

## ABSTRACT

TARIGO, JAIME. The *Cytauxzoon felis* Genome: A Guide To Vaccine Candidate Antigen Discovery For Cytauxzoonosis. (Under the direction of Dr. Adam Birkenheuer, Chair, and Dr. Gregg Dean, Vice Chair).

Cytauxzoonosis is an emerging tick transmitted disease of domestic cats (*Felis catus*) caused by the apicomplexan protozoan parasite *Cytauxzoon felis*. There are currently no effective means to prevent cytauxzoonosis, and even with treatment costing thousands of dollars, up to 40% of cats still succumb. First described in 1976, the geographic range of *C. felis* is expanding and it has now been diagnosed in domestic cats in one third of US states. The high mortality, growing epidemic and cost of care point to vaccination as the most practical control strategy. Prior studies documenting the development of a protective immune response against *C. felis* imply that vaccine development is feasible. Unfortunately, the causative agent has yet to be cultured continuously *in vitro*, rendering traditional vaccine development approaches beyond reach. To overcome these limitations, we sequenced, assembled, and annotated the entire 9.1 Mbp *C. felis* genome and identified approximately 4,300 protein-coding genes, each of which represents a potential protective antigen. Here we report the use of comparative apicomplexan genomics to computationally and experimentally interpret the *C. felis* genome to identify novel candidate vaccine antigens for cytauxzoonosis. We used three bio-informatic strategies to accelerate vaccine antigen discovery for cytauxzoonosis.

Whole genome alignment revealed considerable conserved synteny with other apicomplexan relatives. In particular, alignments with the bovine parasite *Theileria parva* revealed that a *C. felis* gene, cf76, is syntenic to p67 (the leading vaccine candidate for bovine Theileriosis), despite a lack of significant sequence similarity. Recombinant subdomains of cf76 were challenged with survivor-cat antiserum and found to be highly seroreactive. Geographically diverse samples demonstrated 91-100% amino acid sequence identity across cf76. Transcription of cf76 was documented in the schizogenous stage of parasite replication, the life stage that is believed to be the most important for development of a protective immune response. Collectively, these data point to identification of the first potential vaccine candidate antigen for cytauxzoonosis.

We identified and assessed *C. felis* orthologues to leading vaccine candidates from closely related genera for recognition by the feline immune system. Recombinant *C. felis* orthologues to the *Theileria* spp. antigens Tp2 and TaD, and the *Plasmodium* spp. antigens thrombospondin related adhesive protein (TRAP, also known as thrombospondin related anonymous protein and surface sporozoite protein 2 [SSP2]), and apical membrane antigen 1 (AMA-1) were challenged with hyperimmune sera and *C. felis* AMA-1 was found to be mildly seroreactive. Given the importance of AMA-1 as a vaccine candidate for the causative agent of malaria, *P. falciparum*, our data provides promising evidence that *C. felis* AMA-1 may also represent a vaccine candidate for cytauxzoonosis.

Finally, we report the first application of heterologous protein microarray immunoscreening across related genera. We screened hyperimmune sera from *C. felis* survivors using a pre-fabricated microchip containing 500 *P. falciparum* antigens which are known to induce a humoral immune response in humans. Sera from *C. felis* survivors demonstrated significant serologic cross-reactivity against five *P. falciparum* antigens compared to naive cat sera. Recombinant *C. felis* orthologues to these antigens were challenged with hyperimmune sera and one was found to be highly seroreactive. This novel approach allowed for the rapid identification of a previously uncharacterized *C. felis* antigen which represents a new vaccine candidate for cytauxzoonosis.

These bioinformatic strategies emphasize the use of comparative genomics and proteomics as an accelerated path to antigen discovery for vaccine development against experimentally intractable pathogens.

© Copyright 2013 by Jaime Tarigo

All Rights Reserved

The *Cytauxzoon felis* Genome: A Guide To Vaccine Candidate Antigen Discovery  
For Cytauxzoonosis

by  
Jaime Tarigo

A dissertation submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

Comparative Biomedical Science

Raleigh, North Carolina

2013

APPROVED BY:

---

Adam Birkenheuer  
Committee Chair

---

Gregg Dean  
Vice Chair

---

David Bird

---

Michael Levy

## **BIOGRAPHY**

Jaime received her Bachelor's of Science Degree from the State University of New York at Binghamton, Doctor of Veterinary Medicine from the University of Georgia, and completed a residency in clinical pathology at North Carolina State University.

## TABLE OF CONTENTS

LIST OF TABLES.....	v
LIST OF FIGURES .....	vii
LITERATURE REVIEW .....	1
INTRODUCTION.....	1
TAXONOMY.....	2
LIFE CYCLE.....	5
HOSTS.....	8
VECTORS.....	12
EPIDEMIOLOGY.....	15
EXPERIMENTAL PATHOGENESIS OF CYTAUXZONOSIS.....	20
CLINICAL AND PATHOLOGIC FINDINGS IN NATURALLY OCCURRING CYTAUXZONOSIS.....	23
TREATMENT .....	29
IMMUNITY AND VACCINE DEVELOPMENT.....	32
REFERENCES.....	33
The <i>Cytauxzoon felis</i> genome: A guide to vaccine candidate antigen discovery for Cytauxzoonosis .....	41
ABSTRACT .....	41
INTRODUCTION.....	42
MATERIALS AND METHODS .....	43
RESULTS AND DISCUSSION.....	45

CONCLUSIONS.....	49
REFERENCES.....	50
The <i>Cytauxzoon felis</i> genome: A novel candidate vaccine for Cytauxzoonosis inferred from comparative apicomplexan genomics.....	
	52
ABSTRACT .....	52
INTRODUCTION.....	54
MATERIALS AND METHODS .....	56
RESULTS AND DISCUSSION.....	62
CONCLUSIONS.....	73
REFERENCES.....	74
The <i>Cytauxzoon felis</i> genome: Identification of <i>C. felis</i> orthologues to leading vaccine candidates of related apicomplexans.....	
	77
ABSTRACT .....	77
INTRODUCTION.....	78
MATERIALS AND METHODS .....	81
RESULTS AND DISCUSSION.....	84
CONCLUSIONS.....	90
REFERENCES.....	91
The <i>Cytauxzoon felis</i> genome: Characterization of vaccine candidate antigens identified by heterologous immunoscreening of <i>Plasmodium falciparum</i> protein microarrays .....	
	94
ABSTRACT .....	94

INTRODUCTION.....	96
MATERIALS AND METHODS .....	98
RESULTS AND DISCUSSION.....	102
CONCLUSIONS.....	109
REFERENCES.....	110
CONCLUSIONS.....	112
APPENDICES.....	113
APPENDIX A. <i>CYTAUXZOOM FELIS</i> LIFESTAGES.....	114
APPENDIX B. NATURAL AND ABBERANT HOSTS OF <i>C. FELIS</i> ....	117
APPENDIX C. TRANSMISSION OF <i>C. FELIS</i> - EXPERIMENTAL STUDIES.....	128

## LIST OF TABLES

The *Cytauxzoon felis* genome: A guide to vaccine candidate antigen discovery for Cytauxzoonosis.

Table 1.	Sequence assembly of <i>Cytauxzoon felis</i> genomic and cDNA ..	45
Table 2.	Comparison of gene predictions of the <i>Cytauxzoon felis</i> genome with related apicomplexans.....	46

The *Cytauxzoon felis* genome: A novel candidate vaccine for Cytauxzoonosis inferred from comparative apicomplexan genomics.

Table 1.	Primer sequences for amplification of cf76.....	58
----------	---	----

The *Cytauxzoon felis* genome: Identification of *C. felis* orthologues to leading vaccine candidates of related apicomplexans.

Table 1.	PCR primers for amplification of <i>C. felis</i> orthologues. ....	82
Table 2.	<i>Cytauxzoon felis</i> orthologues to vaccine candidates for <i>Theileria</i> spp. and <i>Plasmodium</i> spp .....	84
Table 3.	Signal peptide, transmembrane domain and GPI anchor motifs in <i>C. felis</i> orthologues.....	85
Table 4.	Apparent and expected molecular masses of <i>C. felis</i> orthologues .....	86

The *Cytauxzoon felis* genome: Characterization of vaccine candidate antigens identified by heterologous immunoscreening of *Plasmodium falciparum* protein microarrays.

Table 1.	PCR primers for amplification of <i>C. felis</i> orthologues .....	100
Table 2.	Identification of five <i>C. felis</i> orthologues to <i>P. falciparum</i> proteins seroreactive to <i>C. felis</i> survivor serum.....	103
Table 3.	Molecular mass of <i>C. felis</i> orthologues detected on Western blot.....	107

## LIST OF FIGURES

### LITERATURE REVIEW

Figure 1.	Distribution of Cytauxzoonosis in the United States .....	1
Figure 2A.	Life cycle of <i>Cytauxzoon felis</i> .....	7
Figure 2B.	Widespread parasitic thrombus formation in the acute schizogenous phase of cytauxzoonosis .....	8
Figure 3.	Approximate distribution of the lone star tick, <i>Amblyomma americanum</i> .....	13
Figure 4.	<i>Cytauxzoon felis</i> merozoites.....	24
Figure 5.	<i>Cytauxzoon felis</i> schizonts .....	25

The *Cytauxzoon felis* genome: A guide to vaccine candidate antigen discovery for Cytauxzoonosis.

Figure 1.	<i>Cytauxzoon felis</i> genome browser (GBrowse) .....	47
Figure 2.	Four way Venn Diagram: Protein coding genes of <i>Cytauxzoon felis</i> , <i>Babesia bovis</i> , <i>Theileria parva</i> , and <i>Plasmodium falciparum</i> ....	48

The *Cytauxzoon felis* genome: A novel candidate vaccine for Cytauxzoonosis inferred from comparative apicomplexan genomics.

Figure 1.	Conserved gene synteny between <i>T. parva</i> p67 and <i>C. felis</i> cf76.....	62
Figure 2.	GeneMark <i>in silico</i> prediction of <i>C. felis</i> cf76.....	63

Figure 3.	Polymerase chain amplification of predicted cf76 intron and exon junctions .....	64
Figure 4.	Assessment of purified cf76 and cf76 fragments by Western blot.....	65
Figure 5.	Assessment of feline sero-reactivity to cf76 and cf76 fragments by Western Blot.....	66
Figure 6.	Amino acid sequences of syntenic gene cf76 from geographic isolates across the southeastern and southwestern United States .....	68
Figure 7.	<i>In situ</i> hybridization to identify transcription of cf76 in <i>C.felis</i> -infected lung tissue .....	72

The *Cytauxzoon felis* genome: Identification of *C. felis* orthologues to leading vaccine candidates of related apicomplexans.

Figure 1.	Assessment of purified <i>C. felis</i> orthologues by Western blot....	87
Figure 2.	Assessment of feline sero-reactivity to <i>C. felis</i> orthologues by Western blot .....	88
Figure 3.	Assessment of feline sero-reactivity to <i>C. felis</i> orthologues by Immuno-dot blot .....	88
Figure 4.	Additional assessment of feline sero-reactivity to <i>C. felis</i> orthologues by Western blot.....	89

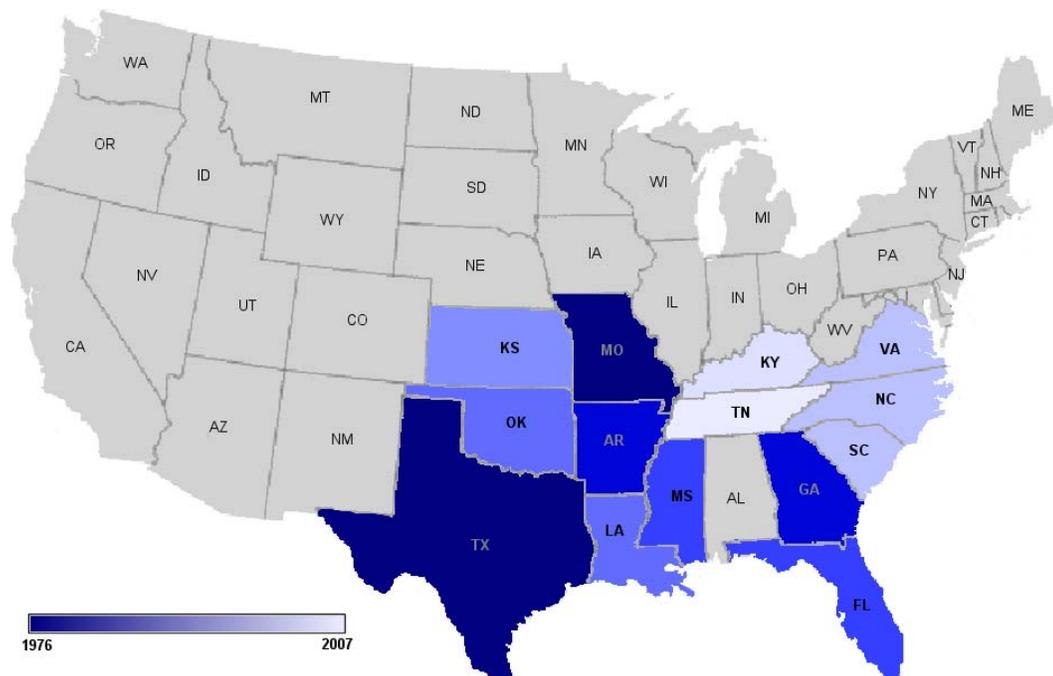
The *Cytauxzoon felis* genome: Characterization of vaccine candidate antigens identified by heterologous immunoscreening of *Plasmodium falciparum* protein microarrays.

Figure 1. Seroreactivity of <i>C. felis</i> immune sera against <i>P. falciparum</i> proteins.....	102
Figure 2. Assessment of purified <i>C. felis</i> orthologues by Western blot.....	107
Figure 3. Assessment of feline sero-reactivity to <i>C. felis</i> orthologues by Western blot.....	108
Figure 4. Assessment of feline sero-reactivity to <i>C. felis</i> orthologues by Immuno-dot blot.....	108

## LITERATURE REVIEW

### INTRODUCTION

Cytauxzoonosis is a life-threatening emerging infectious disease of domestic cats [*Felis catus*] caused by the tick-transmitted protozoan parasite *Cytauxzoon felis*<sup>1-6</sup>. Since its discovery in Missouri in the mid-1970s, the distribution of *C. felis* has been expanding. Cytauxzoonosis is now diagnosed in domestic cats in 35% of the states in the continental USA with *C. felis* as a newly recognized infection in six of these states in the last five years (Figure 1)<sup>1,6-14</sup>. Anecdotal reports of *C. felis* infection in domestic cats in additional states include Alabama, southern Illinois (2003) and Ohio (2008).



**Figure 1. Distribution of Cytauxzoonosis in the United States.**

It is transmitted by the Lone Star Tick [*Amblyomma americanum*] to domestic cats, the most common aberrant host<sup>15</sup>. The natural host for *C. felis* is thought to be the bobcat [*Lynx rufus*]<sup>16</sup> and reservoir hosts of the parasite include bobcats and domestic cats that survive infection.

## **TAXONOMY**

The first case of *C. felis* infection in a domestic cat in 1973 in Missouri was the first report of cytauxzoonosis in the U.S. and was thought to be the first known infection of a carnivore with a parasite from within the Cytauxzoon genus. Later it was discovered that felids are the only species infected with Cytauxzoon spp.

*Cytauxzoon* spp was initially reported in 1948 in the African gray duiker [*Sylvicapra grimmia*]<sup>17</sup> and has been documented in several ungulates indigenous to Africa including kudu [*Tragelaphus strepsiceros*]<sup>18</sup>, eland [*Taurotragus oryx pattersonianunus*]<sup>19</sup>, giraffe [*Giraffa camelopardalis*]<sup>20</sup>, roan antelope [*Hippotragus equines*], sable antelope [*Hippotragus niger*]<sup>21</sup>, and spring bok [*Antidorcas marsupialis*]<sup>16</sup>. Further molecular characterization of infections in kudu, the African gray duiker, giraffe, sable antelope, and roan antelope identified the etiologic agent to be *Theileria* spp<sup>22,23</sup>.

*C. felis* is classified within the phylum Apicomplexa, class Sporozoea, order Piroplasmida, and family Theileridae. The Apicomplexans consist of over 5000 species of organisms, most of which undergo three distinct phases of replication

(sporogony, merogony, and gametogony) and have infective forms which share in common a complex of organelles at their apical end critical for host invasion, hence the name 'Api' 'complexan'. Members of the family Theileridae include two genera, *Cytauxzoon* spp and *Theileria* spp and share in common an intra and extra-erythrocytic phase of organism. Cytauxzoon has a notably unique life cycle in mammalian hosts shared by only two other genera, *Theileria* spp and *Plasmodium* spp, implying that that each might be used as a model to facilitate discoveries relevant to treatment and prevention of the others.

*C. felis* is most closely related to *Theileria* spp. with 18s ribosomal RNA sequence differentiation ranging from 5.52-7.90% across different species<sup>24</sup>. Similar data for *Babesia* spp. has reported 18s rRNA sequence differentiations between 8.66-14.32% with *C. felis*<sup>24,25</sup>. Recent molecular characterizations have resulted in some dispute about the taxonomic status of *Cytauxzoon* and synonymization with *Theileria* has been suggested<sup>22</sup>. Several major differences in pathogenesis provide a strong argument for maintenance in separate genus. Sporozoite infected cells differ in origin and in the number of sporozoites infecting the host cell. A single *C. felis* sporozoite infects a cell of mononuclear phagocytic origin whereas *Theileria* spp. sporozoites infect cells of lymphoid origin. *Theileria* uniquely induces lymphoblastogenesis with division of schizonts into daughter cells via mitosis, a reversible phenomenon with treatment that is not seen with *Cytauxzoon*. *Cytauxzoon* schizonts adhere to the lining of small blood vessels resulting formation

of massive numbers of disseminated parasitic thrombi which does not occur with *Theileria* infection <sup>26</sup>. In 2013, a study evaluating mitochondrial genome sequences resulted in improved phylogenetic analyses of *Piroplasmida* and reported that *C. felis* was in a clade with other *Theileria* species, but may represent a distinct clade as more *Cytauxzoon* spp. *cox1* sequences are characterized <sup>27</sup>.

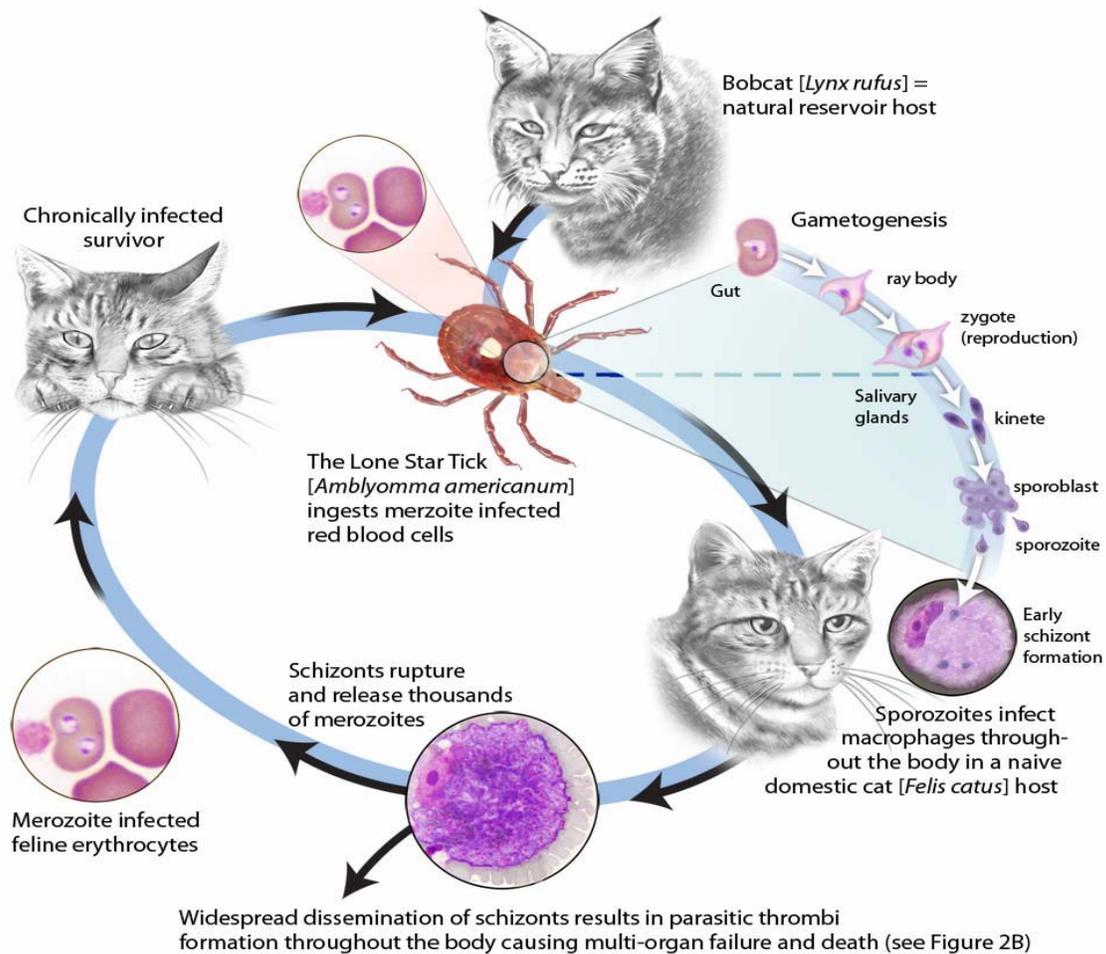
Comparative sequence analysis of *C. felis* 18S rRNA from geographically diverse regions reveals a high degree of homology. In a subset of samples collected from 1998-2006 nearly full length *C. felis* 18S rRNA (~1.7 kilobases) sequences from four cats in NC, SC, and VA were ~99.9% homologous to sequences previously reported for cats that died and survived of cytauxzoonosis (GenBank accession Nos. L19080 and AF399930) <sup>1</sup>. Analyses of rRNA is commonly used for sequence comparisons however they do not always detect differentiation between closely related species, subspecies, or strains <sup>28,29</sup>. The rRNA subunits are highly conserved under the constraint of maintaining structure to preserve coding for functional RNA molecules. Some studies have included comparisons of the first and second internal transcriber spacer regions of the rRNA operon (ITS1, ITS2) which are in non-coding regions and may be more likely to reflect variability across *C. felis* isolates. Potential variability in these regions may or may not correlate with variability of other traits of the parasite, for example pathogenicity.

In 2005 sequence comparisons of the small subunit ribosomal RNA (ssu-RNA) gene and internal transcribed spacer-1 (ITS-1) gene for 1,362 field-collected ticks in Missouri reported minimal sequence variation<sup>30</sup>. Polymerase chain reactions (PCR) were performed on pooled samples from *A. americanum* (764 adults and 65 nymphs), *D. variabilis* (293 adults), and *R. sanguineus* (157 adults) collected in Missouri and only 3 *A. americanum* nymphs tested positive (3 ssu-RNA PCR positive, 2 of the 3 ITS-1 PCR positive). Greater than 99.8% ssu-RNA sequence similarity was reported between each sequence and previously published sequence (Genbank L19080). One hundred percent homology for ITS-1 gene was seen between the two positive nymphs and with a blood derived sequence (GenBank AY158898) with the exception of 2 bases (of 474bp) that were ambiguous in the sequence data. Given the use of pooled samples, high sequence similarity reported in this study may not reflect polymorphisms in different gene cassettes as has been reported in *Babesia* spp.<sup>31,32</sup>.

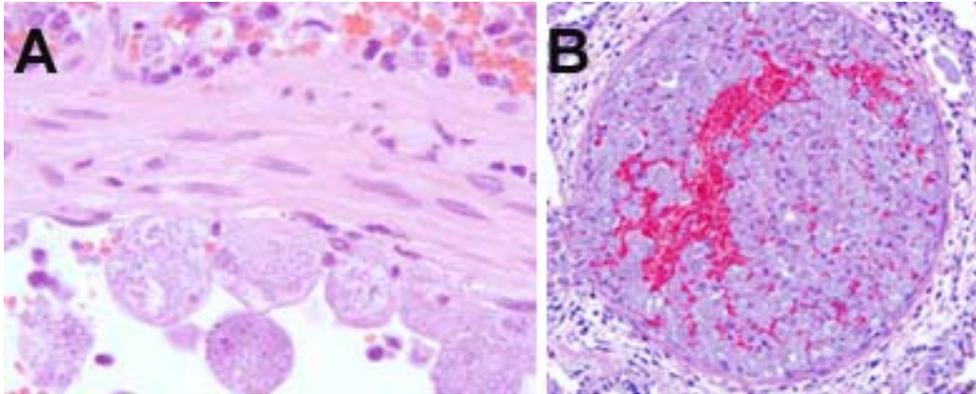
## **LIFE CYCLE**

*C. felis* has a complicated life cycle with three life stages in the mammalian host: sporozoites, schizonts, and merozoites (Figure2a). Infection begins with sporozoites which are transmitted to the bobcat or domestic cat by a tick vector, the Lone Star Tick [*Amblyomma americanum*]<sup>15</sup>. Sporozoites then infect feline mononuclear phagocytes (myeloid dendritic cells or hematopoietic macrophages<sup>33</sup>) throughout the body and form schizonts that fill and expand the host cell cytoplasm increasing

cell size from ~12um to up to 250um. Schizonts adhere to the endothelial lining of organs throughout the body forming parasitic thrombi resulting in ischemia, necrosis and multi-organ failure (Figure 2B). The wide spread dissemination of schizonts results in a typically fatal acute tissue phase of disease. Schizonts then rupture, releasing merozoites which enter erythrocytes via endocytosis within a parasitophorous vacuole leaving no notable damage to the erythrocyte membrane <sup>26</sup>. If the host survives the acute schizogenous phase, a chronic, fairly innocuous erythroparasitemia ensues. The arthropod vector will then ingest merozoites from the chronically parasitemic host and gametogony and sporogony occur to form infective sporozoites in the salivary gland which will be transmitted upon feeding on a mammalian host. Several descriptive studies on the ultrastructure, immunophenotype, and histopathology of sporozoites, schizonts, and merozoites are summarized in Appendix A.



**Figure 2. Life cycle of *Cytauxzoon felis*.** The acute tissue stage of disease (the schizogenous phase) is characterized by wide spread dissemination of schizonts which form parasitic thrombi throughout the body resulting in a disease course that is typically fatal. Hosts that survive this acute tissue phase develop a chronic yet fairly innocuous erythroparasitemia with merozoite-infected red cells.



**Figure 2B. Widespread parasitic thrombus formation in the acute schizogenous phase of cytauxzoonosis.** *Cytauxzoon felis* schizonts adhere to the vascular endothelium (A) and result in vascular occlusion throughout the body (B).

## HOSTS

### Natural Host: The Bobcat [*Lynx rufus*]

In the 1980s, experimental studies substantiated the bobcat as the natural reservoir host of *C. felis*<sup>16,34,35</sup>. Once infected, bobcats typically remain clinically asymptomatic during a transient schizogenous phase and then become chronically parasitemic<sup>16,26,34,36,37</sup>. Natural infection in bobcats was suspected when parasitemia ranging between 0.5-5% with *C. felis*-like piroplasms was detected in clinically asymptomatic wild-trapped bobcats in Oklahoma<sup>37,38</sup>. Although most bobcats remain clinically asymptomatic, mild to moderate clinicopathologic changes have been reported. A moderate regenerative anemia was seen in one bobcat with a 5% parasitemia and lower red blood cell counts, calcium, and albumin levels, and higher glucose and total proteins levels were seen in one group of infected bobcats

suggesting that stress (hyperglycemia) and immune-mediated changes (anemia, hyperglobulinemia) may occur in chronic infection <sup>37</sup>.

Three cases of fatal cytauxzoonosis in bobcats have been reported including one with natural infection <sup>39</sup> and two with experimental infection <sup>36,40</sup>. A free ranging bobcat cub presented moribund to the Veterinary Teaching Hospital at Kansas State University and histopathologic lesions consistent with acute cytauxzoonosis were confirmed post-euthanasia <sup>39</sup>. Two fatal infections in bobcats resulted after experimental transmission of *C. felis* by *D. variabilis* ticks or parenteral inoculation with virulent *C. felis* tissues from a domestic cat. Atypical vulnerability to acute cytauxzoonosis in these bobcats may be secondary to lack of immunocompetence (particularly in the cub), strain virulence, dose and/or route of experimental inoculum. It is possible that free-ranging bobcats infected with *C. felis* may become ill more frequently than we recognize.

### **Aberrant Host: The Domestic Cat**

*C. felis* was first reported in domestic cats by J.E. Wagner in Missouri in 1976 <sup>14</sup>. Four fatal cases of cytauxzoonosis were identified, one in 1973 from a household in which three other cats had died previously with similar clinical signs, a second in 1974 followed by two more cases in 1975. Since then, as previously noted, cytauxzoonosis is now diagnosed in 35% of the states of the continental U.S. (Figure 1). Without treatment less than 1% of domestic cats survive and intensive and costly

treatment results in a 60% survival rate at best <sup>1,41</sup>. Of over 500 experimentally infected domestic cats, only four have survived cytauxzoonosis <sup>5,42,43</sup>.

Interestingly, domestic cats surviving infection without treatment have been reported on rare occasion <sup>44,45</sup>. A study of *C. felis* in northwestern Arkansas and northeastern Oklahoma indicated survival of natural infection in 18 cats with and without treatment. Fourteen cats presented with acute cytauxzoonosis, one received an anti-parasiticide (imidocarb dipropionate) and the remaining cats were treated with supportive care and antimicrobials. It was noted that the cats in this study seemed 'less sick' initially, did not have temperatures exceeding 106 degrees Fahrenheit, and never became hypothermic. Similar sporadic reports in other areas exist. Some hypotheses for survival in these cats have included the following: 1) an atypical route of infection, 2) innate immunity in certain cats, 3) increased detection of carriers, 4) decreased virulence with strain attenuation or occurrence of a new strain, 5) lower dose of infectious inoculum, 6) timing and type of treatment, and 7) mechanical inoculation of the merozoite stage inducing chronic parasitemia in the absence of the acute tissue stage.

### **Other Wild Felids**

Cytauxzoonosis has been reported in several wild felids in the U.S. and other countries with both fatal and non-fatal outcomes. Prevalence and consequence of *C.*

*felis* infection in wild felids will be important to monitor for future protection, especially of endangered species.

In the U.S., *C. felis* infection has been confirmed in tigers [*Panthera tigris*] and Florida panthers [*Puma concolor coryi*]<sup>25,46,47</sup>. Two suspected but unconfirmed cases of cytauxzoonosis have been reported in cheetahs [*Acinonyx jubatus*] however, infection with small piroplasms of Babesia sp. and Theileria-like origin has been reported in cheetahs and cannot be excluded<sup>48,49</sup>. Two reports of infection with other small piroplasms that are morphologically indistinguishable from, and may be misdiagnosed as *C. felis* include *B. pantherae* in Florida Panthers and Texas Cougars [*Puma concolor*], and *C. manul* in Pallus Cats [*Otocolobus manul*] imported from Mongolia<sup>24,25,50</sup>. Molecular characterization is recommended when piroplasmiasis is documented to ensure accurate identification of the etiologic agent.

Several other countries have reported *C. felis* infection in wild felids including: 1) Brazil (lions [*Panthera leo*], jaguars [*Panthera onca*], pumas [*Puma concolor*], ocelots [*Leopardus pardalis*], and little spotted cats [*Leopardus tigrinis*]) and 2) Germany (lions)<sup>51-55</sup>. Epidemiologies of other small piroplasms that infect wild felids is important to consider when identifying *C. felis* to avoid phenotypic misdiagnosis. Reports of *C. felis* in wild felids including discussion of other small piroplasms in the reported regions are summarized in Appendix A.

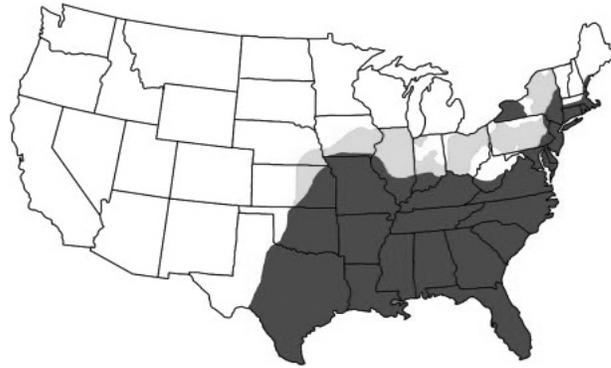
## **Wildlife, Laboratory Animals, and Domestic Farm Animals**

In the early 1980s interspecies transmission of *C. felis* was investigated to identify additional potential natural and aberrant hosts among 91 wildlife, laboratory, and domestic farm animals<sup>40</sup>. Bobcats were the only animals confirmed as hosts of *C. felis*. One Florida bobcat developed fatal cytauxzoonosis and an Eastern bobcat developed chronic parasitemia in the absence of overt disease. Inoculation with tissues from either bobcat induced fatal cytauxzoonosis in domestic cats. A low parasitemia was detected in two clinically asymptomatic sheep, however tissues from these animals did not induce disease in domestic cats and artifact or infection with a different small piroplasm cannot be excluded. Details of this study are summarized in Appendix A.

## **VECTORS**

### **Natural Transmission**

Early studies suggested that the American Dog Tick [*Dermacentor variabilis*] was the arthropod vector of *C. felis* however, based on geographic distribution of potential tick vectors and the distribution of cytauxzoonosis, it was suggested that the Lone Star Tick be investigated as the most likely biologic vector (Figure 3)<sup>8</sup>.



**Figure 3. Approximate distribution of the lone star tick, *Amblyomma americanum*.** Historical and current range shown in dark gray while areas where populations have recently been established are light gray.

Other suggestive evidence existed including reports of *A. americanum* and *A. cajennense* ticks in the presence of confirmed cases of fatal cytauxzoonosis in a tiger in the U.S. (1996) and two lions in Brazil (1998) respectively <sup>46,54</sup>.

*Cytauxzoon felis* infection in the Lone Star Tick was confirmed in 2005 in three partially engorged nymphs recovered from a fatal case of acute cytauxzoonosis in a domestic cat <sup>30</sup>. At that time, it was uncertain whether the nymphs ingested pre-existing merozoites in circulation from the cat or whether they were a biologic vector. In 2009 and 2010 two studies demonstrated transmission of *C. felis* to a total of five domestic cats by *A. americanum* in the absence of transmission by other tick vectors under the same conditions (including *D. variabilis*), substantiating that the Lone Star Tick [*A. americanum*] is likely to be the main arthropod vector for *C. felis* under natural conditions <sup>15,56</sup>. The latter study also determined the minimum infection rate

of *C. felis* in wild-collected *A. americanum* ticks from an enzootic area to be 0.5% (1/178) in males, 0.8% (3/393) in nymphs and 1.5% (3/197) in females while infection was not detected in any wild-collected *D. variabilis* ticks (n = 160) further supporting *A. americanum* as the main biologic vector for *C. felis*<sup>56</sup>.

### **Experimental Transmission**

Early experimental *C. felis* transmission studies documented the following: 1) fatal *C. felis* infection can be transmitted from a chronically infected splenectomized bobcat by *D. variabilis* to splenectomized domestic cats, 2) *D. variabilis* can transmit a nonfatal *C. felis* infection from bobcat to bobcat, 3) parenteral inoculation of domestic cats with bobcat tissues lacking schizonts results in chronic parasitemia in the absence of acute and fatal disease, 4) parenteral inoculation of domestic cats with schizont containing bobcat tissues induces fatal cytauxzoonosis, 5) fatal *C. felis* infection can be transmitted to naive domestic cats with inoculation of tissues from cats with acute cytauxzoonosis by all routes of parenteral administration (subcutaneous, intravenous, intraperitoneal) but not through oral administration, 6) *C. felis* was not transmitted through intercat exposure, 7) *C. felis* is transmitted transstadially (between lifestages) of the tick vector, 8) fatal *C. felis* infection has been induced by iatrogenic transmission from a Florida Panther to a domestic cat, 9) transmission of *C. manul* merozoites from Pallas Cats to domestic cats resulted in a nonfatal persistent parasitemia in the absence of overt disease, and 10) *C. felis* was successfully transmitted to naive domestic cat by *A. americanum* in the absence of

transmission by *D. variabilis*, *Rhipicephalus sanguineus*, *Ixodes scapularis* ticks under the same conditions suggesting that these are not likely vectors of *C. felis* under natural conditions<sup>5,15,16,26,34-36,57-59</sup>. Detailed summaries of these experimental studies are presented in Appendix A. In 2012, a study failed to document perinatal transmission of *C. felis* from two chronically infected dams to 14 healthy kittens in three litters<sup>60</sup>. The results from this study do not exclude the potential for vertical transmission of *C. felis*, however the results suggest that it is not likely to occur commonly.

## **EPIDEMIOLOGY**

*Cytauxzoon felis* was reported in domestic cats in Missouri, Texas, Georgia, Arkansas, Mississippi, Florida, Louisiana, and Oklahoma through the mid 1980s and then in Kentucky, Kansas, Tennessee, North Carolina, South Carolina, and Virginia by 2008<sup>1,6,9-14,61,62</sup>. The geographic distribution of *C. felis* is likely to continue its expansion and threaten even more cats as the most likely primary vector, the lone star tick, extends its distribution further north and east (Figure 3)<sup>8,15,56</sup>.

*Cytauxzoon felis* infection was also reported in domestic cats in Brazil and Spain however all of the Brazilian cats tested negative by PCR<sup>63-65</sup>. In 2009, *C. felis* infection was reported to be present in 11 of 50 (22%) stray cats from Mosul Iraq however bloodsmears, tissue touch imprints, and biopsies presented to diagnose *C. felis* in this study are inconclusive in the authors opinion<sup>66</sup>. In 2012, *Cytauxzoon* sp.

infection with was reported to be present in 19 of 63 (30.2%) of colony cats and 5 of 52 (9.6%) of owned cats tested by PCR in Trieste Italy<sup>67</sup>. The 18S rRNA gene sequences were reported to be 99% identical to GenBank sequences for previously *Cytauxzoon* sp. infections in Europe in four Iberian lynx and two domestic cats<sup>55,63,68</sup>.

In 2007, the prevalence of *C. felis* in 961 healthy free-roaming domestic cats in three states (FL, NC, TN) was reported to be 0.3%<sup>10</sup>. Surveys in Brazil reported remarkably higher prevalence in 33 stray domestic cats from a colony which rose from 15.8% in 2002 to 48.5% in 2004; it is likely that the area surveyed represented a high-risk 'hyper-endemic foci of *C. felis*'<sup>64,65</sup>. In 2010, prevalence of *C. felis* infection in asymptomatic cats residing specifically in high risk areas for *C. felis* exposure in Arkansas and Georgia was reported to be 41.9% and 19.6% respectively<sup>69</sup>.

The earliest bobcat surveys reported circulating *C. felis*-like piroplasms in 61.9% (13/21) and 31.2% (5/16) of clinically asymptomatic wild-trapped bobcats in Oklahoma in 1982 and 1985 respectively<sup>37,38</sup>. In 2008, the prevalence of *C. felis* in wild-trapped bobcats, in NC and PA was reported to be 33% (10/30) and 7% (5/70) respectively and this was the first report of infection in bobcats in PA where cytauxzoonosis is not [yet] recognized in domestic cats<sup>8</sup>. In 2010, the prevalence of *C. felis* in bobcats residing specifically in high risk areas for *C. felis* exposure in

Arkansas, Florida, and Georgia was 25.6%<sup>69</sup>. In 2011, the prevalence of *C. felis* in bobcats residing in areas with high *A. americanum* presence including MO, NC, OK, SC, KY, FL and KS was 79%, 63%, 60%, 57%, 55%, 44%, and 27% respectively. In that same study the the prevalence of *C. felis* in bobcats residing in areas with low or no presence of *A. americanum* including GA, ND, OH, WV, CA, and CO was 9%, 2.4%, 0%, 0%, 0%, and 0% respectively<sup>70</sup>. Variation in reported prevalence rates of *C. felis*, may be due to epidemiologic variation, differential vector presence, and/or to specific sensitivity levels of the PCR testing methods used.

Several temporal and environmental risk factors for cytauxzoonosis have been documented. It is typically diagnosed during April through September which correlates with climate dependent seasonal activity of the Lone Star Tick vector. A study in Oklahoma assessing risk factors for cytauxzoonosis in 232 cases reported a bimodal pattern of occurrence with a peak in April, May, and June, followed by a second smaller peak in August and September<sup>3</sup>. Cats at highest risk for infection include those living near heavily wooded, low density residential areas particularly closest to natural or unmanaged habitats where both ticks and bobcats may be in close proximity<sup>1-3</sup>. Presence of 'hyperendemic foci' has been suggested based on the occurrence of large numbers of cases reported by certain clinics and the high frequency of multiple cases occurring within the same household<sup>1,44,71</sup>. Recently, two different approaches to ecological and niche modeling were reported to

determine the potential distribution of *C. felis* in three states where infection is common<sup>72</sup>.

### **Molecular Epidemiology**

In 2009, genetic variability of ITS1 and ITS2 regions of *C. felis* from 88 cats with acute cytauxzoonosis in Arkansas and Georgia was reported and correlated with survival outcome<sup>69</sup>. The following findings were reported: 1) ITS1 sequences revealed 8 single nucleotide polymorphisms (SNPs) and one single nucleotide insertion, 2) ITS2 sequences contained 4 SNPs and one 40bp nucleotide insertion, 3) a total of 11 different sequences and 3 unique genotypes (ITSA, ITSB- present only in GA, ITSC- present only in AR), 4) presence of either co-infection with two genotypes or multiple rRNA genes with polymorphic unit based on two nucleotide substitutions at the same position in 14 cats, and 5) survival rates of 79.2% (38/48), 19% (4/21), 0% (0/5) for cats with ITSA, ITSB, and ITSC respectively. The author's concluded that ITS genotype may correlate with pathogenicity of *C. felis* isolates. However, in 2010, the same author's reported the same three ITS genotypes in 61 asymptomatic *C. felis* infected domestic cats from AR and GA with a distribution similar distribution to the initial study and concluded that evaluation of the ITS region did not appear to correlate with pathogenicity of *C. felis*<sup>69</sup>. In samples collected from 25 bobcats, eleven different ITS1 and ITS2 sequence types were reported, three of which have been reported in domestic cats and eight which have not<sup>69</sup>. The most common ITS sequence found in bobcats, ITSg, had not been detected in domestic

cats, however ITSg samples were collected from bobcats in a region of northern Florida where samples from domestic cats had not been collected and it was also the region from which the highest number of bobcat samples originated. A recent study in 2012 investigated the intraspecific variation of the ITS-1 and ITS-2 rRNA regions from 139 bobcats and 6 pumas from 11 southcentral and southeastern states and found that while 43.8 and 45% of ITS-1 and ITS-2 sequences respectively from bobcats were identical to those previously reported in domestic felines, the remainder of sequences were unique. Five different genotypes were identified in the bobcats and pumas and similarly to previous reports ITSa was the most common genotype <sup>73</sup>.

In 2011, a comprehensive study reporting efficacy of combined atovaquone and azithromycin in the treatment of acute cytauxzoonosis also compared ITS sequence and geographic location with survival and found that: 1) despite previous findings of certain genotypes (ITSa/ITSc) correlated with lower pathogenicity than other genotypes (ITSC), it is unlikely that ITS genotype can predict pathogenicity of *C. felis* <sup>41,69,74,75</sup>, and 2) similar proportions of cats from 5 different states suffered nonfatal versus fatal disease suggesting that there is a lack of evidence for association between geographic location and virulence of *C. felis*.

## EXPERIMENTAL PATHOGENESIS OF CYTAUXZONOSIS

Following the initial report of cytauxzoonosis in the U.S., a large study involving experimental infection of domestic cats with *C. felis* was conducted by the Animal and Plant Health Inspection Service (APHIS) and the Plum Island Animal Disease Center (PIADC) of the United States Department of Agriculture (USDA)<sup>5,76</sup>. Over 500 cats were parenterally inoculated with blood, triturates of homogenized spleen, liver, and lung, or lymph homogenates from *C. felis* infected cats. Onset of clinical signs including anorexia and depression occurred 5-7 days post-infection (PI). Body temperature increased gradually for 3-4 days to as high as 106 degrees Fahrenheit and then decreased to euthermic or hypothermic levels. Cats died between 9-15 days PI on average with the longest survival of 20 days PI. On necropsy and histopathology changes included: 1) splenic enlarged and hemorrhage with marked erythrophagocytosis in red pulp 2) petechiation and hemorrhage of lymph nodes most prominent in the cervical region, 3) pulmonary hemorrhage and consolidation, and 4) pericardial effusion, 5) icterus, 6) widespread parasitic thrombosis characterized by infected swollen reticuloendothelial cells of the histiocytic series, and 7) up to 4% parasitemia with 1% seen most commonly. All cats in infected with the schizogenous tissue phase of *C. felis* died of acute cytauxzoonosis in this study with the exception of one, "Number 4538". After being "vaccinated" with *C. felis* infected cells that were harvested post-mortem from an experimentally infected cat then "co-cultured" and attenuated with African green monkey kidney cells, 4538 was resistant to infection despite 10 subsequent challenges with virulent inoculums. This

study discovered that solid immunity to *C. felis* can be developed if a cat survives infection with the acute schizogenous tissue stage of cytauxzoonosis.

Comprehensive clinicopathologic data post *C. felis* infection was collected in study of 48 domestic cats inoculated subcutaneously with virulent *C. felis* tissues in 1987<sup>77</sup>.

Findings in this study included: 1) merozoites in erythrocytes 10 days PI with morphology characterized by round, oval, anaplasmod, bipolar (binucleated) and rod shapes that ranged in size from 0.3um width x 0.7um length to 1.2um x 2.4um in size with an occasional maltese cross forms 2) schizonts in low numbers at 12 days PI within germinal centers of secondary and tertiary nodules in lymph nodes and spleen and in the bone marrow ranging in size from 15-20um with high numbers seen widespread intravascularly and occasionally in interstitium 19 days PI increasing in size from 80-250um (uninfected host cells were approximately 10umx11um), 3) onset of fever 14days PI which correlated with a significant increase in parasitemia up to 7% and a decline in leukocytes, 4) onset of clinical signs of depression, lethargy, anorexia, dehydration +/- icterus 16-19 days PI, 5) gross findings of splenomegaly within 1 day PI, mesenteric and popliteal lymphadenopathy with edema as early as 2 days PI, generalized lymphadenopathy by day 12 PI, increase in abdominal veins (mesenteric, renal, posterior vena cava) by three times the normal size by day 19 PI, renomegaly, hepatomegaly, pulmonary edema and congestion, and 6) histopathologic findings of erythrophagocytosis of merozoite containing red blood cells in splenic red pulp by day 15PI, increased

numbers of Kupffer cells with schizonts seen in hepatic cords lining hepatic and central veins by day 12 PI, diffuse vasculitis, thrombosis, perivascular edema, hemosiderosis, and severe congestion and edema of the liver, lungs, and lymph nodes. Parasitemia in this study correlated strongly with rise in temperature, presence of schizonts, and leukopenia. Leukopenia may occur due increased consumption or decreased production secondary to widespread ischemia and necrosis, toxic parasitic byproducts, infection of blast cells in the bone marrow, or suppressive effects of parasitic byproducts in the bone marrow.

In 1988 a smaller study reported hematologic findings in acute cytauxzoonosis following inoculation of 7 cats intraperitoneally with virulent *C. felis* tissue homogenates<sup>78</sup>. Findings included: 1) circulating piroplasms <2% first seen 6-8d PI, 2) anemia (mean PCV 20.2%) onset by 6d PI with a steady decline thereafter, 3) no change in MCV, MCHC, or reticulocytes, 4) hypoproteinemia (mean total protein 6.2g/dl) by 8d PI with no change in fibrinogen, 5) thrombocytopenia (mean  $121 \times 10^3/\text{ul}$ ) by 8d PI with no change seen in PTT or APTT, and 6) lymphopenia (mean  $524 \times 10^3/\text{ul}$ ) and eosinopenia (mean  $16 \times 10^3/\text{ul}$ ). Discrepancies between these findings and findings reported in naturally occurring cytauxzoonosis (ie. normal total leukocyte counts) may be due to type, dose, and route of inoculum generating an accelerated disease course.

## **CLINICAL AND PATHOLOGIC FINDINGS IN NATURALLY OCCURRING CYTAUXZONOSIS**

### **Clinical presentation**

Onset of clinical signs for cats infected with *C. felis* usually occurs 5-14 days (~10 days on average) after infection by tick transmission. In a recent study including 80 cases of acute cytauxzoonosis non-specific signs including lethargy (n=78) and anorexia (60) were most common <sup>41</sup>. Common physical exam findings in this study included hyperthermia (n=78) > 102.5degrees Fahrenheit (mean 104.5 +/-1.15), icterus (31), elevated nictitans (31), dehydration (22), presence of ticks (22), tachypnea >40rpm (20), tachycardia (13), pallor (9), murmur (8), vocalization (5), discomfort on abdominal palpation (5), lymphadenomegaly (5), and splenomegaly (5). Temperature tends to gradually rise and in this study reached as high as 107.0 degrees Fahrenheit. In extremis cats are often hypothermic, dyspneic, and vocalize as if in pain. Without treatment death typically occurs within 2-3 days following peak in temperature.

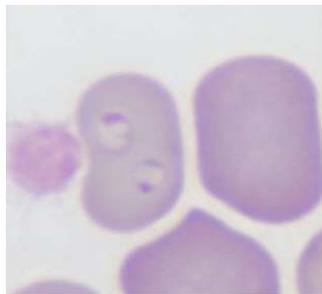
### **Cytologic Findings**

Merozoites. Rapid diagnosis requires microscopic observation of merozoites or schizonts. Observation of merozoites on bloodsmear is variable; they are seen in association with increasing body temperature and typically become apparent approximately 1 to 3 days prior to death. On a well-prepared, well-stained (Wright's Giemsa, Giemsa, Diff-Quik most commonly) bloodsmear, when detectable,

merozoites may be seen ranging from 1 to 4% on average with extremely high percentages up to 25% reported in some cases<sup>9,14,61,79,80</sup>. They are pleomorphic and may be round, oval, anaplasmoid, bipolar (binucleated), or rod-shaped, however the round and oval piroplasm forms are most commonly seen. The round forms are 1.0-2.2  $\mu\text{m}$  in diameter, while oval forms are 0.8-1.0  $\mu\text{m}$  to 1.5-2.0  $\mu\text{m}$ . They are **pale centrally and contain a small magenta round to crescent shaped nucleus on one side**. Once the parasitemia is  $>0.5\%$ , Maltese cross and paired piriforms may be seen.

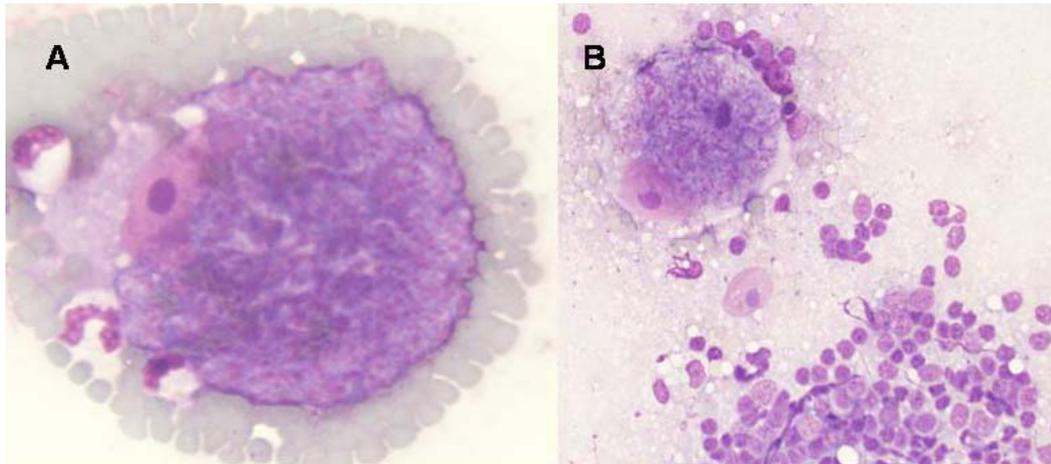
Careful observation by the clinician must be taken to exclude *Mycoplasma haemofelis*, Howell-Jolly bodies, stain precipitate, and water artifact.

Morphologically, intra-erythrocytic piroplasms may be difficult to distinguish between Theileria sp. and small Babesia sp. (*B. felis* and *B. leo*), however there is only one report of feline babesiosis (detected in Florida panthers) in the United States.<sup>25</sup>



**Figure 4. *Cytauxzoon felis* merozoites.** Two *C. felis* merozoites are seen within a feline erythrocyte on peripheral blood smear (100X) of a domestic cat.

Schizonts. The schizont tissue stage precedes the formation of the red blood cell phase. Occasionally, schizonts may be observed in peripheral blood smears, particularly at the feathered edge, and may be mistaken for large platelet clumps at low power. In the absence of detection of red blood cell piroplasms or schizonts on bloodsmear a rapid diagnosis should be pursued by performing fine needle aspiration of a peripheral lymph node, spleen, or liver to identify schizonts cytologically. These phagocytes are 15-250  $\mu\text{m}$  in diameter and contain an ovoid nucleus with a distinctive prominent large dark nucleolus. The cytoplasm is often greatly distended with numerous small deeply basophilic particles representing developing merozoites (Figure 5).



**Figure 5. *Cytosporidium felis* schizonts.** *Cytosporidium felis* schizonts on the feathered edge of a peripheral bloodsmear (**A**, 50X) and in a touch imprint of peripheral lymph node (**B**, 20X) from a domestic cat with acute cytosporidiosis.

## **Clinicopathologic findings**

A comprehensive study assessing clinicopathologic findings in naturally occurring cases of acute cytauxzoonosis reported common abnormalities to include leukopenia (white blood cell counts  $<5 \times 10^3/\text{ul}$ ; 43 of 73), anemia (PCV  $<26\%$ ; 40 of 74), hyperbilirubinemia (t.bili  $>0.5\text{mg/dl}$ ; 37 of 50), hyperglycemia (glucose  $>150\text{mg/dl}$ ; 35 of 55), hypocalcemia (Ca  $<9.0\text{mg/dl}$ ; 32 of 47), and hypoproteinemia (t.solids or t.protein  $<6.0\text{g/dl}$ ; 12 of 51 cats)<sup>41</sup>. Thrombocytopenia was also reported in this study however artifactually low values secondary to platelet clumping could not be excluded. In a retrospective study from 2006 including 34 cases of natural infection with *C. felis* the most common clinicopathologic abnormalities reported were pancytopenia with a mean of 43,100 +/- 30,200 platelets/ul (reference range, 300,000 to 800,000 platelets/ul) and hyperbilirubinemia with a mean of 4.6 +/- 3.7mg/dl (reference range, 0.0 to 0.5mg/dl)<sup>1</sup>.

## **Post-Mortem Findings**

Major findings on necropsy include splenomegaly, hepatomegaly, enlarged lymph nodes, pulmonary edema with petechial hemorrhage and icterus often seen on serosal surfaces<sup>2</sup>. There is progressive venous distension (secondary to diffuse parasitic thrombosis), especially the mesenteric and renal veins and the posterior vena cava. Hydropericardium is often seen with petechial hemorrhage of the epicardium<sup>59</sup>. A severe interstitial pneumonia is seen on microscopic exam characterized by edema and neutrophilic infiltrates that has been suggested to result

in acute respiratory distress syndrome (ARDS) associated with peri-mortem dyspnea seen in many cats <sup>81</sup>.

### **Molecular Diagnostic Testing**

A *C. felis*-specific diagnostic PCR test amplifying a 284bp segment of the 18S rRNA gene sequence was developed in 2006 that is able to detect 10 gene copies of DNA/ul (50copies per PCR reaction) with 100% sensitivity <sup>7</sup>. This test is currently available through the NCSU Vector Borne Diagnostic Disease Laboratory. Reaction components for this assay include: 25pmol each of primer: 5'-GCGAATCGCATTGCTTTATGCT-3' and 5'-CCAAATGATACTCCGGAAAGAG-3', 1X concentration of PCR Buffer II (Applied Biosystems, Foster City, CA), 1.25U of Taq Polymerase, 5ul of DNA template extracted from 200ul of whole using an automated workstation according to manufacturers instructions (QIAmp DNA Blood Mini Kit or Magattract DNA Blood Mini M48 Kit, Qiagen Inc., Valencia, CA), 1.5mM MgCl<sub>2</sub>, and 200uM of each dNTP. Thermal cycling parameters included an initial denaturation at 95°C for 5min, followed by 40 amplification cycles (95°C for 45sec, 59°C for 45sec, and 72°C for 1min) <sup>7</sup>.

### **Experimental Fluorescent Antibody Testing**

An indirect fluorescent antibody test using antiserum to *C. felis*-like parasites on fresh frozen sections of *C. felis* infected spleens was developed in 1978 <sup>76</sup>.

Hyperimmune sera from a domestic cat that survived infection was used as primary

antibody with a fluorescein conjugated rabbit anti-feline IgG secondary antibody on fresh frozen splenic sections from 21 cats euthanized at the terminal stage of cytauxzoonosis in addition to 10 control cats. Indirect fluorescent antibody testing detected *C. felis*-like parasites in all of the infected cat tissue sections and the control cats were negative. Circulating piroplasms were only detected in 64.3% of the *C. felis* infected cats.

A microfluorometric immunoassay to detect serum IgG antibodies to *C. felis* was developed in 1988<sup>82</sup>. Two splenectomized and two non-splenectomized cats were infected with the merozoite stage of *C. felis* and the following results were reported: 1) non-splenectomized and splenectomized cats developed detectable antibodies two and four weeks PI respectively, 2) non-splenectomized cats had lower parasitemia (<6% vs. >30%) and less severe anemia (>23% vs. <13%) than splenectomized cats, 3) non-splenectomized cats developed detectable antibody levels sooner (2weeks vs. 4weeks) than splenectomized cats, 4) non-splenectomized cats developed lower antibody levels than non-splenectomized cats.

An ELISA (enzyme linked immunoabsorbent assay) for use by primary care veterinary facilities would be helpful for early detection but has not yet been developed. Development of an ELISA would involve: 1) identification of *C. felis*-specific antigens that induce an antibody response in the feline host, 2) recombinant production of these antigens given the lack of ability to culture the parasite stages to

date, and 3) inclusion of early life stage antigens (ie of sporozoite and schizont origin). We have identified at least one *C. felis* candidate antigen that fulfills these criteria.

## **TREATMENT**

Several drugs have been investigated for efficacy in treating cytauxzoonosis over the last two decades including parvaquone (Clexon®), buparvaquone (Butalex®), diminazine acetate, imidocarb dipropionate, atovaquone, and azithromycin.

Parvaquone and buparvaquone are naphthoquinones that were investigated as treatments for cytauxzoonosis because they are effective against *Theileria* spp., the closest relative to *Cytauxzoon* spp.<sup>83,84</sup>. Buparvaquone is a second-generation hydroxynaphthoquinone and although its mechanism of action is not well established, it has been shown to cause parasite-specific degenerative changes on electron microscopy in bovine theileriosis. Seventeen cats were experimentally infected with a virulent *C. felis* splenic inoculum and were treated with 20 or 30mg/kg of parvaquone (n=7) or 5 or 10mg/kg of buparvaquone (n=8) once daily beginning on the first or second day of detectable parasitemia; two cats were left untreated<sup>42</sup>. Administration of supportive care (i.e. heparin, intravenous fluid therapy) was not addressed. One untreated cat and one cat receiving the higher dose of parvaquone survived and both cats were immune to subsequent challenge with virulent *C. felis* inoculum. This study concluded that at the doses and time intervals used,

parvaquone and buparvaquone were not effective treatments for acute cytauxzoonosis.

Diminazene aceturate and imidocarb proprionate were investigated because of their effectiveness against *Babesia* spp., another apicomplexan hematoprozoan related to *Cytauxzoon* spp.<sup>85-87</sup>. Diminazine aceturate is an aromatic diamidine analog that inhibits parasite DNA synthesis by binding mitochondrial topoisomerase II and kinetoplast DNA rich in adenosine and thymidine nucleotide bases<sup>88</sup>. Imidocarb is a related aromatic diamidine with similar mechanisms of action. In the 1990s, 7 naturally occurring cases of acute cytauxzoonosis presenting to the University of Georgia's Veterinary Teaching Hospital were treated either with 2mg/kg diminazene aceturate IM (6cats) or 2mg/kg imidocarb proprionate IM (1cat); each cat was given one injection at presentation and a second injection 3-7days later. Supportive therapy including heparin (100-150 U/kg subcutaneously q. 8hrs) and intravenous fluids were also administered. Five of the 6 cases treated with diminazine and the one case treated with imidocarb were in critical stages of acute cytauxzoonosis as indicated by presence of disseminated intravascular coagulation (DIC). Five of the 6 cases treated with diminazine and the one case treated with imidocarb survived.

This study concluded that diminazine aceturate or imidocarb prorionate may be used as treatments for acute cytauxzoonosis and further studies assessing efficacy were needed. However, diminazine aceturate is not currently approved for use in the United States. In a recent study, the ability of diaminazene diacetate to eliminate

parasitemia was assessed and at a dose of 3mg/kg given IM twice seven days apart, the drug was unable to eliminate or reduce parasite burden <sup>89</sup>.

The largest and most successful clinical trial to date investigating the treatment of acute cytauxzoonosis was reported in 2010 comparing the efficacy of imidocarb dipropionate versus atovaquone co-administered with azithromycin <sup>41</sup>. Atovaquone is a ubiquinone analog that binds cytochrome b (complex III) and selectively decreases electron transport decreasing adenosine triphosphate (ATP) and pyrimidine synthesis. Azithromycin is a macrolide antimicrobial that blocks prokaryotic protein synthesis binding the 50S rRNA subunit. In apicomplexans, it has been suggested that antimicrobials effective in eukaryotes may be due to targeting of a primitive, the apicoplast thought to have been obtained via endosymbiosis with green algae <sup>90</sup>. The combination therapy of the antimalarial drug atovaquone with the antibacterial drug azithromycin was investigated because of successful management of other hematoparasites refractory to individual treatments <sup>91,92</sup>. In addition, preliminary data from an uncontrolled trial treating naturally infected cats with atovaquone and azithromycin suggested efficacy as well as a reduction in parasitemia in chronically infected cats compared with imidocarb <sup>93,94</sup>. In this study, 80 cats from 5 states (MO, TN, NC, AR, OK) with acute cytauxzoonosis were randomly assigned to receive either atovaquone (15mg/kg PO q. 8hrs) and azithromycin (10mg/kg PO q. 24hrs) or imidocarb (3.5mg/kg IM) <sup>41</sup>. Patients also received heparin (200U/kg SQ q.8hrs when indicated), IV fluid therapy, and

supportive care. Survival rate was significantly higher in cats treated with atovaquone and azithromycin at 60.4% (32/53) compared with 25.9% (7/27) when treated with imidocarb. This study demonstrated that combination therapy using atovaquone and azithromycin is currently the most effective treatment for acute cytauxzoonosis. This study also reported that cats with lower parasitemia were more likely to survive regardless of treatment type.

## **IMMUNITY AND VACCINE DEVELOPMENT**

Early experimental *C. felis* studies discovered that solid immunity to *C. felis* is obtained if a cat survives infection with the acute schizogenous tissue stage of cytauxzoonosis<sup>5,76</sup>. Of over 500 experimentally infected cats, the four that have survived were immune to subsequent challenges with virulent inoculum<sup>5,42,43</sup>. This has been supported anecdotally to date as there have been no reports of disease reoccurrence in survivors of fulminant cytauxzoonosis despite new infections in naive animals in the same area and occasionally within the same household. This suggests that survivors of natural infection are likely being re-exposed but are protected against *C. felis*. Importantly, these findings provide evidence that **vaccine development is possible and the schizont specific antigens are a rationale target for vaccine candidate antigen discovery.**

## REFERENCES

1. Birkenheuer AJ, Le JA, Valenzisi AM, et al. Cytauxzoon felis infection in cats in the mid-Atlantic states: 34 cases (1998-2004). *J Am Vet Med Assoc* 2006;228:568-571.
2. Meinkoth JH, Kocan AA. Feline cytauxzoonosis. *Vet Clin North Am Small Anim Pract* 2005;35:89-101, vi.
3. Reichard MV, Baum KA, Cadenhead SC, et al. Temporal occurrence and environmental risk factors associated with cytauxzoonosis in domestic cats. *Vet Parasitol* 2008;152:314-320.
4. Cowell RL, Panciera RJ, Fox JC, et al. Feline Cytauxzoonosis. *Compendium Small Animal* 1988;10:731-736.
5. Ferris DH. A progress report on the status of a new disease of American cats: cytauxzoonosis. *Comp Immunol Microbiol Infect Dis* 1979;1:269-276.
6. Kier A, Morehouse L, Wagner J. Feline cytauxzoonosis: An update. *Missouri Vet* 1979;29:15-18.
7. Birkenheuer AJ, Marr H, Alleman AR, et al. Development and evaluation of a PCR assay for the detection of Cytauxzoon felis DNA in feline blood samples. *Vet Parasitol* 2006;137:144-149.
8. Birkenheuer AJ, Marr HS, Warren C, et al. Cytauxzoon felis infections are present in bobcats (*Lynx rufus*) in a region where cytauxzoonosis is not recognized in domestic cats. *Vet Parasitol* 2008;153:126-130.
9. Glenn BL, Stair EL. Cytauxzoonosis in domestic cats: report of two cases in Oklahoma, with a review and discussion of the disease. *J Am Vet Med Assoc* 1984;184:822-825.
10. Haber MD, Tucker MD, Marr HS, et al. The detection of Cytauxzoon felis in apparently healthy free-roaming cats in the USA. *Vet Parasitol* 2007;146:316-320.
11. Hauck WN, Snider TG, 3rd, Lawrence JE. Cytauxzoonosis in a native Louisiana cat. *J Am Vet Med Assoc* 1982;180:1472-1474.
12. Jackson CB, Fisher T. Fatal cytauxzoonosis in a Kentucky cat (*Felis domesticus*). *Vet Parasitol* 2006;139:192-195.

13. Meier HT, Moore LE. Feline cytauxzoonosis: a case report and literature review. *J Am Anim Hosp Assoc* 2000;36:493-496.
14. Wagner JE. A fatal cytauxzoonosis-like disease in cats. *J Am Vet Med Assoc* 1976;168:585-588.
15. Reichard MV, Meinkoth JH, Edwards AC, et al. Transmission of *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum*. *Vet Parasitol* 2009;161:110-115.
16. Glenn BL, Kocan AA, Blouin EF. Cytauxzoonosis in bobcats. *J Am Vet Med Assoc* 1983;183:1155-1158.
17. Neitz WO, Thomas AD. *Cytauxzoon sylvicaprae* gen. nov., spec. nov., a protozoon responsible for a hitherto undescribed disease in the duiker, *Sylvicapra grimmia* (Linne). *Onderstepoort J Vet Sci Anim Ind* 1948;23:63-76.
18. Neitz WO. Theileriosis, gonderiosis, and cytauxzoonosis: A review. *Onderstepoort J Vet Res* 1957;27:275-430.
19. DW B. *Cytauxzoon taurotragi*, a piroplasm of the eland (*Taurotragus oryx pattersonianus*). *Res Vet Sci* 1962;3:334-344.
20. McCully RM, Keep ME, Basson PA. Cytauxzoonosis in a giraffe (*Giraffa camelopardalis* (Linnaeus, 1758)) in Zululand. *Onderstepoort J Vet Res* 1970;37:7-9.
21. Wilson DE, Bartsch R.C., Bigalke R.D., et al. Observations on mortality rates and disease in roan and sable antelope on nature reserves in the Transalvaal. *J S Afr Wildl Manage Assoc* 1974;4:203-206.
22. Nijhof AM, Pillay V, Steyl J, et al. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. *J Clin Microbiol* 2005;43:5907-5911.
23. Oosthuizen MC, Allsopp BA, Troskie M, et al. Identification of novel *Babesia* and *Theileria* species in South African giraffe (*Giraffa camelopardalis*, Linnaeus, 1758) and roan antelope (*Hippotragus equinus*, Desmarest 1804). *Vet Parasitol* 2009;163:39-46.
24. Ketz-Riley CJ, Reichard MV, Van den Bussche RA, et al. An intraerythrocytic small piroplasm in wild-caught Pallas's cats (*Otocolobus manul*) from Mongolia. *J Wildl Dis* 2003;39:424-430.

25. Yabsley MJ, Murphy SM, Cunningham MW. Molecular detection and characterization of *Cytauxzoon felis* and a *Babesia* species in cougars from Florida. *J Wildl Dis* 2006;42:366-374.
26. Kocan AA, Kocan KM, Blouin EF, et al. A redescription of schizogony of *Cytauxzoon felis* in the domestic cat. *Ann N Y Acad Sci* 1992;653:161-167.
27. Downey M, Marr H, Tarigo J, et al. Mitochondrial genome sequences result in improved phylogenetic analyses of Piroplasmida. *Parasitology* 2013;In submission.
28. Birkenheuer AJ, Breitschwerdt EB, Alleman AR, et al. Differentiation of *Haemobartonella canis* and *Mycoplasma haemofelis* on the basis of comparative analysis of gene sequences. *Am J Vet Res* 2002;63:1385-1388.
29. Fox GE, Wisotzkey, J.D., Jurtshuk, P. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* 1992;42:166-170.
30. Bondy PJ, Jr., Cohn LA, Tyler JW, et al. Polymerase chain reaction detection of *Cytauxzoon felis* from field-collected ticks and sequence analysis of the small subunit and internal transcribed spacer 1 region of the ribosomal RNA gene. *J Parasitol* 2005;91:458-461.
31. Dalrymple BP. Cloning and characterization of the rRNA genes and flanking regions from *Babesia bovis*: use of the genes as strain discriminating probes. *Mol Biochem Parasitol* 1990;43:117-124.
32. Reddy GR, Chakrabarti D, Yowell CA, et al. Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in erythrocyte culture. *Nucleic Acids Res* 1991;19:3641-3645.
33. Susta L, Torres-Velez F, Zhang J, et al. An in situ hybridization and immunohistochemical study of cytauxzoonosis in domestic cats. *Vet Pathol* 2009;46:1197-1204.
34. Blouin EF, Kocan AA, Glenn BL, et al. Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). *J Wildl Dis* 1984;20:241-242.
35. Kier AB, Wagner JE, Morehouse LG. Experimental transmission of *Cytauxzoon felis* from bobcats (*Lynx rufus*) to domestic cats (*Felis domesticus*). *Am J Vet Res* 1982;43:97-101.

36. Blouin EF, Kocan AA, Kocan KM, et al. Evidence of a limited schizogonous cycle for *Cytauxzoon felis* in bobcats following exposure to infected ticks. *J Wildl Dis* 1987;23:499-501.
37. Glenn BL, Rolley RE, Kocan AA. Cytauxzoon-like piroplasms in erythrocytes of wild-trapped bobcats in Oklahoma. *J Am Vet Med Assoc* 1982;181:1251-1253.
38. Kocan AA, Blouin EF, Glenn BL. Hematologic and serum chemical values for free-ranging bobcats, *Felis rufus* (Schreber), with reference to animals with natural infections of *Cytauxzoon felis* Kier, 1979. *J Wildl Dis* 1985;21:190-192.
39. Nietfeld JC, Pollock C. Fatal cytauxzoonosis in a free-ranging bobcat (*Lynx rufus*). *J Wildl Dis* 2002;38:607-610.
40. Kier AB, Wightman SR, Wagner JE. Interspecies transmission of *Cytauxzoon felis*. *Am J Vet Res* 1982;43:102-105.
41. Cohn LA, Birkenheuer AJ, Brunker JD, et al. Efficacy of atovaquone and azithromycin or imidocarb dipropionate in cats with acute cytauxzoonosis. *J Vet Intern Med* 25:55-60.
42. Motzel SL, Wagner JE. Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. *Vet Parasitol* 1990;35:131-138.
43. Uilenberg G, Franssen FF, Perie NM. Relationships between *Cytauxzoon felis* and African piroplasmids. *Vet Parasitol* 1987;26:21-28.
44. Meinkoth J, Kocan AA, Whitworth L, et al. Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997-1998). *J Vet Intern Med* 2000;14:521-525.
45. Walker DB, Cowell RL. Survival of a domestic cat with naturally acquired cytauxzoonosis. *J Am Vet Med Assoc* 1995;206:1363-1365.
46. Garner MM, Lung NP, Citino S, et al. Fatal cytauxzoonosis in a captive-reared white tiger (*Panthera tigris*). *Vet Pathol* 1996;33:82-86.
47. Lewis KM, Cohn LA, Downey ME, et al. Evaluation of *Cytauxzoon felis* infection status in captive-born wild felids housed in an area endemic for the pathogen. *J Am Vet Med Assoc* 241:1088-1092.

48. Bosman AM, Oosthuizen MC, Peirce MA, et al. *Babesia lengau* sp. nov., a novel *Babesia* species in cheetah (*Acinonyx jubatus*, Schreber, 1775) populations in South Africa. *J Clin Microbiol* 48:2703-2708.
49. Zinkl JG, McDonald SE, Kier AB, et al. Cytauxzoon-like organisms in erythrocytes of two cheetahs. *J Am Vet Med Assoc* 1981;179:1261-1262.
50. Dennig HK, Brocklesby DW. *Babesia pantherae* sp.nov., a piroplasm of the leopard (*Panthera pardus*). *Parasitology* 1972;64:525-532.
51. Andre MR, Adania CH, Machado RZ, et al. Molecular detection of Cytauxzoon spp. in asymptomatic Brazilian wild captive felids. *J Wildl Dis* 2009;45:234-237.
52. Jakob W, Wesemeier HH. A fatal infection in a Bengal tiger resembling cytauxzoonosis in domestic cats. *J Comp Pathol* 1996;114:439-444.
53. Millan J, Naranjo V, Rodriguez A, et al. Prevalence of infection and 18S rRNA gene sequences of Cytauxzoon species in Iberian lynx (*Lynx pardinus*) in Spain. *Parasitology* 2007;134:995-1001.
54. Peixoto PV, Soares CO, Scofield A, et al. Fatal cytauxzoonosis in captive-reared lions in Brazil. *Vet Parasitol* 2007;145:383-387.
55. Luaces I, Aguirre E, Garcia-Montijano M, et al. First report of an intraerythrocytic small piroplasm in wild Iberian lynx (*Lynx pardinus*). *J Wildl Dis* 2005;41:810-815.
56. Reichard MV, Edwards AC, Meinkoth JH, et al. Confirmation of *Amblyomma americanum* (Acari: Ixodidae) as a vector for *Cytauxzoon felis* (Piroplasmorida: Theileriidae) to domestic cats. *J Med Entomol* 47:890-896.
57. Butt MT, Bowman D, Barr MC, et al. Iatrogenic transmission of *Cytauxzoon felis* from a Florida panther (*Felis concolor coryi*) to a domestic cat. *J Wildl Dis* 1991;27:342-347.
58. Joyner PH, Reichard MV, Meinkoth JH, et al. Experimental infection of domestic cats (*Felis domesticus*) with *Cytauxzoon manul* from Pallas' cats (*Otocolobus manul*). *Vet Parasitol* 2007;146:302-306.
59. Wagner JE, Ferris DH, Kier AB, et al. Experimentally induced cytauxzoonosis-like disease in domestic cats. *Vet Parasitol* 1980;6:305-311.

60. Lewis KM, Cohn LA, Birkenheuer AJ. Lack of evidence for perinatal transmission of *Cytauxzoon felis* in domestic cats. *Vet Parasitol* 188:172-174.
61. Wightman SR, Kier AB, Wagner JE. Feline cytauxzoonosis: Clinical features of a newly described blood parasite disease. *Feline Prac* 1977;7:23-26.
62. Bendele RA, Schwartz WL, Jones LP. Cytauxzoonosis-like disease in Texas cats. *The Southwest Veterinarian* 1976;29:244-246.
63. Criado-Fornelio A, Gonzalez-del-Rio MA, Buling-Sarana A, et al. The "expanding universe" of piroplasms. *Vet Parasitol* 2004;119:337-345.
64. Mendes-de-Almeida F, Faria MC, Branco AS, et al. Sanitary conditions of a colony of urban feral cats (*Felis catus* Linnaeus, 1758) in a zoological garden of Rio de Janeiro, Brazil. *Rev Inst Med Trop Sao Paulo* 2004;46:269-274.
65. Mendes-de-Almeida F, Labarthe N, Guerrero J, et al. Follow-up of the health conditions of an urban colony of free-roaming cats (*Felis catus* Linnaeus, 1758) in the city of Rio de Janeiro, Brazil. *Vet Parasitol* 2007;147:9-15.
66. Suliman EG. Detection the infection with *Babesia* spp. *Cytauxzoon felis* and *Haemobaronella felis* in stray cats in Mosul. *Iraqi Journal of Veterinary Sciences* 2009;23:49-55.
67. Carli E, Trotta M, Chinelli R, et al. Cytauxzoon sp. infection in the first endemic focus described in domestic cats in Europe. *Vet Parasitol* 183:343-352.
68. Millan J, Candela MG, Palomares F, et al. Disease threats to the endangered Iberian lynx (*Lynx pardinus*). *Vet J* 2009;182:114-124.
69. Brown HM, Berghaus RD, Latimer KS, et al. Genetic variability of *Cytauxzoon felis* from 88 infected domestic cats in Arkansas and Georgia. *J Vet Diagn Invest* 2009;21:59-63.
70. Shock BC, Murphy SM, Patton LL, et al. Distribution and prevalence of *Cytauxzoon felis* in bobcats (*Lynx rufus*), the natural reservoir, and other wild felids in thirteen states. *Vet Parasitol* 175:325-330.
71. Greene CE, Latimer K, Hopper E, et al. Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. *J Am Vet Med Assoc* 1999;215:497-500, 482.

72. Mueller EK, Baum KA, Papes M, et al. Potential ecological distribution of *Cytauxzoon felis* in domestic cats in Oklahoma, Missouri, and Arkansas. *Vet Parasitol* 192:104-110.
73. Shock BC, Birkenheuer AJ, Patton LL, et al. Variation in the ITS-1 and ITS-2 rRNA genomic regions of *Cytauxzoon felis* from bobcats and pumas in the eastern United States and comparison with sequences from domestic cats. *Vet Parasitol* 190:29-35.
74. Beare PA, Chen C, Bouman T, et al. Candidate antigens for Q fever serodiagnosis revealed by immunoscreening of a *Coxiella burnetii* protein microarray. *Clin Vaccine Immunol* 2008;15:1771-1779.
75. Brown HM, Modaresi SM, Cook JL, et al. Genetic variability of archived *Cytauxzoon felis* histologic specimens from domestic cats in Georgia, 1995-2007. *J Vet Diagn Invest* 2009;21:493-498.
76. Shindel N, Dardiri AH, Ferris DH. An indirect fluorescent antibody test for the detection of *Cytauxzoon*-like organisms in experimentally infected cats. *Can J Comp Med* 1978;42:460-465.
77. Kier AB, Wagner JE, Kinden DA. The pathology of experimental cytauxzoonosis. *J Comp Pathol* 1987;97:415-432.
78. Franks PT, Harvey JW, Shields RP, et al. Hematologic Findings in Experimental Feline *Cytauxzoonosis*. *J Am Anim Hosp Assoc* 1988;24:395-401.
79. Hoover JP, Walker DB, Hedges JD. *Cytauxzoonosis* in cats: eight cases (1985-1992). *J Am Vet Med Assoc* 1994;205:455-460.
80. Simpson CF, Harvey JW, Carlisle JW. Ultrastructure of the intraerythrocytic stage of *Cytauxzoon felis*. *Am J Vet Res* 1985;46:1178-1180.
81. Snider TA, Confer AW, Payton ME. Pulmonary histopathology of *Cytauxzoon felis* infections in the cat. *Vet Pathol* 47:698-702.
82. Cowell RL, Fox JC, Panciera RJ, et al. Detection of anticytauxzoon antibodies in cats infected with a *Cytauxzoon* organism from bobcats. *Vet Parasitol* 1988;28:43-52.
83. Hawa N, Rae DG, Younis S, et al. Efficacy of parvaquone in the treatment of naturally occurring theileriosis in cattle in Iraq. *Trop Anim Health Prod* 1988;20:130-136.

84. Muraguri GR, Ngumi PN, Wesonga D, et al. Clinical efficacy and plasma concentrations of two formulations of buparvaquone in cattle infected with East Coast fever (*Theileria parva* infection). *Res Vet Sci* 2006;81:119-126.
85. McHardy N, Woollon RM, Clampitt RB, et al. Efficacy, toxicity and metabolism of imidocarb dipropionate in the treatment of *Babesia ovis* infection in sheep. *Res Vet Sci* 1986;41:14-20.
86. Penzhorn BL, Lewis BD, de Waal DT, et al. Sterilisation of *Babesia canis* infections by imidocarb alone or in combination with diminazene. *J S Afr Vet Assoc* 1995;66:157-159.
87. Schwint ON, Ueti MW, Palmer GH, et al. Imidocarb dipropionate clears persistent *Babesia caballi* infection with elimination of transmission potential. *Antimicrob Agents Chemother* 2009;53:4327-4332.
88. Peregrine A.S. MM. Pharmacology of diminazene: a review. . *Acta Tropica* 1993;54:185-203.
89. Lewis KM, Cohn LA, Marr HS, et al. Diminazene Diacetate for Treatment of Chronic *Cytauxzoon felis* Parasitemia in Naturally Infected Cats. *J Vet Intern Med* 26:1490-1493.
90. Fichera ME, Roos DS. A plastid organelle as a drug target in apicomplexan parasites. *Nature* 1997;390:407-409.
91. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Efficacy of combined atovaquone and azithromycin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. *J Vet Intern Med* 2004;18:494-498.
92. Krause PJ, Lepore T, Sikand VK, et al. Atovaquone and azithromycin for the treatment of babesiosis. *N Engl J Med* 2000;343:1454-1458.
93. Birkenheuer AJ, Cohn LA, Levy MG, et al. Atovaquone and azithromycin for the treatment of *Cytauxzoon felis*. *J Vet Intern Med* 2008;22.
94. Cohn LA, Birkenheuer AJ, Ratcliff ER. Comparison of two drug protocols for clearance of *Cytauxzoon felis* infections. *J Vet Intern Med* 2008;22.

## **The *Cytauxzoon felis* genome: A guide to vaccine candidate antigen discovery for Cytauxzoonosis.**

### **ABSTRACT**

Cytauxzoonosis is an emerging infectious disease of domestic cats (*Felis catus*) caused by the apicomplexan protozoan parasite *Cytauxzoon felis*. The growing epidemic, with its high morbidity and mortality points to the need for a protective vaccine against Cytauxzoonosis. Unfortunately, the causative agent has yet to be cultured continuously *in vitro*, rendering traditional vaccine development approaches beyond reach. To overcome these limitations we sequenced, assembled, and annotated the *C. felis* genome and the proteins it encodes. Whole genome alignment revealed considerable conserved synteny with other apicomplexans including *Theileria*, *Plasmodium*, and *Babesia* spp. Here we report the *C. felis* genome and propose the use of comparative genomics to computationally and experimentally interpret the *C. felis* genome to identify candidate vaccine antigens.

## INTRODUCTION

*Cytauxzoon felis* is a protozoan parasite of felids that causes cytauxzoonosis, an emerging disease in domestic cats. Without treatment nearly all cats die within three to five days of the onset of clinical symptoms. There are currently no effective means to prevent cytauxzoonosis, and even with treatment costing thousands of dollars, up to 40% of cats still succumb<sup>1,2</sup>. First described in Missouri in 1976, the geographic range of *C. felis* is expanding and it has now been diagnosed in domestic cats in one third of US states (Chapter 1, Figure 1)<sup>1,3-11</sup>. Expansion of the geographic range is presumed to be due to changes in climate, urbanization, and increased exposure to the bobcat [*Lynx rufus*] reservoir host and the tick vector [*Amblyomma americanum*]. The disease is characterized by a lethal acute schizogenous tissue phase followed by a fairly innocuous chronic erythoparasitemia (Chapter 1, Figure 2). The high mortality, growing epidemic and cost of care point to vaccination as the most practical control strategy. Prior studies documenting the development of a protective immune response against *C. felis* imply that vaccine development is feasible. However the inability to culture *C. felis in vitro* has been a major barrier to discovery of protective antigens<sup>12,13</sup> and no vaccines against *C. felis* exist. In order to overcome experimental limitations and facilitate the rapid identification of vaccine candidate antigens we sequenced the entire 9.1 Mbp *C. felis* genome and identified approximately 4,300 protein-coding genes, each of which represents a potential protective antigen.

## **MATERIALS AND METHODS**

### **Extraction of *Cytauxzoon felis* DNA**

Whole blood (80 ml) was collected by sterile methods into citrate phosphate dextrose adenine (CPDA-1) anticoagulant immediately post-mortem from a domestic cat that died of acute *C. felis* infection. Acute infection was confirmed by microscopic observation of numerous *C. felis* schizonts in tissue imprints of liver, lung, and spleen. The blood was leuko-reduced using a Purecell NEO Neonatal High Efficiency Leukocyte Reduction Filter for Red Cell Aliquots (PALL Corp., Port Washington, NY)<sup>14</sup>. *C. felis* genomic DNA was purified from leukoreduced blood using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA).

### **Sequencing, assembly and annotation of the *C. felis* genome**

We sequenced the *C felis* genome using a 454 Genome Sequencer FLX (Roche, Indianapolis, IN) with Titanium chemistry and the standard Roche protocol. The sequence was assembled using Newbler 2.0 with a minimum overlap requirement of 90% identity over 30 bases. Resulting contigs were compared to the *Felis catus* genome and contaminating cat sequences (<2% of total reads) were removed.

*Cytauxzoon felis* tRNA and mRNA were isolated from purified merozoites using the Ribopure Blood Kit and PolyAPurist Mag Kit respectively (Ambion, Grand Island, NY)<sup>14</sup>. A cDNA library was constructed using the SMARTer PCR cDNA Synthesis Kit (Clontech, Mountain View, CA) and generation of expressed sequence tags

(ESTs) was completed at the NCSU Genome Sequencing Laboratory, using standard procedures. ESTs were assembled with Newbler and the EST assembly was aligned to the genome using GeneDetective (Time Logic, Carlsbad, CA).

GeneMark-ES 2.5 ([http://exon.gatech.edu/genemark\\_prok\\_gms\\_plus.cgi](http://exon.gatech.edu/genemark_prok_gms_plus.cgi)), which utilizes a Gibbs sampling algorithm to self-train for gene prediction, was deployed to create an initial computationally derived proteome. A combination of hand curated EST data and GeneMark results were used to create a training set for GlimmerHMM (<http://www.cbcb.umd.edu/software/glimmer>) to provide a second predicted proteome. Results from the EST comparisons, GlimmerHMM and GeneMark as well as homology searches against protein data from *B. bovis*, *T. parva*, *P. falciparum* and NCBI's non-redundant protein dataset were integrated into a generic Genome Browser (GBrowse).

## RESULTS AND DISCUSSION

### Sequence and assembly of the *Cytauxzoon felis* genome and comparison of the *C. felis* genome with related apicomplexans

The *C. felis* sequence assembled into 361 contigs spanning 9.1 mega-bases (MB) of genomic DNA post decontamination of *F. catus* sequence (Table 1). The largest contiguous stretch of genomic sequence was 183kb, with an N<sub>50</sub> of greater than 70kb. This genomic data was used to establish an initial computationally predicted proteome of 4,323 genes using the self-training program GeneMark.hmm-ES (v2.5).

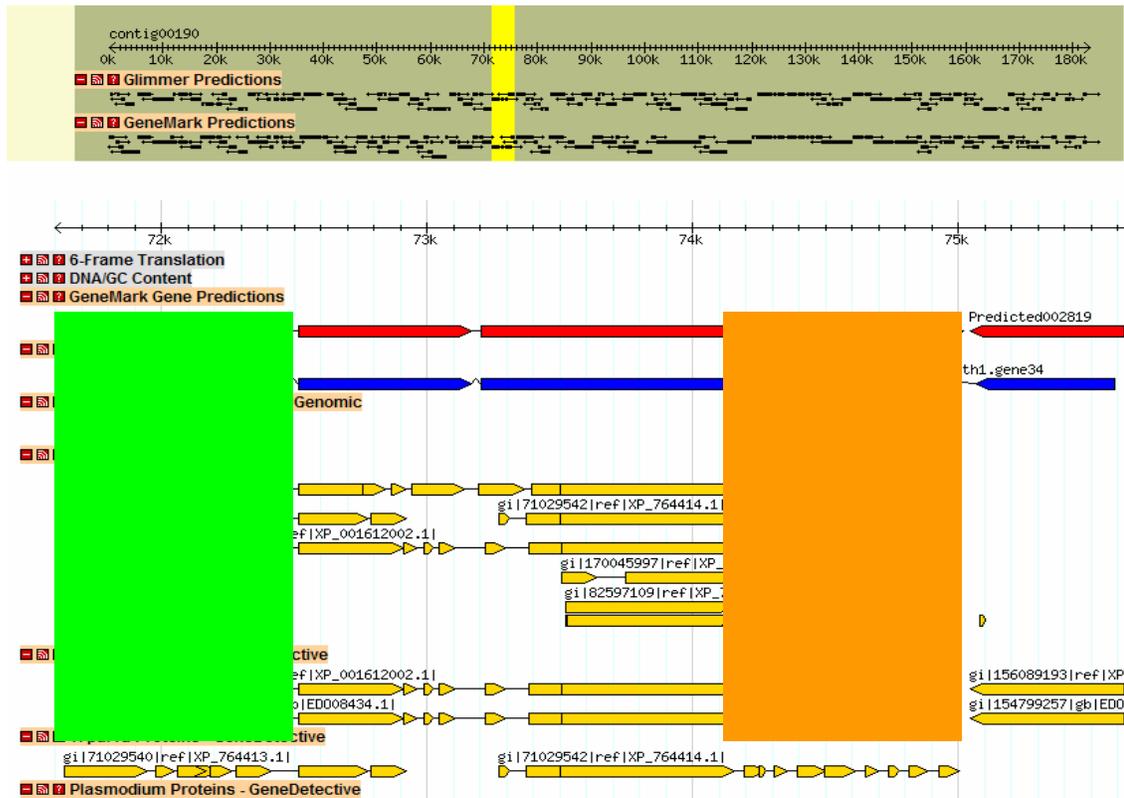
The *C. felis* EST data assembled to 962 contigs covering 547kb of gene space (Table 1). These contigs were used in a BLASTX (basic local alignment search tool) search against the NCBI non-redundant database to identify contigs that likely represent close to full-length genes. A GeneDetective search of the ESTs against the genomic data provided information about gene structure (Time Logic GeneDetective, Carlsbad, CA).

<b>Table 1. Sequence assembly of <i>Cytauxzoon. felis</i> genomic and cDNA</b>		
	<b>Genomic</b>	<b>ESTs<sup>a</sup></b>
<b>Raw Reads</b>	603,160	202,774
<b>Feline Contamination (%)</b>	4.9	53.9
<b><i>C. felis</i> Contigs</b>	361	962
<b>Total # Bases in Contigs</b>	9,110,259	547,540
<b>Largest Contig (bp)</b>	183,236	4,132
<b>Contig N50<sup>b</sup></b>	70,451	639
<b>G+C Composition (%)</b>	31.8	36.2
<sup>a</sup> Expressed Sequence Tags		
<sup>b</sup> The length of contigs comprising >=50% of the <i>C. felis</i> genome sequence		

A set of 100 randomly selected GeneMark predictions and 57 hand-curated full-length ESTs were then used as a training set for GlimmerHMM (v.3.02). Based on that training set, GlimmerHMM predicted 4,378 genes (Table 2). Although there was some slight variation between the two computationally derived gene sets (Table 2), approximately 25% of the genes are identical between the two, and a further 50% differ only in ascribing the most 5' or most 3' exons; such discrepancies were typically straightforward to resolve with manual curation.

<b>Table 2. Comparison of gene predictions of the <i>Cytauxzoon felis</i> genome with related apicomplexans</b>					
	<i>C. felis</i>		<i>T. parva</i> <sup>rf</sup>	<i>B. bovis</i> <sup>rf</sup>	<i>P. falciparum</i> <sup>rf</sup>
	GeneMark	Glimmer			
<b>Genome Size (Mbp)</b>	<b>9.1</b>	<b>9.1</b>	8.3	8.2	22.8
<b>G+C Composition (%)</b>	<b>31.8</b>	<b>31.8</b>	34.1	41.8	19.4
<b>Protein Coding Genes</b>	<b>4,314</b>	<b>4,373</b>	4,035	3,671	5,268
<b>Average Protein (aa)</b>	<b>466</b>	<b>409</b>	469	505	761
<b>% Genes with Introns</b>	<b>68.7</b>	<b>61.7</b>	73.6	61.5	53.9

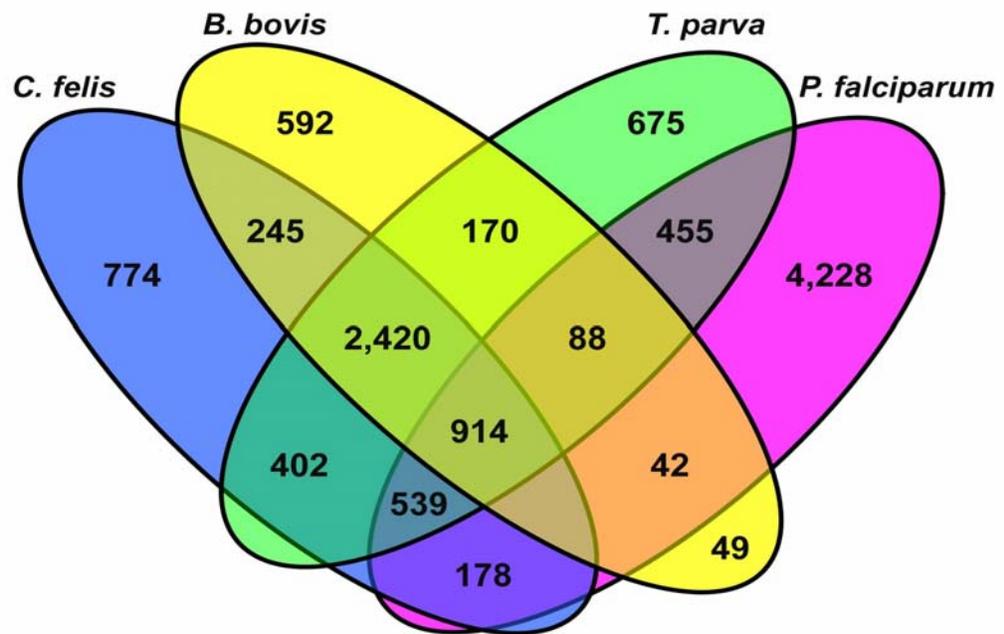
In some instances, expressed sequence tag data was used to resolve the correct structure of a gene when software predictions diverged (orange box) but some ambiguities remain (green box) (Figure 1). When gene predictions were divergent for genes of high interest, genes were amplified by PCR from *C. felis* cDNA and sequenced bi-directionally.



**Figure 1. *Cytauxzoon felis* genome browser (GBrowse).**

In comparing the *C. felis* genome to the genomes of three related apicomplexans, *T. parva*, *B. bovis* and *P. falciparum*, attributes such as genome size, %GC content, average protein length and number of protein-coding genes most closely resemble *T. parva* and are most different from *P. falciparum* (Table 2). A comparison of predicted genes between these sets reveals more genes in common with *T. parva*. A total of 914 predicted genes are present in all four apicomplexans, and 2,420 are shared by *C. felis*, *B. bovis* and *T. parva* but are not found in *P. falciparum*. Note that

the numbers in each sector of the Venn Diagram are not strictly additive due to the variation in size of different gene families within each of the respective genomes, but provide an overall indication as to the relatedness between the organisms as a whole (Figure 2).



**Figure 2. Four way Venn Diagram: Protein coding genes of *Cytauxzoon felis*, *Babesia bovis*, *Theileria parva*, and *Plasmodium falciparum*.**

## CONCLUSIONS

The considerable conserved synteny in addition to a substantial number of genes in common with *T. parva*, *P. falciparum*, and *B. bovis* suggests that the use of comparative apicomplexan genomics may accelerate vaccine antigen discovery for Cytauxzoonosis. We proposed three bio-informatic strategies using the *C. felis* genome for vaccine antigen discovery including: 1) identification of vaccine candidates through the use of genome synteny, 2) identification of *C. felis* orthologues to leading vaccine candidate antigens from the closely related parasites *Plasmodium*, *Theileria* and *Babesia* spp., and 3) heterologous microarray immunoscreening across related genera. The first two strategies have been shown to increase the chances of detecting antigens and third strategy is novel and unique.

## REFERENCES

1. Birkenheuer AJ, Le JA, Valenzisi AM, et al. Cytauxzoon felis infection in cats in the mid-Atlantic states: 34 cases (1998-2004). *J Am Vet Med Assoc* 2006;228:568-571.
2. Cohn LA, Birkenheuer AJ, Brunner JD, et al. Efficacy of atovaquone and azithromycin or imidocarb dipropionate in cats with acute cytauxzoonosis. *J Vet Intern Med* 2011;25:55-60.
3. Birkenheuer AJ, Marr H, Alleman AR, et al. Development and evaluation of a PCR assay for the detection of *Cytauxzoon felis* DNA in feline blood samples. *Vet Parasitol* 2006;137:144-149.
4. Birkenheuer AJ, Marr HS, Warren C, et al. *Cytauxzoon felis* infections are present in bobcats (*Lynx rufus*) in a region where cytauxzoonosis is not recognized in domestic cats. *Vet Parasitol* 2008;153:126-130.
5. Glenn BL, Stair EL. Cytauxzoonosis in domestic cats: report of two cases in Oklahoma, with a review and discussion of the disease. *J Am Vet Med Assoc* 1984;184:822-825.
6. Haber MD, Tucker MD, Marr HS, et al. The detection of *Cytauxzoon felis* in apparently healthy free-roaming cats in the USA. *Vet Parasitol* 2007;146:316-320.
7. Hauck WN, Snider TG, 3rd, Lawrence JE. Cytauxzoonosis in a native Louisiana cat. *J Am Vet Med Assoc* 1982;180:1472-1474.
8. Jackson CB, Fisher T. Fatal cytauxzoonosis in a Kentucky cat (*Felis domesticus*). *Vet Parasitol* 2006;139:192-195.
9. Kier A, Morehouse L, Wagner J. Feline cytauxzoonosis: An update. *Missouri Vet* 1979;29:15-18.
10. Meier HT, Moore LE. Feline cytauxzoonosis: a case report and literature review. *J Am Anim Hosp Assoc* 2000;36:493-496.
11. Wagner JE. A fatal cytauxzoonosis-like disease in cats. *J Am Vet Med Assoc* 1976;168:585-588.
12. Birkenheuer AJ, Breitschwerdt E.B., Levy M.G., Tarigo J.L. Unpublished data. In: North Carolina State University Vector Borne Diagnostic Disease Laboratory, Raleigh North Carolina.

13. Shindel N, Dardiri AH, Ferris DH. An indirect fluorescent antibody test for the detection of Cytauxzoon-like organisms in experimentally infected cats. *Can J Comp Med* 1978;42:460-465.
14. Machado RZ, Valadao CA, Melo WR, et al. Isolation of *Babesia bigemina* and *Babesia bovis* merozoites by ammonium chloride lysis of infected erythrocytes. *Braz J Med Biol Res* 1994;27:2591-2598.

**The *Cytauxzoon felis* genome: A novel candidate vaccine for Cytauxzoonosis inferred from comparative apicomplexan genomics.**

**ABSTRACT**

Cytauxzoonosis is an emerging infectious disease of domestic cats (*Felis catus*) caused by the apicomplexan protozoan parasite *Cytauxzoon felis*. The growing epidemic, with its high morbidity and mortality points to the need for a protective vaccine against cytauxzoonosis. Unfortunately, the causative agent has yet to be cultured continuously *in vitro*, rendering traditional vaccine development approaches beyond reach. Here we report the use of comparative genomics to computationally and experimentally interpret the *C. felis* genome to identify a novel candidate vaccine antigen for cytauxzoonosis. As a starting point we sequenced, assembled, and annotated the *C. felis* genome and the proteins it encodes. Whole genome alignment revealed considerable conserved synteny with other apicomplexans. In particular, alignments with the bovine parasite *Theileria parva* revealed that a *C. felis* gene, cf76, is syntenic to p67 (the leading vaccine candidate for bovine Theileriosis), despite a lack of significant sequence similarity. Recombinant subdomains of cf76 were challenged with survivor-cat antiserum and found to be highly seroreactive. Comparison of eleven geographically diverse samples from the south-central and southeastern USA demonstrated 91-100% amino acid sequence identity across cf76, including a high level of conservation in an immunogenic 226 amino acid (24kDa) carboxyl terminal domain. Using *in situ* hybridization, transcription of cf76

was documented in the schizogenous stage of parasite replication, the life stage that is believed to be the most important for development of a protective immune response. Collectively, these data point to identification of the first potential vaccine candidate antigen for cytauxzoonosis. Further, our bioinformatic approach emphasizes the use of comparative genomics as an accelerated path to developing vaccines against experimentally intractable pathogens.

## INTRODUCTION

*Cytauxzoon felis* is a protozoan parasite of felids that causes cytauxzoonosis, an emerging disease in domestic cats. Without treatment nearly all cats die within three to five days of the onset of clinical symptoms. There are currently no effective means to prevent cytauxzoonosis, and even with treatment costing thousands of dollars, up to 40% of cats still succumb<sup>1,2</sup>. First described in Missouri in 1976, the geographic range of *C. felis* is expanding and it has now been diagnosed in domestic cats in one third of US states (Chapter 1, Figure 1)<sup>1,3-11</sup>. Anecdotal reports of *C. felis* in domestic cats in additional states include Arkansas, southern Illinois (2003), and Ohio (2008). Expansion of the geographic range is presumed to be due to changes in climate, urbanization, and increased exposure to the bobcat [*Lynx rufus*] reservoir host and the tick vector [*Amblyomma americanum*]. The disease is characterized by a lethal acute schizogenous tissue phase followed by a fairly innocuous chronic erythoparasitemia (Chapter 1, Figure 2). The high mortality, growing epidemic and cost of care point to vaccination as the most practical control strategy. Prior studies documenting the development of a protective immune response against *C. felis* imply that vaccine development is feasible. However the inability to culture *C. felis in vitro* has been a major barrier to discovery of protective antigens<sup>12,13</sup> and no vaccines against *C. felis* exist. In order to overcome experimental limitations and facilitate the rapid identification of vaccine candidate antigens we sequenced the entire 9.1 Mbp *C. felis* genome and identified approximately 4,300 protein-coding genes, each of which represents a potential protective antigen.

We used leading vaccine candidates from other apicomplexans as a guide to search for orthologues within the *C. felis* gene complement. *Cytauxzoon felis* is closely related to the apicomplexans *Theileria parva* and *Theileria annulata*, the etiologic agents of East Coast Fever (ECF) and Tropical Theileriosis in cattle, respectively <sup>14</sup>. The leading vaccine candidate for *T. parva*, p67, has conferred substantial protection against ECF in clinical trials. Immunization of cattle with p67 reduced the incidence of severe ECF by 49% during field tick challenge trials in Kenya<sup>15</sup>. The *T. annulata* homologue of p67, SPAG-1, includes neutralizing epitopes on the carboxy terminus that are cross-reactive with p67, and SPAG-1 has been shown to confer protection to homologous species challenge <sup>16,17</sup>.

Although *T. parva* p67 shares only 47% amino acid sequence identity with SPAG-1 these two loci reside within a syntenic block of genes highly conserved between the two *Theileria* species, consistent with their orthology <sup>18</sup>. We searched for *C. felis* orthologues of p67 and SPAG-1 but found no sequences with significant amino acid similarity. Therefore, guided by the approach used to identify the p67/SPAG-1 orthologue (bov57) in *Babesia bovis*, we used conserved genome synteny to expose the *C. felis* orthologue of p67/SPAG-1, which we call cf76 <sup>19</sup>. Here we report our assessment of three criteria likely to be important in determining suitability of cf76 as a vaccine candidate: 1) recognition by the feline immune system 2) degree of sequence similarity among *C. felis* isolates and 3) expression in the *C. felis* life stage that is believed to be critical for the development of a protective immune response.

## MATERIALS AND METHODS

### Identification and amplification *C. felis* cf76

A predicted three exon *C. felis* gene, cf76, syntenic to p67/SPAG-1/Bbov57 was identified *in silico*. Total RNA was extracted from *C. felis* merozoite and schizont-laden splenic tissue collected immediately post-mortem from a domestic cat that died of acute *C. felis* infection using the Trizol LS reagent (Sigma, St. Louis, MO), following the manufacturer methods. Total RNA (10µg/reaction) was treated twice with DNA-free DNase Treatment and Removal Reagent (Ambion, Grand Island, NY). Prior to generation of cDNA, the absence of contaminating DNA in the purified RNA was confirmed by PCR for *C. felis* 18S rRNA genes *Cytauxzoon felis* tRNA and mRNA were isolated from purified merozoites using the Ribopure Blood Kit and PolyAPurist Mag Kit respectively (Ambion, Grand Island, NY)<sup>21</sup>. *C. felis* cDNA was produced using random hexamer primers (Promega, Madison WI) and Smartscribe reverse transcriptase (Clontech, Mountain View, CA).

PCR to amplify predicted cf76 intron 1 (412bp) and intron 2 (587bp) with primers designed from flanking exon sequences was performed using the following conditions: 25pmol each of primer (INTRON 1 forward 5' ATGCCATTACTGTACCTTC 3', INTRON 1 reverse 5' ACCAATCGGTAAACCATCC 3', INTRON 2 forward 5' TACTGCTGATGAATCCAATAC 3', INTRON 2 reverse 5' AACTAGTGTTAATGATAACAATAATGTAGCGATTATTTTAATG 3'), 1X concentration of SYBR® Green Master Mix (Applied Biosystems, Foster City, CA),

template (50ng of splenic or liver RNA, or 1ul of splenic or liver cDNA, 16ng of *C. felis* gDNA, or 1ul of water). Thermal cycling parameters included an initial denaturation at 95°C for 5min, followed by 40 amplification cycles (95°C for 45sec, 59°C for 45sec, and 72°C for 1min). PCR products were analyzed by protein gel electrophoresis.

PCR to amplify the *C.felis* syntenic gene ORF with primers designed from the predicted flanking sequences was performed using previously published conditions with 25pmol each of primer (5' ATTGGATAGTAAATTAGGTTATAAG 3' and 5' GGAATTAATTCAGTTGGAATTTG 3') and template (50ng of *C. felis* splenic RNA, 1µl of *C. felis* splenic cDNA, 16ng of *C. felis* gDNA, or 1µl of water)<sup>21</sup>.

### **Cloning and *in vitro* expression of cf76 and cf76 fragments**

The cf76 ORF (2172bp) and three overlapping subdomains of cf76 including the N-terminal region (720bp), the central region (828bp), and the C-terminal region (675bp) were amplified from *C. felis* cDNA using primer pairs (Table 1) with a 20bp adapter sequence at the 5' and 3' ends homologous to cloning sites of a linearized acceptor vector pXT7, to allow for directional cloning. PCR was performed with previously published conditions using 0.05U/µl High Fidelity Expand Plus Taq DNA polymerase (Roche, Indianapolis, IN), 25pmol of each primer (Table 1), and 5ng of *C. felis* cDNA template<sup>22-24</sup>.

<b>Table 1. Primer sequences for amplification of cf76.</b>		
Primer	Sequence	Product
cf76 Forward ORF	5' ACGACAAGCATATGCTCGAG-ATGAAATTTTTATTAATGTTTGTGGTGCCTTTG 3'	Full length Cf76 (2172bp)
cf76 Reverse ORF	5' TCCGGAACATCGTATGGGTA-AACTAGTGTTAATGATAACAATAATGTAGC 3'	Full length Cf76 (2172bp)
cf76 Forward Fragment 1	5' ACGACAAGCATATGCTCGAG-ATGAAATTTTTATTAATGTTTGTGGTGCCTTTG 3'	C-terminal region (720bp)
cf76 Reverse Fragment 1	5' TCCGGAACATCGTATGGGTA-TTCCACTTGAGGTCCAGTGA CTATAC 3'	C-terminal region (720bp)
cf76 Forward Fragment 2	5' ACGACAAGCATATGCTCGAG-GATCGTGCGGGAAGTATAGTCACTG 3'	Central region (828bp)
cf76 Reverse Fragment 2	5' TCCGGAACATCGTATGGGTA-AGCTATTGAATGTTCTTCTTGTAATGAA TT 3'	Central region (828bp)
cf76 Forward Fragment 3	5' ACGACAAGCATATGCTCGAG-GAAGAACATTCAATAGCTAATTCATTA 3'	N-terminal region (675bp)
cf76 Reverse Fragment 3	5' TCCGGAACATCGTATGGGTA-AACTAGTGTTAATGATAACAATAATGTAGC 3'	N-terminal region (675bp)

Each amplified cf76 PCR product was cloned into a pXT7 vector containing an N-terminus 10x histidine (HIS) tag and a C-terminus hemagglutinin (HA) tag using homologous recombination as previously described<sup>22-24</sup> and all clones were sequenced bi-directionally. *In vitro* transcription and translation reactions (IVTT) were performed with purified recombinant plasmids using the RTS 100 E. coli HY kit (5 PRIME, Gaithersburg, MD).

### **Purification of cf76 and cf76 subdomains**

IVTT reaction components containing cf76 and cf76 subdomains were purified using the N-terminal HIS tag under native and denaturing conditions with Qiagen Ni-NTA Magnetic Agarose Beads (Qiagen, Valencia, CA). Purity and quantity was assessed via western blot analysis in duplicate using secondary antibodies against the N-terminal HIS tag and the C-terminal HA tag using mouse anti-poly-HIS monoclonal IgG<sub>2a</sub> antibody or mouse anti-poly-HA monoclonal antibody (Anti-His<sub>6</sub> (2) and anti-HA clone 12CA5 respectively (Roche, Indianapolis, IN).

### **SDS-PAGE and immunoblot analysis**

cf76 and cf76 subdomain IVTT reactions and purified proteins were analyzed by western blot analysis. Proteins were subjected to SDS-PAGE (4-12% Bis-Tris Gel NuPAGE, Invitrogen, Grand Island, NY) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). After blocking (1X phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST), 2% nonfat milk, 2% bovine serum albumin (BSA), 2% gelatin from cold water fish skin for 1h at room temperature (RT), membranes were incubated with mouse anti-poly-HIS monoclonal antibody or mouse anti-poly-HA monoclonal antibody (Anti-His<sub>6</sub> (2) and anti-HA clone 12CA5; Roche, Indianapolis, IN) overnight at 4°C. After three consecutive washes for 5min at RT in PBST, membranes were incubated with horse radish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin (H + L IgG; Biorad, Hercules, CA) at RT for 1h and washed 3X with PBST at RT. Immobilon Western

Chemiluminescent HRP Substrate (Millipore, Billerica, MA) was used for signal detection.

The immune response to purified cf76 and cf76 subdomains was assessed by western blotting using pooled sera from 10 domestic cats that survived natural *C. felis* infection, as well as 10 naïve cats. To determine *C. felis* infection status, genomic DNA (gDNA) was purified using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and real-time PCR for *C. felis* 18S and for the feline house-keeping gene GAPDH was performed using previously published methods<sup>3,21</sup>. Western blots of purified protein were prepared and blocked as described with the addition of 5% goat serum. Cat sera was diluted 1:500 in blocking buffer containing 1.5mg/ml *E. coli* lysate (MCLAB, South San Francisco, CA) and incubated for 2h at RT. Membranes were incubated with pre-adsorbed cat sera for 2h at RT, washed 5x 5min in PBST, incubated for 1h at RT with goat anti-cat HRP antibody (H + L IgG; Jackson ImmunoResearch, West Grove, PA), washed 5x 5min in PBST, and a chemiluminescent signal was detected with a luminometer (Perkin Elmer, Massachusetts, NY).

### **Conservation of cf76 sequence from diverse geographic regions**

Genomic DNA was extracted from 11 *C. felis* infected whole blood samples collected from geographically diverse regions using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). cf76 was amplified by PCR under the following conditions: 0.05U/ $\mu$ l

High Fidelity Expand Plus Taq DNA polymerase (Roche, Indianapolis, IN), 0.2mM of each dNTP, 1X reaction buffer, 25pmol of each primer (FOR- 5' ATTGGATAGTAAATTAGGTTATAAG 3' and REV- 5' GGAATTAATTCAGTTGGAATTTG 3'), 5µl gDNA, initial denaturation at 95°C for 5min; 40 cycles of 95°C for 30sec, 54°C for 1.5min, and 72°C for 2min; and a final extension at 72°C for 10min. The cf76 sequences from these samples and sequences from *Babesia bovis* (BOV57, GenBank ACY08791.1), *T. parva* (p67, GenBank U40703.1) and *T. annulata* (SPAG-1, GenBank M63017.1) were aligned (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

### **Transcription of cf76 in schizonts**

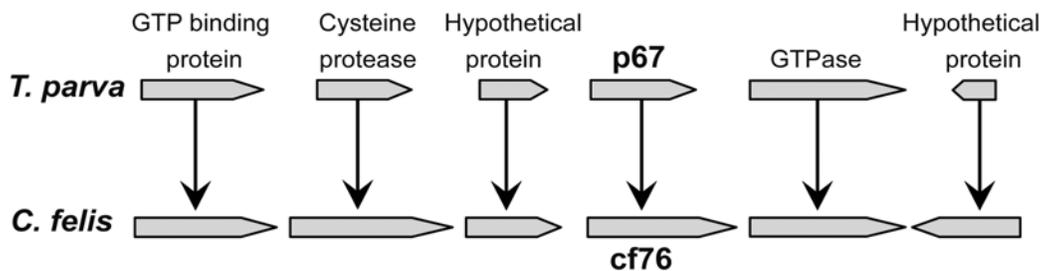
*C. felis* infected lung tissues were harvested and formalin fixed immediately post-mortem from a cat that died of acute cytauxzoonosis. Hematoxylin and eosin (H&E) stained sections were examined for the presence of schizonts. The C-terminal region (678bp) of cf76 was amplified by PCR, cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced bi-directionally. An anti-sense riboprobe was generated and *in situ* hybridization was performed as previously described on infected lung tissue including the use of a negative control nonsense probe <sup>25</sup>.

## RESULTS AND DISCUSSION

### Identification and characterization of *C. felis* cf76

Given that *C. felis* is most closely related to *Theileria* spp, a BLAST search was used to identify a *C. felis* orthologue to p67/SPAG-1. However, no *C. felis* genes with significant identity to p67 or SPAG-1 were identified within the *C. felis* genome.

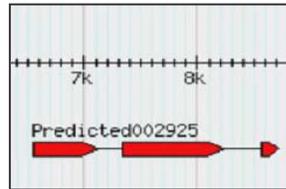
Therefore we used genome synteny as a guide, and identified a 2172bp single copy *C. felis* gene syntenic to p67. *Theileria parva* p67, *T. annulata* SPAG-1, and *B. bovis* bov57 antigens are encoded by genes that reside within a syntenic block that is highly conserved between the three species and a similar syntenic block of *C. felis* genes was identified *in silico* (Figure 1).



**Figure 1. Conserved gene synteny between *T. parva* p67 and *C. felis* cf76.** cf76 is identified *in silico* within a highly conserved syntenic block of genes similarly to the leading vaccine candidate for *T. parva*, p67.

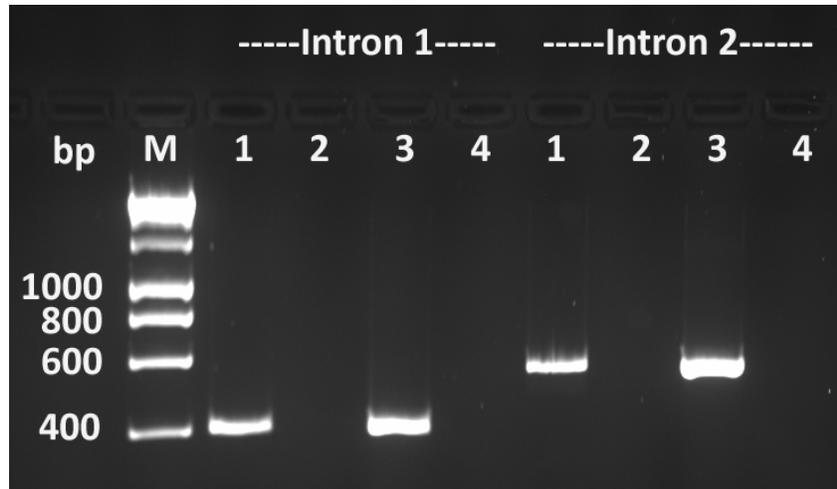
*T. parva* p67, *T. annulata* SPAG-1, and *B. bovis* bov57 antigens are encoded by genes that reside within a syntenic block that is highly conserved between the

*Cytauxzoon felis* cf76 was predicted to be a multi-exon gene by GeneMark gene prediction software (Figure 2).



**Figure 2. *In silico* prediction of *C. felis* cf76.** cf76 is predicted by GeneMark to possess three exons (red arrows) and two introns (lines).

In order to determine if *C. felis* cf76 was predicted accurately as a three exon gene, two PCR reactions were performed with primers designed to amplify regions including the first and second predicted introns (412bp and 587bp respectively) using *C. felis* cDNA and gDNA as template. If the intron predictions were correct larger amplicons would be expected using gDNA as template compared with cDNA. Amplicons of equal size were observed which resolved that cf76 is a single exon gene.



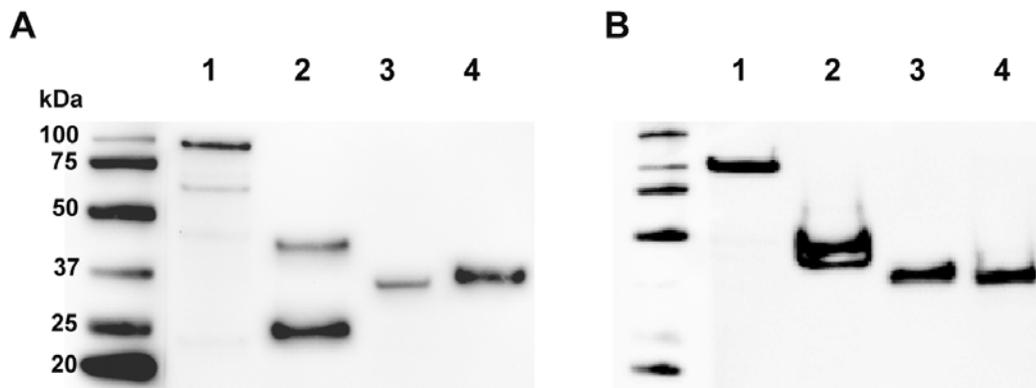
**Figure 3. PCR amplification of predicted cf76 intron and exon junctions.** Amplification of the first predicted cf76 intron yields products of equal size using *C. felis* cDNA (Lanes 1) and *C. felis* gDNA (Lane 3). Amplification of the second predicted cf76 intron also yields products of equal size using *C. felis* cDNA (Lane 1) and *C. felis* gDNA (Lane 3). RNA and no DNA controls were negative for both intron PCR reactions (Lanes 2 and 4 respectively).

Consistent with the BLAST result, cf76 only shared 45% and 42% nucleotide identities with p67 and SPAG-1 respectively. Based on the mean predicted molecular weight across isolates sequenced (75,557.78 Da) we designated the gene that is syntenic to p67/SPAG-1 as cf76. Similar to p67, SPAG-1, and *B. bovis* bov57, cf76 encodes a protein with a predicted signal peptide sequence at the amino terminus, suggesting this protein may be secreted. In contrast to p67/SPAG-1, cf76 does not have a transmembrane domain, suggesting that it is unlikely to be membrane bound. Also unique to cf76 is a putative of a glycosylphosphatidylinositol (GPI) anchor. GPI anchors are glycolipids that anchor membrane proteins and have been associated with immunoreactivity in some protozoan pathogens<sup>26</sup>.

Collectively, these data point to cf76 being an orthologue of the *Theileria* genes. We speculate that conserved synteny combined with a lack of conserved sequence identity may indicate a gene that is under extreme pressure from the host immune response.

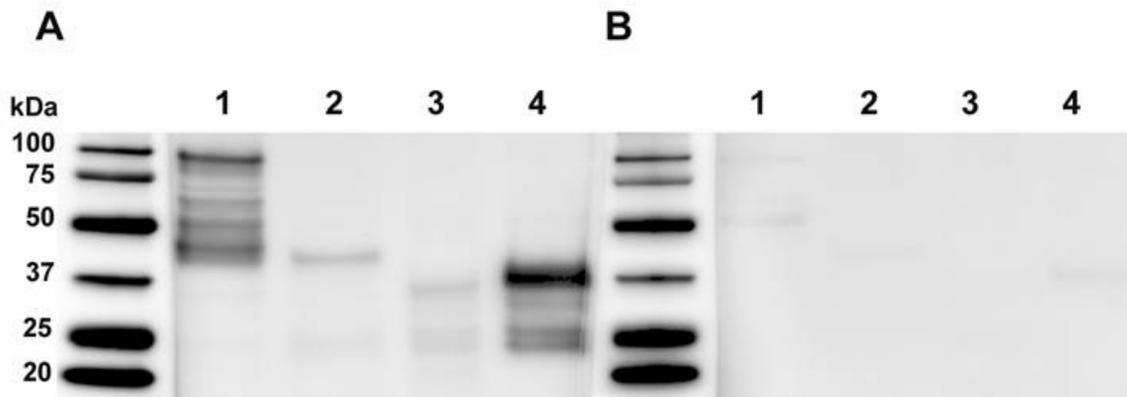
### Feline humoral immune response to recombinant cf76

The apparent molecular mass of full length cf76, the N-terminal region, the central region, and the C-terminal region were approximately 100 kDa, 42 kDa, 35 kDa and 37 kDa, despite predicted molecular mass of 81.6 kDa, 27.1 kDa, 33 kDa, and 26.8 kDa respectively (Figure 4). Production and co-purification of partial transcripts as well as putative degradation products were observed for western blots probed with anti-HIS antibodies while only complete proteins were observed on blots probed with anti-HA antibodies.



**Figure 4. Assessment of purified cf76 and cf76 fragments by Western Blot.** Purified full length cf76 (1), the N-terminal region (2), the central region (3), and the C-terminal region (4) were probed with anti-HIS N-terminal tag (A) and anti-HA C-terminal tag antibodies (B).

Western blot analysis using pooled sera from 10 cats surviving *C. felis* infection revealed strong seroreactivity to His-purified recombinant cf76 and the C-terminal region. In comparison, lower intensity signal was detected against the central and N-terminal regions of cf76 with immune sera (Figure 5A). Substantial reactivity was not observed using pooled sera from 10 cats that tested negative for *C. felis*, and observed signal was attributed to low levels of cross-reacting antibodies unrelated to *C. felis* infection (Figure 5b).



**Figure 5. Assessment of feline sero-reactivity to cf76 and cf76 fragments by Western Blot.** Purified full length cf76 (1), the N-terminal region (2), the central region (3), and the C-terminal region (4) were probed probed with pooled sera (1:500) from cats surviving *C. felis* infection (A) or naive cats (B).

Collectively these data support that the C-terminus of cf76 is highly immunogenic during natural infection with *C. felis*.

### **cf76 sequence is conserved between samples from different geographic regions**

In order to assess the degree of conservation amongst *C. felis* parasite samples from different geographic regions, we amplified and sequenced cf76 from eleven different samples from eight states in the southeastern and south-central United States, revealing a high degree of conservation (92.2 to 100% identity) (Figure 6). Preliminary epitope mapping revealed that high levels of feline antibodies are developed against linear epitopes present in the C-terminal region (Figure 5). This region is highly conserved amongst samples. The only variation in this region was that ten of eleven samples had a tandem repeat of 30bp sequence while the remaining sample only had this 30bp sequence once.

**Figure 6. Amino acid sequences of syntenic gene cf76 from geographic isolates across the southeastern and southwestern United States.**



```

AR1      258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
MO       258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
AR2     258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
OK       258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
OH       258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
TN       258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
KS       258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
AR3     258  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
NC1     255  DGNIIITDGLPIGTVNSASSEGDITTSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
VA       275  DGNIIITDGLPIGTVNSASSESDIATSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
NC2     275  DGNIIITDGLPIGTVNSASSESDIATSRILLGIGNEMSLIVDEILVKLEELKVLEDKKLVGNTQKLESIRE
consensus 281  ***** * .***** ** *****

```

```

AR1      328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
MO       328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
AR2     328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
OK       328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
OH       328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
TN       328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
KS       328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
AR3     328  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
NC1     325  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
VA       345  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
NC2     345  SIITEYQKFIQEI TEIENS DENTKMDGIQSSDIAQTLRYKYDASVKNIMANVMKILNTKGKYDGAILAYN
consensus 351  *****

```

```

AR1      398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
MO       398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
AR2     398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
OK       398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
OH       398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
TN       398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
KS       398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
AR3     398  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
NC1     395  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
VA       415  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
NC2     415  YIKDKVQSIKNGIKNPSSEYLKLRIDIDFSADNIIDPMINNEEKVGIQLKDAKSKI FGLLSNNTNNITY
consensus 421  *****

```

```

-----cf76 carboxy terminus region-----
AR1      468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
MO       468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
AR2     468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
OK       468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
OH       468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
TN       468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
KS       468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
AR3     468  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
NC1     465  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
VA       485  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
NC2     485  DLKKKII EHFNSLQEEHSIANSLINGAKKFSNKLEHLTNKLLKISISKYVATADESNTIKFIHQASNALEK
consensus 491  *****

```

```

-----
AR1      538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
MO       538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
AR2      538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
OK       538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
OH       538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
TN       538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
KS       538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
AR3      538  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
NC1      535  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
VA       555  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
NC2      555  TNNTQIIMNTTNDSSNAVKSTSDVQSMVPLAESSNLLSQMGRATPRDRGGNEGSDGMKSSTGPQVEPAA
consensus 561  *****

```

```

-----
AR1      608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
MO       608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
AR2      608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
OK       608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
OH       608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
TN       608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
KS       608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
AR3      608  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
NC1      605  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
VA       625  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
NC2      625  DLREAEGEVV[REDACTED]KTSSLEDNTWNYGGINTENTKAKGNLKGKEEGELKLV
consensus 631  *****

```

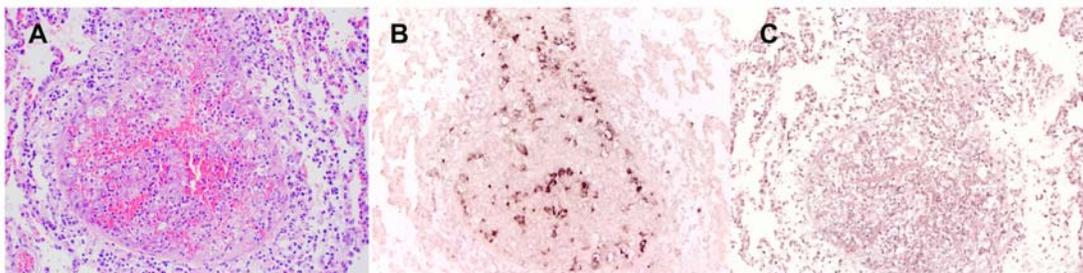
```

-----
AR1      678  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
MO       667  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
AR2      678  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
OK       678  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
OH       678  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
TN       678  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
KS       678  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
AR3      678  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
NC1      675  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
VA       695  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
NC2      695  DDEDEEEAVKDGFNHIKI IATLLLSLTLV
consensus 701  *****

```

### **cf76 is expressed in the *C. felis* life-stage associated with immune protection**

*Cytauxzoon* spp. has a complex life cycle with three life stages in the mammalian host: sporozoites, schizonts, and merozoites (Chapter 1, Figure 2). Of these, schizonts have been associated with a protective immune response. Solid immunity to *C. felis* was observed in cats that had previously survived the schizogenous phase of cytauxzoonosis<sup>13,27,28</sup>. These cats survived challenge infection with no signs of illness while naïve control cats died of cytauxzoonosis. In contrast direct inoculation with *C. felis* merozoites alone has not conferred protective immunity. Collectively, these data suggest antigens associated with schizonts are vaccine targets for *C. felis*. Based on these findings we investigated expression of cf76 in the schizont stage of *C. felis* using *in situ* hybridization. We found robust levels of cf76 transcripts in the schizogenous tissue stage of *C. felis* (Figure 7) further supporting consideration of this antigen as a vaccine candidate.



**Figure 7. *In situ* hybridization to identify transcription of cf76 in *C.felis*-infected lung tissue. A.** Hematoxylin and eosin stained lung tissue demonstrating shizonts forming a parasitic thrombus within a pulmonary vessel, 20X, **B.** Antisense probe, hematoxylin and eosin counterstain, demonstrating numerous positive cells 20X, **C.** Nonsense probe (negative control), hematoxylin and eosin counterstain, 20X.

## CONCLUSIONS

Prior to our work no protein coding genes from *C. felis* had been characterized. Based on a full genome sequence we have now identified  $\approx 4,300$  protein coding genes and characterized the first vaccine candidate for *C. felis*. Specifically, our work demonstrates the potential of cf76 as a vaccine candidate antigen for cytauxzoonosis as it is: 1) recognized by the feline humoral immune system, 2) highly conserved amongst isolates and 3) transcribed in the life stage of *C. felis* shown to confer protective immunity. To substantiate the efficacy of cf76 as a vaccine antigen, significant reduction in morbidity and mortality of cytauxzoonosis must be demonstrated in immunization and challenge trials.

Our bioinformatic approach provides an example of how comparative genomics can provide an accelerated path to identify vaccine candidates in experimentally intractable pathogens. In addition to identification of specific candidate genes, this approach provides a valuable resource for future comparative genomic and proteomic studies to accelerate identification of additional vaccine candidates and drug targets for *C. felis* and related apicomplexans.

## REFERENCES

1. Birkenheuer AJ, Le JA, Valenzisi AM, et al. Cytauxzoon felis infection in cats in the mid-Atlantic states: 34 cases (1998-2004). *J Am Vet Med Assoc* 2006;228:568-571.
2. Cohn LA, Birkenheuer AJ, Brunker JD, et al. Efficacy of atovaquone and azithromycin or imidocarb dipropionate in cats with acute cytauxzoonosis. *J Vet Intern Med* 25:55-60.
3. Birkenheuer AJ, Marr H, Alleman AR, et al. Development and evaluation of a PCR assay for the detection of *Cytauxzoon felis* DNA in feline blood samples. *Vet Parasitol* 2006;137:144-149.
4. Birkenheuer AJ, Marr HS, Warren C, et al. *Cytauxzoon felis* infections are present in bobcats (*Lynx rufus*) in a region where cytauxzoonosis is not recognized in domestic cats. *Vet Parasitol* 2008;153:126-130.
5. Glenn BL, Stair EL. Cytauxzoonosis in domestic cats: report of two cases in Oklahoma, with a review and discussion of the disease. *J Am Vet Med Assoc* 1984;184:822-825.
6. Haber MD, Tucker MD, Marr HS, et al. The detection of *Cytauxzoon felis* in apparently healthy free-roaming cats in the USA. *Vet Parasitol* 2007;146:316-320.
7. Hauck WN, Snider TG, 3rd, Lawrence JE. Cytauxzoonosis in a native Louisiana cat. *J Am Vet Med Assoc* 1982;180:1472-1474.
8. Jackson CB, Fisher T. Fatal cytauxzoonosis in a Kentucky cat (*Felis domesticus*). *Vet Parasitol* 2006;139:192-195.
9. Kier A, Morehouse L, Wagner J. Feline cytauxzoonosis: An update. *Missouri Vet* 1979;29:15-18.
10. Meier HT, Moore LE. Feline cytauxzoonosis: a case report and literature review. *J Am Anim Hosp Assoc* 2000;36:493-496.
11. Wagner JE. A fatal cytauxzoonosis-like disease in cats. *J Am Vet Med Assoc* 1976;168:585-588.
12. Birkenheuer AJ, Breitschwerdt E.B., Levy M.G., Tarigo J.L. Unpublished data. In: North Carolina State University Vector Borne Diagnostic Disease Laboratory, Raleigh North Carolina.

13. Shindel N, Dardiri AH, Ferris DH. An indirect fluorescent antibody test for the detection of Cytauxzoon-like organisms in experimentally infected cats. *Can J Comp Med* 1978;42:460-465.
14. Ketz-Riley CJ, Reichard MV, Van den Bussche RA, et al. An intraerythrocytic small piroplasm in wild-caught Pallas's cats (*Otocolobus manul*) from Mongolia. *J Wildl Dis* 2003;39:424-430.
15. Musoke A, Rowlands J, Nene V, et al. Subunit vaccine based on the p67 major surface protein of *Theileria parva* sporozoites reduces severity of infection derived from field tick challenge. *Vaccine* 2005;23:3084-3095.
16. Boulter N, Knight PA, Hunt PD, et al. *Theileria annulata* sporozoite surface antigen (SPAG-1) contains neutralizing determinants in the C terminus. *Parasite Immunol* 1994;16:97-104.
17. Hall R, Boulter NR, Brown CG, et al. Reciprocal cross-protection induced by sporozoite antigens SPAG-1 from *Theileria annulata* and p67 from *Theileria parva*. *Parasite Immunol* 2000;22:223-230.
18. Brayton KA, Lau AO, Herndon DR, et al. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. *PLoS Pathog* 2007;3:1401-1413.
19. Freeman JM, Kappmeyer LS, Ueti MW, et al. A *Babesia bovis* gene syntenic to *Theileria parva* p67 is expressed in blood and tick stage parasites. *Vet Parasitol* 173:211-218.
20. Machado RZ, Valadao CA, Melo WR, et al. Isolation of *Babesia bigemina* and *Babesia bovis* merozoites by ammonium chloride lysis of infected erythrocytes. *Braz J Med Biol Res* 1994;27:2591-2598.
21. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. *J Clin Microbiol* 2003;41:4172-4177.
22. Davies DH, Liang X, Hernandez JE, et al. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc Natl Acad Sci U S A* 2005;102:547-552.
23. Doolan DL, Mu Y, Unal B, et al. Profiling humoral immune responses to *P. falciparum* infection with protein microarrays. *Proteomics* 2008;8:4680-4694.

24. Vigil A, Ortega R, Jain A, et al. Identification of the feline humoral immune response to *Bartonella henselae* infection by protein microarray. *PLoS One* 2010;5:e11447.
25. Susta L, Torres-Velez F, Zhang J, et al. An in situ hybridization and immunohistochemical study of cytauxzoonosis in domestic cats. *Vet Pathol* 2009;46:1197-1204.
26. Debierre-Grockiego F, Schwarz RT. Immunological reactions in response to apicomplexan glycosylphosphatidylinositols. *Glycobiology* 20:801-811.
27. Wagner JE, Ferris DH, Kier AB, et al. Experimentally induced cytauxzoonosis-like disease in domestic cats. *Vet Parasitol* 1980;6:305-3111.
28. Ferris DH. A progress report on the status of a new disease of American cats: cytauxzoonosis. *Comp Immunol Microbiol Infect Dis* 1979;1:269-276.

## The *Cytauxzoon felis* genome: Identification of *C. felis* orthologues to leading vaccine candidates of related apicomplexans.

### ABSTRACT

Cytauxzoonosis, caused by the protozoan parasite *Cytauxzoon felis*, is an emerging infectious disease of high morbidity and mortality in domestic cats (*Felis catus*) that is expanding in territory. Currently there is no effective means to prevent *C. felis* infection and a growing epidemic points to the need for a protective vaccine against Cytauxzoonosis. We recently sequenced, assembled and annotated the *C. felis* genome sequence and the proteins it encodes. Whole genome alignment revealed considerable conserved synteny with other apicomplexans including *Theileria*, *Plasmodium*, and *Babesia* spp.. Using a bioinformatic approach emphasizing comparative genomics as an accelerated path to vaccine antigen discovery for *C. felis* we identified and characterized cf76, the first vaccine candidate for cytauxzoonosis. Here we report the identification and assessment of additional *C. felis* orthologues to leading vaccine candidates for *Theileria* and *Plasmodium* spp.. including Tp2, TaD, thrombospondin related adhesive protein (TRAP, also known as thrombospondin related anonymous protein and surface sporozoite protein 2 [SSP2]), and apical membrane antigen 1 (AMA-1). *C. felis* orthologues to Tp2, TaD, TRAP and AMA-1 were recombinantly expressed and assessed for recognition by the feline humoral immune response using Western blot and immuno-dot blot.

## INTRODUCTION

*Cytauxzoon felis* is a protozoan parasite of felids that causes cytauxzoonosis, an emerging disease in domestic cats. Without treatment nearly all cats die within days and there are currently no effective means to prevent cytauxzoonosis. Treatment costing thousands of dollars results in a 60% survival rate at best<sup>1,2</sup>. The high mortality and growing epidemic point to vaccination as the most practical control strategy. In order to overcome experimental limitations and facilitate the rapid identification of vaccine candidate antigens we sequenced the entire 9.1 Mbp *C. felis* genome and identified approximately 4,300 protein-coding genes, each of which represents a potential protective antigen (Table 2, Chapter 2). Whole genome alignments revealed considerable conserved synteny and shared features between *C. felis* and *Theileria*, *Plasmodium*, and *Babesia* spp. (Table 2, Chapter 2). Using conserved genome synteny we identified cf76, the first vaccine candidate for cytauxzoonosis. To rapidly identify additional vaccine candidates within this large pool of antigens we searched for *C. felis* orthologues to leading vaccine candidates for related apicomplexans.

*Cytauxzoon felis* orthologues were identified to vaccine candidates for *Theileria* and *Plasmodium* spp. including Tp2, TaD, thrombospondin related adhesive protein (TRAP, also known as thrombospondin related anonymous protein and surface sporozoite protein 2 [SSP2]), and apical membrane antigen 1 (AMA-1).

Tp2 is a *T. parva* antigen expressed in schizonts which is recognized by immune bovine cytotoxic T lymphocytes in animals with East Coast Fever and has been investigated as a candidate for subunit vaccine development<sup>3-5</sup>. The *T. annulata* orthologue to Tp2 is TaD which is constitutively expressed in sporozoites, schizonts, and merozoites and has been established as an immunodominant protein in animals with Tropical Theileriosis<sup>6,7</sup>. Two *C. felis* orthologues sharing significant amino acid similarity to Tp2 and TaD were identified adjacent to one another within the *C. felis* genome. The gene that shared the highest similarity with Tp2 was called cTp2 and the gene that shared the highest similarity with TaD was called cTaD.

TRAP is an adhesion protein involved in the recognition and invasion of host cells. It is a transmembrane antigen expressed in *Plasmodium* spp. sporozoites that is essential for invasion of hepatocytes and as such has been investigated as a vaccine candidate for malaria<sup>8-11</sup>. TRAP is also expressed in merozoites in *Babesia* spp. and is important for invasion of erythrocytes by *B. gibsoni* and *B. bovis*<sup>12,13</sup>. TRAP is recognized by the host humoral immune system resulting in antibody production that has been useful for serodiagnosis of *B. gibsoni* infection in dogs<sup>14</sup>.

AMA-1 is an antigen unique to apicomplexans that allows for efficient invasion of host cells. It is present in both sporozoite and merozoite life stages of *Plasmodium* spp. and antibodies against AMA-1 have demonstrated inhibition of sporozoite invasion of hepatocytes in vitro<sup>15</sup> and merozoite invasion of erythrocytes in vitro and

in vivo <sup>15,16</sup>, reviewed in <sup>17</sup>. Within *Plasmodium* spp., AMA-1 has shown significant polymorphism that is proposed to occur due to immunological pressure. However, the ectodomain of AMA-1 which contains subdomains DI, DII, and DIII has maintained significant structural amino acid conservation within *Plasmodium* spp..<sup>18</sup> Conservation of this ectodomain has also been observed in orthologues from other genera including *Babesia* spp. <sup>19</sup>, *Toxoplasma gondii* <sup>20</sup>, and *Theileria parva* <sup>3</sup>. Blockade of AMA-1 in these genera has also been associated with inhibition of host cell invasion. AMA-1 has been studied extensively as a leading vaccine candidate for *Plasmodium* spp. and the host immune response to the ectodomain of *P. falciparum* AMA-1 in particular has been well documented <sup>21</sup>.

*Cytauxzoon felis* orthologues to Tp2, TaD, TRAP, and the AMA-1 ectodomain were identified, recombinantly expressed, purified, and assessed for recognition by the feline immune system.

## MATERIALS AND METHODS

### Identification, cloning and *in vitro* expression of *C. felis* orthologues to vaccine candidates of closely related apicomplexans

Using our *C. felis* genome resource, *C. felis* orthologues to several of the leading candidates from *Theileria* and *Plasmodium* were identified *in silico* for recombinant expression and screening. The open reading frames (ORFs) for four orthologous regions of predicted *C. felis* genes including 1) cfTp2 (492bp), 2) cfTaD (501bp), 3) cfTRAP (1233bp) and 4) cfAMA-1 which included the full ectodomain (Domains I through III, 1304bp), were amplified from *C. felis* cDNA using primer pairs (Table 1) with a 20bp adapter sequence at the 5' and 3' ends homologous to cloning sites of a linearized acceptor vector pIVEX 2.3 (cfTp2, cfTaD, cfTRAP) or pXT7 (cfAMA-1 ectodomain and subdomains), to allow for directional cloning. PCR was performed with previously published conditions using 0.05U/ $\mu$ l High Fidelity Expand Plus Taq DNA polymerase (Roche, Indianapolis, IN), 25pmol of each primer (Table 1), and 5ng of *C. felis* cDNA template<sup>22-24</sup>. Each amplified PCR product was cloned into a pIVEX 2.3 (cfTp2, cfTaD, cfTRAP) or pXT7 (cfAMA-1) vectors containing an N-terminus 10x (pXT7) or 6x (pIVEX 2.3) histidine (HIS) tag and a C-terminus hemagglutinin (HA) tag (pXT7) using homologous recombination as previously described<sup>22-24</sup> and all clones were sequenced bi-directionally. *In vitro* transcription and translation reactions (IVTT) were performed with purified recombinant plasmids using the RTS 100 E. coli HY kit (5 PRIME, Gaithersburg, MD).

**Table 1 . PCR primers for amplification of *C. felis* orthologues**

Primer	Sequence	Product
cfTp2 Forward	5' TAAGAAGGAGATATACCATG-AAGATAATCAGCATCATTTCC 3'	cfTp2, 492bp
cfTp2 Reverse	5' TGATGATGATGAGATCCAGA-ATCAAGTTTGGGTAACCTTGTAC 3'	cfTp2, 492bp
cfTaD Forward	5' TAAGAAGGAGATATACCATG-GAGGGCATAAAATTAATTTTCAGTC 3'	cfTaD, 501bp
cfTaD Reverse	5' TGATGATGATGAGATCCAGA-ATTAGGACTGACTGGATCGTTAGG 3'	cfTaD, 501bp
cfTRAP Forward	5' TAAGAAGGAGATATACCATG-TCGGGTCTAGAACTCTCCTCGAAC 3'	cfTRAP, 1233bp
cfTRAP Reverse	5' TGATGATGATGAGATCCAGA-ATTTTCTTCATCTAGATGCATTTTCATTTG 3'	cfTRAP, 1233bp
cfAMA-1 Forward	5' ACGACAAGCATATGCTCGAG-AGGGGCGGTTTTGATAATG 3'	cfAMA-1, 1304bp
cfAMA-1 Reverse	5' TCCGGAACATCGTATGGGTA-CCTCCTGATGAGTGCGAATA 3'	cfAMA-1, 1304bp

### **Purification of *C. felis* orthologues to Tp2, TaD, TRAP, AMA1**

IVTT reaction components containing cfTp2, cfTaD, cfTRAP, cfAMA-1 were purified using the N-terminal HIS tag under native and denaturing conditions with Qiagen Ni-NTA Magnetic Agarose Beads (Qiagen, Valencia, CA). Purity and quantity was assessed via western blot analysis in duplicate using secondary antibodies against the N-terminal HIS tag and the C-terminal HA tag using mouse anti-poly-HIS monoclonal IgG<sub>2a</sub> antibody or mouse anti-poly-HA monoclonal antibody (Anti-His<sub>6</sub> (2) and anti-HA clone 12CA5 respectively (Roche, Indianapolis, IN).

### **SDS-PAGE and immunoblot analysis**

cfTp2, cfTaD, cfTRAP, and cfAMA-1 IVTT reactions and purified proteins were analyzed by western blot analysis as previously described (Chapter 3, Materials and Methods). In addition, equal amounts of purified protein were directly blotted onto nitrocellulose membrane using a dotblot vacuum manifold (Bio-Dot®, Biorad Laboratories, Hercules, CA) and processed in tandem with Western blots.

The immune response to purified cfTp2, cfTaD, cfTRAP, cfAMA-1 was assessed by western blotting and immuno-dot blotting using pooled sera from 5 domestic cats that survived natural *C. felis* infection, as well as 5 naïve cats as previously described (Chapter 3, Materials and Methods).

## RESULTS AND DISCUSSION

### Identification of *C. felis* orthologues to vaccine candidates of closely related apicomplexans

To execute a search for *C. felis* vaccine candidates, 413 vaccine candidate antigens from the closely related apicomplexans *Plasmodium* (n=382), *Theileria* (n=19) and *Babesia* (n=12) were identified<sup>25-27</sup>. Using our *C. felis* genome resource, *C. felis* orthologues to several of the leading vaccine candidates for *Theileria* and *Plasmodium* were identified *in silico* for recombinant expression and screening (Table 2).

<b>Table 2 . <i>Cytauxzoon felis</i> orthologues to vaccine candidates for <i>Theileria</i> spp. and <i>Plasmodium</i> spp.</b>							
	<b>Protein</b>	<b>Organism/ Size (bp/aa)</b>	<b>Subcellular Location/Functi on</b>	<b># Exons</b>	<b>Size</b>	<b>E-value</b>	<b>Identities/ Positives (%)</b>
1	TP2 <i>C. felis</i> Contig:00028:16251-16742	<i>T. parva</i>	Hypothetical protein Secreted	1	174aa		
				1	164aa	2e-22	30/53
2	TaD <i>C. felis</i> Contig00028:17571..18071	<i>T. annulata</i>	Immunodominant Surface Expressed		178aa		
				1	167aa	2e-24	36/54
3	SSP-2 (TRAP) <i>C. felis</i> Contig00429:15505-19351	<i>P. falciparum</i>	Surface Expressed	7	808aa	2e-29	33/50
4	AMA1 <i>C. felis</i> Contig:00447:23576-25580	<i>P. falciparum/vivax</i>	Apical Membrane Ag Secreted	2	526aa		
				2	563aa	1e-57	32/48

### Characterization of *C. felis* cfTp2, cfTaD, cfTRAP, and cfAMA-1

*C. felis* orthologues were assessed for motifs which may facilitate an immune response in the host including: 1) presence of a signal peptide (SignalP Server v. 4.0) and/or transmembrane domains (TMHMM Server v.2.0) to allow for exposure of an antigen to the host immune system and 2) presence of a glycosylphosphatidylinositol (GPI) anchor which has been associated with immunoreactivity in some protozoan pathogens<sup>28</sup> (Table 3).

**Table 3. Signal peptide, transmembrane domain and GPI anchor motifs in *C. felis* orthologues.**

Orthologue	Signal Peptide	Transmembrane Domain	GPI Anchor
cfTp2	Yes	0	No
cfTaD	Yes	0	No
cfTRAP	No	1	No
cfAMA-1	No	2	No

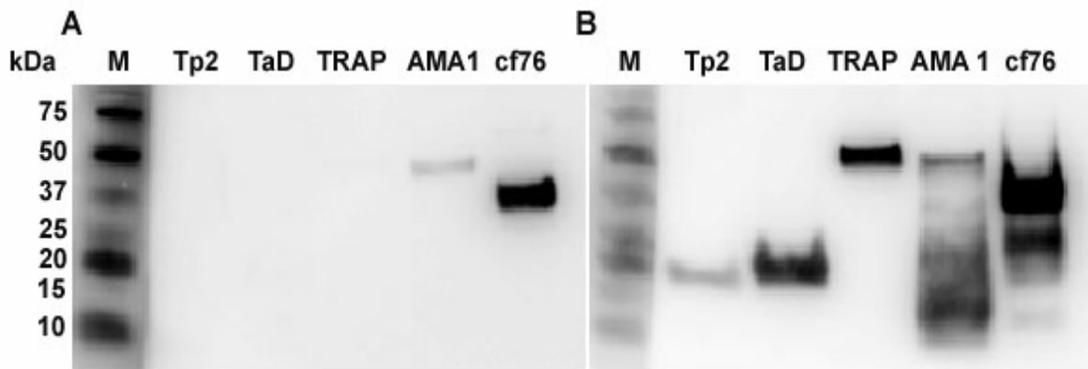
### Feline humoral immune response to recombinant cfTp2, cfTaD, cfTRAP, and cfAMA-1

Apparent molecular mass of the *C. felis* orthologues differed from predicated molecular mass (Table 3, Figure 1). Production and co-purification of partial transcripts as well as putative degradation products were observed for western blots probed with anti-HIS antibodies while only complete proteins were observed on blots probed with anti-HA antibodies for cfAMA-1 and cf76. Note, cfTp2, cfTaD, and

cfTRAP were cloned into the pIVEX 2.3 vector which does not contain a hemagglutinin tag.

**Table 4. Apparent and expected molecular masses of *C. felis* orthologues.**

Orthologue	Apparent molecular Mass	Expected molecular mass (kDa)
cfTp2	18 kDa	20.9 kDa
cfTaD	19 kDa	21.2 kDa
cfTRAP	46 kDa	48.3 kDa
cfAMA-1	49 kDa	51.8 kDa



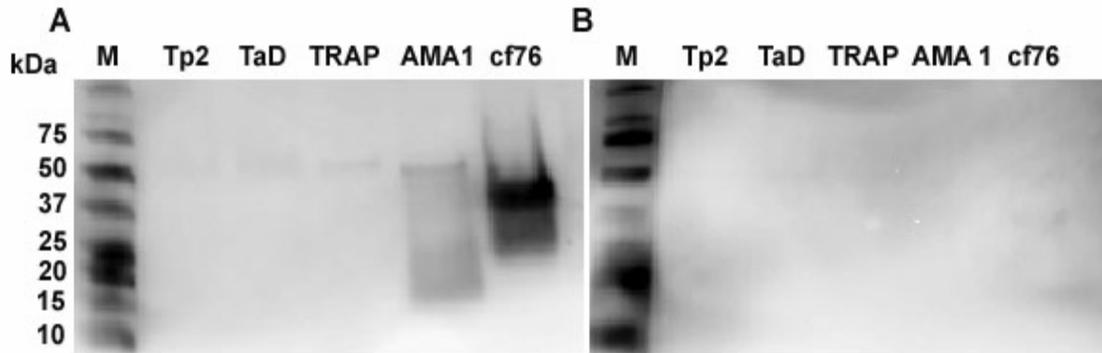
**Figure 1. Assessment of purified *C. felis* orthologues by Western blot.** Purified *C. felis* orthologues of Tp2, TaD, TRAP, AMA-1, and the carboxy terminus of cf76 (positive control) were probed with anti-HA C-terminal tag antibodies (A) anti-HIS N-terminal tag (B).

**cfTp2 and cfTaD.** Western blot and immuno-dot blot analysis using pooled sera from 5 cats surviving *C. felis* infection revealed no detectable signal to His-purified recombinant cfTp2 and cfTaD (Figure 2, panel A). Collectively these data support

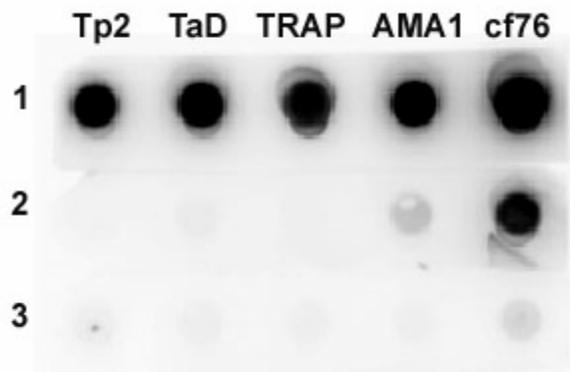
that the *C. felis* orthologues to Tp2 and TaD do not contain epitopes detectable by Western blot analysis under denaturing conditions or immuno-dot blot analysis under native conditions when probed with *C. felis* immune sera.

**cfTRAP.** Western blot and immuno-dot blot analysis using pooled sera from 5 cats surviving *C. felis* infection revealed an equivocal faint signal to His-purified recombinant cfAMA-1 on Western blot that is not observed on immuno-dot blot. No signal observed using pooled sera from 5 naive cats (Figures 2 and 3). This result has been observed on additional Western blots (Figure 4). Further investigation of cfTRAP is warranted.

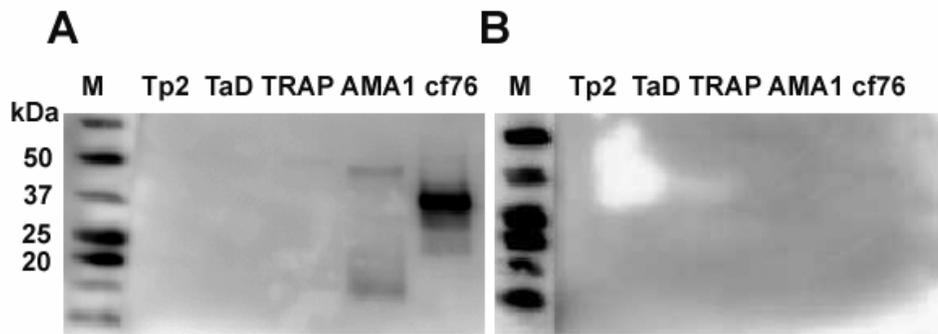
**cfAMA-1.** Western blot and immuno-dot blot analysis using pooled sera from 5 cats surviving *C. felis* infection revealed a faint signal to His-purified recombinant cfAMA-1 on Western blot and immuno-dot blot that exceeds a very faint signal observed using pooled sera from 5 naive cats on immuno-dot blot (Figures 2 and 3). This observation has been made previously Western blot challenged with pooled *C. felis* immune sera and naive sera (Figure 4). The ectodomain of *P. falciparum* AMA1 requires disulfide linkages for production of a conformational epitope<sup>21</sup>. It is plausible that cfAMA-1 also requires tertiary structure for antibody binding to conformational epitopes. Given the status of AMA-1 as a leading vaccine candidate for *Plasmodium* spp., further investigation of cfAMA-1 is warranted.



**Figure 2. Assessment of feline sero-reactivity to *C. felis* orthologues by Western blot.** Purified *C. felis* orthologues of Tp2, TaD, TRAP, AMA-1, and the carboxy terminus of cf76 (positive control) were probed with pooled sera (1:500) from cats surviving *C. felis* infection (A) or naive cats



**Figure 3. Assessment of feline sero-reactivity to *C. felis* orthologues by Immuno-dot blot.** Purified *C. felis* orthologues of Tp2, TaD, TRAP, AMA-1, and the carboxy terminus of cf76 (positive control) were probed with with anti-HIS N-terminal tag antibodies (1), pooled sera (1:500) from cats surviving *C. felis* infection (2) or naive cats (3).



**Figure 4. Additional assessment of feline sero-reactivity to *C. felis* orthologues by Western blot.** Purified *C. felis* orthologues of Tp2, TaD, TRAP, AMA-1, and the carboxy terminus of cf76 (positive control) were probed with pooled sera (1:500) from cats surviving *C. felis* infection (**A**) or naive cats

### Limitations and Future Directions

In this study we only investigated the feline humoral immune response to antigens using Western and immuno-dot blots. One limitation of these techniques is the inability to detect conformational epitopes. Most cell free protein synthesis systems typically do not produce tertiary protein structure which is required for conformational epitope formation. Future studies could include recombinant production of these proteins using *Escherichia coli*, yeast cell lines, and/or mammalian cell lines which allow for conformational epitope formation. There are also cell-free synthesis systems available which allow for formation of disulfide bonds to yield recombinant proteins with tertiary structure.

## CONCLUSIONS

We used a bioinformatic approach emphasizing comparative genomics as an accelerated pathway for vaccine candidate identification. We identified *C. felis* orthologues to leading vaccine candidates for *Theileria* and *Plasmodium* spp. including Tp2, TaD, TRAP and AMA-1. Preliminary assessment of these orthologues for recognition by the feline humoral immune response reveals promising evidence that the *C. felis* orthologue to AMA-1 represents an additional vaccine candidate. Future studies cfTp2, cfTaD, cfTRAP, and cfAMA-1 that investigate conformational epitopes are warranted.

## REFERENCES

1. Birkenheuer AJ, Le JA, Valenzisi AM, et al. Cytauxzoon felis infection in cats in the mid-Atlantic states: 34 cases (1998-2004). *J Am Vet Med Assoc* 2006;228:568-571.
2. Cohn LA, Birkenheuer AJ, Brunker JD, et al. Efficacy of atovaquone and azithromycin or imidocarb dipropionate in cats with acute cytauxzoonosis. *J Vet Intern Med* 25:55-60.
3. Gardner MJ, Bishop R, Shah T, et al. Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. *Science* 2005;309:134-137.
4. Graham SP, Pelle R, Yamage M, et al. Characterization of the fine specificity of bovine CD8 T-cell responses to defined antigens from the protozoan parasite *Theileria parva*. *Infect Immun* 2008;76:685-694.
5. Graham SP, Pelle R, Honda Y, et al. *Theileria parva* candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* 2006;103:3286-3291.
6. Schneider I, Haller D, Seitzer U, et al. Molecular genetic characterization and subcellular localization of a putative *Theileria annulata* membrane protein. *Parasitol Res* 2004;94:405-415.
7. Seitzer U, Beyer D, Kullmann B, et al. Evaluation of *Theileria annulata* recombinant immunodominant proteins for the development of ELISA. *Transbound Emerg Dis* 2008;55:244-248.
8. Chenet SM, Tapia LL, Escalante AA, et al. Genetic diversity and population structure of genes encoding vaccine candidate antigens of *Plasmodium vivax*. *Malar J* 11:68.
9. Curtidor H, Arevalo-Pinzon G, Bermudez A, et al. Binding activity, structure, and immunogenicity of synthetic peptides derived from *Plasmodium falciparum* CelTOS and TRSP proteins. *Amino Acids* 43:365-378.
10. Rogers WO, Malik A, Mellouk S, et al. Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Proc Natl Acad Sci U S A* 1992;89:9176-9180.
11. Ferry G. First DNA malaria vaccine on trial in Africa. *Curr Biol* 2000;10:R810-811.

12. Zhou J, Fukumoto S, Jia H, et al. Characterization of the Babesia gibsoni P18 as a homologue of thrombospondin related adhesive protein. *Mol Biochem Parasitol* 2006;148:190-198.
13. Gaffar FR, Yatsuda AP, Franssen FF, et al. A Babesia bovis merozoite protein with a domain architecture highly similar to the thrombospondin-related anonymous protein (TRAP) present in Plasmodium sporozoites. *Mol Biochem Parasitol* 2004;136:25-34.
14. Goo YK, Jia H, Aboge GO, et al. Babesia gibsoni: Serodiagnosis of infection in dogs by an enzyme-linked immunosorbent assay with recombinant BgTRAP. *Exp Parasitol* 2008;118:555-560.
15. Silvie O, Franetich JF, Charrin S, et al. A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites. *J Biol Chem* 2004;279:9490-9496.
16. Kennedy MC, Wang J, Zhang Y, et al. In vitro studies with recombinant Plasmodium falciparum apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. *Infect Immun* 2002;70:6948-6960.
17. Remarque EJ, Faber BW, Kocken CH, et al. Apical membrane antigen 1: a malaria vaccine candidate in review. *Trends Parasitol* 2008;24:74-84.
18. Hodder AN, Crewther PE, Matthew ML, et al. The disulfide bond structure of Plasmodium apical membrane antigen-1. *J Biol Chem* 1996;271:29446-29452.
19. Gaffar FR, Yatsuda AP, Franssen FF, et al. Erythrocyte invasion by Babesia bovis merozoites is inhibited by polyclonal antisera directed against peptides derived from a homologue of Plasmodium falciparum apical membrane antigen 1. *Infect Immun* 2004;72:2947-2955.
20. Hehl AB, Lekutis C, Grigg ME, et al. Toxoplasma gondii homologue of plasmodium apical membrane antigen 1 is involved in invasion of host cells. *Infect Immun* 2000;68:7078-7086.
21. Lalitha PV, Ware LA, Barbosa A, et al. Production of the subdomains of the Plasmodium falciparum apical membrane antigen 1 ectodomain and analysis of the immune response. *Infect Immun* 2004;72:4464-4470.

22. Davies DH, Liang X, Hernandez JE, et al. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc Natl Acad Sci U S A* 2005;102:547-552.
23. Doolan DL, Mu Y, Unal B, et al. Profiling humoral immune responses to *P. falciparum* infection with protein microarrays. *Proteomics* 2008;8:4680-4694.
24. Vigil A, Ortega R, Jain A, et al. Identification of the feline humoral immune response to *Bartonella henselae* infection by protein microarray. *PLoS One* 2010;5:e11447.
25. Chaudhuri R, Ahmed S, Ansari FA, et al. MalVac: database of malarial vaccine candidates. *Malar J* 2008;7:184.
26. Hu J, Chen Z, Gu J, et al. Safety and immunogenicity of a malaria vaccine, *Plasmodium falciparum* AMA-1/MSP-1 chimeric protein formulated in montanide ISA 720 in healthy adults. *PLoS One* 2008;3:e1952.
27. Yang B, Sayers S, Xiang Z, et al. Protegen: a web-based protective antigen database and analysis system. *Nucleic Acids Res* 2011;39:D1073-1078.
28. Debierre-Grockiego F, Schwarz RT. Immunological reactions in response to apicomplexan glycosylphosphatidylinositols. *Glycobiology* 20:801-811.

**The *Cytauxzoon felis* genome: Characterization of vaccine candidate antigens identified by heterologous immunoscreening of *Plasmodium falciparum* protein microarrays.**

**ABSTRACT**

*Cytauxzoon felis* is an emerging tick-transmitted pathogen of domestic cats in the United States that is related to the causative agent of malaria, *Plasmodium falciparum*. To further accelerate vaccine antigen discovery for cytauxzoonosis we utilized heterologous protein microarray immunoscreening across these related genera. When pathogen genome sequences are available, high throughput approaches are ideal for antigen discovery. Protein microarray technology allows for rapid screening of thousands of proteins to identify seroreactive antigens that may represent vaccine candidates. Using a cost-effective and novel approach, we screened hyperimmune sera from *C. felis* survivors using a pre-fabricated microchip containing 500 *P. falciparum* antigens which are known to induce a humoral immune response in humans. Sera from *C. felis* survivors demonstrated significant serologic cross-reactivity against five *P. falciparum* antigens compared to naive cat sera. We identified orthologues to these five antigens within the *C. felis* genome. We amplified, cloned and expressed these proteins using an *in vitro* transcription and translation system. Recombinantly expressed *C. felis* orthologues were challenged with survivor-cat antiserum and one was found to be highly seroreactive. These

results validate the potential of heterologous protein microarray immunoscreening across genera as a tool for rapid identification of vaccine candidates.

## INTRODUCTION

*Cytauxzoon felis* is a tick-transmitted protozoan pathogen of domestic cats and is related to the etiologic agent of human malaria, *Plasmodium falciparum*. It is a growing epidemic of high morbidity and mortality for which there is no prevention and current treatment protocols offer a 60% survival rate at best <sup>1,2</sup>. In turn, we have focused our efforts on vaccine development for cytauxzoonosis. In order to facilitate the rapid identification of vaccine candidate antigens we have recently sequenced the entire *C. felis* genome and identified ~4300 protein coding genes <sup>3</sup>. In order to identify vaccine candidates within this gene pool, we utilized several bio-informatic strategies. In addition to these previously described strategies (Chapters 3 and 4) we hypothesized that heterologous protein microarray immunoscreening across related genera would result in rapid identification of *C. felis* candidate vaccine antigens. Protein microarray profiling involves rapid high throughput cloning and protein microarray chip fabrication for profiling immunoreactivity on a large scale <sup>4,5</sup>. Immunoscreening of protein microarrays permits the screening of microliter volumes of serum (1-5ul of sera per patient) from large numbers of individual patients against thousands of potential antigens. It provides unique capabilities such as parallelism and a high-throughput format which are ideally suited for comprehensive investigation of the antibody repertoire generated in response to infection or exposure. Protein microarray immunoscreening has been applied to more than 25 medically important infectious agents from a wide range of organisms, including viral, bacterial, protozoal, and fungal pathogens <sup>reviewed in 6</sup>. At this time, it was not

cost-effective to produce a protein microarray containing *C. felis* proteins. Therefore, we took advantage of existing resources and immunoscreened a protein microarray printed with 500 proteins from the closely related organism *P. falciparum*. In order to ensure that seroreactivity was specific to *C. felis* infection, we screened the *P. falciparum* array with sera from cats that have survived *C. felis* infection and sera from naïve cats. We identified five *P. falciparum* proteins against which *C. felis* hyperimmune serum was seroreactive and identified the orthologues to these antigens within the *C. felis* genome. These orthologues were further assessed for recognition by the feline humoral immune system.

## **MATERIALS AND METHODS**

### **Protein Microarray Screening (University of California, Irvine)**

A pre-fabricated protein microarray printed with 500 proteins from *P. falciparum* was probed with sera from cats (n=3) that had survived *C. felis* infection and from naive cats (n=3). Feline serum was pre-absorbed against *Escherichia coli*-lysate to block anti-*E. coli* antibodies. After washing three washes with 10 mM Tris (hydroxymethyl) aminomethane buffer (pH 8.0) containing 0.05% (v/v) Tween-20 (TTBS), slides were incubated in biotin-conjugated, goat anti-cat immunoglobulin-G (IgG) diluted 1/200 in blocking buffer. Slides were washed three times in TTBS and incubated with streptavidin-conjugated SureLight® P-3 (Columbia Biosciences) and washed again three times in TTBS followed by three times in Tris buffer without Tween-20 followed by a final water wash. The slides were air dried after brief centrifugation and analyzed using a Perkin Elmer ScanArray Express HT microarray scanner.

### **Statistical analysis (University of California, Irvine)**

Signal intensities were quantified using QuantArray software utilizing automatic background subtraction for each spot. Proteins were considered to be expressed if either tag's signal intensity was greater than the average signal intensity of the IVTT reaction without plasmid, plus 2.5-times the standard deviation. "No DNA" controls consisting of IVTT reactions without the addition of plasmid were averaged and used to subtract background reactivity from the unmanipulated raw data. All results were expressed as signal intensity. The "vsn" package in the Bioconductor suite

(<http://Bioconductor.org/>) in the R statistical environment (<http://www.R-project.org>) was used to calculate seroreactivity. In addition to the variance correction, this method calculates maximum likelihood shifting and scaling calibration parameters for different arrays, using known non-differentially expressed spots. This calibration has been shown to minimize experimental effects <sup>7</sup>. Raw values were used for the positive and negative controls to calibrate, and then normalize, the entire data set using the vsn package. Differential analysis of the normalized signals was performed using a Bayes-regularized t-test adapted from Cyber-T for protein arrays <sup>8-10</sup>. Benjamini-Hochberg p-value adjustments were applied to account for multiple test conditions. All p-values determined were Benjamini-Hochberg corrected for false discovery <sup>11</sup>. Multiple antigen classifiers were built using Support Vector Machines (SVMs). The “e1071” and “ROCR” packages in R were utilized to train the SVMs and to produce receiver operating characteristic curves, respectively <sup>12</sup>.

### **Identification, cloning and *in vitro* expression of *C. felis* orthologues to *P. falciparum* antigens**

*Cytauxzoon felis* orthologues for proteins to which cats (n=3) that had survived *C. felis* infection were significantly more seroreactive than naïve cats on the *P. falciparum* array were searched for within the *C. felis* genome. Full or partial gene segments representing the homologous regions of *C. felis* to the *P. falciparum* proteins were amplified from *C. felis* cDNA using primer pairs (Table 1), cloned into the pXT7 plasmid by *in vivo* homologous recombination, and proteins were

recombinantly expressed using an *in vitro* transcription and translation system as previously described (Chapter 3, Materials and Methods).

**Table 1 . PCR primers for amplification of *C. felis* orthologues**

Primer	Sequence	Product
Cf1 Forward	5' ACGACAAGCATATGCTCGAG – ATGTCTACACTTATGGAAGACC 3'	1371bp
Cf1 Reverse	5' TCCGGAACATCGTATGGGTA – TGAAGTTTCATTAATAATCTGAT A 3'	1371bp
Cf2 Forward	5' ACGACAAGCATATGCTCGAG - AATACATTACAAGAGGAGG 3'	1145bp
Cf2 Reverse	5' TCCGGAACATCGTATGGGTA – GAGATCTTGTTGTGTGAATAATGG 3'	1145bp
Cf3 Forward	5' ACGACAAGCATATGCTCGAG - ATGGCAGATGCTATGAAAACCTTAG 3'	734bp
Cf3 Reverse	5' TCCGGAACATCGTATGGGTA- ATCATCAACTGAGTCACTTTGAATATC 3'	734bp
Cf4 Forward	5'ACGACAAGCATATGCTCGAG- ATGTTACAGTATATTGTACATCAAT 3'	1599bp
Cf4 Reverse	5'TCCGGAACATCGTATGGGTA- AATGAATTCCAAGTCATCATTAGATGG 3'	1599bp
Cf5 Segment 1 Forward	5'ACGACAAGCATATGCTCGAG- GTACTCGCTACTGGTGGTGAAG- 3'	692bp
Cf5 Segment 1 Reverse	5'TCCGGAACATCGTATGGGTA- TTGATCAGAACCTAATGATGC- 3'	692bp
Cf5 Segment 2 Forward	5' ACGACAAGCATATGCTCGAG- AATCGTTGCATATTTCTATGG- 3'	1810bp
Cf5 Segment 2 Reverse	5' TCCGGAACATCGTATGGGTA- AGATAGTTCTCTTTTATTGAAG- 3'	1810bp

### **Assessment of the feline humoral immune response to *C. felis* orthologues of *P. falciparum* antigens**

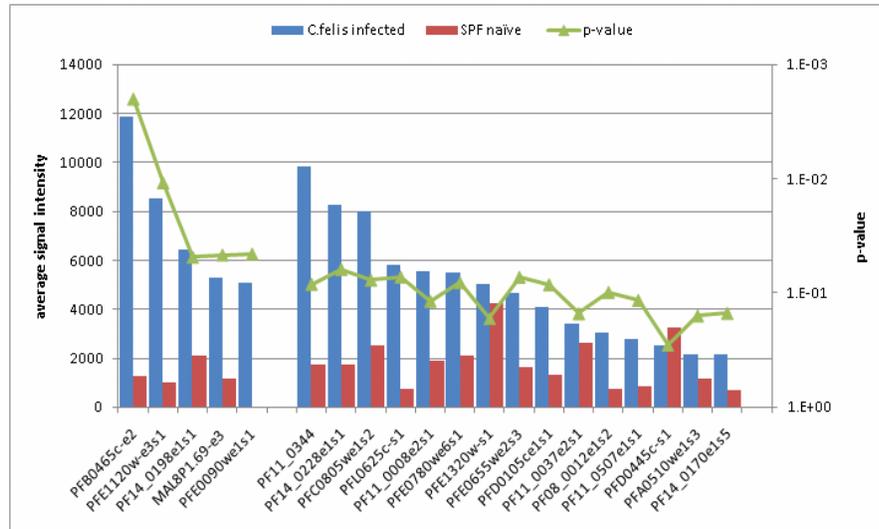
IVTT reaction components containing *C. felis* orthologues Cf1-Cf5 were purified using the N-terminal HIS tag under native conditions with Qiagen Ni-NTA Magnetic

Agarose Beads (Qiagen, Valencia, CA). Purity and quantity was assessed via Western blot analysis in duplicate using secondary antibodies against the N-terminal HIS tag and the C-terminal HA tag using mouse anti-poly-HIS monoclonal IgG<sub>2a</sub> antibody or mouse anti-poly-HA monoclonal antibody as previously described (Materials and Methods, Chapter 3).

The immune response to purified *C. felis* orthologues was assessed by Western blotting and immuno-dot blotting using pooled sera from 5 domestic cats that survived natural *C. felis* infection, as well as 5 naïve cats as previously described (Materials and Methods, Chapter 3).

## RESULTS AND DISCUSSION

### Protein microarray profiling



**Figure 1. Seroreactivity of *C. felis* immune sera against *P. falciparum* proteins.**

Five *P. falciparum* antigens to which cats that had survived *C. felis* infection were significantly ( $p \leq 0.05$ ) more seroreactive than the naïve cats were detected.

*Cytauxzoon felis* orthologues to the five *P. falciparum* antigens against which *C. felis* survivor sera was reactive were identified *in silico* (Table 2). These included *P. falciparum* monocarboxylate transporter (Cf1), *P. falciparum* glycine tRNA ligase (Cf2), *P. falciparum* 14-3-3 protein (Cf3), a conserved *Plasmodium* protein of unknown function (Cf4), and *P. falciparum* chromosome assembly factor 1 (Cf5).

<b>Table 2. Identification of five <i>C. felis</i> orthologues to <i>P. falciparum</i> proteins seroreactive to <i>C. felis</i> survivor serum</b>			
<b><i>C. felis</i> orthologue</b>	<b><i>P. falciparum</i> protein</b>	<b>Amino Acid Sequence Identity/Similarity %</b>	<b>Exons (single or multiple)</b>
<b>Cf1</b> Genemark Predicted 001090	PFB0465c (Monocarboxylate transporter)	33/55	Multiple
<b>Cf2</b> Genemark Predicted 002184	PF14_0198 (Glycine tRNA Ligase)	53/68	Single
<b>Cf3</b> Genemark Predicted 00120	MAL8P1.69 (14-3-3 Protein)	75/86	Multiple
<b>Cf4</b> Genemark Predicted 003345	PFE1120w (Conserved <i>Plasmodium</i> Protein Unknown Function)	47/65	Multiple
<b>Cf5</b> Genemark Predicted 000167	PFE0090W (Chromosome Assembly Factor 1)	33/47	Multiple

### **Assessment of the feline humoral immune response to *C. felis* orthologues of *P. falciparum* antigens**

The apparent molecular mass of Cf1, Cf2, Cf3, Cf4, Cf5 segment 1, Cf5 segment 2, and cf76 (carboxy terminus) observed with an HA tag on the carboxy terminus differed from predicted molecular mass (Table 3). Production and co-purification of partial transcripts as well as putative degradation products were observed for western blots probed with anti-HA and anti-HIS antibodies (Figure 2).

Western blot and immunodot blot analysis using pooled sera from 5 cats surviving *C. felis* infection revealed strong seroreactivity to several HIS-purified recombinant *C. felis* antigens.

**Cf1.** Cf1 represents the *C. felis* orthologue to a *P. falciparum* monocarboxylate transporter (MCT). This protein exports molecules such as lactate and pyruvate across the plasma membrane. *Plasmodium falciparum* relies heavily on glycolytic pathways to meet energy requirements which results in high quantities of intracellular lactic acid. Transport of lactate out of the cytosol is vital to maintaining intracellular pH and osmolality compatible with cell survival. Lactate transport represents a housekeeping process in most cells and the presence of a host immune response to the *C. felis* orthologue of *P. falciparum* MCT in *C. felis* warrants further investigation as a vaccine candidate, drug target or diagnostic marker.

**Cf2.** The *C. felis* orthologue to *P. falciparum* glycine tRNA ligase was recombinantly expressed and observed at the appropriate size on Western blot (Figure 2) and did not demonstrate seroreactivity on Western blot or immuno-dot blot when probed with *C. felis* hyperimmune sera (Figures 3 and 4). Glycine tRNA ligase binds ATP and catalyzes the formation of glycyl-tRNA which is required for incorporation of glycine into polypeptides. *Plasmodium falciparum* glycine tRNA ligase stimulates a measurable host humoral immune response in humans. Further investigation of Cf2 may be warranted to identify conformational epitopes.

**Cf3.** The *C. felis* orthologue to *P. falciparum* 14-3-3 protein was recombinantly expressed and observed at the appropriate size on Western blot in addition to a partial transcript or degradation product (Figure 2). Cf3 did not demonstrate

seroreactivity when probed with *C. felis* hyperimmune sera on Western blot or on immuno-dot blot (Figures 3 and 4). 14-3-3 is a highly conserved protein that functions as a mediator in signal transduction and cell cycle regulation. In one study, high levels of host autoantibodies to 14-3-3 generated in asymptomatic *P. falciparum* malaria were correlated with low levels of parasitemia implicating a mechanism for protection<sup>13</sup>. Further investigation of Cf3 for conformational epitopes is warranted.

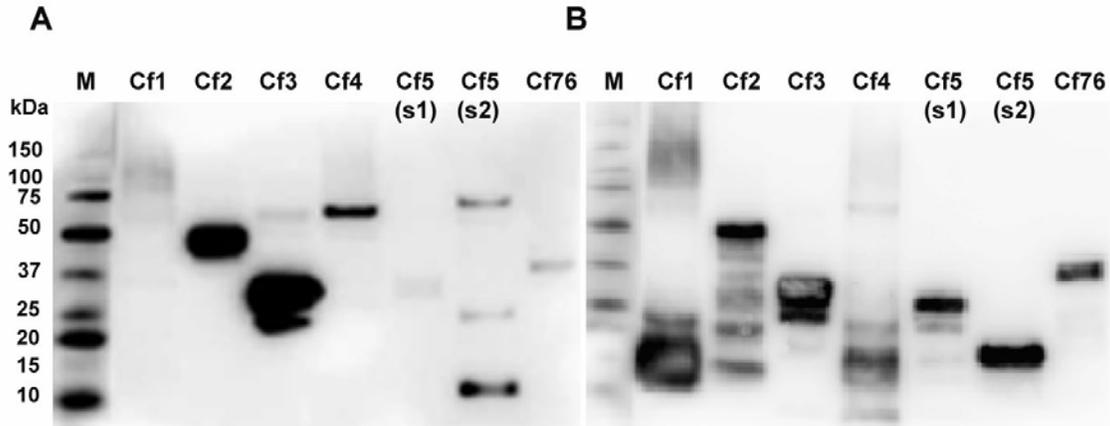
**Cf4.** Cf4 was recombinantly expressed and observed at the appropriate size (~61 kDa) on Western blot when probed with anti-HA antibody (Figure 2A). When probed with anti-HIS antibody on the carboxy terminus, the 61 kDa band is faintly visible with stronger bands observed for smaller partial transcripts or degradation products (Figure 2B). A robust signal was detected at 61 kDa and on immuno-dot blot when probed with *C. felis* hyperimmune sera (Figures 3A and 4). Significant seroreactivity was not observed when Cf4 was probed with sera from naive cats (Figure 3B). This protein is orthologue to a conserved *P. falciparum* protein of unknown function that is known to stimulate a host immune response to malaria infection. Our findings suggest that Cf4 should be considered as a vaccine candidate against *C. felis*.

**Cf5.** The *C. felis* orthologue to *P. falciparum* chromosome assembly factor was recombinantly expressed and purified in two segments. The first segment, Cf5s1, was observed at the appropriate size on Western blot in addition to a partial transcript or degradation product (Figure 2). Cf5s1 did not demonstrate

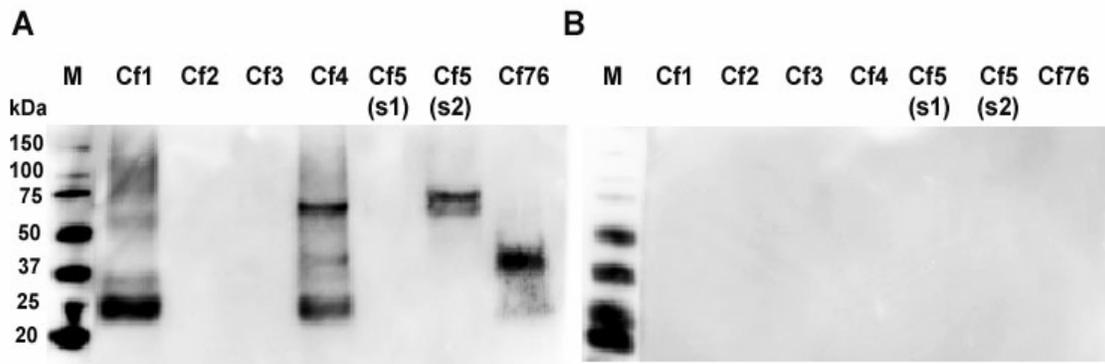
seroreactivity when probed with *C. felis* immune sera on Western blot or on immuno-dot blot (Figures 3 and 4). The second segment of Cf5, Cf5s2, was observed at ~70 kDa with two smaller transcripts when probed with anti-HA antibody. When probed with anti-HIS antibody only a small protein (~18 kDa) was observed. We identified a premature stop codon which would be consistent with the 18 kDa HIS-tagged protein seen. An alternative start site was identified downstream of the stop codon which would produce a protein of ~55 kDa and may represent the larger protein observed. It is well known that HIS purification is not a high fidelity system and it is possible that this larger protein was co-purified despite the absence of a HIS tag. Cf5s2 demonstrated strong seroreactivity at approximately 70kDa and on immuno-dot blot when probed with *C. felis* immune sera (Figures 3 and 4). Substantial reactivity was not observed using pooled sera from 5 cats that tested negative for *C. felis* on Western Blot (Figure 3B). Observed signal on immuno-dot blot (Figure 4) was attributed to low levels of cross-reacting antibodies unrelated to *C. felis* infection. Further studies are needed to resolve the discrepancy in protein size observed in this study. The next step is direct sequencing of the PCR product to rule out an error during amplification or plasmid replication. If the sequence appears correct, partial gene segments can be amplified and cloned and re-assessed for a feline humoral immune response.

**Table 3. Molecular mass of *C. felis* orthologues detected on Western blot.**

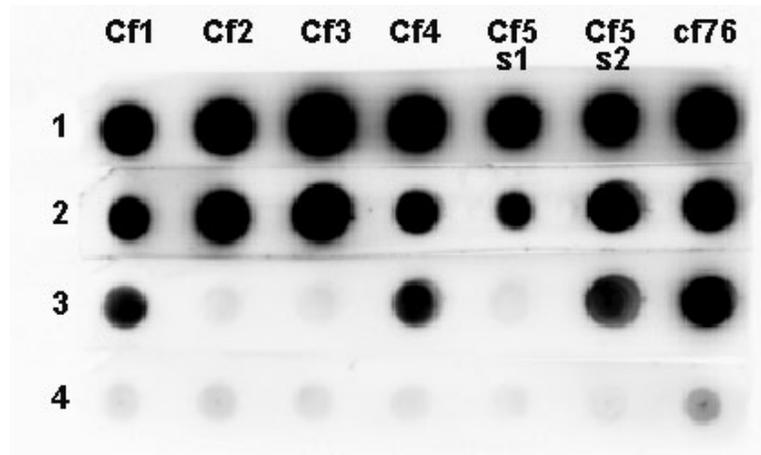
Orthologue	Approximate Apparent Molecular Mass		Expected Molecular Mass	Approximate Apparent Molecular Mass
	Anti-HA	Anti-HIS		<i>C. felis</i> Sera
<b>Cf1</b>	125 kDa	15, 22.5, and 125 kDa	53 kDa	25kDa
<b>Cf2</b>	47 kDa	47 kDa	47 kDa	no signal
<b>Cf3</b>	22 and 30 kDa	22 and 30 kDa	29.4 kDa	no signal
<b>Cf4</b>	61 kDa	<10-25 and 61 kDa	61 kDa	25kDa, 61kDa
<b>Cf5 segment 1</b>	29 kDa	25 and 29 kDa	29.6 kDa	no signal
<b>Cf5 segment 2</b>	12, 22, 70 kDa	18 kDa	70 kDa	70kDa
<b>cf76 (carboxy terminus)</b>	35 kDa	35 kDa	26.8 kDa	35kDa



**Figure 2. Assessment of purified *C. felis* orthologues by Western blot.** Purified orthologues Cf1-Cf5 and the carboxy terminus of cf76 (positive control) were probed with anti-HA C-terminal tag antibodies (**A**) anti-HIS N-terminal tag (**B**).



**Figure 3. Assessment of feline sero-reactivity to *C. felis* orthologues by Western blot.**



**Figure 4. Assessment of feline sero-reactivity to *C. felis* orthologues by Immuno-dot blot.** Purified orthologues Cf1-Cf5 and the carboxy terminus of cf76 (positive control) were probed with with anti-HIS N-terminal tag antibodies (1) anti-HA C-terminal tag (2), pooled sera (1:500) from cats surviving *C. felis* infection (3) or naive cats (4).

## CONCLUSIONS

Collectively these data support that *P. falciparum*-reactive sera from cats surviving *C. felis* infection reacted in kind to the *C. felis* orthologue of one *P. falciparum* antigen, a conserved Plasmodium protein of unknown function that has been demonstrated to stimulate a humoral response in malaria patients. Additional criteria to assess as a vaccine candidate include conservation of the protein across diverse geographic isolates and expression in schizonts, the life stage of *C. felis* previously demonstrated to confer protective immunity against cytauxzoonosis. Two additional *C. felis* antigens assessed in this study (orthologues to *P. falciparum* monocarboxylate transporter and chromosome assembly factor 1) require further characterization to accurately interpret the observed seroreactivity.

Here we report the first application of heterologous protein microarray immunoscreening across related genera. Heterologous resources developed for malaria served as a platform for *C. felis* antigen discovery. This novel approach allowed for the rapid identification of *C. felis* antigens recognized by the feline humoral immune system representing at least one new vaccine candidate antigen for cytauxzoonosis.

## REFERENCES

1. Birkenheuer AJ, Le JA, Valenzisi AM, et al. Cytauxzoon felis infection in cats in the mid-Atlantic states: 34 cases (1998-2004). *J Am Vet Med Assoc* 2006;228:568-571.
2. Cohn LA, Birkenheuer AJ, Brunner JD, et al. Efficacy of atovaquone and azithromycin or imidocarb dipropionate in cats with acute cytauxzoonosis. *J Vet Intern Med* 2011;25:55-60.
3. Tarigo JL, Scholl E.H, Bird D.McK., Cohn L.A, Levy M.G., Dean G.A., Birkenheuer A.J. The Cytauxzoon felis genome: A guide to preventing a fatal unculturable parasite of domestic cats. In: International Congress of Parasitology (ICOPA) XII 2010.
4. Davies DH, Liang X, Hernandez JE, et al. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc Natl Acad Sci U S A* 2005;102:547-552.
5. Davies DH, Wyatt LS, Newman FK, et al. Antibody profiling by proteome microarray reveals the immunogenicity of the attenuated smallpox vaccine modified vaccinia virus ankara is comparable to that of Dryvax. *J Virol* 2008;82:652-663.
6. Vigil A, Davies DH, Felgner PL. Defining the humoral immune response to infectious agents using high-density protein microarrays. *Future Microbiol* 2010;5:241-251.
7. Kreil DP, Karp NA, Lilley KS. DNA microarray normalization methods can remove bias from differential protein expression analysis of 2D difference gel electrophoresis results. *Bioinformatics* 2004;20:2026-2034.
8. Baldi P, Long AD. A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes. *Bioinformatics* 2001;17:509-519.
9. Long AD, Mangalam HJ, Chan BY, et al. Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. Analysis of global gene expression in Escherichia coli K12. *J Biol Chem* 2001;276:19937-19944.
10. Sundaresh S, Randall A, Unal B, et al. From protein microarrays to diagnostic antigen discovery: a study of the pathogen Francisella tularensis. *Bioinformatics* 2007;23:i508-518.

11. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat Med* 1990;9:811-818.
12. Sing T, Sander O, Beerenwinkel N, et al. ROCR: visualizing classifier performance in R. *Bioinformatics* 2005;21:3940-3941.
13. Duarte J, Herbert F, Guiyedi V, et al. High levels of immunoglobulin E autoantibody to 14-3-3 epsilon protein correlate with protection against severe *Plasmodium falciparum* malaria. *J Infect Dis* 206:1781-1789.

## CONCLUSIONS

The work performed in this study resulted in the rapid identification and characterization of the first two vaccine candidates for cytauxzoonosis. Using a newly created *C. felis* genome resource, we employed three bio-informatic strategies to accelerate antigen discovery. The high mortality, growing epidemic and cost of care point to vaccination as the only practical control strategy. The *C. felis* genome resource and *C. felis* antigens identified in this study will have a substantial impact on the diagnosis and prevention of cytauxzoonosis.

## APPENDICES

## APPENDIX A

### **CYTAUXZOOM FELIS LIFESTAGES**

#### **Ultrastructure- sporozoites, schizonts, & merozoites**

A transmission electron microscopy study provided a detailed description of schizogony which included the following sequence of events: 1) sporozoite infection of a monocytic cell, 2) presence of early underdeveloped schizonts characterized by an indistinct electron dense mass filling the cytoplasm, 3) visualization of nuclei with nuclear membranes within the mass, 4) nuclear division resulting in production of thousands of nuclei, 5) formation of a large multilobulated and multinucleated sporont syncytium containing interconnecting cytomeres attached by cytoplasmic bridges, 6) increase in size and complexity of the organisms with organelles forming at parasite apical margins, 7) sequential, rapid marginal fission of the sporont releasing individual merozoites with each containing a visible nucleus, mitochondria, rhoptry, and microneme at the apical end, 8) formation of residual bodies consisting of a rosette-like arrangement of merozoites, 9) release of free merozoites with rupture of the host cell, 10) visualization of a 'fuzzy' coating around the merozoite surface, and 11) endocytosis of the merozoite into a parasitophorous vacuole within host erythrocytes, loss of the merozoite 'fuzzy coating' and no residual damage to the host erythrocyte membrane seen<sup>1</sup>.

Additional ultrastructural morphologic assessment of *C. felis* merozoites on electron microscopy reported the following features: 1) 1 or 2 organisms per erythrocyte with

up to 4 seen less commonly, 2) a typical size of 0.5um to 1.5um in diameter, occasionally ranging up to 2.5um in diameter, 3) ring forms with dark staining peripheral basophilic nuclei and pale bluish cytoplasm, 4) occasional bipolar (binucleated) elongated forms, 5) occasional 'trailing' (budding) of a smaller parasite at one end of the larger or primary parasite, 6) a poorly defined double membrane bound nucleus with delicate chromatin, 1 or 2 strands of rough endoplasmic reticulum, ribosomes, nonplicated mitochondria, food vacuoles, and a cytostome <sup>2</sup>.

### **Immunophenotypic characterization of schizont infected feline cells**

Schizont infected feline host cells have demonstrated increased proliferating cell nuclear antigen (PCNA)/p53 activity, presence of lysozyme activity and absence of MAC387 (calprotectin) activity on immunohistochemistry <sup>3</sup>. Increased PCNA activity represents heightened replicative cycles and may confer increased survival of the parasite. Presence of lysozyme activity is consistent with myeloid origin. Calprotectin is a member of a family of calcium and zinc binding protein involved in diapedesis and the author's suggested that it's absence may allow for the a lack of diapedesis seen in the majority of schizont infected feline cells and/or a decrease in chemotaxis. The current information suggests that schizont infected feline host cells are likely of myeloid dendritic or hematopoietic (splenic or bone marrow origin) macrophages.

There are two immunophenotypic markers from the Leukocyte Antigen Biology Laboratory at the University of California at Davis that may help make the distinction:

1) 'cd1c', an Ig superfamily.type I transmembrane protein involved in non-

classical/non-major histocompatibility complex (MHC) antigen presentation that is expressed strongly in feline cells of dendritic origin and 2) 'cd11d' a member of the integrin  $\alpha$  chain family (the  $\alpha$  integrin subunit combines with CD18, an integrin  $\beta$ 2 subunit, to form the integrin  $\alpha$ D $\beta$ 2 which binds CD53/intercellular adhesion molecule (ICAM3) which is expressed in feline hematopoietic macrophages and is typically negative in cells of dendritic origin <sup>4</sup>.

## REFERENCES

1. Kocan AA, Kocan KM, Blouin EF, et al. A redescription of schizogony of *Cytauxzoon felis* in the domestic cat. *Ann N Y Acad Sci* 1992;653:161-167.
2. Simpson CF, Harvey JW, Carlisle JW. Ultrastructure of the intraerythrocytic stage of *Cytauxzoon felis*. *Am J Vet Res* 1985;46:1178-1180.
3. Susta L, Torres-Velez F, Zhang J, et al. An in situ hybridization and immunohistochemical study of cytauxzoonosis in domestic cats. *Vet Pathol* 2009;46:1197-1204.
4. Affolter VK, Moore PF. Feline progressive histiocytosis. *Vet Pathol* 2006;43:646-655.

## APPENDIX B

### NATURAL AND ABERRANT HOSTS OF *C. FELIS*

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

Small piroplasms (<1.5um) from *Cytauxzoon* spp., *Babesia* spp., and *Theileria* spp. are difficult to reliably differentiate between on morphologic criteria alone. It is important to consider the epidemiology of morphologically indistinguishable organisms when diagnosing feline piroplasmosis and molecular confirmation is recommended whenever possible.

*C. felis* vs. other small piroplasm infection in the United States: *C. felis* infection has been confirmed in domestic cats and bobcats as previously cited as well as tigers, and Florida panthers<sup>1,2</sup>. Two suspected but unconfirmed cases of cytauxzoonosis have been reported in cheetahs<sup>3</sup>. Infection of domestic cats and bobcats with small piroplasms other than *C. felis* has not been reported in the United States. Two reports of infection with other small piroplasms include *B. felis* in Florida Panthers and Texas Cougars, and *C. manul* in Pallus Cats (imported from Mongolia)<sup>2,4</sup>.

*C. felis* vs. other small piroplasm infection in other countries: *C. felis* infection has been reported in Brazil (domestic cats, lions, jaguars, pumas, ocelots, and little spotted cats), Germany (lions) and Spain (lynx)<sup>5-8</sup>. Infection of felids with other small piroplasms includes: 1) *B. felis* in Africa (the domestic cat, the African wild cat [*Felis sylvestrus ocreata*], and the caracal [*F. caracal*], *B. microti*-like organisms in Portugal

(a domestic cat), 2) *B. leo* in South Africa (in lions), and 3) *C. manul* in Mongolia, and 4) several reports of unidentified piroplasms in a variety of felid hosts <sup>4,9-14</sup>.

### **Cheetahs** (*Acinonyx* sp.)

**Infection with *C. felis*-like organisms in cheetahs has been reported but was not confirmed.** Two sibling cheetahs [*Acinonyx jubatus*] from Oregon were reported to have intra-erythrocytic *C. felis*-like piroplasms in 1978 <sup>3</sup>. The cheetahs were captive born, transported to Africa in 1977 at 9 months of age for habitat reintroduction and were returned to the U.S. 2 months later following development of lameness in the male. Upon admission to the Veterinary Medical Teaching Center at University of California at Davis at 1.5 years of age for orthopedic disease, *C. felis*-like piroplasms were seen in less than 0.1% of erythrocytes on examination of bloodsmears over a 9 month period. Examination of the female sibling cheetah's bloodsmear revealed similar organisms. Intra-peritoneal inoculation of a domestic cat with whole blood from the male cheetah immediately post-collection failed to result in detectable disease. Infection with small piroplasms of *Babesia* sp. and *Theileria*-like origin has been reported in cheetahs from Africa and cannot be excluded <sup>15</sup>.

### **Cougars & Panthers** (*Puma concolor*)

Prevalence of cytauxzoonosis in free-ranging Florida Panthers [*Puma concolor coryi*] and Texas Cougars in Florida was reported to be 35% and 39% respectively in

samples collected between 1983 and 1997 based on detection of small piroplasm on bloodsmear examination<sup>16</sup>. However, **the prevalence of *C. felis* in a molecular survey of free-ranging Florida Panthers and Texas Cougars in Florida was 13% (2/37 panthers and 2/2 cougars)<sup>2</sup>**. In this study 82% (32/39) of Florida Panthers tested positive for Babesia sp. in samples collected between 1989 and 2005 and co-infection with *C. felis* was detected in two panthers. **This was the first report of feline babesiosis and co-infection of *C. felis* with Babesia sp. in Florida Panthers in the United States.** Given the higher prevalence of Babesia in this population and difficulty in distinguishing small piroplasms on bloodsmear examination alone, it is possible that the prevalence of cytauxzoonosis was over-estimated in the earlier report. The earlier study also reported that cytauxzoonosis did not have a negative effect on hematologic parameters in chronically infected Florida Panthers and Texas Cougars although confirmation of *C. felis* infection was not confirmed. Hematologic findings from three cougars from the same area acutely infected with *C. felis* were later reported to include anemia (regenerative in two cats), thrombocytopenia and leukopenia in one cat, and increased bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST)<sup>17</sup>. In 2011, the first report of an infected cougar outside of the Florida panther population was identified in Louisiana<sup>18</sup>.

## **Lions (Panthera sp.)**

Between 1991 and 1992, small intra-erythrocytic *C. felis*-like piroplasms were found in bloodsmears from 47 lions [*Panthera leo*] at a national park in the Republic of South Africa<sup>19</sup>. Immunofluorescent antibody (IFA) testing for *C. felis* and *B. felis* on all samples were negative and these organisms were most likely *B. leo*. Blood from 16 of the lions was sub-inoculated into another lion, 2 leopards, and a domestic cat and parasitemia was only observed following splenectomy of the domestic cat 42d. PI; this domestic cat remained *C. felis* IFA negative. The results from this study were inconclusive. Similar inconclusive results were reported in 2004 when bloodsmears from 2 out of 7 mountain lions [*Puma concolor*] at a rehabilitation center in Brazil revealed *C. felis*-like parasitemias between 10-20% in the absence of clinical disease<sup>20</sup>. Sub-inoculation of 2 domestic cats with blood from these panthers did not result in detectable disease. In 2012, *C. felis* was identified by PCR in four of eight clinically asymptomatic captive born tigers in a heavily wooded region of northern Arkansas<sup>21</sup>.

**Two cases of fatal *C. felis* infection in lions occurred in 1998 in Brazil<sup>8</sup>.** An 8 year old lioness died acutely 45 days after her 6month old cub was found dead in his zoo exhibit and histopathologic lesions consistent with acute cytauxzoonosis were confirmed in both cases. High numbers of *A. cajennese* ticks were found in the lion exhibits as well as in the exhibits of two other felids (a jaguar [*Panthera onca*] and a Little Spotted Cat [*Leopardus tigrinus*]) in which *C. felis*-like intra-erythrocytic

piroplasms were identified two weeks later. Molecular characterization of 18SrRNA from these two felids revealed sequences (DQ382276 and DQ382277) with 99% similarity to a *C. felis* sequence in Genbank from the U.S. (AF 399930). *Amblyomma americanum* has been identified as the biologic vector for *C. felis* and it is possible that *A. cajennense* may have been a competent vector in these cases<sup>22</sup>.

### **Iberian Lynx** (*Lynx pardinus*)

**Cytauxzoon sp. infection has been reported in Iberian lynx in Spain**<sup>7,23,24</sup>. In two studies the prevalence of Cytauxzoonosis in free-ranging Iberian lynx in Spain as determined by *C. felis* 18SrRNA PCR testing was 15%<sup>7,24</sup>. The 18SrRNA sequences from four different lynx in Spain (EF094468-EF094470, AY496273) shared between 95.37%-99.76% sequence identity and sequences identities between these lynx, a domestic cat in Spain (AY309956), and domestic cats in the U.S. (AF399930, AY531524) were between 98.90-99.13% and 95.37-95.85% respectively<sup>7</sup>.

### **Pallus Cats** (*Otocolobus manul*)

Although ***C. felis* infection has not been reported in Pallus cats, infection with a closely related species, *C. manul* was discovered in 2000**<sup>4</sup>. Bloodsmear examination of samples taken from four clinically asymptomatic Mongolian Pallus cats transported to a zoo in Oklahoma revealed a <1% parasitemia with small piroplasms indistinguishable from *C. felis*. At that time, sequence differentiation of

18srRNA between the organisms from one of these cats and *C. felis* (L19080) was 3.6%. A later study reported a corrected mean of 1.69% sequence differentiation between *C. manul* from the remaining three Pallus cats from Mongolia and the New World isolate of *C. felis* (L19080, AY309956) <sup>25</sup>.

### **Tigers** (*Panthera tigris*)

**Two fatal cases of cytauxzoonosis have been reported in tigers** <sup>1,6</sup>. The first fatal *C. felis* infection in a tiger occurred in 1994 at a zoo in Germany <sup>6</sup>. An 18 month old, female Bengal tiger born at German zoo presented with anorexia, lethargy and dyspnea. She died within hours of presentation and histopathologic and electron microscopy findings consistent with acute cytauxzoonosis were confirmed.

Interestingly, the onset of her disease occurred 14months after three young bobcats from the United States were imported to the zoo. A twin brother sharing her enclosure grew to adulthood without development of disease. Examination of a bloodsmear from this sibling for *C. felis* piroplasms was not reported and occurrence of a non-fatal infection cannot be excluded.

A second fatal *C. felis* infection in a tiger was diagnosed at University of Florida Veterinary Teaching Hospital 1996 <sup>1</sup>. A 7 year old, female, captive bred white tiger with a feline leukemia and feline immunodeficiency virus negative status presented febrile (105.2°F) with clinicopathologic findings including mild to moderate anemia, moderate leukopenia, severe thrombocytopenia, presence of fibrin degradation

products and a 5% intra-erythrocytic parasitemia with *C. felis*-like piroplasms. The tiger died on day 2 and histopathologic findings consistent with acute cytauxzoonosis were confirmed. Two female *A. americanum* ticks were removed from this tiger which is consistent with a recent report suggesting that *A. americanum*, and not *D. variabilis* as believed at the time this case occurred, is the most likely biologic vector for *C. felis*<sup>22</sup>. Intra-erythrocytic piroplasms consistent with *C. felis* were found in wild bobcats, a Texas cougar [*Felis concolor*], and three Florida panthers [*Felis concolor coryi*] within the same housing facility in Northern Florida.

#### **Additional Various Wild Cats**

In 2009, a survey of asymptomatic wild felids in **Brazil reported *C. felis*** positive 18SrRNA PCR results in 2 out of 9 **pumas** [*Puma concolor*], 6 out of 29 **ocelots** [*Leopardus pardalis*], and 1 out of 9 **jaguars** [*Panthera onca*] tested<sup>5</sup>. In the same survey 14 little spotted cats [*Leopardus tigrinus*], 2 margays [*Leopardus wiedii*], 3 pampas cats [*Oncifelis colocolo*], and 6 jaguarundis [*Puma yagouaroundi*] tested negative for *C. felis*. The sequences from the ocelots, pumas and jaguar (GenBank accession numbers EU376525, EU376525, and EU376525 respectively) were 99% similar to a GenBank accession from a Brazilian *Leopardus tigrinus* (DQ382277) and 98% similar to a sequence from North American cats (AF399930).

## Wildlife, Laboratory, and Domestic Farm Animals

In the early 1980s interspecies transmission of *C. felis* was investigated to identify additional potential natural and aberrant hosts among 91 wildlife, laboratory, and domestic farm animals<sup>26</sup>. The animals tested included:

- 17 different species of wildlife - 2 coyotes [*Canis latrans*], 1 red fox [*Vulpes fulva*], 3 striped skunks [*Mephitis mephitis*], 3 raccoon [*Procyon lotor*], 1 woodchuck [*Marmota monax*], 2 yellow bellied marmots [*Marmota flaviventris*], 2 opossum [*Didelphis marsupialis*], 2 ground squirrels [*Citellus tridecemlineatus*], 1 eastern grey squirrel [*Sciurus carolinensis*], 1 prairie meadow vole [*Microtus ochrogaster*], 3 little brown bats [*Myotis lucifugus*], 7 prairie white footed mice [*Peromyscus maniculatus*], 2 cotton tail rabbit [*Sylvilagus floridanus*], 2 white-tailed deer [*Odocoileus virginianus*], 1 ocelot [*Leopardus pardalis*], 1 mountain lion [*Felis concolor*], 1 eastern bobcat [*Lynx rufus rufus*] and one Florida bobcat [*Lynx rufus floridanus*]
- 9 different species of laboratory animals - 7 mice [*Mus musculus*], 12 nude mice [*M. musculus*, BALB/c-nu], 4 rats [*Rattus norvegicus*], 5 hamsters [*Mesocricetus auratus*], 2 gerbils [*Meriones unguiculatus*], 6 guinea pigs [*Cavia procellus*], 4 rabbits [*Oryctolagus cuniculus*], 3 squirrel monkeys [*Saimiri sciureus*], 2 chinchillas [*Chinchilla laniger*], 2 dogs [*Canis familiaris*], and 1 cat [*Felis domesticus*]
- 4 species of domestic farm animals including 2 swine [*Sus scrofa*], 2 Angus cattle [*Bos taurus*], 2 sheep [*Ovis aries*], and 2 goats [*Capra hircus*].

All animals were inoculated parenterally with fresh tissues from a domestic cat with acute cytauxzoonosis containing a lethal dose of *C. felis*, observed for clinical signs, held from 7 to up to 365 days (the eastern bobcat), euthanized and necropsied with histopathologic examination. Many of the tissues from these animals were later subinoculated into naive domestic cats. The Florida bobcat was the only animal to develop acute cytauxzoonosis and die within 14 days with similar presentation and histologic findings as previously described for the domestic cat. The Eastern bobcat developed a persistent parasitemia in the absence of overt disease. No other species experienced clinical signs or parasitemia with the exception of the 2 sheep in which parasitemia was detected (<0.01%). However sub-inoculation of domestic cats with tissues from the sheep and all animals except the bobcats did not induce cytauxzoonosis and the possibilities that the inclusions seen were artifact or that the sheep had a pre-existing infection with a different hemaprotzoan such as Babesia or Theileria cannot be excluded.

## REFERENCES

1. Garner MM, Lung NP, Citino S, et al. Fatal cytauxzoonosis in a captive-reared white tiger (*Panthera tigris*). *Vet Pathol* 1996;33:82-86.
2. Yabsley MJ, Murphy SM, Cunningham MW. Molecular detection and characterization of *Cytauxzoon felis* and a *Babesia* species in cougars from Florida. *J Wildl Dis* 2006;42:366-374.
3. Zinkl JG, McDonald SE, Kier AB, et al. Cytauxzoon-like organisms in erythrocytes of two cheetahs. *J Am Vet Med Assoc* 1981;179:1261-1262.

4. Ketz-Riley CJ, Reichard MV, Van den Bussche RA, et al. An intraerythrocytic small piroplasm in wild-caught Pallas's cats (*Otocolobus manul*) from Mongolia. *J Wildl Dis* 2003;39:424-430.
5. Andre MR, Adania CH, Machado RZ, et al. Molecular detection of *Cytauxzoon* spp. in asymptomatic Brazilian wild captive felids. *J Wildl Dis* 2009;45:234-237.
6. Jakob W, Wesemeier HH. A fatal infection in a Bengal tiger resembling cytauxzoonosis in domestic cats. *J Comp Pathol* 1996;114:439-444.
7. Millan J, Naranjo V, Rodriguez A, et al. Prevalence of infection and 18S rRNA gene sequences of *Cytauxzoon* species in Iberian lynx (*Lynx pardinus*) in Spain. *Parasitology* 2007;134:995-1001.
8. Peixoto PV, Soares CO, Scofield A, et al. Fatal cytauxzoonosis in captive-reared lions in Brazil. *Vet Parasitol* 2007;145:383-387.
9. Averbeck GA, Bjork KE, Packer C, et al. Prevalence of hematozoans in lions (*Panthera leo*) and cheetah (*Acinonyx jubatus*) in Serengeti National Park and Ngorongoro Crater, Tanzania. *J Wildl Dis* 1990;26:392-394.
10. Davis LJ. On a piroplasm of the Sudanese wild cat (*Felis ocreata*). *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1929;22:523-534.
11. Dennig HK. [Babesia infections in exotic cats and the significance of these blood parasites for veterinary research]. *Acta Zool Pathol Antverp* 1969;48:361-367.
12. Dennig HK, Brocklesby DW. *Babesia pantherae* sp.nov., a piroplasm of the leopard (*Panthera pardus*). *Parasitology* 1972;64:525-532.
13. Jackson C, Dunning FJ. Biliary fever (Nutalliosis) of the cat: A case in the Stellen-bosch District. *Journal of the South African Veterinary Medical Association* 1937;8:83-88.
14. Penzhorn BL, Kjemtrup AM, Lopez-Rebollar LM, et al. *Babesia leo* n. sp. from lions in the Kruger National Park, South Africa, and its relation to other small piroplasms. *J Parasitol* 2001;87:681-685.
15. Bosman AM, Oosthuizen MC, Peirce MA, et al. *Babesia lengau* sp. nov., a novel *Babesia* species in cheetah (*Acinonyx jubatus*, Schreber, 1775) populations in South Africa. *J Clin Microbiol* 48:2703-2708.

16. Rotstein DS, Taylor SK, Harvey JW, et al. Hematologic effects of cytauxzoonosis in Florida panthers and Texas cougars in Florida. *J Wildl Dis* 1999;35:613-617.
17. Harvey JW, Dunbar MR, Norton TM, et al. Laboratory findings in acute *Cytauxzoon felis* infection in cougars (*Puma concolor* cougar) in Florida. *J Zoo Wildl Med* 2007;38:285-291.
18. Shock BC, Murphy SM, Patton LL, et al. Distribution and prevalence of *Cytauxzoon felis* in bobcats (*Lynx rufus*), the natural reservoir, and other wild felids in thirteen states. *Vet Parasitol* 175:325-330.
19. Lopez-Rebollar LM, Penzhorn BL, de Waal DT, et al. A possible new piroplasm in lions from the Republic of South Africa. *J Wildl Dis* 1999;35:82-85.
20. Soares JR, Izabel SA, Tercio N, et al. *Cytauxzoon felis* in the Mountain Lion (*Puma concolor*): A Case Report. *Journal of Animal and Veterinary Advances* 2004;3:820-823.
21. Lewis KM, Cohn LA, Downey ME, et al. Evaluation of *Cytauxzoon felis* infection status in captive-born wild felids housed in an area endemic for the pathogen. *J Am Vet Med Assoc* 241:1088-1092.
22. Reichard MV, Meinkoth JH, Edwards AC, et al. Transmission of *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum*. *Vet Parasitol* 2009;161:110-115.
23. Luaces I, Aguirre E, Garcia-Montijano M, et al. First report of an intraerythrocytic small piroplasm in wild Iberian lynx (*Lynx pardinus*). *J Wildl Dis* 2005;41:810-815.
24. Millan J, Candela MG, Palomares F, et al. Disease threats to the endangered Iberian lynx (*Lynx pardinus*). *Vet J* 2009;182:114-124.
25. Reichard MV, Van Den Bussche RA, Meinkoth JH, et al. A new species of *Cytauxzoon* from Pallas' cats caught in Mongolia and comments on the systematics and taxonomy of piroplasmids. *J Parasitol* 2005;91:420-426.
26. Kier AB, Wightman SR, Wagner JE. Interspecies transmission of *Cytauxzoon felis*. *Am J Vet Res* 1982;43:102-105.

## APPENDIX C

### TRANSMISSION OF *C. FELIS* - EXPERIMENTAL STUDIES

#### **Bobcat to domestic cat via *D. variabilis***

The first study implicating the bobcat as a natural reservoir host and demonstrating an arthropod-borne mechanism of transmission of *C. felis* to domestic cats was conducted in 1979 <sup>1</sup>. *C. felis* was experimentally transmitted to domestic cats by American Dog ticks [*D. variabilis*] that were fed on an infected bobcat. Laboratory reared *D. variabilis* nymphs were allowed to feed on a wild-trapped, splenectomized Oklahoma bobcat that had a 40% parasitemia with *C. felis*-like piroplasms. The engorged nymphs were allowed to molt and were fed on 2 splenectomized naive domestic cats which then died of acute cytauxzoonosis 13 and 17 days PI. Tissue lesions consistent with cytauxzoonosis were confirmed post-mortem. Blood from the same parasitemic bobcat was later inoculated into a domestic cat and a chronic parasitemia developed in the absence of clinical symptoms. This study demonstrated that fatal *C. felis* infection can be transmitted from a chronically infected splenectomized bobcat by *D. variabilis* to splenectomized domestic cats.

#### **Bobcat to bobcat via *D. variabilis***

The second study investigating transmission of *C. felis* by *D. variabilis*, revealed a transient, non-lethal schizogenous tissue phase in bobcats <sup>2</sup>. A chronically infected bobcat was splenectomized and schizonts were not seen on histopathology. When

homogenates from this spleen were administered parenterally to a domestic cat a chronic parasitemia in the absence of acute cytauxzoonosis ensued. *D. variabilis* nymphs were fed to repletion on the splenectomized bobcat and following molting the adult ticks were fed on two naive bobcats. Schizonts were seen in lymph nodes from both bobcats at 11days PI and one bobcat died 19days PI of acute cytauxzoonosis. Schizonts were no longer detected in lymph node tissue from the surviving bobcat at 30days PI. Lymph node homogenates from both bobcats 11days PI induced fatal cytauxzoonosis in domestic cats. Lymph node homogenates from one bobcat 30days PI induced a chronic parasitemia in the absence of acute cytauxzoonosis. This study concluded that: 1) *D. variabilis* transmits *C. felis* from bobcat to bobcat 2) a short-lived non-fatal schizogenous *C. felis* tissue phase occurs in bobcats, 3) parenteral inoculation of domestic cats with bobcat tissues lacking schizonts results in chronic parasitemia in the absence of fatal disease, and 4) parenteral inoculation of domestic cats with schizont containing bobcat tissues induces fatal cytauxzoonosis.

### **Bobcat to domestic cat via *C. felis* infected tissues**

The next study providing strong evidence that the bobcat is a natural reservoir host for *C. felis* was reported in 1983<sup>3</sup>. Blood from four asymptomatic, wild trapped bobcats with *C. felis*-like piroplasms in circulation at less than 2.5% was injected subcutaneously and intraperitoneally into 4 domestic cats. Three of the domestic cats developed a chronic parasitemia and one developed acute and fatal

cytauxzoonosis. One of the surviving cats later developed acute and fatal cytauxzoonosis when inoculated with blood from a domestic cat with acute cytauxzoonosis. The remaining two surviving cats were euthanized 2 months PI and showed no tissue lesions consistent with *C. felis*, however the occurrence of a transient schizogenous phase cannot be excluded. Two of the bobcats in this study were inoculated with virulent *C. felis* tissue from a domestic cat and both bobcats remained asymptomatic and had no histopathologic lesions consistent with cytauxzoonosis on necropsy 30 days PI. This was the first study substantiating the bobcat as the natural reservoir host by showing that naive domestic cats could develop fatal cytauxzoonosis when inoculated with blood from clinically asymptomatic parasitemic bobcats and that bobcats were resistant to disease when given a virulent *C. felis* inoculum.

Another study looking at transmission of *C. felis* from bobcats to domestic cats suggested that the ability to transmit *C. felis* and the vulnerability to develop cytauxzoonosis was different for different species of bobcats <sup>4</sup>. A Florida bobcat and an eastern bobcat were administered virulent *C. felis* inoculum from a domestic cat. Both bobcats developed parasitemia however the Florida bobcat developed fatal cytauxzoonosis and the eastern bobcat did not. Sub-inoculation of blood or tissue from the Florida bobcat produced fatal disease in domestic cats however similar inoculation from the eastern bobcat resulted in parasitemia in the absence of clinical disease. Interestingly, five of the domestic cats inoculated with eastern bobcat blood

and tissues were splenectomized. All domestic cats were later challenged with virulent *C. felis* inoculum of domestic cat origin and developed fatal cytauxzoonosis. The authors suggested subspecies variability in susceptibility to cytauxzoonosis between bobcats however with only one animal representing each group, an atypical individual resistance cannot be excluded. This study also demonstrated increased parasitemia and a moderate regenerative anemia developed in the eastern bobcat with splenectomy, administration of steroids or co-delivery of *C. felis* inoculum with Freund's adjuvant.

### **Domestic cat to domestic cat**

Non-arthropod transmission of *C. felis* to domestic cats was investigated in several experimental studies in the late 1970s and early 1980s<sup>5,6</sup>. In 1980, one study investigated multiple potential routes including parenteral, oral, and intercat transmission of *C. felis* to domestic cats<sup>6</sup>. One hundred thirty one domestic cats were infected intravenously, intraperitoneally, or subcutaneously with fresh and frozen preparations of EDTA whole blood and tissue homogenates of lymph node, liver, lung and spleen from acutely infected domestic cats. Inoculum doses were standardized with subcutaneous administration of 'full strength' tissue homogenates (1 part tissue to 9 parts cryoprotective fluid) and serial dilutions from 1:10 to 1:10e6. Disease was not established with dilutions greater than 1:10 and inoculums were effective after six months of storage in liquid nitrogen. Following parenteral infection, all cats developed acute cytauxzoonosis with the most rapid onset of disease

occurring in two splenectomized cats. However, infection was not induced when these tissues were administered intra-gastrically or when non-infected cats were housed together with infected cats in the absence of arthropod vectors. This study concluded that *C. felis* can be successfully transmitted to domestic cats and induce acute cytauxzoonosis using tissues from cats with acute cytauxzoonosis by all routes of parenteral administration (subcutaneous, intravenous, intraperitoneal) but not through oral administration or intercat exposure<sup>6</sup>.

In another study, *D. variabilis* nymphs were fed to repletion on infected domestic cats, allowed to molt, and then fed on naive domestic cats in which infection was successfully established identifying that *C. felis* is transmitted transstadially (between lifestages) of the tick vector<sup>7</sup>.

### **Wild felid to domestic cat**

**iatrogenic transmission of *C. felis* from a florida panther to domestic cat**<sup>8</sup>. A domestic cat developed fatal cytauxzoonosis following inoculation with mononuclear cells harvested from a *C. felis* infected Florida panther. A wild Florida panther [*Felix concolor coryi*] with conflicting test results for Feline Immunodeficiency Virus (FIV) while under care for orthopedic injuries. To determine FIV status, mononuclear cells isolated from whole blood from this panther were injected intra-peritoneally into a domestic cat to look for seroconversion. The domestic cat developed acute cytauxzoonosis and died 12days PI. Later, piroplasms consistent with *C. felis* were

detected on bloodsmear exam of the panther who had previously received two blood transfusions from other Florida panthers.

### **Transmission of *C. manul* merozoites from Pallas cats to domestic cats <sup>9</sup>.**

Six domestic cats were inoculated intravenously with *C. manul* infected blood from 2 Pallas cats [*Otocolobus manul*] containing  $>1 \times 10^6$  merozoites. Within 14 days PI, all 6 cats developed parasitemia ( $<0.1-0.7\%$ ) and when later challenged with virulent *C. felis* infected tissues, all 6 cats developed acute cytauxzoonosis and died. This study demonstrated that domestic cats can be persistently infected with *C. manul* merozoites and this infection does not confer immunoprotection to *C. felis* induced cytauxzoonosis. Of particular interest would be to investigate the ability to establish acute cytauxzoonosis in domestic cats with infection of *C. manul* sporozoites and/or schizonts and assess the resulting disease course and potential immunoprotection upon survival.

### **Domestic cat to domestic cat via *A. americanum***

*Cytauxzoon felis* was transmitted by *A. americanum* adult ticks to a domestic cat <sup>10</sup>.

One hundred nymphs each of *A. americanum*, *D. variabilis*, *Ixodes scapularis*, and *Rhipicephalus sanguineus* were fed to repletion on a domestic cat chronically infected with *C. felis* harboring a parasitemia of approximately 0.15%. Infected nymphs were allowed to molt and four naive domestic cats were infested with the surviving *A. americanum* (100), *D. variabilis* (95), *I. scapularis* (16) or *R. sanguineus*

(64) adult ticks. Only the cat infested with *A. americanum* became ill showing signs of anorexia, fever, depression, lethargy, pale mucous membranes, splenomegaly, icterus, and dyspnea. *C. felis*-specific PCR on whole blood collected throughout the study was positive only for the cat infested with *A. americanum* ticks. Schizonts were detected in splenic aspirates from the *C. felis* infected cat. The three additional cats remained asymptomatic, however given the previous experimental infection of domestic cats with *D. variabilis*, the cat infested with these ticks was euthanized and *C. felis* infection was not detected on microscopic examination <sup>1</sup>.

## REFERENCES

1. Blouin EF, Kocan AA, Glenn BL, et al. Transmission of *Cytauxzoon felis* Kier, 1979 from bobcats, *Felis rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). *J Wildl Dis* 1984;20:241-242.
2. Blouin EF, Kocan AA, Kocan KM, et al. Evidence of a limited schizogonous cycle for *Cytauxzoon felis* in bobcats following exposure to infected ticks. *J Wildl Dis* 1987;23:499-501.
3. Glenn BL, Kocan AA, Blouin EF. *Cytauxzoonosis* in bobcats. *J Am Vet Med Assoc* 1983;183:1155-1158.
4. Kier AB, Wagner JE, Morehouse LG. Experimental transmission of *Cytauxzoon felis* from bobcats (*Lynx rufus*) to domestic cats (*Felis domesticus*). *Am J Vet Res* 1982;43:97-101.
5. Ferris DH. A progress report on the status of a new disease of American cats: *cytauxzoonosis*. *Comp Immunol Microbiol Infect Dis* 1979;1:269-276.
6. Wagner JE, Ferris DH, Kier AB, et al. Experimentally induced *cytauxzoonosis*-like disease in domestic cats. *Vet Parasitol* 1980;6:305-311.
7. Kocan AA, Kocan KM, Blouin EF, et al. A redescription of schizogony of *Cytauxzoon felis* in the domestic cat. *Ann N Y Acad Sci* 1992;653:161-167.

8. Butt MT, Bowman D, Barr MC, et al. Iatrogenic transmission of *Cytauxzoon felis* from a Florida panther (*Felis concolor coryi*) to a domestic cat. *J Wildl Dis* 1991;27:342-347.
9. Joyner PH, Reichard MV, Meinkoth JH, et al. Experimental infection of domestic cats (*Felis domesticus*) with *Cytauxzoon manul* from Pallas' cats (*Otocolobus manul*). *Vet Parasitol* 2007;146:302-306.
10. Reichard MV, Meinkoth JH, Edwards AC, et al. Transmission of *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum*. *Vet Parasitol* 2009;161:110-115.