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Short communication

Prevalence of *Anaplasma phagocytophilum* in domestic felines in the United States

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Abstract

Anaplasma phagocytophilum is among the more common tick-borne disease agents in the United States. It is of veterinary and public health significance as dogs, cats, and human beings are known to be susceptible. *A. phagocytophilum* is transmitted transstadially by either nymphs or adults of either the black-legged tick (*Ixodes scapularis*) or the western black-legged tick (*Ixodes pacificus*). Little information is available regarding either the prevalence of this agent in cats or the dynamics of vector transmission. Four hundred and sixty feline blood samples from sites throughout the United States were assayed for antibodies to *A. phagocytophilum* using an indirect immunofluorescence assay (IFA). Results of the prevalence study showed that 20 samples (4.3%) were positive for *A. phagocytophilum* antibodies by IFA at a 1:50 dilution, however these results could not be confirmed by PCR analysis. PCR analysis for other cross-reacting *Ehrlichia/Anaplasma* spp. was also negative. These results demonstrate that natural infection of *A. phagocytophilum* in cats is uncommon.
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1. Introduction

Ixodes scapularis, the deer/black-legged tick, and *I. pacificus*, the western black-legged tick, may transmit multiple pathogens, including *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Babesia microti*, and possibly *Bartonella* spp. (Magnarelli et al., 1995; Mitchell et al., 1996; Chang et al., 2001; Eskow et al., 2001). *I. scapularis* is distributed throughout the eastern and Midwestern United States; *I. pacificus* is found principally in western coastal United States. *I. scapularis* in the northeast feed primarily on mammals, while *I. scapularis* in the southeast feed on mammals, birds, and lizards (Keirans et al., 1996). Larval *I. scapularis* prefer to feed on the white-footed mouse, *Peromyscus leucopus*, or other small rodents (Mather et al., 1989; Telford et al., 1996). However, as nymphs and adults, they are likely to attach to a wider variety of large hosts, such as white-tailed deer (*Odocoileus virginianus*), dogs, cats, and humans.

A. phagocytophilum and *B. burgdorferi*, the agent of Lyme disease, are the leading vector-borne transmitted diseases in the United States. *A. phagocytophilum* is an intracellular bacterium that resides within the

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granulocytes of its host. Originally, this agent was referred to as the Human Granulocytic Ehrlichiosis agent (HGE) in humans, *Ehrlichia equi* in horses, and *Ehrlichia phagocytophila* in sheep and other ruminants (Dumler et al., 2001), however molecular studies showed them to be the same organism. General clinical symptoms of anaplasmosis and borreliosis are similar—fatigue, headache, arthralgias, myalgias, and fever (Steere et al., 1983; Aguero-Rosenfeld et al., 1996; Bakken et al., 1996; Petrovec et al., 1997).

Secondary infections may occur, however both anaplasmosis and borreliosis are easily treated with doxycycline (Foggie, 1951). These infections may be particularly severe in immunocompromised individuals. The identification of animals that serve as reservoir hosts is of key importance in the control of these diseases.

Research involving felines and vector-borne diseases is limited, with the first publication of *A. phagocytophilum* in domestic felines being reported in 2004 (Lappin et al., 2004). Clinical disease due to anaplasmosis in felines can present with the following signs; fever, thrombocytopenia, hyperglobulinemia, lethargy, anorexia, weight loss, vomiting, polyarthritis, lameness and ocular discharge (Stubbs et al., 2000).

There have been very few reports of infections with *A. phagocytophilum* in domestic cats in the United States, but nationwide prevalence studies have not been conducted (Hackett et al., 2006). In a recent study of 553 feral cats in Florida, none of the cats tested by PCR were positive for the presence of *A. phagocytophilum* DNA (Luria et al., 2004). Serological surveys, however, were not conducted for the presence of antibodies to this organism. As a study on the prevalence of antibodies to *B. burgdorferi* has already been published (Magnarelli et al., 1990), the purpose of this study was to examine the seroprevalence of *A. phagocytophilum* in cats from different regions of the United States, with attempted follow-up confirmation by PCR.

2. Materials and methods

Feline serum samples were obtained by collaborators in Auburn, Alabama ($n = 175$, all stray or feral), Gainesville, Florida ($n = 90$, all stray or feral), San Diego, California ($n = 23$, from clinics or rescue organizations [indoor/outdoor status not provided]), Verona and Oregon, Wisconsin ($n = 96$, 1 indoor/outdoor; 30 stray; 65 feral), East Lansing, Michigan ($n = 45$, 3 indoor/outdoor; 42 from rescue organizations—indoor/outdoor status unknown), and Providence, Rhode Island ($n = 31$, 11 strays; 18 indoor/outdoor; 2 feral). Collaborators were required to collect serum and whole blood samples from felines that were indoor/outdoor, stray, or feral. Furthermore, stipulations were made that all cats must be greater than a year in age to increase the likelihood of contact with tick vectors. The above information, in addition to the geographical location of the cat, was included with each sample.

Plasma/serum was separated from whole blood prior to shipment. Samples of plasma or serum and whole blood were sent via overnight courier to Auburn University's College of Veterinary Medicine. Samples were frozen immediately upon arrival and maintained at −20 °C until immunofluorescence assays (IFA) were performed using *A. phagocytophilum* IFA commercial substrate slides (VMRD Inc., Pullman, WA). A goat anti-cat IgG FITC conjugate was used as the secondary antibody. The recommended staining procedure from VMRD Inc. was followed except that the conjugate was diluted at 1:50 (20 μl conjugate, 63 μl Evans blue, and 917 μl PBS). Sera were screened at a 1:50 dilution (as is the routine in our laboratory) for the presence of antibodies to *A. phagocytophilum*. Any positive sera were titered out to end point dilutions. Mounting fluid was added (glycerol/FA rinse buffer) and slides were viewed with a fluorescence microscope at 400×. Samples were evaluated concurrently with positive and negative controls. Only intracellular organisms demonstrating bright green fluorescence were interpreted as positive as per manufacturer's instructions.

DNA for *A. phagocytophilum* PCR analysis was extracted from the feline whole blood samples using a modified method from Sambrook et al. (1989) as follows: 200 μl of each blood sample was added to 100 μl lysis buffer (0.25 M EDTA, 1% SDS, 10 mM Tris, pH 8.0) containing 100 μg/ml Proteinase K and incubated at 42 °C for 3–4 h. The Proteinase K was inactivated by incubating the tubes at 65 °C for 30 min. Thereafter 150 μl cold Solution III (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml distilled water) was added and the samples were kept on ice for 30 min. The tubes were then centrifuged at 13,000 rpm for 5 min and the supernatant was transferred to clean tubes. Isopropanol was added to give a total volume of 1 ml and the tubes were kept at room temperature for 5 min before centrifugation as described above. The isopropanol was removed and the DNA pellet was washed in cold 70% ethanol and allowed to air dry. The DNA pellet was resuspended in Tris–EDTA buffer. Only samples that were positive at the 1:50 dilution using the indirect immunofluorescence assay were subjected to polymerase chain reaction

Table 1
Prevalence results of *Anaplasma phagocytophilum* from feline samples

State	Number of samples received	Number of serum samples positive by IFA at a 1/50 dilution	Whole blood PCR results on IFA positive samples
Alabama	175	0	Not done
Florida	90	12	All negative
California	23	3	All negative
Wisconsin	96	0	Not done
Michigan	45	3	All negative
Rhode Island	31	2 (1/200 titer)	Not supplied
Total	460	20 (4.3%)	0

(PCR) analysis for the presence of *A. phagocytophilum* specific DNA.

A total volume of 50 μl was used to carry out DNA amplification: 0.2 μM of each primer, 100 μM of each deoxynucleotide triphosphate, and 1.25 U of *Taq* polymerase. To amplify the 16S rDNA gene of *A. phagocytophilum*, a nested PCR was performed using the methods of Massung et al. (1998) and Massung and Slater (2003), which had a detection limit of 0.25 infected cells per reaction. Primers used for the primary amplification were ge3a (5′CACATGCAAGTCGAACGGATTATTC) and ge10r (5′ TTCCGTTAAGAAGGATCTAATCTCC). Primers used in the nested PCR consisted of ge9f (5′AACGGATTATTCTTTATAGCTTGCT) and ge2 (5′GGCAGTATTAAAAGCAGCTCCAGG).

Ten microliters (10–20 ng/ul) of DNA from each sample was used in the PCR under the following cycling conditions: an initial 2 min denaturation at 95 °C, followed by 40 cycles, each consisting of a 30 s denaturation at 94 °C, a 30 s annealing at 55 °C, and a 1 min extension at 72 °C. These 40 cycles were followed by a 5 min extension at 72 °C. Reaction products were maintained at 4 °C until they were analyzed. Positive control DNA was obtained from naturally infected ticks (kindly supplied by Dr. Kirby Stafford, Connecticut Agricultural Experiment Station, New Haven, Connecticut) and water served as the negative control. All reactions were performed in an Eppendorf thermocycler or Strategene RoboCycler Gradient 96 and final products were analyzed by 2% agarose gel electrophoresis.

Samples that were seropositive for *A. phagocytophilum* but negative on PCR analysis were further subjected to an *Ehrlichia/Anaplasma* spp. 16Sr gene PCR according to the following protocol. DNA was extracted from frozen whole blood samples on a Qiagen Bio Robot M48 using a Qiagen MagAttract DNA blood Mini M48 kit and suspended in 200 μl RNase free water. A 25 μl reaction containing 12.5 μl of TaKaRa Premix Ex Taq (Perfect Real Time, Takara Mirus Bio, Madison, Wisconsin), 6.75 pmol of each primer (GEPs 5′CTGGCGGCAAGCYTAACAC-ATGCAAGTCGAACGGA3′ and GEPr 5′CTTCTRTR-CTTCTRTRGGTACCGTCATTATCTTCCCYAYTG3′) and 5 μl of template was utilized. PCR was performed in an Eppendorf Mastercycler ep gradient as follows: one cycle of 95 °C for 10 s followed by 55 cycles of 95 °C for 15 s, 66 °C for 15 s and 72 °C for 18 s, and one final cycle of 72 °C for 2 min. Positive control DNA was obtained from an *Ehrlichia chaffeensis* isolate from culture and water served as the negative control. Feline GAPDH served as the housekeeping gene.

3. Results

Twelve of 90 feline samples from Florida appeared positive by IFA at a titer of 1:50. The whole blood samples from these cats were negative in both PCR analyses. Three of 45 samples from Michigan also appeared positive by IFA at a titer of 1:50, however, bacterial DNA was not detected by either PCR. Likewise, three of 23 samples from California were positive by IFA and, again, bacterial DNA could not be detected by either PCR. One hundred seventy-five samples from Alabama and 96 samples from Wisconsin were negative by IFA. Two of 31 samples from Rhode Island were positive by IFA. These samples remained positive at titers up to 1:200, however, whole blood was not provided for PCR analysis (Table 1).

4. Discussion

Results of the seroprevalence survey suggest that cats are not commonly infected with *A. phagocytophilum* and are thus unlikely to serve as maintenance hosts for this parasite. Approximately 4.3% of samples tested by immunofluorescence assay were positive. Of 460 serum samples, two from Providence, Rhode Island

appeared positive at a dilution of 1:200. There were 18 samples positive by IFA at dilution of 1:50 that tested negative by PCR.

Prevalence results might have been higher if more samples had been collected from survey areas where the parasite is naturally more common (northeastern United States). Two hundred and sixty-five samples of the 460 total were collected from the southeast, which is known to have a lower prevalence of *A. phagocytophilum* than the northeast. In a recent study from the northeast, it was shown that 30% of cats were positive for *A. phagocytophilum* antibodies by IFA, or 38% by ELISA (Magnarelli et al., 2005).

It was originally speculated that the 18 samples positive on IFA, in this study, at a titer of 1:50, were cross-reactive with similar species, for example *Ehrlichia canis*, as cats have been shown to have antibodies to this organism (Breitschwerdt et al., 2002). However, the follow-up PCR showed that this was not the case.

A serologic and molecular survey performed in Spain found that six of 122 cats were positive by immunofluorescence assay for *A. phagocytophilum*, but were negative by PCR (Aguirre et al., 2004). It was speculated that organisms were sequestered within other tissues, and that parasites were not present in blood specimens. It has also been previously shown that antibodies persisted in clinically ill cats that were treated while PCR analysis was negative (Lappin et al., 2004). It was also speculated in this study that organisms may have been sequestered in low numbers in tissues such as the spleen (Lappin et al., 2004).

Another consideration would be that the cats were infected but cleared the organisms and hence remained antibody positive while becoming antigen negative. It is likely that one or both of those factors were also responsible for the negative PCR results obtained in the present study.

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