Identifying *Cytauxzoon felis* infections through detection and evaluation of immunodominant *C. felis* antigens

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ABSTRACT

Cytauxzoon felis is a tick-transmitted apicomplexan parasite that infects mononuclear cells and erythrocytes of domestic cats. The mortality rate of cytauxzoonosis in domestic cats is close to 90%, with the cats that survive remaining persistently infected for life without displaying clinical signs. These carrier cats can serve as additional infection sources of *C. felis* for naïve ticks and further increase the risk of domestic cat exposure to *C. felis*. Our goal is to develop a diagnostic assay to identify carrier cats whose low level of circulating parasitemia lead to inconsistent results with nucleic acid detection methods. Our experimental approach includes probing protein extracts from *C. felis* infected tissues with serum from carrier cats, identifying immunodominant *C. felis* antigens using LC-MS/MS analysis, followed by expressing, purifying and evaluating select immunodominant antigens by western blot and ELISA. Currently, we have developed a real-time quantitative PCR assay against Cf76 to quantify infection levels in *C. felis* infected tissues and probed protein extracts from *C. felis* infected tissues with serum from carrier cats. We are also expressing and purifying Cf76 to use as a positive control to compare with novel immunodominant proteins identified by LC-MS/MS. The ideal immunoassay will detect carrier cats, including those with low levels of parasitemia that may go undetected by using molecular assays, and rapidly diagnose *C. felis* infections earlier in the parasite's lifecycle so that the veterinarian can make informed decisions regarding patient treatment and welfare.

INTRODUCTION

- *C. felis* can cause anemia, pyrexia, icterus and hepatosplenomegaly in domestic cats.[1]
- *C. felis* has a mortality rate of 90% in domestic cats. Cats that survive acute clinical disease develop a protective immune response against the schizont stage and become lifelong carriers.[1]
- Carrier cats remain persistently infected with *C. felis*. Although they stop displaying clinical signs, they have low circulating parasitemia and serve as reservoirs of infection.[1]
- *C. felis* is transmitted by ticks, primarily *Amblyomma americanum*, also known as the Lone Star tick, with transmission most common in the southern and central U.S. states (**Fig 1, 2**).[2]
- Survival of infected cats depends on early detection and treatment. A combination of atovaquone and azithromycin has been demonstrated to be the most effective treatment.[1]
- To prevent *C. felis* infections, regular application of a tick preventative is highly recommended.[1]

STUDY OBJECTIVES

1. **Identify promising immunodominant antigens against *C. felis* using serum from known carrier cats and tissues from cats that died of clinical cytauxzoonosis.**
2. **Develop a real-time PCR assay for detection of acutely infected and *C. felis* carrier cats to compare diagnostic assays.**

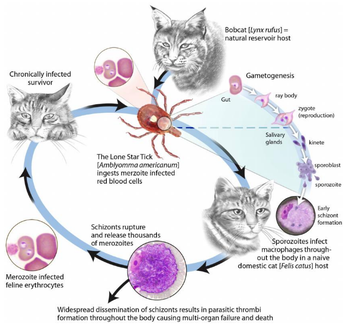


FIGURE 1. *Cytauxzoon felis* life cycle. The *C. felis* life cycle involves felids (intermediate host) and ticks (definitive hosts).[1]

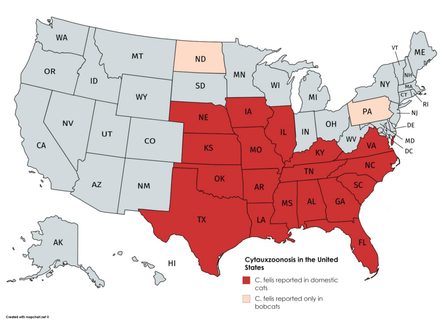


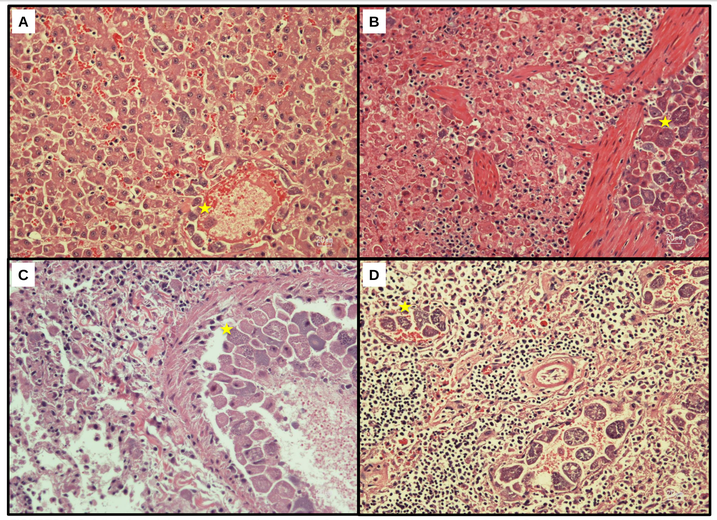
FIGURE 2. Distribution of *C. felis* cases in U.S. felids. Reports of *C. felis* infection in domestic cats and bobcats primarily occur in southern and central states.[3]

METHODS

- **Serum Samples**
  - Carrier cat serum was provided by Dr. Reichard from Oklahoma State University.
  - Pooled serum samples were used for western blots.
- **Tissue Samples**
  - Tissue samples from cats that died of naturally-acquired *C. felis* infection were used for DNA isolation and total protein isolation.
  - Tissue samples were analyzed histologically to verify infection and visualize parasite life stage(s).
- ***Cox3, 18S, Ama1, Cf76* Real-Time PCR**
  - Total genomic DNA was extracted from liver, lung, spleen and lymph node samples to confirm infection and determine parasite burden by real-time PCR.
  - Using *Cox3* as a positive control multi-copy gene, tissue samples were analyzed against other genes to confirm infection and find a conserved single-copy gene using real-time PCR.
  - The copy number of *Cf76* will be used to quantify parasite burden in tissue samples.
- **SDS-PAGE Gels and Western Blots**
  - Total protein, extracted from infected tissue samples, was loaded onto a gel, transferred to a nitrocellulose membrane, and exposed to carrier cat serum followed by goat anti-cat IgG to identify immunodominant bands to submit for LCMS/MS analysis.

RESULTS

PROTEIN EXTRACTION FROM ACUTELY INFECTED FELINE TISSUE SAMPLES



FIGURES 3. *C. felis* infected tissue samples. Haemotoxylin & eosin-stained histological sections of *C. felis*-infected (A) Liver, (B) Spleen, (C) Lung, (D) Lymph Node. Yellow stars indicate *C. felis* schizonts. All images are 20x magnification. Micrographs prepared by Dr. Wikander.

❖ **The spleen was the heaviest infected tissue type by histopathology (Fig 3) and real-time PCR (Tables 1&2). Total protein was extracted from *C. felis* acutely infected spleen samples.**

IDENTIFYING A CONSERVED SINGLE-COPY *C. FELIS* GENE

TABLE 1. Cycle thresholds of real-time PCR primers from *C. felis*-infected cat tissues: lymph node (LN), lung, liver, and spleen. Positive control was *C. felis*-infected blood. A cycle threshold (Ct) of >39 is considered weakly positive.

C. felis-infected cat tissue samples						
Primer	Positive Control Ct	Non-Template Control CT	LN Ct	Lung Ct	Liver Ct	Spleen Ct
Cox3	19	-	20	22	34	25
18S	32	-	33	31	-	37
Ama (1)	33	-	34	31	-	36
Ama (2)	33	-	33	31	39	36
Ama (3)	33	-	33	31	-	37
Ama (4)	31	-	34	31	-	38
Cf76 (A)	25	-	28	25	36	31
Cf76 (B)	27	-	28	23	32	30

TABLE 2. Cycle thresholds of real-time PCR primers from uninfected cat tissues: lymph node (LN), lung, liver, and spleen. Positive control was *C. felis*-infected blood. A cycle threshold (Ct) of >39 is considered weakly positive. A lymph node (LN) sample was not available from the uninfected cat.

Uninfected cat tissue samples						
Primer	Positive Control Ct	Non-Template Control CT	LN Ct	Lung CT	Liver CT	Spleen CT
Cox3	24	-	n/a	-	-	-
18S	33	-	n/a	39	-	-
Ama (1)	33	-	n/a	42	-	-
Ama (2)	32	39	n/a	42	-	44
Ama (3)	33	-	n/a	40	-	-
Ama (4)	34	-	n/a	42	-	-
Cf76 (A)	30	-	n/a	-	-	-
Cf76 (B)	29	-	n/a	-	-	-

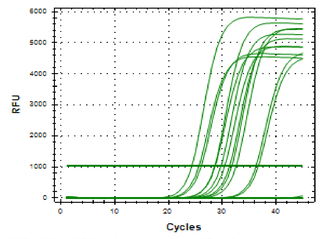


FIGURE 4. Cf76 primer set A amplification curve. Amplification of Cf76 from multiple cat tissue types.

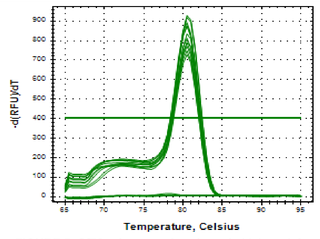


FIGURE 5. Cf76 primer set A melt peak. Melt peak for Cf76 primer set A is ~81°C.

❖ **Cox3 is a multi-copy mitochondrial gene while 18S is a ribosomal RNA gene of *C. felis*.[4]**

❖ **Ama1 is an apical membrane antigen of *C. felis*, with 99% sequence identity between domestic cat- and bobcat-derived samples.[5]**

❖ **Cf76 is expressed during the schizogenous stage, with >90% sequence identify among geographically diverse samples.[2,6]**

❖ **Cf76 primer set A, performed best at correctly identifying *C. felis*-infected samples (Tables 1&2, Fig 4) and yielded a single amplification product as determined by melt curve analysis (Fig 5).**

IDENTIFYING IMMUNODOMINANT BANDS USING SDS-PAGE GELS AND WESTERN BLOTS

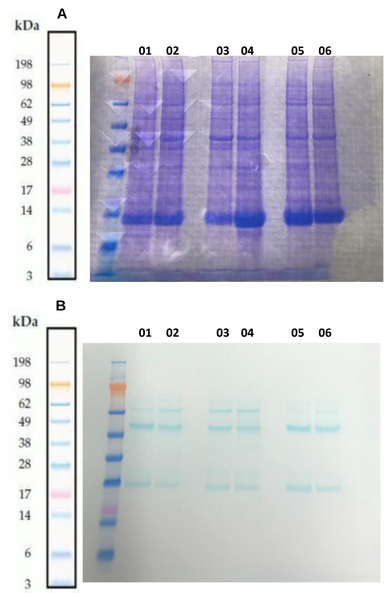


FIGURE 8. Immunodominant *C. felis* bands identified from spleen samples of multiple infected cats. (A) Gel of six unrelated *C. felis*-infected cat (Cat ID: 01-06) spleen protein extracts. (B) Using pooled serum from known *C. felis* carrier cats, several consistent immunodominant bands were visible by western blot among infected spleen protein extracts.

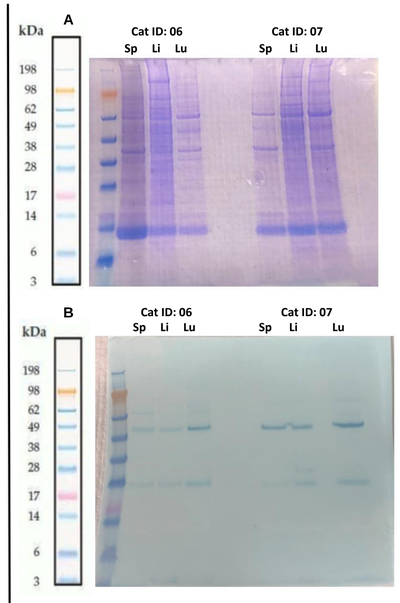


FIGURE 9. Immunodominant *C. felis* bands identified from multiple tissues of a cat that died of acute cytauxzoonsis. (A) Gel of spleen (Sp), liver (Li), and lung (Lu) protein extracts from a cat that died of acute cytauxzaoonosis (Cat ID: 06) and from a cat that died of *C. felis*-unrelated reasons (Cat ID: 07). (B) Using pooled serum from known *C. felis* carrier cats, a consistent immunodominant band was visible by western blot among tissues from the *C. felis*-infected cat but not the uninfected cat.

❖ **Three immunodominant bands (62-, 52- and 28-kDa) were consistently identified across total protein extracted spleen samples from six unrelated cats who died from cytauxzoonsis (Fig 8).**

❖ **Of these three bands, one immunodominant band (62-kDa) was identified from multiple *C. felis*-infected tissues from the same cat that was not visible on the uninfected tissue samples (Fig 9).**

CONCLUSIONS

- **The spleen is heavily infected in cats during acute cytauxzoonsis.**
- **Cf76 is a promising conserved single-copy gene that can be used to identify *C. felis* carrier cats and determine parasite burden.**
- **One immunodominant band (62-kDa) was identified by western blot across tissue types of unrelated cats that died of acute cytauxzoonsis that was not present on *C. felis* uninfected tissue samples.**

ON-GOING WORK and FUTURE DIRECTIONS

- Perform bioinformatics on LCMS/MS results to compare protein sequences against the *C. felis* and feline genome.
- Express, purify and evaluate promising candidates against *C. felis* carrier cat serum.
- Use the identified antigen in a serologic-based assay to diagnose *C. felis* carrier cats and compare the antigen's performance against nucleic acid-based detection methods.

ACKNOWLEDGEMENTS

- This work was supported by the National Center for Veterinary Parasitology Research Grant 2019/2020.
- AJC was supported by the Morris Animal Foundation Veterinary Student Scholar Program (D20FE-602) and KSU Veterinary Research Scholars Program.

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