percentage (10.5%) of CD8+ lymphocytes, and a very low percentage (1.78%) of CD25+ lymphocytes during acute cytauxzoonosis. Although there was no healthy control cat data available for comparison in the current study, these numbers are subjectively not dramatically different when compared to data healthy control cats from other studies. [30] In addition to reporting the lymphocyte proportion, CBC results from the same analyzer should ideally be used to calculate the actual numbers of these lymphocytes to provide more objective interpretation of the alteration of different immunophenotypes during acute cytauxzoonosis. However, this was not available in the current study due to deteriorating sample quality.

Alterations of several parameters were observed over time in all cats that received treatment for cytauxzoonosis. Absolute parasite loads decreased significantly over time, which

likely resulted from therapeutic effects of azithromycin and atovaquone (similar to what has previously been reported [16]). Although dexamethasone did not result in a significant difference in parasite loads over time between treatment groups, this may be due to the fact that the dosage used and treatment duration was not sufficient enough to result in inflammatory suppression. An increase in CD8+ lymphocyte population was noted over time, and the percentage of B220+ lymphocytes in study cats diverged between the two treatment groups at day 3 after treatment (around 24 hours after the last dose of dexamethasone in the group that received it). The percentage of B220+ cells are higher in the cats that received dexamethasone. B220 is a heavily glycosylated isoform of CD45R primarily expressed in immature and mature naïve B lymphocytes. Previous study has showed that prednisolone causes dose dependent suppression of lymphocyte populations. [30] At higher dosage, prednisolone resulted in suppression in both T and B lymphocytes whereas a lower dosage, it caused a selective decrease in B lymphocytes. [30] Dexamethasone is a corticosteroid structurally similar to which of prednisolone, with an addition of a methyl group on the cyclopentane ring and a fluorine atom in the 9α position. [43] These modifications made dexamethasone more potent as a glucocorticoid with minimal mineralocorticoid activity. Previous study has shown that dexamethasone is 16 times more potent glucocorticoid activity compared to prednisolone against lymphoblasts *in vitro*. [44,45] Considering the difference between the two drugs and previous research, it is interesting finding that the B220+ lymphocyte percentage did not decrease but increase in the cats that received dexamethasone in the current study. It could be that we only assessed the percentage but not the actual number of these cells, which could have introduced bias. In addition, lymphopenia and the nature of the disease have made flow cytometry analyses challenging, which could also have contributed to the conflicting results.

Regardless of treatment groups, Flt-3L and IL-12 decreased over time while PGDF-BB increased over time, and the possible explanations for these trends were well established in

previous paragraphs. However, it is worth noting that in the group of cats that received dexamethasone, a marked suppression of PDGF-BB was noted as compared to the group that did not receive dexamethasone. Considering multiple human studies, there may be a link between PDGF-BB level and survival from sepsis, and it is speculated that the use of dexamethasone might have negative effects in cats with acute cytauxzoonosis. Although the survival did not differ between groups in the current study, no obvious harm was noted either. Thus, whether to include dexamethasone into the routine regimen of cytauxzoonosis therapy still needs to be further evaluated.

Simple logistic regression was carried out and identified no factor associated with survival. This is likely due to the small sample size. However, patient's geographic location is subjectively related to survival by reviewing available medical records. Similar as previously reported, [16,18,19] cats from Oklahoma are less likely to survive as compared to cats from Missouri or Arkansas.

There are several limitations of the current study. One is the small sample size, which may have contributed to a lack of statistical significance in several parameters evaluated including survival and factors associated with survival. Another one is nature of the disease and shipping of the blood samples, which resulted in suboptimal sample quality for MIA and flow cytometry analysis. As compared to experimental infection study, the clinical study design allowed potential introduction of biases in many aspects such as supportive therapies, blood analyzer machines and different treating veterinarians. Although an improved survival was not noted in the current study in cats receiving dexamethasone as hypothesized, it is still valuable information that dexamethasone did not worsen the survival used at the proposed dosage and duration as an adjunctive therapy to treat acute cytauxzoonosis in cats. In addition, the study demonstrated a more complete immunologic profile of feline acute cytauxzoonosis, and the

alterations observed in peripheral cytokine levels and immunophenotypes can serve as a foundation for potential therapeutic targets in future studies.

Table 3.1. Patient data. Information on study cats regarding demographic information, signalment, clinical history, physical exam, and laboratory findings were listed and compared by treatment group. No significant difference was noted between the two treatment groups.

	Group A (A&A + Dexamethasone)	**Group B (A&A)**
Demography	Oklahoma (n = 1) Arkansas (n = 9) Missouri (n = 1)	Oklahoma (n = 1) Arkansas (n = 8) Missouri (n = 3)
Signalment		
Age (years)	$3.4 \pm 2.4\ (1.0 - 9.0)$	$3.2 \pm 3.1\ (0.3 - 11)$
Sex	MC (n = 7) MI (n = 0) FS (n = 4) FI (n = 0)	MC (n = 8) MI (n = 1) FS (n = 3) FI (n = 0)
Breed	DSH (n = 10) Other breeds (n = 1)	DSH (n = 10) Other breeds (n = 2)
History		
Access to outdoor	Yes (n = 10) No (n = 1)	Yes (n = 11) No (n = 1)
Regular tick prevention	Yes (n = 0) No (n = 10) N/A (n = 1)	Yes (n = 0) No (n = 12)
Duration of illness (days)	$2.1 \pm 0.8\ (1 - 3)$	$2.9 \pm 1.5\ (1 - 6)$
Vital Parameters		
Body Weight (kg)	$4.73 \pm 1.14\ (3.44 - 7.00)$	$4.45 \pm 1.23\ (1.93 - 5.64)$
Body Temperature (°F)	$104.8 \pm 0.9\ (103.0 - 106.1)$	$105.0 \pm 0.7\ (103.7 - 106.1)$
Heart Rate (bpm)	$166 \pm 27\ (120 - 220)$	$169 \pm 36\ (100 - 210)$
Respiratory Rate (brpm)	$35 \pm 14\ (20 - 60)$	$37 \pm 12\ (20 - 66)$

A&A, atovaquone and azithromycin; MC, male castrated; MI, male intact; FS, female spayed; FI, female intact; DSH, domestic short hair; N/A, not available; kg, kilogram; bpm, beats per minute; brpm, breaths per minute.

Table 3.2. Common physical examination and laboratory abnormalities in the study cats.

Abnormality	Percentage of study cats
History & Physical Examination	
Fever	95.6% (22/23)
Lethargy	93% (14/15)
Depression	75% (6/8)
Inappetence	64.2% (9/14)
Pallor	64.7% (11/17)
Muscle pain	56.2% (9/16)
Icterus	50% (8/16)
CBC abnormalities	
Thrombocytopenia (< 200 10^3/μL)	100% (16/16)
Lymphopenia (< 2000 /μL)	93% (14/15)
Leukopenia (< 5.0 10^3/μL)	66.6% (10/15)
Neutropenia (< 2500 μL)	60% (9/15)
Anemia (HCT < 29%)	31.2% (5/16)
Chemistry abnormalities	
Hyperbilirubinemia (> 0.4 mg/dL)	88.9% (8/9)
Hyperglycemia (>170 mg/dL)	50% (4/8)
Hypokalemia (< 3.7 mEq/L)	50% (4/8)
Hyponatremia (<145 mEq/L)	50% (4/8)
Hypoalbuminemia (< 2.5 g/dL)	37.5% (3/8)

CBC, complete blood count; HCT, hematocrit.

Figure 3.1. Study design. Cats diagnosed acute cytauxzoonosis were randomly assigned to one of the following treatment groups. Group A received atovaquone, azithromycin and dexamethasone and group B received atovaquone and azithromycin. Blood samples were collected at day 0, day 3 and day 10 after initiation of treatment. All cats received fluid therapy and supportive care.

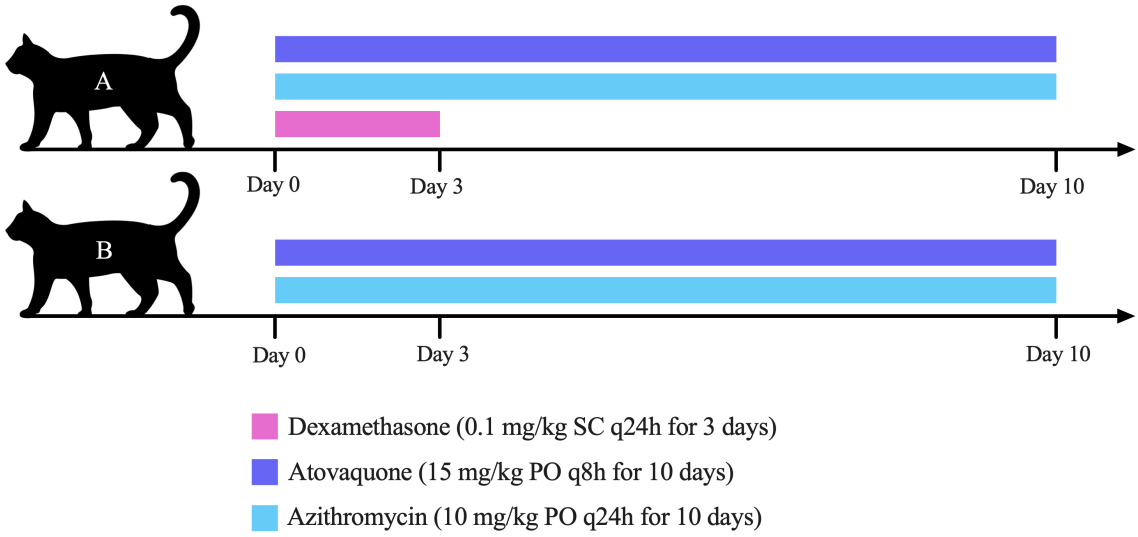


Figure 3.2. Immunophenotypic profile in cats with acute cytauxzoonosis. During acute infection, the cats on average presented with a predominant proportion of B220+ and CD4+ lymphocytes, a low percentage of CD8+ lymphocytes and very low percentage of CD25+ lymphocytes.

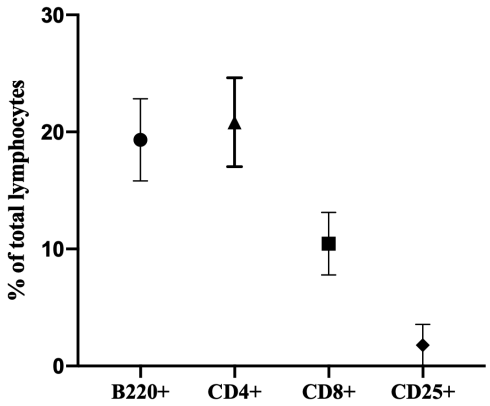


Figure 3.3. Alterations in systemic immunophynotypes under treatments with or without dexamethasone in cats with acute cytauxzoonosis. Over the course of treatment, an increased percentage of CD8+ lymphocytes was noted 10 days after treatment. Additionally, a significant difference was noted in the percentage of B220+ lymphocytes between the cats treated with and without dexamethasone at 3 days after treatment. Cats that received dexamethasone had a higher percentage of B220+ lymphocytes as compared to cats that did not receive dexamethasone. (* = $P < 0.05$)

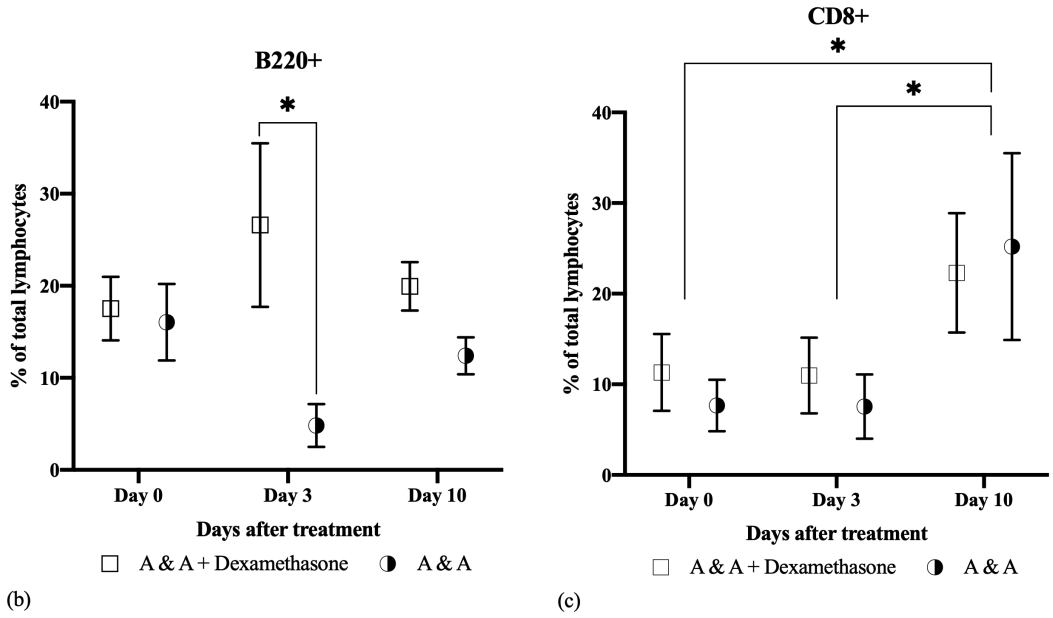
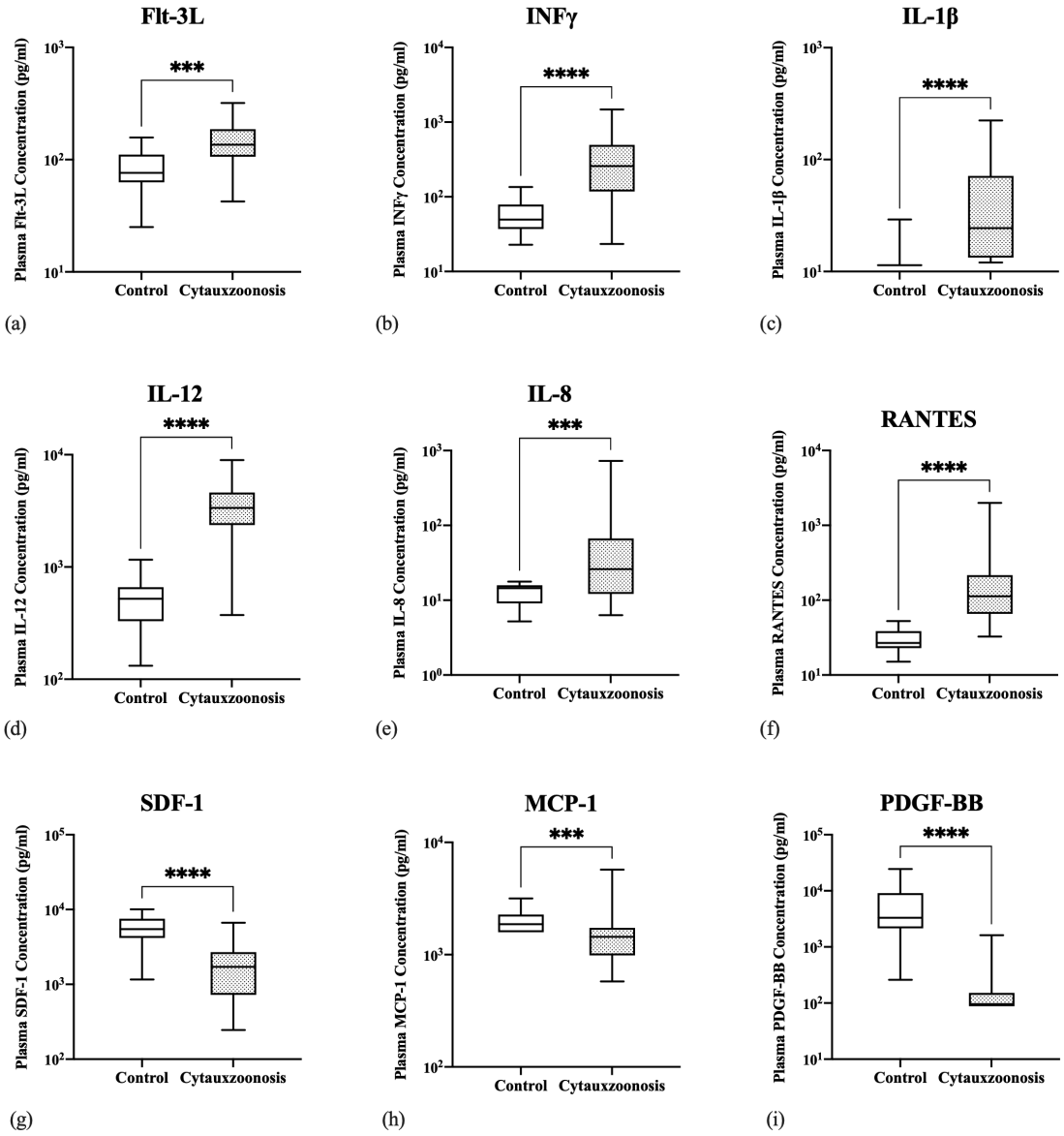
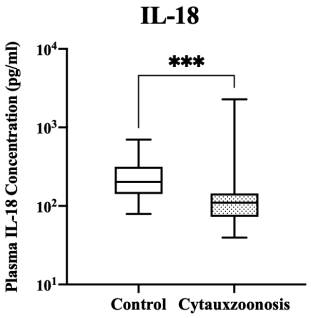


Figure 3.4. Peripheral cytokine concentrations in cats with acute cytauxzoonosis vs. healthy control cats. Elevation of pro-inflammatory cytokines including IL-1β (c), INFγ (b), IL-12 (d), IL-8 (e), Flt-3L (a) and RANTES (f), and decrease of PDGF-BB (i), SDF-1 (g), MCP-1 (h) and IL-18 (j) were noted in plasma samples of cats with acute cytauxzoonosis as compared to 18 healthy uninfected cats.




IL-18
***
Plasma IL-18 Concentration (pg/ml)
10^4
10^3
10^2
10^1
Control
Cytauxzoonosis

(j)

Figure 3.5. *C. felis* ddPCR parasite load. (a) Parasite loads decreased significantly over time in cats with acute cytauxzoonosis that received antiprotozoal therapy with or without dexamethasone. (b) Dexamethasone did not show significant effect in alteration of parasite loads during treatment. (*** = P < 0.005)

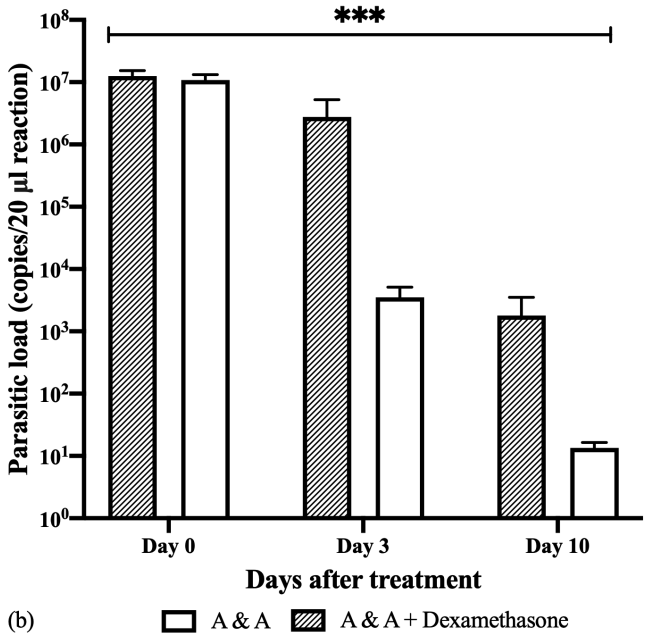


Figure 3.6. Alterations in peripheral cytokine levels in cats with acute cytauxzoonosis receiving treatment. Significant decreases in Flt-3L (a) and IL-12 (b) over time and at individual time points was observed in study cats, while a significant increase in PDGF-BB (c) was observed over time (* = $P < 0.05$; ** = $P < 0.005$) during treatment.

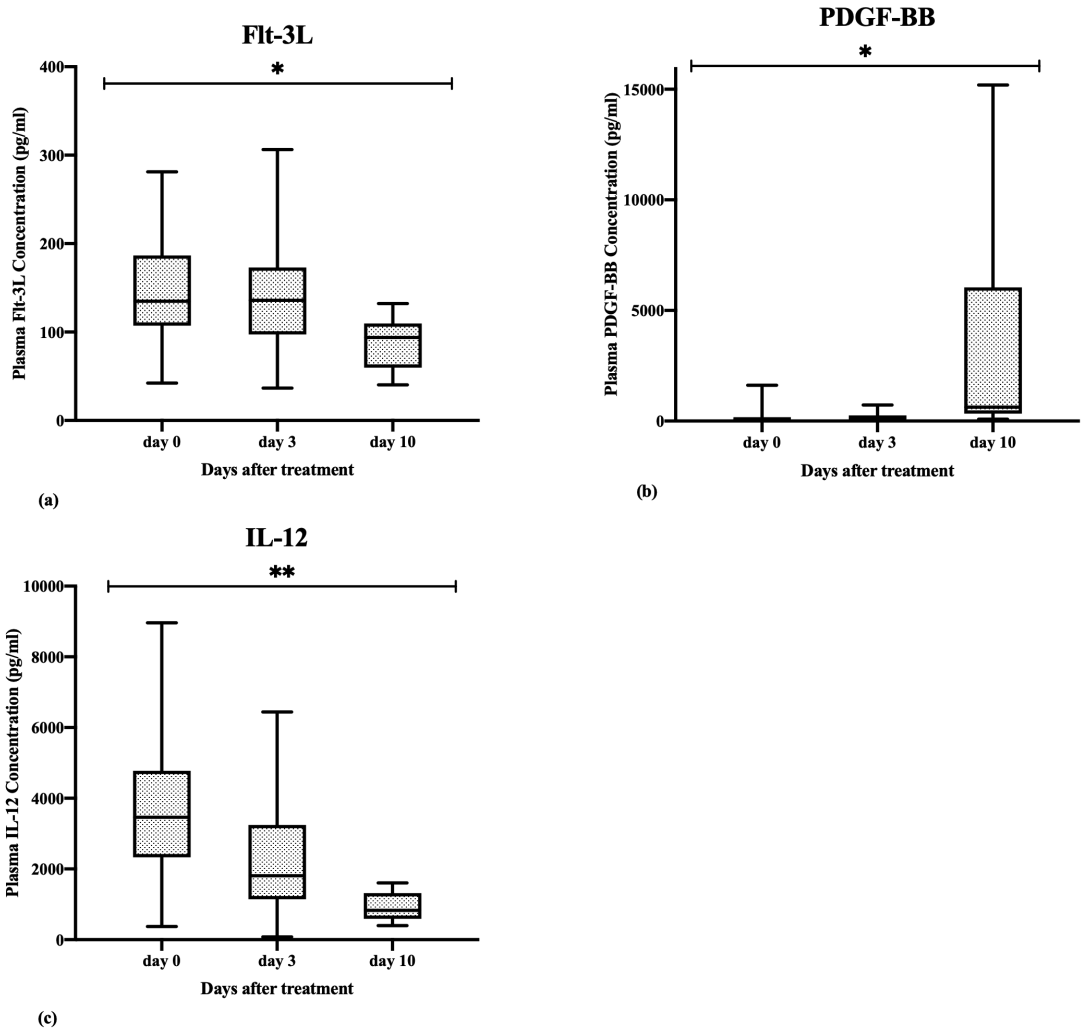


Figure 3.7. Comparison of peripheral PDGF-BB levels over time in cats with acute cytauxzoonosis that received antiprotozoals with or without dexamethasone. At day 10 after initiation of therapy, cats with acute cytauxzoonosis that did not receive dexamethasone exhibited a greater increase in peripheral PDGF-BB concentrations ($p = 0.0012$).

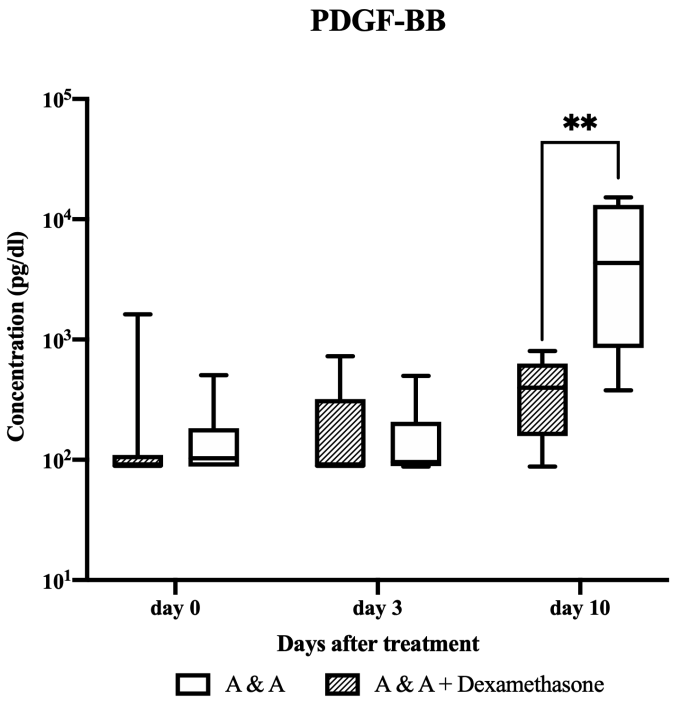
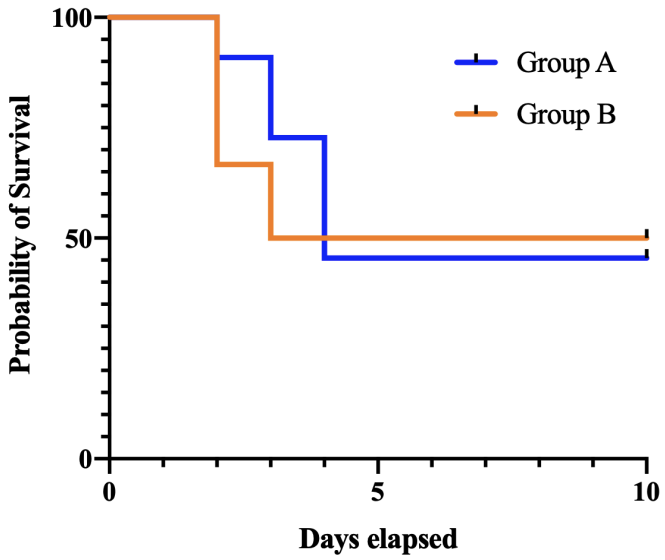


Figure 3.8. Kaplan–Meier survival curve. The Kaplan–Meier curve was generated for group A (A & A + dexamethasone) and group B (A & A only) cats to compare the survival rate. No difference in survival was noted in *C. felis* infected cats that received A & & and dexamethasone or A & A alone.



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CHAPTER IV

EVALUATION OF THE PUTATIVE CYTAUXZOON FELIS SURFACE PROTEIN C88 IN DEVELOPING AN IGM ELISA TO DETECT FELINE ACUTE CYTAUXZOONOSIS

Introduction

Cytauxzoonosis is a highly-fatal disease of domestic cats with limited treatment modalities and a narrow therapeutic window. During acute infection, cats develop severe clinical illness with rapid disease progression associated with the schizogenous phase of parasite replication, in which large schizont-laden monocytes occlude vascular lumina in vital organs.[1,2] Unfortunately, there is no vaccine to prevent *C. felis* infection, and attempts to develop effective anti-protozoal therapies have been met with limited success.[3-6] In the southcentral and southeastern United States, fatal cases of cytauxzoonosis in domestic cats are fairly common and mortality in this region is estimated to be around 40% even with treatment and potentially worse in some areas. [3,7] Reviewing available medical records from the Boren Veterinary Medical Hospital of Oklahoma State University (OSU-VTH) from 2014 to 2020, there were 52 cats diagnosed with acute cytauxzoonosis with only 2 cats survived to hospital discharge (mortality rate 96.2%, unpublished data). Because of the rapid disease progression and severe clinical consequence, early detection and therapeutic implementation is crucial for effective treatment.

Although numerous diagnostic methods are available to aid veterinarians in diagnosing

cytauxzoonosis, each technique has its limitations. Currently, diagnosis of cytauxzoonosis relies on cytology and confirmatory molecular testing such as quantitative polymerase chain reaction (real-time PCR). Cytologic diagnosis of cytauxzoonosis is achieved by identifying intra-erythrocytic piroplasms and/or schizont-laden macrophages in blood smears preparations from infected cats. Although blood smear evaluation is cost efficient and easily performed in a clinical setting, normal structures like Howell-Jolly bodies or water artifacts resulting from slide preparation can be mistaken as piroplasms by underexperienced individuals. In addition, schizont-laden macrophages are not readily found in blood smears, and the piroplasm phase is considered a later stage of infection.[4,8] Schizont-laden macrophages can also be identified in tissue aspirates from peripheral lymph nodes, spleen, liver and the lungs, but there are inherent risks and difficulties in acquiring these samples for analysis, and the sparse availability of ultrasound equipment might hinder veterinarians from utilizing this diagnostic method. [4,8,9]

Alternatively, blood samples from cats with suspected infection may be submitted to diagnostic laboratories that offer real-time PCR for the detection of *C. felis* DNA. Although considered a "gold-standard" in diagnosing active *C. felis* infection, real-time PCR and other available PCR assays can be time- and cost-prohibitive, and PCR is not readily available in the clinical setting; highlighting a crucial need for a rapid, clinical test for definitive diagnosis in order to improve therapeutic outcomes.

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay technique designed for the detection and quantification of soluble proteins or peptides and is widely used in the biomedical field to detect various substances of interests including antigens from pathogens, antibodies and hormones. The principle of ELISA involves the high affinity of antigen-antibody interaction and is accomplished by immobilizing a target of interest to a solid surface (microplate), which is then complexed to an antibody linked to a reporter enzyme. Detection is achieved by measuring the activity of the reporter enzyme via incubation with appropriate

substrate to produce a measurable product. Many commonly used bedside rapid diagnostic test kits in veterinary clinics are designed based on ELISA methodology such as the SNAP® 4DX Test (IDEXX Laboratories, Inc. Westbrook, Maine, USA) for common canine vector-borne diseases, SNAP® FIV/FeLV Combo Test (IDEXX Laboratories, Inc. Westbrook, Maine, USA) for feline retroviruses, WITNESS® Lepto Rapid Test (Zoetis LLC, Kalamazoo, MI, USA) for leptospirosis and the Cardiopet® proBNP Test (IDEXX Laboratories, Inc. Westbrook, Maine, USA) for detection of preclinical cardiac diseases. These rapid tests have provided tremendous advantages to the veterinary practitioners in disease screening and implementation of appropriate treatment strategies for veterinary patients in a timely fashion.

Recent studies in *C. felis* genome sequencing and subsequent protein microarrays have revealed several putative *C. felis* antigens including contig00088:95434-96586(−) (c88) and cf76.[10] Preliminary data from our lab [11] have shown that a significant IgM and IgG antibody response to both c88 and cf76 is generated during acute experimental infection with *C. felis*, indicating potential targets to detect *C. felis* in peripheral blood of acutely infected cats. Because IgM antibodies play an important role in generating early immunity and are the first antibodies elicited in an immune response during acute infection, the objective of this study was to develop a *C. felis* specific IgM ELISA for early detection of acute cytauxzoonosis in domestic cats.

Materials and Methods

Control samples. EDTA-anticoagulated plasma sample from a cat presented to Oklahoma State University (OSU-VMH) with signs of acute cytauxzoonosis was utilized as positive control plasma. Cytauxzoonosis was confirmed both cytologically and via ddPCR. EDTA-anticoagulated plasma samples from 18 specific pathogen-free cats housed at Oklahoma State University Animal Resources and from another 4 specific pathogen-free cats from another study were available as

negative control plasma. These samples (except the positive control plasma) were classified into group 1 (n=22).

Clinical samples (group 2 and 3). 36 EDTA-anticoagulated plasma samples were available from the clinical study described in Chapter III. These samples were classified into two groups: Cats diagnosed with acute cytauxzoonosis by ddPCR (group 2, n=29) and *C. felis*-negative cats (negative ddPCR and blood smear evaluation) with systemic illnesses (group 3, n=7).

Samples from chronic *C. felis* carriers (group 4). EDTA-anticoagulated plasma samples from 3 *C. felis* survivor cats housed at Oklahoma State University Animal Resources, and from a cat presented to OSU-VMH Small Animal Internal Medicine service for hepatobiliary disease and suspected cytauxzoonosis relapse (incidental finding of intra-erythrocytic piroplasms on serial blood smear evaluation throughout hospitalization) were available. *C. felis* infection was confirmed by ddPCR in all 4 samples. These samples were classified into group 4 (n=4).

c88 recombinant protein. The recombinant protein of the *C. feli*s putative surface antigen contig00088:95434-96586(-), or c88, was synthesized by GenScript Biotech (Piscataway, NJ). According to the manufacturer, the target DNA sequence of c88 was synthesized and cloned to vector pET30a with His tag for protein expression in *E. coli*. The cells were harvested by centrifugation. SDS-PAGE and Western blot were utilized to determine the protein purity and molecular weight.

***C. felis* IgM ELISA.** An indirect ELISA was designed as previously published [12,13] with modifications in order to detect *Cytauxzoon felis*-specific IgM antibodies in plasma samples from cats with acute cytauxzoonosis. Briefly, Corning® 96-well Clear Flat Bottom Polystyrene High Bind Microplates (Corning Inc. Glendale, AZ) were coated with diluted c88 recombinant protein (5 μg/ml in 0.1 M carbonate buffer, 100 μl/well) and incubated overnight at 4°C. After discarding the coating solution, the plate was blocked in 2% bovine serum albumin in TEN buffer (200

μl/well) for 4 hours at room temperature. The blocking solution was discarded upon completion of blocking. Samples diluted 1:500 in ELISA diluent, positive controls, negative controls and blank were added (100 μl/well) and incubated at 37°C for one hour. The plate was washed 5 times with 0.2% Tween in TEN buffer after incubation. HRP conjugated goat anti-feline IgM antibodies were diluted 1:20000 in ELISA diluent (4% v/v fetal bovine serum, 0.5% v/v Triton X-100, 2% bovine serum albumin in TEN buffer) and added into wells (100 μl/well). The plate was incubated at 37°C for one hour. After incubation, the plate was washed 5 times with 0.2% Tween in TEN buffer. 100 μl 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoFisher™Scientific. Waltham, MA) was added into each well and incubated at room temperature for 10 minutes. 75 μl ELISA Stop Solution (ThermoFisher™Scientific. Waltham, MA) was added in to each well to terminate the reaction. Absorbance was measured immediately at 450 nm (OD450) by SpectraMax® Plus 384 Microplate Reader (Molecular Devices, LLC. San Jose, CA). The concentration of coating antigen, detection antibody and positive/negative controls were optimized before testing clinical plasma samples. All samples were run in duplicates.

Statistical analysis. The data was plotted on Microsoft Excel 16.16.27 software (Redmond, WA, USA) to calculate mean, standard deviation, and percentage of coefficient of variation (% CV) between duplicate samples within the same plate to ensure acceptable intra-plate consistency (% CV < 10%). Each sample was run in duplicates on two separate plates whenever possible to obtain mean absorbance values. The mean absorbance value for each sample was transformed into percent positivity (PP) using a previous published formula [14-16] to eliminate inter-plate variability. Appendix 2 lists the formula used to calculate PP value in the current study. The PP values were imported into GraphPad Prism 8.0 software (La Jolla, California, USA) for receiver operating characteristic (ROC) curve analysis and determination of the assay cut-off value for optimal sensitivity and specificity. The differences in mean PP value between control cats, cats

with acute cytauxzoonosis, cats with non-*C. felis* systemic illnesses and chronic *C. felis* carriers were compared by one-way ANOVA and Dunnett's multiple comparison test. $P < 0.05$ was considered significant in all analyzes.

Results

Cats with acute cytauxzoonosis had significantly higher detectable plasma anti-c88 IgM antibodies compared to cats in other groups. As outlined in methods, optical density (OD) values of the samples were compared to the OD of positive controls to obtain percent positivity (PP) to allow inter-plate comparisons. Mean PP values were compared by one-way ANOVA and revealed statistically significant differences between the four study groups. Post-hoc analysis (Dunnett's multiple comparison test) revealed significant differences ($P < 0.0001$) between healthy control cats (group 1) and cats with acute cytauxzoonosis (group 2) samples, as shown in Figure 4.1. While compared to healthy control cats (group 1), *C. felis* PCR negative cats with systemic illnesses (group 3) and chronic *C. felis* carriers (group 4) were not significantly different.

***C. felis* IgM ELISA is highly specific and can successfully detect infection in cats with acute cytauxzoonosis.** When compared to healthy control cats (group 1) and *C. felis* negative cats with systemic illnesses (group 3), the majority of cats with acute cytauxzoonosis (group 2) had high PP values easily distinguished from *C. felis* PCR-negative cats (groups 1 and 3, Figure 4.2). Based on the results generated from the receiver operating curve analysis (Figure 4.3) and the area under the curve, the *C. felis* IgM ELISA can reliably differentiate cats with and without acute cytauxzoonosis with 100% specificity above 28.05 percent positive. Sample values greater than this cut-off value yields an assay sensitivity is 86.21% and is indicative of a "Strong Positive" result. Although there are no false positive results, infection was not detectable in 4 of the 29 cats

with acute cytauxzoonosis at this cut-off value. However, when the limit of detection was lowered to 8.35 percent positive, infection could be detected in these 4 cats with 100% sensitivity and 75.86% specificity. Thus, samples with PP values between 8.35 – 28.05 are indicative of a "Weak Positive" result. The range of PP and interpretation of the current ELISA is summarized Table 4.1.

Weak positive ELISA result can be evaluated in combination with other diagnostics to reach a definitive diagnosis. To assess the utility of the current ELISA design in a veterinary general practice setting and increase the sensitivity of disease detection in samples with weak positive ELISA results, the combined use of the ELISA and blood smear evaluation were evaluated. The results of blood smear evaluation from Chapter II were modified to suit the average veterinarian practitioner. Briefly, the samples that had less than or equal to 3 piroplasms per 10 high power field were considered negative, whereas samples with more than 3 piroplasms per 10 high power field or at least 2 schizont-laden macrophages were considered positive. According to the modified blood smear results (Table 4.2), 8 of the 29 cats with acute cytauxzoonosis (group 2) and 7 of the 7 cats with non-*C. felis* illnesses (group 3) had negative blood smear results. The sensitivity and specificity of the modified blood smear method was calculated and listed in Table 4.3. The sensitivity and specificity for strong positive results of ELISA was also listed in the same table using the results acquired from cut-off of 28.05 percent positive previously. The combined sensitivity and specificity of utilizing the ELISA and modified blood smear method could be obtained utilizing the following formula:

$$(A + B)_{sen} = (A)_{sen} + (B)_{sen} - [(A)_{sen} \times (B)_{sen}]$$

$$(A + B)_{spec} = (A)_{spec} \times (B)_{spec}$$

Where A = A diagnostic test; B = B diagnostic test; $(A)_{sen}$ = Sensitivity of A; $(B)_{sen}$ = Sensitivity of B; $(A)_{spec}$ = Specificity of A; $(B)_{spec}$ = Specificity of B; $(A + B)_{sen}$ = Combined sensitivity of

using A or B test; $(A + B)_{spec}$ = Combined specificity of using A or B test. The combined sensitivity and specificity of ELISA and modified blood smear are 96.19% and 100% respectively (Table 4.3). Applying the combined method, infection in 1 of the 4 cats that had negative ELISA in group 2 could be detected (Table 4.4).

Asymptomatic chronic *C. felis* carriers. While most of the *C. felis* chronic carrier cats (group 4) had low PP values similar to values in healthy control cats (group 1) and negative *C. felis* PCR cats with systemic illnesses (group 2), a high PP value (50.9) was observed in one chronic carrier (Figure 4.2). However, this cat was apparently healthy and did not have any clinical signs of cytauxzoonosis. Thus, with proper clinical context such as cats presented with clinical signs suspicious of acute cytauxzoonosis, the ELISA was able to detect acute cytauxzoonosis in the majority of the infected cats as described previously.

Discussion

The current study documents the potential of the *C. felis* anti-c88 IgM ELISA in detecting infection in cats with acute cytauxzoonosis. Overall, the ELISA has excellent performance in differentiating cats with or without acute cytauxzoonosis based on the receiver operating curve characteristic. With the proposed cut-off value (PP = 28.05) to obtain Strong Positive results, the sensitivity and specificity of the assay is 86.21% and 100% respectively. These results indicate that the *C. felis* anti-c88 IgM ELISA is highly specific in diagnosing acute cytauxzoonosis therefore can be used as a rule-in test. However, although the sensitivity at this cut-off is acceptable, some cats with disease present could still be missed (false negatives). The receiver operating curve analysis provides a trade-off between sensitivity and specificity of a given diagnostic test. Based on the context of the data to be analyzed, a cut-off value is set to reach optimal performance of the assay. If we select a cut-off value that carried a higher

sensitivity and slightly lower specificity, some cats without acute cytauxzoonosis could be misdiagnosed (false positives) based on ELISA only. Due to the historically guarded prognosis of the disease (as described in Chapter I, very low survival rate despite aggressive medical intervention) in some endemic areas of the United States, many cats diagnosed with cytauxzoonosis would be humanly euthanized. Considering the relatively serious consequences (euthanasia) for false positive results, we classified the positive ELISA results into "Strong Positive" and "Weak Positive" based on different ranges of cut-off PP values. With the "Strong Positive" cut-off, we maximized the assay specificity (100%) to eliminate possibilities of a false positive result. With the "Weak Positive" cut-off, we increased the assay sensitivity and lowered the assay specificity, which allowed us to detect all PCR-positive cats with acute cytauxzoonosis.

Within the "Weak Positive" cut-off value range of 8.35 – 28.05 PP, 4 *C. felis* PCR-positive cats (group 2) (Table 4.4) and 2 *C. felis* negative cats with systemic illnesses (group 3) were detected, indicating that ELISA results of these cats should be interpreted with caution. Thus, in the event of a "Weak Positive" result, follow-up diagnostics such as blood smear evaluation and/or PCR is warranted to confirm diagnosis. We therefore evaluated the combination use of ELISA (Strong Positive cut-off) and blood smear evaluation to increase overall diagnostic sensitivity and assess the potential for application of this assay in veterinary general practices. We numerated blood smear evaluation in samples used in the current study based on identification and quantification of intra-erythrocytic piroplasms and schizont-laden macrophages. As outlined above, the blood smear of a given sample was considered negative if there was less than or equal to 3 piroplasms per 10 high power field and less than 2 schizont-laden macrophages per blood smear. Based on this modification, the sensitivity and specificity of blood smear evaluation were calculated. As a result, the overall sensitivity of the combined diagnostics was raised to 96.19% with 100% specificity, indicating excellent performance while using these two diagnostic tests as a combination.

Even with the combination use of ELISA and blood smear evaluation, infection was still not detectable in a small number of cats (n=2; cat 10 and cat 16). As shown in Table 4.4, cat 10 and cat 16 not only had PP values similar to healthy control cats, but parasitic organisms were also challenging to find on their blood smears. Since IgM is the antibody type generated firstly during infection, it is commonly used as an indicator of acute infection with proper clinical context.[17,18] Our preliminary study [11] used magnetic beads to detect anti-*C. felis* IgM and IgG antibodies in cats with experimental *C. felis* infection. Four (4) specific-pathogen free cats were infested with *C. felis*-positive *Amblyomma americanum* adult ticks and the female ticks were allowed to feed until repletion (approximately 7 days). Serial blood samples were collected from these cats at day 0, 7, 10, 12, 15 and 19 post-infestation. IgM antibodies were first detected in 75% (3/4) cats 12 days post-infestation and IgG antibodies were detectable 15 days post-infection. Although IgM is the first detectable antibody type during acute *C. felis* infection, there is still a patent period when infection is not detectable via testing IgM. Additionally, intra-erythrocytic piroplasms only become evident in later stage of infection considering the *C. felis* life cycle. Therefore, it is likely that cat 10 and cat 16 were presented to the veterinary hospital very early in the course of infection. Thus, in cats with weak positive ELISA results, we recommend PCR as an ultimate confirmatory diagnostic test. Due to the turnaround time of currently available *C. felis* PCR assays and the negative outcome of the disease if treatments were not initiated promptly, we also recommend empirical treatment of anti-protozoal therapy before the PCR result becomes available.

One cat in group 4 (chronic *C. felis* carriers) had very high PP value (Figure 4.2.), comparable to many cats in group 2 (acute cytauxzoonosis). While this cat is apparently asymptomatic, it has historically tested positive on various *C. felis* PCR assays,[2,19] and its parasite load is known to be high (10430 copies/reaction). A possible explanation could be that its immune system is not able to clear out the majority of the parasites. Thus, the continuous

replication and release of the parasites could have caused continuous re-infection and intermittent production of IgM antibodies.[20, 21] During human malarial infection, the protozoal parasite *Plasmodium vivax* is known to alter the host B-cell profile and therefore result in a persistent malarial-specific IgM response.[22] Thus, it is reasonable to speculate that *C. felis* could similar response in host immune system which could be accountable for the high anti-*C. felis* IgM level observed in the current study. Due to this scenario, we recommend that interpretation of ELISA results should be considered in the proper clinical context such as clinical history, symptoms, or other laboratory findings suspicious of acute cytauxzoonosis. This could be of particular importance in endemic areas where known *C. felis* survivors or cats with incidental finding of previous exposure are present.

One major limitation of this study is the small sample size particularly in group 3 and group 4 cats. The types of sample applicable for this assay could be another limitation. Due to sample availability, we were only able to use EDTA-anticoagulated plasma for this study. However, considering the potential for this ELISA to become a bedside test in clinical setting, it is worthwhile to expand the testing to other sample types such as whole blood, heparinized plasma, serum, or citrated plasma for further validation of the assay on these samples. Other than applying this ELISA to more clinical cats and further validating it to the use of other types of sample preparation, it is also valuable to investigate and evaluate the same ELISA design using other *C. felis* putative antigens discovered previously[10] to compare their capabilities in the early detection of feline acute cytauxzoonosis.

In conclusion, the C. felis anti-c88 IgM ELISA is a highly specific and sensitive test in diagnosing acute cytauxzoonosis in clinical cats presented with comparable history and symptoms. It is relatively non-invasive as compared to sedated fine needle aspirations and it has the potential to be developed into a bedside screening test with advantageous diagnostic turnaround as compared to PCR.

Table 4.1. Interpretation and comments of the *C. felis* IgM ELISA.

PP	Specificity /sensitivity	Interpretation	Comments
> 28.05	100% / 86.21%	Strong Positive	Cytauxzoonosis.
8.35 – 28.05	75.87% / 100%	Weak Positive	Possible cytauxzoonosis if clinical history is suggestive. Additional diagnostics are recommended (i.e. Blood smear evaluation and/or PCR). Empirical therapy should be initiated while awaiting other diagnostic results.
< 8.35	N/A	Negative	Cytauxzoonosis is unlikely.

PP, percent positivity; N/A, not applicable.

Table 4.2. Results of modified blood smear evaluation in cats with acute cytauxzoonosis (group 2) or with non-*C. felis* illnesses (group 3)

Group 2 Cat ID	Piroplasms/10 HPF	Schizont-laden macrophages/smear	Modified blood smear method
2	344	0	Positive
3	346	0	Positive
6	0	0	Negative
7	26	1	Positive
8	61	2	Positive
9	7	0	Positive
10	2	0	Negative
11	2	0	Negative
12	33	0	Positive
15	1	0	Negative
16	0	0	Negative
17	14	0.5	Positive
18	3	0	Negative
21	208	0	Positive
22	11	0	Positive
23	11	0	Positive
25	542	0	Positive
26	10	5.5	Positive
27	2	0	Negative
28	8	0	Positive
29	7	0	Positive
30	28	0	Positive
31	10	1	Positive
33	7	0	Positive
34	21	1	Positive
35	21	0	Positive
36	0	1	Negative
38	14	2	Positive
Group 3 Cat ID	**Piroplasms/10 HPF**	**Schizont-laden macrophages/smear**	**Modified blood smear method**
13	0	0	Negative
14	0	0	Negative
19	0	0	Negative
20	0	0	Negative
37	0	0	Negative
39	0	0	Negative
40	0	0	Negative

Table 4.3. Sensitivity and specificity of C. felis anti-c88 IgM ELISA for strong positive results, blood smear evaluation, and two diagnostics combined in detecting acute cytauxzoonosis.

Diagnostic test	Sensitivity	Specificity
ELISA	86.21%	100%
Blood smear	72.4%	100%
ELISA + Blood smear	96.19%	100%

Table 4.4. Clinical history and laboratory assessments of the *C. felis*-positive cats with weak positive *C. felis* IgM ELISA results (PP value between 8.35 and 28.05).

Cat ID	ELISA average PP	Blood smear results (Piroplasms/10 HPF)	Blood smear interpretation using modified method	Duration of illness before presentation (days)	ddPCR parasite load (copies/µl)
6	19.7	<1	Negative	2	79100
10	8.7	2	Negative	3	9650
16	9.0	<1	Negative	N/A	26360
21	24.1	208	Positive	3	81400

Figure 4.1. Comparison between the average percent positivity of the C. felis anti-c88 IgM ELISA in cat plasma samples. Average optical absorbance values (presented as PP) between different groups of cats are shown as box and whiskers. When compared to healthy control cats, only cats with acute cytauxzoonosis had significantly higher average PP values ($P < 0.0001$). The average PP values were not significantly different in cats with non-*C. felis* illnesses or *C. felis* chronic carriers.

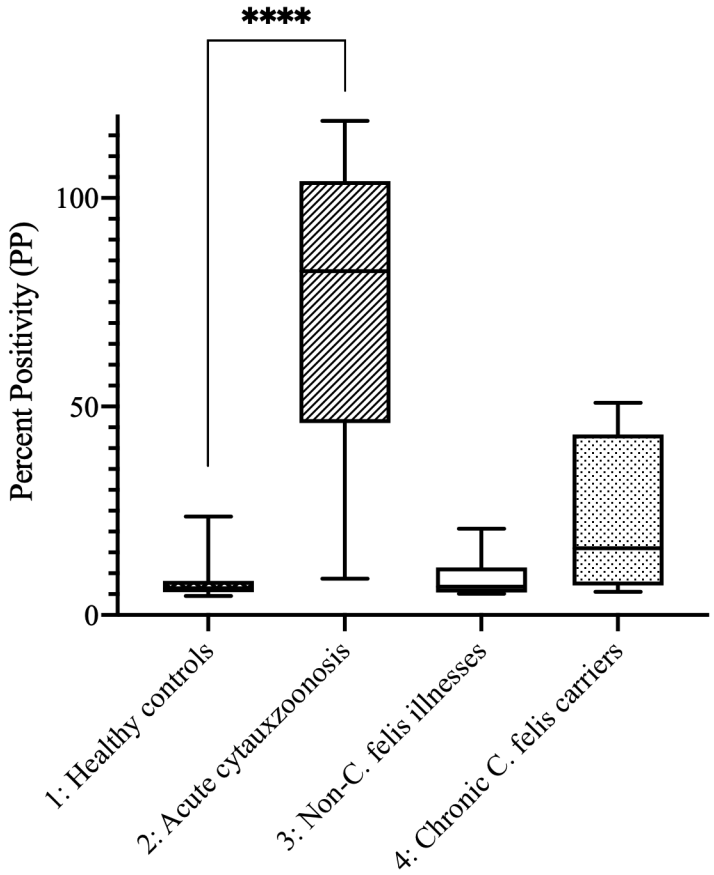


Figure 4.2. Individual PP values from the *C. felis* anti-c88 IgM ELISA from cats in different study groups. When the cut-off PP value was set at 28.05 (the pink line), The ELISA can confidently detect infection in 86.2% (25/29) of the cats in group 2. No false positives were noted in either group 1 or group 3 cats. Samples with PP above 28.05 are considered "Strong Positives". When the cut-off PP value was lowered to 8.35 (the blue line), all cats in group 2 could be detected. Samples with PP value between 8.35 – 28.05 are considered "Weak Positives". Group 1: healthy control cats; Group 2: cats with acute cytauxzoonosis; Group 3: cats with non-*C. felis* illnesses; Group 4: chronic *C. felis* carriers; Each dot represents a different sample; The thin black line in each group represents the mean PP values within the group; The pink line represents the cut-off PP value with 100% specificity and 86.21% sensitivity. The blue line represents the cut-off PP value with 75.86% specificity and 100% sensitivity.

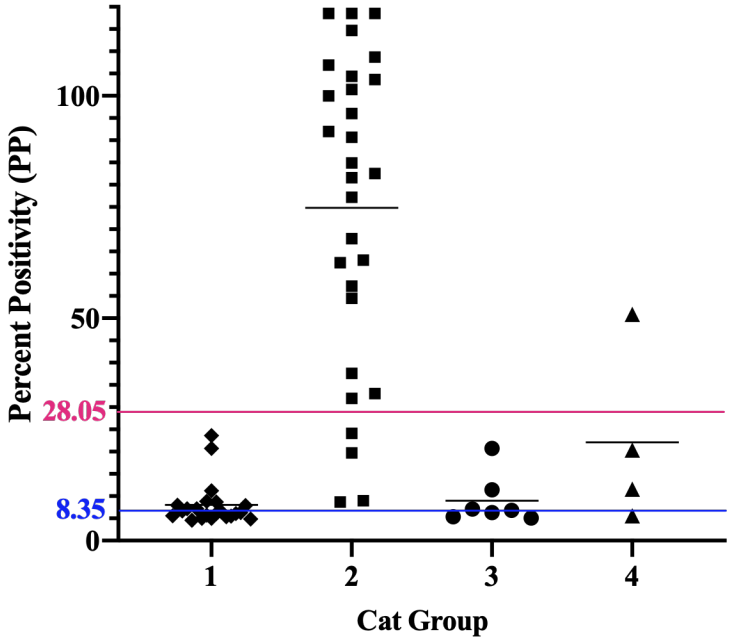
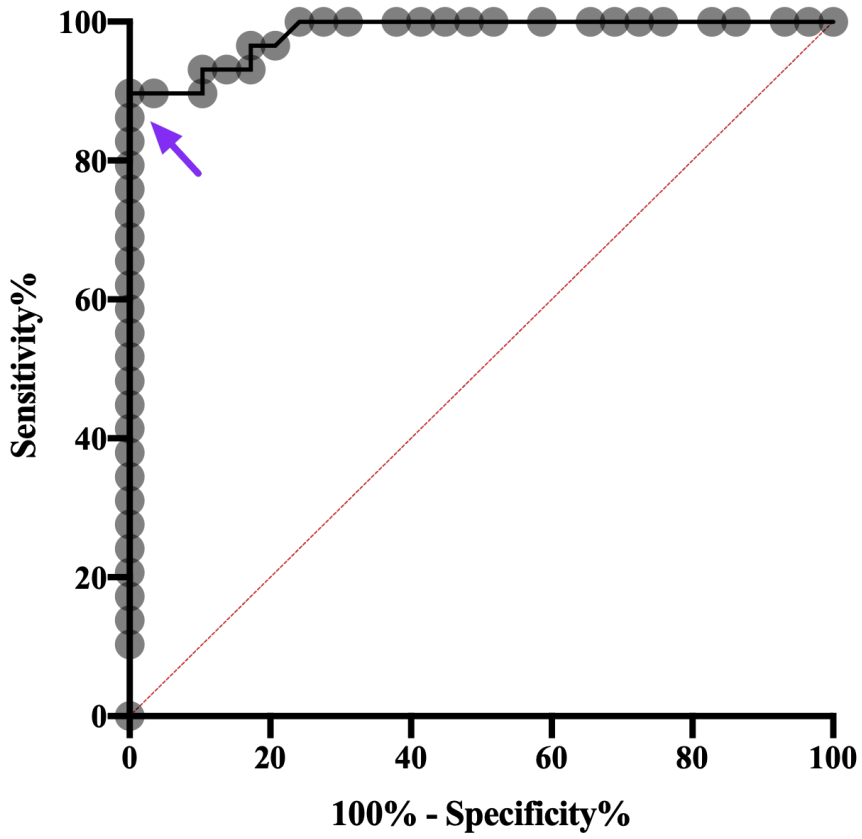


Figure 4.3. Receiver operating characteristic curve of the *C. felis* anti-c88 IgM ELISA. The receiver operating characteristic curve demonstrates the performance of the ELISA in differentiating cats with or without acute cytauxzoonosis (the chronic carrier cats were not included in this analysis). The AUC (area under the curve) was 0.9828 at 95% confidence interval (0.9584 – 1.000). The arrow indicated the cut-off for strong positive results (PP > 28.05) with 100% specificity and 86.21% sensitivity.



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APPENDICES

Appendice 1. Sample submission form

Contact information:

Clinic name:	Clinic/veterinarian phone #:
Treating veterinarian:	Clinic/veterinarian email:

General information:

Owner name:	Sex: ☐ M/I ☐ F/I ☐ M/C ☐ F/S
Cat name:	Time point of blood collection: ☐ day 0 ☐ day 3 ☐ day 10
Age: y/o	The first treatment was administered at _____ (time) on _____ date
Breed:	Is the cat dead? ☐ yes ☐ no; if yes, the cat ☐ arrested ☐ was euthanized at _____ (time) on _____ date

History: *(Please leave this section blank for day 3 and day 10 blood sample submissions)*

Duration of illness prior to presentation:
Living environment: ☐ indoors ☐ outdoors (please check both if both apply)
Tick/flea prevention? ☐ yes ☐ no; if yes, answer the three subsequent questions: • What kind of preventative was used? _____ • How often was it applied? _____ • When was it last applied? _____
Other housemates? ☐ yes ☐ no; if yes, answer the subsequent questions: • What species and how many? _____ • Have any been ill recently? _____

Vital Parameters: *(Please record the most recent parameters relevant to this blood collection)*

Body temperature:	Heart Rate:
Respiratory Rate:	Body Weight:

Physical Examination: *(Please record the most recent findings relevant to this blood collection)*

☐ Lymphadenomegaly ☐ Splenomegaly ☐ Hepatomegaly ☐ Icterus ☐ Pallor ☐ Respiratory distress ☐ Muscle pain ☐ Others _____

Laboratory Abnormalities: *(If performed; please attach all laboratory results with medical records and send to Dr. Eva Kao at yunfank@okstate.edu)*

CBC: ☐ Anemia ☐ Thrombocytopenia ☐ Thrombocytosis
☐ Leukocytosis
☐ Leukopenia ☐ Neutrophilia ☐ Neutropenia ☐ Other abnormalities: _____
Biochemistry abnormalities: _____
Electrolyte abnormalities: _____
FIV/FeLV: ☐ FIV positive ☐ FeLV positive ☐ Both negative ☐ Unknown
Coagulation abnormalities: _____
Urinalysis abnormalities: _____

Please include this submission form with the blood sample submitted each time. Feel free to contact Dr. Eva Kao (yunfank@okstate.edu) if you have any questions!

Appendice 2. Formula of ELISA percent positivity [a]

ELISA percent positivity (PP) = OD_{450} of sample / OD_{450} of positive control × 100%

OD_{450}, optic density (absorbance) at 450 nm

[a] Wright PF, Nilsson E, Van R, et al. Standardization and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev Sci Tech 1993;12:435-450.

VITA

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