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Identification and genetic characterization of *Cytauxzoon felis* in asymptomatic domestic cats and bobcats

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ABSTRACT

The objectives of the current study were to assess the prevalence of *Cytauxzoon felis* infection among a population of domestic cats that were clinically healthy but at higher risk for parasite exposure and to determine if the strains present in these asymptomatically infected cats were genetically unique as compared to those present both in domestic cats that were fatally infected and in the natural reservoir host, the bobcat. Using real-time PCR analysis targeting a portion of the parasite 18S rRNA gene specific for *C. felis*, 27/89 (30.3%) high-risk asymptomatic domestic cats from Arkansas and Georgia, and 34/133 (25.6%) bobcats from Arkansas, Georgia and Florida, were identified as positive for *C. felis* infection. Conventional PCR analysis was performed on all positive samples, targeting the *C. felis* ribosomal internal transcribed spacer regions 1 and 2 (ITS1, ITS2) in order to utilize the ITS sequences as markers to assess the genotype variability of the parasite population. Within the asymptomatically infected domestic cat samples, 3 genetically distinct parasite populations were identified. The *C. felis* ITS sequences from asymptomatic cats were identical to those previously reported from clinically ill infected cats, and 2 of the 3 sequence types were also present in infected bobcat samples. While sequence diversity exists, evaluation of the ITS region does not appear to be useful to verify pathogenicity of *C. felis* strains within host species. However, the presence of asymptomatic *C. felis* infections in clinical healthy domestic cats warrants further investigation to determine if these cats can serve as a new reservoir for *C. felis* transmission.

1. Introduction

Cytauxzoonosis is an emerging, tickborne, hemoprotozoal disease of domestic and exotic cats which has historically been considered highly fatal. The causative organism, *Cytauxzoon felis*, is a relatively new pathogen in the United States which was first detected in Missouri in 1973 (Wagner, 1976) and subsequently throughout southeastern and south-central states. The bobcat (*Lynx rufus*) has been identified as the natural reservoir host of the parasite (Glenn et al., 1983; Blouin et al., 1984). In the United States, asymptomatic *C. felis* infection of free-ranging Florida panthers (*Puma concolor coryi*) and Texas cougars (*Puma concolor stanleyana*) transplanted to Florida has also been reported (Rotstein et al., 1999).

In acutely infected domestic cats, the course of disease is rapid and most cats die within 1 week of initial clinical illness (Hoover et al., 1994; Wightman et al., 1997; Greene et al., 2006). The sporadic occurrence, short course of illness, and historic high fatality of cytauxzoonosis in domestic cats has suggested that they likely serve as aberrant or dead-end hosts (Kier et al., 1987; Greene et al., 2006).

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Although *C. felis* infection has historically been viewed as uniformly fatal in domestic cats, recent studies have documented cats that have survived infection (Motzel and Wagner, 1990; Walker and Cowell, 1995; Greene et al., 1999; Meinkoth et al., 2000). Survival of infected cats allows for the possibility of persistent infection, and studies have identified parasitemic cats following treatment for acute cytauxzoonosis in which clinical signs resolved (Walker and Cowell, 1995; Meinkoth et al., 2000; Brown et al., 2008). Some cats have remained detectably parasitemic greater than 1 year after resolution of clinical signs (Meinkoth et al., 2000; Brown et al., 2008) and PCR testing on blood from some clinically healthy cats with undetectable parasitemias has identified *C. felis* DNA (Haber et al., 2007; Brown et al., 2008). Persistently infected cats could serve as new foci of infection, thus greatly increasing the risk of exposure to *C. felis* for other domestic cats.

It is unknown whether persistent *C. felis* infections in domestic cats are genetically distinct from those causing fatal infections. Genetic characterization of *C. felis* from asymptomatically infected domestic cats would allow a comparison of the parasite genotypes infecting both clinically ill and asymptomatic persistently infected cats, as well as genotypes present in the known reservoir wild cat hosts. Very little is currently known about the *C. felis* genome. One previous study compared a sequenced portion of the *C. felis* 18S rRNA gene derived from an asymptomatic experimentally infected cat with a GenBank archived sequence from a cat with fatal infection; both sequences were virtually identical (>99% sequence identity) (Meinkoth et al., 2000). Subsequent studies comparing the non-coding internal transcribed spacer regions 1 and 2 (ITS1, ITS2) of *C. felis* from infected domestic cats in Arkansas and Georgia found nucleotide variability in the range of 0–1.8% (Brown et al., 2009a). In addition, this same study identified a significant association between particular parasite genotypes and whether infected cats survived or died. The detection of genotype variability within the *C. felis* parasite population, and the association of particular genotypes with the clinical outcome of infection, supported the hypothesis that *C. felis* strains varied in virulence, with some strains causing less severe disease and increased survival of infected domestic cats.

Haber et al. (2007) tested free-roaming domestic cats using a *C. felis*-specific PCR assay and found that 3/961 (0.3%) cats were PCR test positive. Although clinical signs in the positive cats were not noted at the time of their capture, there was limited follow-up or other clinical information on these cats to unambiguously document persistent, subclinical infections. Despite the low prevalence, the presence of apparently healthy, infected, free-roaming cats suggested that these cats may have had the capacity to serve as an additional reservoir host for *C. felis*. A recent study by Reichard et al. (2009) demonstrated transmission of clinical cytauxzoonosis from a domestic cat that was a natural survivor of *C. felis* infection to a naïve domestic cat using *Amblyomma americanum* as the tick vector. The ability to transmit *C. felis* infection from domestic cats suggested that subclinically infected domestic cats might be a reservoir for further infection of domestic cats. Previously, it was presumed that domestic cats that became infected after other household cats demonstrated clinical signs of cytauxzoonosis had been exposed to similarly infected ticks in the shared environment that had fed on infected bobcats. In light of Reichard's most recent experimental infection of domestic cats, support now exists for the possibility of cat-to-cat transmission via tick vectors. However the role of chronically infected cats in disease transmission in areas in which cytauxzoonosis appears hyperendemic remains unknown.

The ability of surviving *C. felis*-infected domestic cats to serve as an additional reservoir host for the parasite would alter the currently accepted paradigm of *C. felis* transmission from bobcats (reservoir host) to domestic cats. The objectives of the current study were to assess the prevalence of *C. felis* infection among a population of domestic cats that were clinically healthy but at higher risk for parasite exposure and to determine if the strains present in these asymptomatically infected cats were genetically unique as compared to those present both domestic cats that are fatally infected and to the natural reservoir host, the bobcat. Establishing that there are clinically healthy domestic cats that are persistently infected with *C. felis* and may have the potential to serve as a reservoir host for the parasite has important application to treatment and prevention of disease spread. Unique *C. felis* strains in infected domestic cats that were not detected in bobcats would suggest that the bobcat may not be necessary in the currently accepted paradigm of *C. felis* transmission where wild felids are the only known reservoir hosts.

2. Materials and methods

2.1. Animals and sample collection

Ethylenediamine tetra-acetic acid (EDTA)-anticoagulated whole blood samples from domestic cats were obtained by routine phlebotomy at private veterinary practices with written consent from the cats' owners, when applicable. Participating private practices were identified from previous studies as clinics in counties from which multiple *C. felis*-positive samples were submitted to our collaborating diagnostic laboratories over the previous 3 years. Cases, identified as those considered to be at high risk for *C. felis* exposure, included cats that had clinically recovered from acute cytauxzoonosis, contact cats that co-habitated with cats previously diagnosed with cytauxzoonosis, and feral cats in endemic areas. In each case, the submitting veterinarian found no clinical evidence for infection or illness on physical exam.

Bobcat tissues samples were obtained from multiple sources in Georgia, Arkansas, and Florida. Fresh-frozen spleen sections were submitted from bobcats collected by the United States Department of Agriculture Wildlife Services as part of a study to evaluate the effects of mesomammalian predator removal on bobwhite-quail reproduction in Georgia and Florida. Fresh-frozen spleen samples were also obtained postmortem from the diagnostic service at the Southeastern Cooperative Wildlife Disease Study (Athens, GA) and a private wildlife rehabilitator in Florida. Finally, formalin-fixed paraffin-embedded (FFPE) splenic tissue and fresh-frozen spleen sections were

obtained from the Veterinary Diagnostic Laboratory at the Arkansas Livestock and Poultry Commission (Little Rock, AR) after routine diagnostic necropsy was performed.

2.2. DNA extraction

DNA was extracted from EDTA-anticoagulated whole blood samples using the illustra™ blood genomicPrep™ Mini Spin Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturer's instructions. DNA was extracted from fresh-frozen splenic tissue samples using the QIAamp DNA Mini-Kit (Qiagen, Inc., Valencia, CA). DNA from the FFPE tissue was also extracted using the QIAamp DNA Mini-Kit (Qiagen Inc., Valencia, CA), with a preliminary overnight 56 °C incubation with proteinase K (Sigma–Aldrich, St. Louis, MO).

2.3. Real-time polymerase chain reaction (real-time PCR) amplification

Using multiple sequence alignments (Vector NTI, Invitrogen, Valencia, CA) to compare the 18S rRNA gene sequences from *C. felis* and related piroplasms (*Babesia* spp., *Theileria* spp.), primer sequences were selected to specifically amplify an 82-bp fragment of the *C. felis* 18S rRNA gene. The selected forward primer was 5′ TGC ATC ATT TAT ATT CCT TAA TCG 3′ and the reverse primer was 5′ CAA TCT GGA TAA TCA TAC CGA AA 3′. In order to perform multiplex real-time PCR analysis, a linear fluorescent-labeled probe was designed to anneal within the targeted 18S amplicon. The 18S probe sequence was 5′ TTA TTT ATG TTG TGG CTT TTT CTG GTG ATT 3′ and was labeled with 5′-HEX fluorescence and 3′-BHQ1 quencher. The real-time PCR components consisted of a 2X *Taq* polymerase master mix (iQ Multiplex Powermix, Bio-Rad Laboratories, Hercules, CA) containing DNA polymerase, PCR buffer with 11 mM $MgCl_2$, and deoxyribonucleotide triphosphates (dNTPs); 6 pmol of the forward primer, 4 pmol of the reverse primer, and 2 pmol of the Hex-labeled probe; 1 μl of DNA template; and molecular biology grade water to adjust the reaction mixture to a final volume of 20 μl. A negative template from an uninfected cat and a non-template control consisting of molecular biology grade water were utilized as controls for all amplification reactions. After an initial activation and denaturation step at 95 °C for 3 min, 40 cycles of a 2-step real-time amplification were performed. Each cycle was comprised of 30 s at 95 °C and 1 min at 54 °C, after which point fluorescence data were collected.

2.4. Standard PCR amplification and DNA sequencing

All real-time PCR-positive samples were further analyzed by conventional PCR analysis using forward and reverse oligonucleotide primer sets designed to conserved regions flanking both the ITS1 and ITS2 regions of *C. felis*. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS1 from the domestic cat samples were 5′ CGA TCG AGT GAT CCG GTG AAT TA 3′ and 5′ GCT GCG TCC TTC ATC GAT GTG 3′, respectively. An alternate reverse primer utilized to amplify *C. felis* ITS1 from the bobcat samples was 5′ GGA GTA CCA CAT GCA AGC AG 3′. These primer sets were expected to produce amplicons of 651 and 746 bp, respectively, from genomic *C. felis* DNA that incorporated the 458-bp ITS1 region plus 18S and 5.8S partial flanking regions. The sequences of the forward and reverse oligonucleotide primers used to amplify *C. felis* ITS2 from all samples were 5′ AGC GAA TTG CGA TAA GCA TT 3′ and 5′ TCA GCC GTT ACT AGG AGA 3′, respectively. This primer set was expected to produce an amplicon of 475 bp from genomic *C. felis* DNA that incorporated the 265-bp ITS2 region plus 28S and 5.8S partial flanking regions.

For all reactions, the PCR components consisted of a 2X hot start *Taq* polymerase master mix (HotStar Taq Plus Master Mix, Qiagen Inc., Valencia, CA) containing hot start DNA polymerase, PCR buffer with 3 mmol of $MgCl_2$, and 400 μmol of each deoxyribonucleotide triphosphate (dNTP); 37.5 pmol of each primer; 5 μl of DNA template; and molecular biology grade water to adjust the reaction mixture to a final volume of 50 μl. A negative template consisting of molecular biology grade water was utilized as a control for all amplification reactions. After initial denaturation at 95 °C for 5 min, 35 amplification cycles were performed, each consisting of 30 s at 94 °C, 30 s at 60 °C, and 90 s at 72 °C, with a final extension at 72 °C for 10 min. The PCR products were resolved by electrophoresis (100 V, 30 min), using agarose gels that were prestained with ethidium bromide and subsequently visualized with ultraviolet light.

The PCR products were purified using a commercial kit (QIAquick® PCR Purification Kit, Qiagen Inc., Valencia, CA) and automated sequencing was performed at a university laboratory (Georgia Genomics Facility, University of Georgia, Athens, GA) or a commercial laboratory (MACROGEN USA, Rockville, MD). The forward and reverse primers used for standard PCR were utilized for all sequencing reactions except for the bobcat ITS1 amplicons. For these sequencing reactions, an additional reverse primer was designed to anneal further downstream than the amplification primer, providing higher quality sequencing results. The sequence for this alternate ITS1 reverse primer was 5′ TTC GCA GAA GTC TGC AAG TC 3′.

Sequences and chromatogram data were carefully analyzed and forward and reverse sequence data were assembled and aligned using Geneious Pro 4.6.5 software (Biomatters Ltd., Auckland, NZ) to identify any polymorphisms within the ITS regions of the amplicons. ITS1 and ITS2 sequence data obtained from *C. felis*-infected asymptomatic domestic cats were compared to those from obtained from bobcats and to *C. felis* ITS sequences previously reported from clinically ill domestic cats with cytauxzoonosis (Brown et al., 2009a) to determine if the presence of particular parasite sequences varied in pathogenicity or by host species.

3. Results

Whole blood samples from 89 domestic cats (43 from Arkansas, 46 from Georgia) and tissue samples from 133 bobcats (73 from Georgia, 6 from Arkansas, and 54 from Florida) were included in the study. Using real-time PCR analysis targeting a portion of the parasite 18S rRNA gene specific for *C. felis*, 27/89 (30.3%) blood samples from high-

Table 1
Real-time PCR results for high-risk cats tested for *Cytauxzoon felis*, including type of parasite exposure and state of origin.

Real-time PCR	*n*	Type of *C. felis* exposure			State of origin	
		Survivor	Contact	Feral	Arkansas	Georgia
Positive	27	11	6	10	18	9
Negative	62	3	34	25	25	37
Total	89	14	40	35	43	46

Table 2
Cytauxzoon felis internal transcribed spacer region (ITS1 and ITS2) sequences obtained from asymptomatically infected domestic cats characterized by nucleotide variation, frequency, and state of origin of *C. felis* infection[a].

Sequence	ITS1					ITS2	*n*	State of origin		GenBank accession # (ITS1, ITS2)
	162	337	344	415	444	180		AR	GA	
ITSa	A	–	T	T	A	G	16	15	1	EU450802, EU450804
ITSb	A	–	T	T	A	T	8	1	7	EU450802, EU450805
ITSc	G	A ins	C	C	G	G	1	1	0	EU450803, EU450804

[a] ins = insertion; – = no insertion.

risk domestic cats (Table 1) and tissue samples from 34/133 (25.6%) bobcats were identified as positive for *C. felis* infection.

Conventional PCR analysis and amplicon sequencing performed on all real-time PCR-positive samples resulted in unambiguous *C. felis* ITS1 and ITS2 sequence data for 25/27 (92.6%) infected domestic cats. Within the 458-bp ITS1 region of the *C. felis* genome, all sequences were identical except for one sample with 4 single nucleotide polymorphisms (SNPs) and a single nucleotide insertion. Within the 265-bp ITS2 region of the *C. felis* genome, there was 1 SNP. Taken together, the *C. felis* ITS1 and ITS2 sequence data defined a total of 3 different genetically distinct parasite populations present within asymptomatically infected domestic cats (Table 2).

Unambiguous *C. felis* ITS1 and ITS2 sequence data was obtained for 25/34 (73.5%) infected bobcats. Within the 458-bp ITS1 region of the *C. felis* genome, a total of 5 SNPs and a single nucleotide insertion were identified. Within the 265-bp ITS2 region of the *C. felis* genome, there were a total of 3 SNPs. Taken together, the *C. felis* sequence data from bobcat samples defined a total of 11 different ITS sequence types (Table 3). For several of the SNPs within ITS1 and the majority of the SNPs within ITS2, the chromatogram data revealed the incorporation of 2 nucleotides at a single position. Polymorphic sites were designated with IUPAC codes.

The *C. felis* ITS1–ITS2 sequences derived from infected asymptomatic domestic cats were identical to those previously reported from clinically ill infected cats (Brown et al., 2009a). The *C. felis* ITSa genotype (GenBank accession nos. EU450802 and EU450804) was detected most commonly in both asymptomatically infected cats and clinically ill cats that survived infection, and for both cat populations, the majority of the parasite infections were acquired in Arkansas. The second most common genotype, designated as ITSb (GenBank accession nos. EU450802 and EU450805) was also previously reported in clinically ill domestic cats, the majority of which were fatal infections and, for both cat populations, infection was far more commonly detected in samples from Georgia. The third genotype, designated as genotype ITSc (GenBank accession nos. EU450803 and EU450804) and present in a single asymptomatically infected cat from Arkansas, was previously reported from clinically ill domestic cats from Arkansas, all of which died.

Table 3
Cytauxzoon felis internal transcribed spacer region (ITS1 and ITS2) sequences obtained from infected bobcats characterized by nucleotide variation, frequency, and state of origin of *C. felis* infection[a].

Sequence	ITS1						ITS2			*n*	State of origin			GenBank accession # (ITS1, ITS2)
	338	344	397	415	444	457	180	204	243		AR	GA	FL	
ITSa	G	T	T	T	A	–	G	G	A	5	2	0	3	EU450802, EU450804
ITSd	A	T	T	T	A	–	T	G	A	1	0	0	1	GU581166, EU450805
ITSe	G	C	C	T	G	C ins	G	A	A	1	0	0	1	GU581167, GU581170
ITSf	G	T	T	T	A	–	G/T[b]	G	A/G[b]	1	0	1	0	EU450802, FJ536421
ITSg	G	C	C	T	G	C ins	G	A/G[b]	A	8	0	1	7	GU581167, GU581171
ITSh	G	T	T	T	A	–	G	A/G[b]	A/G[b]	1	0	1	0	EU450802, GU581172
ITSi	G	T	T	T	A	–	G	G	A/G[b]	3	0	1	2	EU450802, FJ536419
ITSj	A/G[b]	T	C/T[b]	C/T[b]	A	–	G	G	A	2	0	0	2	FJ536425, EU450804
ITSk	A/G[b]	T	C/T[b]	C/T[b]	A	–	G	A/G[b]	A	1	0	0	1	FJ536425, GU581171
ITSl	G	C	C/T[b]	T	A/G[b]	C ins	G	A/G[b]	A	1	0	0	1	GU581168, GU581171
ITSm	G	C/T[b]	C/T[b]	T	G	C ins	G	A/G[b]	A	1	0	0	1	GU581169, GU581171

[a] ins = insertion; – = no insertion.
[b] Chromatograms depict the incorporation of 2 nucleotides at this position.

The most common *C. felis* ITSa sequence reported from asymptomatically infected domestic cats was also present in the infected bobcats. In addition, there were two other ITS sequence types reported in bobcats (ITSf, GenBank accession nos. EU450802 and FJ536421; ITSi, GenBank accession nos. EU450802 and FJ536419) that had been previously reported from histologic tissues of *C. felis*-infected domestic cats presented for necropsy (Brown et al., 2009b). The most common *C. felis* ITS sequence type present in bobcat samples, as well as an additional 7 sequences present infrequently in bobcats, had not been previously reported from infected domestic cats.

4. Discussion

A real-time PCR survey of blood samples from domestic cats considered at high risk for *C. felis* exposure detected a significant population of asymptomatically infected animals. The cats included in the survey were selected to increase the likelihood of obtaining parasite samples for genetic comparisons. As cats surviving clinical cytauxzoonosis may remain persistently infected, blood samples from survivors were included when available. Eleven of 14 (78.6%) cats remained *C. felis* positive after clinical treatment for acute cytauxzoonosis. One of these cats has presumably remained infected for greater than 4 years because it has been kept strictly indoors since the initial illness. Domestic cats that co-habitated with those previously diagnosed with infection were included in the study, as they would likely share exposure to the same environment and infected ticks. Having never exhibited clinical signs consistent with infection, it is surprising that 6 of 14 (15%) contact cats were real-time PCR positive for *C. felis*. As Reichard et al. (2009) has shown, cat-to-cat transmission of infection may be possible via a tick vector, which may also contribute to the infection rate of these contact cats. Finally, feral cats from endemic regions identified in our previous work were included in the sample population as they would have a higher likelihood of tick exposure due to their outdoor lifestyle and lack of preventative treatment with acaricides. The previous study by Haber et al. (2007) of free-roaming cats in Tennessee, North Carolina, and Florida detected a *C. felis* infection prevalence of 0.3%. Among feral cats from Arkansas and Georgia included in the current study, 10 of 35 (28.6%) were *C. felis* positive. Geographic differences in the feral cat populations tested may account for higher prevalence of *C. felis* infection among feral cats in the current study. In addition, increased detection may be attributable to a difference in sensitivity of the different PCR testing methods (real-time PCR used in the current study versus conventional PCR used previously).

The overall prevalence of *C. felis* in high-risk cats from Arkansas (41.9%) was twice that of similar cats tested from Georgia (19.6%). These results are not surprising, given that our previous study (Brown et al., 2009a) identified a far greater number of cats from Arkansas surviving acute cytauxzoonosis compared with those infected in Georgia. Although host factors cannot be excluded, geographic disparity in the number of asymptomatic *C. felis*-infected cats likely results from the survival of cats infected with less pathogenic strains of the parasite. If these surviving cats remain persistently infected, further transmission of these selected parasite strains may occur in a more localized area. Conversely, infections with more highly pathogenic strains are likely to result in host fatality. These highly pathogenic *C. felis* genotypes would not be detected among asymptomatic cats and would be unavailable for further transmission among the domestic felid population.

The degree of ITS sequence polymorphism seen in the current set of *C. felis* samples is comparable to that detected among all *C. felis* ITS1 and ITS2 sequences available in GenBank. As observed in previous studies (Brown et al., 2009a,b), detailed review of the sequencing chromatograms revealed the incorporation of 2 nucleotides at a single position within ITS1 or ITS2 for many bobcat samples, though this position varied among samples. This finding is interpreted to reflect coinfection of the host with multiple *C. felis* genotypes, a finding not unexpected in the wild reservoir host which is suspected to be exposed to various parasite strains over a wider geographic area. Alternatively, the mixed nucleotide loci may reflect polymorphisms in multiple copies of the rRNA genes that may exist in the *C. felis* genome.

The *C. felis* ITS sequence types detected in the asymptomatic cats in the current study were identical to genotypes previously detected in clinically ill domestic cats. An earlier study (Brown et al., 2009a) demonstrated a strong association between particular *C. felis* ITS1–ITS2 genotypes and survival of domestic cats after acute infection, suggesting that ITS sequencing may be useful to define markers that are able to detect more or less pathogenic strains among *C. felis*-infected cats. While the most common *C. felis* ITS sequence detected in the asymptomatic cats was that also reported previously in cats surviving acute infection, the second most common ITS genotype in the asymptomatic cats had been previously detected in predominantly fatal infections. In both studies, however, the geographic distribution of the ITS genotypes varied greatly among Arkansas and Georgia samples, as might be expected in the development of different parasite strains. While suitable for examining genetic diversity among *C. felis* populations, the presence of common ITS sequences in asymptomatic, clinically ill, and fatally infected domestic cats demonstrates that ITS1 and ITS2 rDNA does not appear to be useful for discriminating among variably pathogenic strains of the parasite, at least when comparing geographically distributed isolates.

Increased *C. felis* ITS sequence diversity was present in the infected bobcat samples, as might be expected within the wild reservoir host presumed to be exposed to variable parasite strains over a wider geographic area. The most common *C. felis* ITS sequence detected in bobcats had not been previously described in infected domestic cats. While this finding may reflect the identification of a strain that is incapable of infecting domestic cats, it is more likely a reflection of the geographic variability of the sample populations, as this sequence was detected only in bobcats from a particular trapping site in northern Florida from which we had no domestic cat samples.

At present, only the ribosomal genes have been identified and sequenced for *C. felis*, limiting the areas of the genome which can be investigated for sequence variabil-

ity. Ultimately, identification of genetic variability in genes affecting pathogenicity of the parasite would best test the hypothesis that genetically distinct strains of *C. felis* are capable of establishing persistent infections in the domestic cat and help better define the epidemiology of infection. Asymptomatically infected cats that are carriers of the parasite may serve as reservoirs for infection and thus greatly increase the risk of exposure for other domestic cats. Additional studies are warranted to evaluate the potential for these naturally infected cats to serve as a reservoir for further disease transmission. Better understanding of the epidemiology of *C. felis* infection will enhance the ability to prevent this highly fatal infectious disease.

5. Conclusion

The current study identified a population of domestic cats that were asymptomatically infected with *C. felis*. While *C. felis* ITS sequence diversity existed among infected cats and bobcats, utilization of this non-coding region for detection of parasite strain variability by host species and pathogenicity did not appear to be useful. Establishing that there was a population of clinical healthy domestic cats that were asymptomatically, and presumably persistently, infected with *C. felis* has important implications in feline health. Further evaluation of the potential for these asymptomatically infected cats to serve as a new reservoir for *C. felis* transmission is warranted.

Conflict of interest

The authors have no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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