Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Volume 11 Article 5

Fall 2010

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ABSTRACT

The purpose of this study was to determine the prevalence of *Cytauxzoon felis* and gastrointestinal helminth infections in bobcats (*Lynx rufus*) of Northwest Arkansas, an area known to have numerous cases of cytaux in domestic cats. Sixty bobcat carcasses were collected from trappers located in Mulberry and Decatur, Arkansas. Blood samples from the hearts were used to isolate *Cytauxzoon* DNA. Next, a polymerase chain reaction (PCR) procedure coupled with gel-electrophoresis assay for the 18s region of extracted DNA were used to determine the presence of the protozoan in the bobcats at the time of harvest. Out of the 60 bobcats, 54 (90%) were positive for the protozoan's infection. These findings indicate a large reservoir of *Cytauxzoon* for possible infection of domestic cats. Along with the detection of *Cytauxzoon felis*, intestinal helminths of the bobcats were collected and identified. The isolated helminths included *Alaria marcianae, Ancylostoma* sp., *Molineus barbatus, Taenia* sp., *Spirometra mansonoides, Mesocestoides lineatus, Aonchotheca putorii, Physaloptera praeputialis* and *Toxocara cati*. All helminths found were previously shown to be common in omnivorous and carnivorous sylvatic as well as domestic mammals of the region. It is amazing that bobcats are able to withstand both parasitic infections concurrently, as they roam the forests of Northwest Arkansas.

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MEET THE STUDENT-AUTHOR

Emily Hickman

Since graduating from Jonesboro High School in 2006, I have spent the past four years majoring in Animal Science at the University of Arkansas. I am an Honors College Fellow, and I was able to use funding from my scholarship to spend the summer after my sophomore year studying abroad in Florence, Italy. I am an active member in the St. Thomas Aquinas University Parish, leading the Seton's Women's group for two years, serving on the Peer Ministry Team for two years, singing in the choir, and acting as president of the organization last year. I currently work at Animal Medical Clinic in Fayetteville. I was a Pre-Vet club officer for two years, and enjoyed participating in the various hands on activities and lectures provided by the club. I am very grateful for the preparation I have received by being part of the Animal Science program. I will begin my first year of Veterinary School at LSU in Baton Rouge this fall.

I am very thankful to Dr. Jeremy Powell for helping me to find the topic for my honors project. With his assistance, as well as Dr. Yazwinski and Dr. Kreider's, I learned information that will be very valuable to me as I continue through my career.

INTRODUCTION

Cytauxzoonosis is an infectious disease caused by the protozoan parasite *Cytauxzoon felis*. It was first discovered when a domestic cat was inoculated with blood from a Florida panther and in turn developed fatal cytauxzoonosis (Yabsley et al., 2006). Since then, the protozoan has been isolated from domestic cats throughout the southeastern United States. It is difficult to screen for the actual disease by standard veterinary procedures such as blood smears because of the pathogen's rapid switching between blood-borne and tissue phases.

The life cycle of organisms in the *Cytauxzoon* genus has two stages: a phagocytic cell phase followed by a red blood cell phase (Gardiner et al., 1998). The cycle in the cat (domestic or bobcat) begins with transfer of sporozoites from an attached, protozoa-infected tick to the cat. In the cat, the protozoa initially infect phagocytic cells (macrophages, reticuloendothelial cells, dendritic cells, certain leukocytes, etc). Rapidly, the infection spreads from the phagocytic cells to the red blood cells. The more pronounced symptoms of the infection in the domestic cat are due to restricted blood flow (because of distended phagocytic cells) and a lack of "efficient" blood (because of destroyed red blood cells). Ticks become infected by drawing blood from infected animals. It appears that the parasite reproduces sexually in the tick, and asexually in the cat.

Diagnosing *C. felis* infections in cats has proven to be difficult. An infected cat often goes undiagnosed or is incorrectly diagnosed as having *Babesia felis* or *Mycoplasma haemofelis*. Cats infected with *C. felis* show nonspecific signs of infection such as anorexia, lethargy, shortness of breath, jaundice and pallor. Fever occurs early in the infection, while hypothermia indicates an animal close to death. Delirium, seizures and coma can also occur in the later stages of the disease. The disease works rapidly, with most domestic cats surviving for only a week after onset of signs. Postmortem examination of *C. felis* infected cats usually reveals enlarged and reddened lymph nodes, distended abdominal veins, hemorrhage in the abdominal organs, and a darkened spleen. Splenomegaly and/or hepatomegaly may also be observed (Shell and Cohn, 2005).

Because most of the symptoms of cytauxzoonosis are non-specific and shared with other diseases, the likelihood of misdiagnosis is high. As a result, the use of PCR testing has become accepted as the best diagnostic tool in documenting this disease (Birkenheuer et al., 2006a). Once the disease is diagnosed, treatment varies, but with no definite cures. There have been improvements with treatment regimes, with more cats currently surviving infections by the parasite than what was initially observed (Bondy et al., 2005). Treatment with the antiprotozoal drugs diminazene aceturate or imidocarb diproprionate, along with supportive care, has proven successful in some cases (Brooks, 2008). Better prognosis may also be due to a genetic change in the parasite, which has resulted in an attenuation of the protozoa over time (Merck Veterinary Manual, 2008).

Cytauxzoon felis is vectored to cats by the American dog tick (*Dermacentor variabilis*) with bobcats (*Lynx rufus*) as the primary reservoir host. Bobcats inhabit most of Canada and North America, as well as northern Mexico. The bobcats are not adversely affected by an infection, and only experience a mild phase of the disease. The bobcat infections have perhaps allowed the parasite to change to a less pathogenic strain over time. Brown et al. (2009) found that there were two genetically distinct strains of *C. felis* in both Georgia and Arkansas. The ability of *C. felis* to develop strain variation may explain why more cats are surviving today than previously.

The purpose of the following study was to determine the prevalence of cytauxzoonosis and gastrointestinal helminthiasis in bobcats obtained in Northwest Arkansas. The information gained from this study might help us better grasp the extent of possible exposure of domestic cats to bobcat-reservoired protozoan and helminth parasites.

MATERIALS AND METHODS

Collection of Bobcat Specimens. Bobcat carcasses were obtained from two registered trappers located in Mulberry and Decatur, Ark. during the 2009 trapping season. The specific locations of the bobcat trappings were not recorded, but all bobcats were obtained in Northwest Arkansas. After the pelts were removed by the trappers, the carcasses were frozen and stored in a freezer at -20 °C. The total number of carcasses collected was 60: 57 from Mulberry and 3 from Decatur.

The carcasses were thawed after delivery to the University of Arkansas, Department of Animal Science, Parasitology facility. Immediately after thawing, blood and tissue samples were obtained from each carcass. The samples of clotted whole blood collected from the hearts were stored at -20 °C until DNA extraction. A sample of spleen tissue was also collected (and frozen) from each carcass in case the DNA extraction from the clotted blood was not successful.

Intestines were processed via standard protocol for isolation of helminth parasites. In brief, the entire intestinal tract from each bobcat was opened lengthwise, the total contents were collected and sieved (final screen aperture of 0.2 mm), and the sieve residues were examined microscopically (10-40 magnification) for helminth isolation and identification.

Performing DNA Extraction. A Qiagen DNA extraction kit for Blood and Tissues (QIAamp DNA Blood Mini Kit, catalog no. 51104, QIAGEN Sciences, Germatown, Md., 20874) was used to extract DNA from the clotted blood samples. In all cases, the DNA from the blood was of sufficient concentration to run the PCR and electrophoresis procedures. Hence, the spleen tissue samples were not used in this study.

To confirm that a sufficient amount of DNA was extracted from each sample, total DNA concentration was determined on a Hofer Dynaquant 200 flourometer using Hoest 33258 dye and calf thymus DNA as a standard (Cesarone et al., 1979). The goal was to have each sample contain a minimum concentration of 10 ng DNA/µL. If samples proved to contain less than the required DNA concentration after the first extraction, additional blood would have been extracted until an adequate amount of DNA was obtained.

Testing DNA Using Polymerase Chain Reaction. The presence of *C. felis* DNA in individual samples was determined by the use of a PCR assay that has previously been verified as specific for *C. felis* (Birkenheuer et al., 2006a). The specific forward and reverse primers used were 5'-GCGAATCGCATTGCTTTATGCT -3' and 5'- CCAATTGATACTCCGGAAAGAG -3', respectively. The assay amplifies a specific 284 bp segment of the *C. felis* 18s ribosomal DNA. Each assay mixture also included forward and reverse primers for the DNA region coding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a positive control; this was to detect any presence of PCR inhibitors in the extracted DNA. Glyceraldehyde 3-phosphate dehydrogenase is an enzyme that catalyzes the sixth step of glycolysis, and is present in tissues from all oxygen-breathing animals. All samples were also run with a negative control consisting of the complete reaction mixture with both sets of primers, but with water substituted for the DNA templates.

Each set of PCR reactions also included a positive control consisting of DNA obtained from a domestic cat that died from a *C. felis* infection. The positive control DNA was kindly provided by Dr. Mason Reichard of the Oklahoma State University College of Veterinary Medicine.

Conducting Gel Electrophoresis. A 1% agarose gel was prepared to process and view the DNA fragment material that was propagated by PCR and placed for electrophoretic separation. Gels were run at 85 V for one hour, and were then visualized with images recorded using a UVP Epi Chem II imaging system (UVP Inc., Upland, Calif.). A *C. felis* positive result was indicated by two bands at approximately 401 bp and 284 bp, and a negative result had only one band at 401 bp. The 284 bp band indicated the presence of the 18s region of *C. felis*, and the 401 bp represented the band for GAPDH and indicated that PCR inhibitors were not present in cytaux DNA-negative samples.

RESULTS AND DISCUSSION

The assay was able to amplify cytaux-specific DNA when at a concentration >10 ng/µL (Table 1). Of the 60 samples, only 6 were negative for *C. felis*. This means that an astounding 90% of the bobcats tested were positive for

the presence of *C. felis* DNA and hence, infection. An example of a finished gel is given in Fig 1.

The above results have major implications. This study shows that the bobcat provides a considerable reservoir of the *C. felis* parasite. In other studies, infections with *C. felis* were shown to be far less common in the bobcat (Yabsley et al., 2006). This information is important for owners of cats living in Northwest Arkansas. Chance of infection from a tick would appear to be likely when there is close proximity or overlap of regions frequented by bobcats and domestic cats. Reichard et al. (2008) emphasized this point when he stressed that the presence of bobcats in a domestic cat's environment is a predisposing factor in the spread of this disease to the highly susceptible, domestic cat. It is therefore important that cat owners and veterinarians in the southeastern U.S., and especially in Arkansas, remain vigilant in detecting early signs of *Cytauxzoon* infection in cats.

It is also important to continue studying differences in strains from region to region to determine any connection between strain and pathogenicity. Brown et al. (2009) found 2 genotypes of the ITS region of DNA, indicating that there were two genetically distinct strains of *C. felis* in both Georgia and Arkansas. Changes in a strain could be important relative to protozoan virulence in domestic cats. Mutations might lead to the development of strains that are less (or more) virulent. Also, the rate of change in different geographic regions could vary. This would explain the lower incidence of disease in areas outside the southeastern U.S. It is important to determine if changes in this parasite affect its pathogenicity in the cat, and how to apply this knowledge for the future maintenance of healthy cats. The serious nature of *C. felis* infection was demonstrated by Birkenheuer et al. (2006b); a study wherein 32 of 34 infected domestic cats from North Carolina, South Carolina and West Virginia died or were euthanized because of a *Cytauxzoon* infection. The high incidence of *C. felis* infection in Northwest Arkansas bobcats, as demonstrated in this study, suggests a real and present threat to the well-being of area cats. Further studies are also needed to develop laboratory procedures that could determine the genotypes (strains) and pathogenicities of *C. felis*.

While the main focus of this project was to test for the presence of *C. felis*, discovering which intestinal helminths were present was also thought to be noteworthy given the range of alternate hosts that helminths routinely infect. The helminth parasites might be those that have a wide range of natural as well as dead end hosts, including humans. Nine different species of intestinal helminths were found: *Alaria marcianae, Ancylostoma* sp.*, Molineus barbatus, Taenia* sp.*, Spirometra mansonoides, Mesocestoides lineatus, Aonchotheca putorii, Physaloptera praeputialis,* and *Toxocara cati* (Fig. 2). All of the helminths can also infect domestic felines. Only two are considered to be of any realistic threat to humans; *Ancylostoma* sp. and *Toxocara cati*, which precipitate cutaneous larval migrans (CLM) and visceral larval migrans (VLM) in people, respectively.

ACKNOWLEDGEMENTS

I would like to extend many thanks to Hartz Mountain Industries, Inc. of Secaucus, New Jersey, who was the financial sponsor of this project, and Dr. Wesley Shoop of E.I. du Pont de Nemours & Company of Wilmington, Delaware, who provided supplemental helminth identifications. Thanks are also extended to Dr. Mason Reichard of Oklahoma State University College of Veterinary Medicine for providing positive test samples, and Kate Williams, DVM, who provided the negative control samples, and timely support and encouragement.

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Table 1. The initial DNA concentrations in the blood samples and the infection status results as determined by PCR and electrophoresis (Mulberry [M], Decatur [D]).

Sample # (bobcat)	[DNA] ng/µL	Pos	Neg	Sample # (bobcat)	[DNA] ng/µL	Pos	Neg
M-1	40	X		M-31	10	X	
M-2	300	X		M-32	110	X	
M-3	193		X	M-33	393	X	
M-4	163	X		M-34	270		X
M-5	137	X		M-35	69	X	
M-6	20	X		M-36	40	X	
M-7	87	X		M-37	80	X	
M-8	77	X		M-38	103	X	
M-9	50	X		M-39	143	X	
M-10	239	X		M-40	117	X	
M-11	20		X	M-41	60	X	
M-12	23	X		M-42	183	X	
M-13	125	X		M-43	283	X	
M-14	383	X		M-44	23	X	
M-15	13	X		M-45	63	X	
M-16	17	X		M-46	120	X	
M-17	123	X		M-47	196	X	
M-18	117	X		M-48	50	X	
M-19	83	X		M-49	50	X	
M-20	17	X		M-50	37	X	
M-21	53	X		M-51	37	X	
M-22	147	X		M-52	56	X	
M-23	77		X	M-53	420	X	
M-24	118	X		M-54	107		X
M-25	57	X		M-55	27	X	
M-26	196	X		M-56	67	X	
M-27	156	X		M-57	190		X
M-28	93	X		D-1	238	X	
M-29	246	X		D-2	330	X	
M-30	107	X		D-3	78	X	

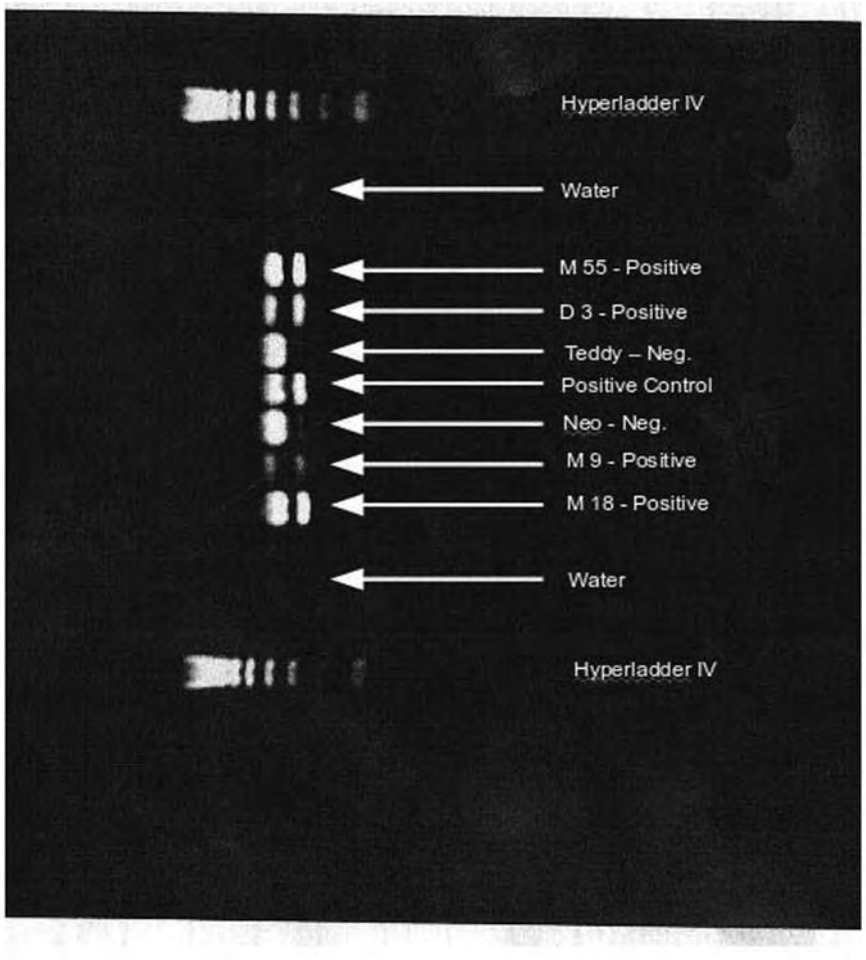

Fig. 1. An example of a finished gel (Neo and Teddy were known negatives, M stands for Mulberry and D stands for Decatur as source regions of the bobcats and Hyperladder IV was a molecular weight marker).

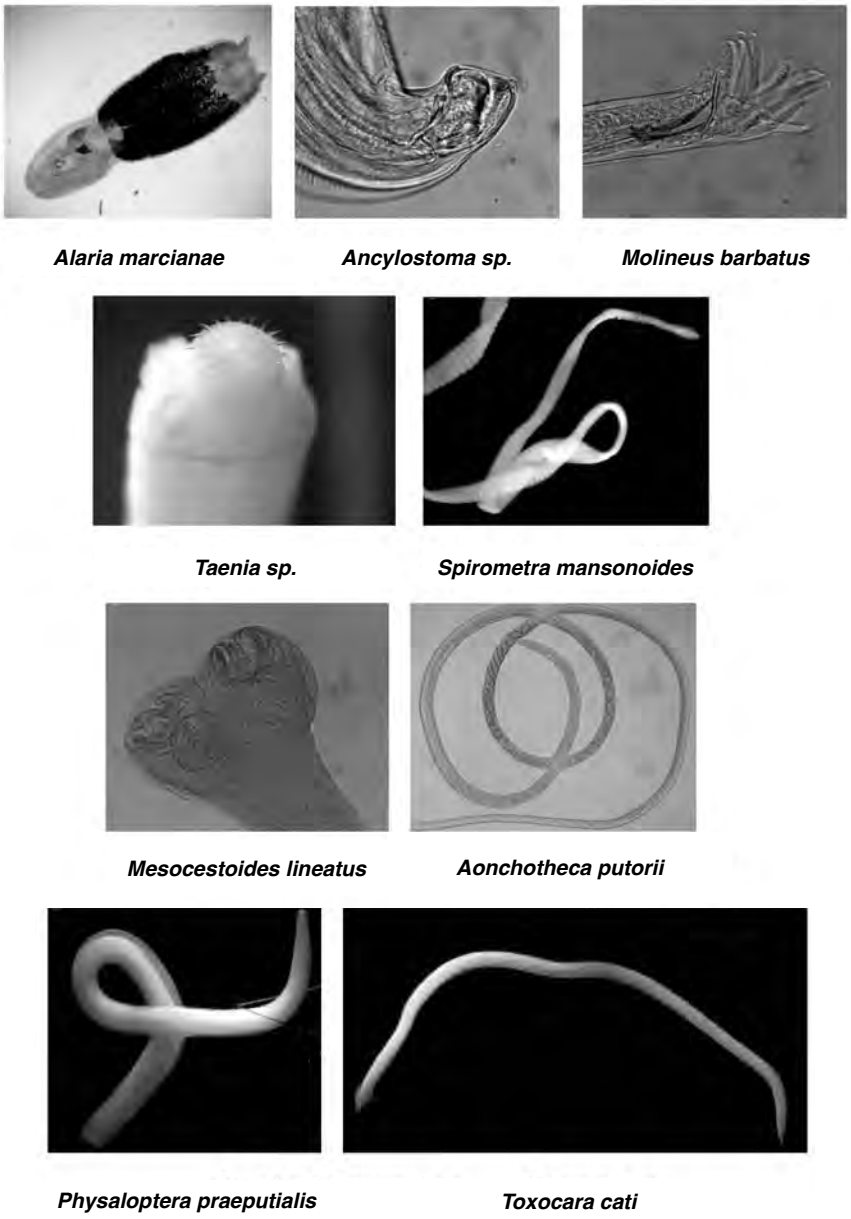

Fig. 2. Photographs of the helminths recovered in this survey.