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Distribution and prevalence of *Cytauxzoon felis* in bobcats (*Lynx rufus*), the natural reservoir, and other wild felids in thirteen states

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ABSTRACT

Cytauxzoon felis, a protozoan parasite of wild and domestic felids, is the causative agent of cytauxzoonosis in domestic and some exotic felids in the United States. The bobcat (*Lynx rufus*) is the natural reservoir for this parasite, but other felids such as Florida panthers (*Puma concolor coryii*) and domestic cats may maintain long-term parasitemias and serve as reservoirs. Experimentally, two tick species, *Dermacentor variabilis* and *Amblyomma americanum*, have demonstrated the ability to transmit *C. felis*. These two tick species have overlapping distributions throughout much of the southeastern United States. The objective of the current study was to determine the distribution and prevalence of *C. felis* in free-ranging bobcat populations from 13 states including California, Colorado, Florida, Georgia, Kansas, Kentucky, Missouri, North Carolina, North Dakota, Ohio, Oklahoma, South Carolina, and West Virginia. These states were selected because of differential vector presence; *D. variabilis* is present in each of these states except for the region of Colorado sampled and *A. americanum* is currently known to be present only in a subset of these states. Blood or spleen samples from 696 bobcats were tested for *C. felis* infection by a polymerase chain reaction

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(PCR) assay which targeted the first ribosomal internal transcribed spacer region (ITS-1). Significantly higher prevalences of *C. felis* were detected from Missouri (79%, $n=39$), North Carolina (63%, $n=8$), Oklahoma (60%, $n=20$), South Carolina (57%, $n=7$), Kentucky (55%, $n=74$), Florida (44%, $n=45$), and Kansas (27%, $n=41$) compared with Georgia (9%, $n=159$), North Dakota (2.4%, $n=124$), Ohio (0%, $n=19$), West Virginia (0%, $n=37$), California (0%, $n=26$), and Colorado (0%, $n=67$). In addition to bobcats, seven cougars (*Puma concolor*) from Georgia, Louisiana, and North Dakota and one serval (*Leptailurus serval*) from Louisiana were tested for *C. felis*. Only one cougar from Louisiana was PCR positive, which represents the first report of an infected cougar outside of the Florida panther population. These data also indicate that *C. felis* is present in North Dakota where infection has not been reported in domestic cats. Based on a nonparametric analysis, prevalence rates were significantly higher in states where there are established populations of *A. americanum*, which supports recent data on the experimental transmission of *C. felis* by *A. americanum* and the fact that domestic cat clinical cases are temporally associated with *A. americanum* activity. Collectively, these data confirm that bobcats are a common reservoir for *C. felis* and that *A. americanum* is likely an epidemiologically important vector.

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1. Introduction

Piroplasms in the genus *Cytauxzoon* (Family Theileriidae) are related to members of the genera *Theileria* and *Babesia*. All three of these genera contain species of veterinary concern and *Cytauxzoon* spp. are increasingly reported in domestic and wild felid species worldwide (Luaces et al., 2005; Criado-Fornelio et al., 2004; Peixoto et al., 2007; Ketz-Riley et al., 2003). The *Cytauxzoon* spp. are distinguished from *Theileria* spp. by the location of schizogenous replication which occurs in mononuclear phagocytes for *Cytauxzoon* and predominantly in lymphocytes for *Theileria* (Nijhof et al., 2005). Currently *Cytauxzoon* spp. infections are restricted to felids; species previously described in African ungulates have been reclassified as *Theileria* spp. (Nijhof et al., 2005). In the United States, only one species, *Cytauxzoon felis*, has been detected and it is an emerging infectious pathogen of domestic cats in Southeastern, Midwestern, and Mid-Atlantic States (Wagner, 1976; Ferris, 1979; Kier et al., 1982b; Birkenheuer et al., 2006). *Cytauxzoon* spp. are transmitted by ixodid ticks, and *C. felis* has been experimentally transmitted by two ticks, *Dermacentor variabilis* and *Amblyomma americanum* (Blouin et al., 1984; Kocan et al., 1992; Reichard et al., 2008; Edwards et al., 2010).

C. felis infection was first described in domestic cats from Missouri in 1976 (Wagner, 1976). Since that time, *C. felis* has been detected in domestic cats from numerous states, including Alabama, Arkansas, Florida, Georgia, Kansas, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, and Virginia (Bendele et al., 1976; Wagner, 1976; Wightman et al., 1977; Ferris, 1979; Glenn and Stair, 1984; Hauck et al., 1982; Kocan and Kocan, 1991; Meier and Moore, 2000; Birkenheuer et al., 2006; Jackson and Fisher, 2006; Haber et al., 2007). Historically, infection with the parasite was considered nearly uniformly fatal for domestic cats due to the development of acute clinical cytauxzoonosis (Ferris, 1979). The pathognomonic sign of cytauxzoonosis in the domestic cat, occlusion of blood vessels by schizont-laden macrophages, is also presumed to be responsible for much of the observed morbidity and mortality. Recently, however, research and surveillance studies have indicated

that some domestic cats can survive infection and become persistently parasitemic (Kier et al., 1982b; Meinkoth and Kocan, 2005; Haber et al., 2007; Brown et al., 2008).

The bobcat (*Lynx rufus*) is considered to be the natural reservoir for *C. felis* in the United States (Kier et al., 1982a,b; Glenn et al., 1983; Glenn and Stair, 1984; Blouin et al., 1984). Naturally infected bobcats rarely display clinical signs and there is only one report from Kansas of a naturally infected young bobcat with acute cytauxzoonosis (Nietfeld and Pollock, 2002). Experimental studies indicate that some bobcats can develop acute cytauxzoonosis when inoculated with schizogenous stages of the parasite, but no clinical signs were observed when the bobcats were inoculated with the intraerythrocytic stages (Kier et al., 1982b; Glenn et al., 1983). Similarly, bobcats experimentally infected via tick transmission typically have a limited schizogenous phase which leads to a long-term subclinical parasitemia (Blouin et al., 1987). Few studies have examined the prevalence of the parasite within bobcat populations, but high prevalences have been reported in Oklahoma (31–60%) and North Carolina (33%) and a low prevalence was reported in Pennsylvania (7%) (Glenn et al., 1982; Glenn et al., 1983; Kocan et al., 1985; Birkenheuer et al., 2008).

In addition to bobcats, *C. felis* has been reported from clinically normal free-ranging Florida panthers (*Puma concolor coryii*) and a captive white tiger (*Panthera tigris*) in Florida which died from acute cytauxzoonosis (Butt et al., 1991; Rotstein et al., 1999; Garner et al., 1996). Detailed analysis of clinical pathology records of four Florida panthers that were recently infected with *C. felis* revealed mild hemolytic anemia and liver damage, but no deaths have been attributed to the parasite (Yabsley et al., 2006; Harvey et al., 2007). These data suggest that free-ranging cougars may be an additional natural reservoir for *C. felis*.

In the current study, we conducted a comprehensive study of the distribution and prevalence of *C. felis* in free-ranging bobcat populations from thirteen states. To determine exposure of alternative feline species, a limited number of cougar samples and one serval (*Leptailurus serval*) from three other states were included in the study. States for sampling were selected to encompass the range of the two demonstrated tick vectors of *C. felis* (*A. ameri-*

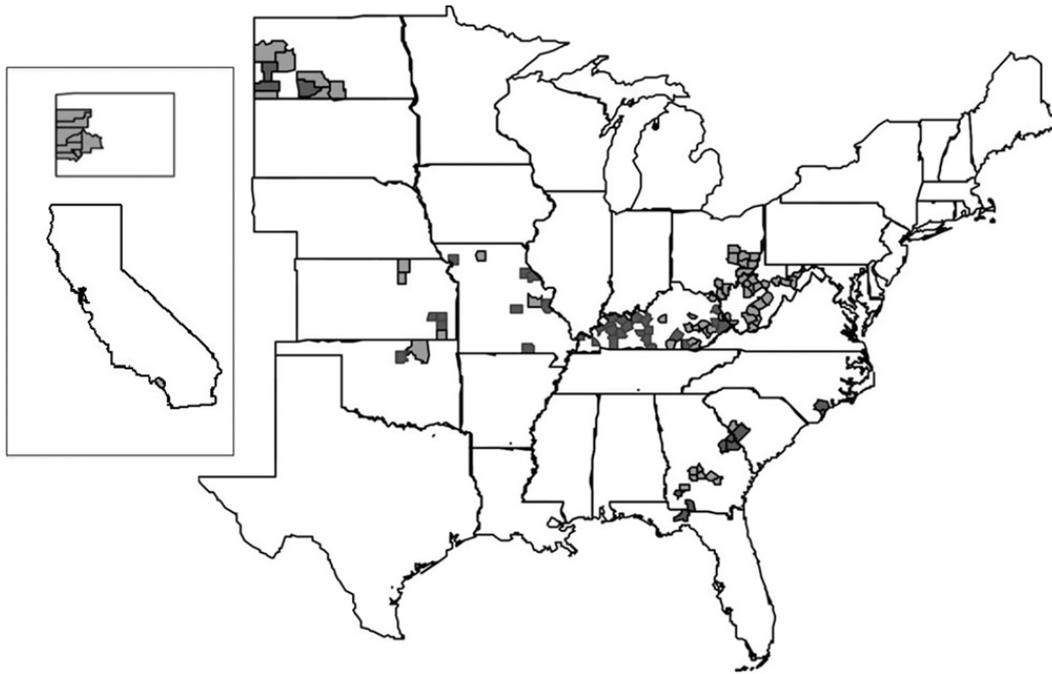


Fig. 1. Distribution of *Cytauxzoon felis* in bobcats from 13 states. For ease of illustration, Colorado (above) and California (below) shown in inset box. Light grey counties represent counties from which bobcats were sampled. Dark grey counties indicate counties in which at least one positive bobcat was detected.

canum and *D. variabilis*). The objective of this project was to gain a better understanding of the natural history of the parasite within wild felid populations with the hope of increasing the health of domestic, exotic, and wild felids in the United States.

2. Materials and methods

2.1. Sample collection

Samples from bobcats were collected opportunistically from a variety of sources including trapper-harvested, vehicle-killed, or clinical case submissions of mortalities. Spleen samples were collected from fresh carcasses or from previously frozen carcasses and frozen at -20°C until processing. From some fresh carcasses, a blood sample was collected from the heart or thoracic cavity. From 1999 to 2010, 696 blood or spleen samples were collected from thirteen states (California, Colorado, Florida, Georgia, Kansas, Kentucky, Missouri, North Carolina, North Dakota, Ohio, Oklahoma, South Carolina, and West Virginia; Fig. 1). Additionally, seven cougar samples were collected from Georgia ($n=1$), Louisiana ($n=1$), and North Dakota ($n=5$) and one escaped exotic serval (*Leptailurus serval*) sample was collected from Louisiana.

2.2. Molecular analysis

Genomic DNA was extracted from 100 μl of whole blood or 10 mg spleen using the Qiagen DNA Purification Kit (Germantown, MD) following the manufacturer's protocol. A nested PCR protocol that amplifies the entire internal transcribed spacer (ITS)-1 rRNA region of most piroplasm including *Cytauxzoon*, *Babesia*, and *Theileria* spp. (Bostrom et al., 2008) was used to detect *C. felis*. For primary ampli-

fication, 5 μl of DNA was added to 20 μl of a master mix containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM each dNTP (Promega, Madison, Wisconsin), 2.5 units Taq DNA Polymerase (Promega), and 0.8 μM of primers ITS-15C (5'-CGATCGAGTGATCCGGTGAATTA) and ITS-13B (5'-GCTGCGTCCTTCATCGTTGTG). Cycling parameters were 94 C for 1 min followed by 35 cycles of 94 C for 30 s, 52 C for 30 s, 72 C for 1 min, and a final extension at 72 C for 5 min. For the nested PCR, 1 μl of primary product was used as template in a 25 μl reaction containing the same PCR components except primers, ITS-15D (5'-AAGGAAGGAGAAGTCGTAACAAGG) and ITS-13C (5'-TTGTGTGAGCCAAGACATCCA) were used. The cycling parameters were the same as the primary reaction except the annealing temperature was 49 C.

To prevent and detect contamination, the DNA extractions, primary and secondary amplification, and product analysis were done in separate dedicated areas. A negative water control was included in each set of DNA extraction, and a different water control was included in each set of primary and secondary PCR reactions. To confirm identity, all amplicons of approximately 550 bp were purified with a Qiagen gel extraction kit (Germantown, MD) and bi-directionally sequenced at the University of Georgia Integrated Biotechnology Laboratory (Athens, GA).

2.3. Data analysis

Chi-square analysis and Fischer's exact test (MiniTab v16) were performed to determine differences in prevalence between states. A nonparametric test for trend (Cuzick, 1985) was performed to determine whether there was an association between tick density categories and the prevalence of *C. felis* within states. States were assigned to one of three tick density levels per tick species, absent,

Table 1
Prevalence of *Cytauxzoon felis* in bobcats based on polymerase chain assay testing.

State	Dv ^a density	Aa ^a density	No. positive/no. tested (%)
Colorado	Absent	Absent	0/67 (0) ^a
California	High	Absent	0/26 (0) ^a
Ohio	High	Low	0/19 (0) ^a
West Virginia	High	Low	0/37 (0) ^a
North Dakota	High	Absent	3/172 (2) ^a
Georgia	High	High	13/143 (9) ^b
Kansas	High	High	12/39 (31) ^c
Florida	High	High	16/45 (36) ^c
Kentucky	High	High	41/74 (55) ^c
South Carolina	High	High	4/7 (57) ^{c,d}
North Carolina	High	High	5/8 (63) ^{c,d}
Oklahoma	High	High	13/20 (65) ^{c,d}
Missouri	High	High	31/39 (79) ^d
			138/696 (20)

^{a–d} States with different letters have significantly different prevalences ($p < 0.05$).

^a Dv, *Dermacentor variabilis*; Aa, *Amblyomma americanum*.

low, or high. Based on published reports, *A. americanum* is absent in the regions of California, Colorado, and North Dakota sampled in the study and *D. variabilis* is absent in the sampled areas of Colorado. The remaining states sampled in the current study have documented populations of *D. variabilis* and were all classified as high density. *A. americanum* densities were classified as high in Georgia, Kansas, Florida, Kentucky, South Carolina, North Carolina, Oklahoma, and Missouri. In Ohio and West Virginia, *A. americanum* were classified as low due to reports of low densities occurring in isolated regions of southern Ohio and western West Virginia (Yabsley et al., 2003; Kelly et al., 2005; Yabsley, 2010).

3. Results

Based on PCR testing and sequence analysis, 138 of the 696 (20%) bobcats were positive for *C. felis* (Table 1). All positives were confirmed to be *C. felis* by sequence analysis. Infected bobcats were detected in all states except California, Colorado, Ohio, and West Virginia. The prevalence in Missouri (79%) was higher than all other states ($p < 0.01$), except for Oklahoma (65%), North Carolina (63%), and South Carolina (57%). Prevalence rates in these states, as well as Kentucky (55%), Florida (36%), and Kansas (31%) were higher ($p < 0.05$) than prevalence rates in Georgia (9%) and North Dakota (2%) (Table 1). A limited number of bobcats from several states had positive amplification with our PCR protocol, but were determined to be infected with other protozoan parasites (e.g. *Heptazoon* spp., *Toxoplasma gondii*, and *Babesia* spp.) based on sequence analysis (data not shown). Of the seven cougars tested, only a single cougar (100%) from Louisiana was positive for *C. felis*. The single serval from Louisiana tested negative.

No difference in *C. felis* infection rate was detected between male and female bobcats ($p = 0.98$). High densities of *A. americanum* in a state were associated with a higher prevalence of *C. felis* in bobcats ($p = 0.007$), while densities of *D. variabilis* were not associated with *C. felis* prevalence ($p = 0.223$). Because 12 of 13 states had a high density of

D. variabilis, however, our ability to evaluate the effect of tick density for this species was limited in the current study.

4. Discussion

Currently, this is the most comprehensive study of the distribution and prevalence of *C. felis* in bobcats, the natural wildlife reservoir of *C. felis* in the United States. A total of 705 wild felids from fourteen states were examined for the parasite. Because *C. felis* prevalence rates are higher in wild felids compared with domestic cats, testing of wild felids provides a more sensitive method to determine the distribution and prevalence of *C. felis* in the United States. Interestingly, we detected a low prevalence of *C. felis* in North Dakota which is the first report of *C. felis* in any felid from this state, as well as the most northerly report of *C. felis*. Previously, the most northerly report was Pennsylvania (Birkenheuer et al., 2008). In addition, we detected *C. felis* in a cougar from Louisiana, which represents the first report of *C. felis* in a cougar outside of the Florida panther population (Butt et al., 1991; Yabsley et al., 2006). Although *C. felis* has been reported in domestic cats from Kentucky and South Carolina, this is the first report of *C. felis* from a free-ranging wild felid in these states (Birkenheuer et al., 2006; Jackson and Fisher, 2006).

We found a significant association between higher prevalence rates of *C. felis* in states where both confirmed tick vectors are known to be present and common. These data are in agreement with reports of clinical cytauxzoonosis in domestic cats which have only been reported from Southeastern, Midwestern, and Mid-Atlantic states and not from California, Colorado, North Dakota, Ohio, or West Virginia. Of the states where *A. americanum* densities are low or the tick is absent, *C. felis* was only detected in North Dakota, but the prevalence was very low (2%). The possibility of uncommon PCR contamination was ruled out by sequence analysis of ITS-1 and the second internal transcribed spacer region of the 18S rRNA gene of the North Dakota *C. felis* samples, which both had unique single nucleotide polymorphisms (data not shown). North Dakota is well outside the known range of *A. americanum*, which is not reported to be north of Iowa or west of Nebraska, although reports of *A. americanum* exist for South Dakota (M. Wimberly, unpublished data). These field data suggest that *A. americanum* may play a more primary role in the maintenance and dissemination of the parasite in wild felids. Recent tick transmission studies have indicated that *A. americanum* may be a more competent vector of *C. felis* than *D. variabilis* as in repeated trials the latter was an unsuccessful vector; whereas *A. americanum* transstadially transmitted the parasite under identical situations (Reichard et al., 2008; Edwards et al., 2010). Additionally, *C. felis* has only been detected from wild-caught questing *A. americanum* from Oklahoma (MIR 0.5–1.5%), while there is no report of *C. felis* from questing *D. variabilis* (Edwards et al., 2010).

Data from this study support previous studies in Oklahoma and North Carolina, both of which had high prevalence rates of *C. felis*. In general, high prevalence rates (31–60%) were reported from bobcats from Oklahoma in

the 1980s (Kier et al., 1982a,b; Kocan et al., 1985; Blouin et al., 1987). Similarly, a relatively high prevalence rate (33%) was reported in North Carolina (Birkenheuer et al., 2008). A significantly higher prevalence was detected in North Carolina (63%) in the current study, but this could be attributed to sampling in areas with higher densities of *A. americanum*. The prevalence in Georgia (9%) was lower than expected; however, the majority of samples for this study were obtained from bobcats removed from various plantations in southwestern Georgia where prescribed burning is commonly used to maintain quail habitat and the long-leaf pine ecosystem (Outcalt, 2000). Prescribed burns can decrease tick densities (Davidson et al., 1994) which theoretically would decrease exposure rates of animals to tick-borne pathogens.

Our data indicates that *C. felis* is widespread and, in some areas, highly prevalent in bobcat populations. The high prevalences observed in presumably healthy, free-ranging wild felids are further evidence that bobcats are the primary reservoir. Although no felids in this study were suspected to have died from clinical cytauxzoonosis, experimental animals and a single fatality report of a naturally infected young bobcat suggest that undetected fatal infections of wild bobcats may occur. Molecular characterization of *C. felis* from domestic cats and a limited number of bobcats from Arkansas, Florida, and Georgia suggest that several strains of *C. felis* circulate in domestic and wild felids (Brown et al., 2009a,b, 2010). Future work on the molecular characterization of *C. felis* should be conducted to determine the genetic variability of this parasite in the natural reservoir.

Conflict of interest statement

The authors have no knowledge of a conflict of interest.

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