SEROPREVALENCE AND ATTEMPTED TRANSMISSION OF *ANAPLASMA*

PHAGOCYTOPHILUM AND/OR *BORRELIA BURGDORFERI*

FROM NATURALLY INFECTED TICKS TO CATS

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Sarah Arnao Billeter

Certificate of Approval:

_____	_____
Jennifer A. Spencer	Byron L. Blagburn, Chair
Research Fellow	Distinguished University Professor
Pathobiology	Pathobiology
_____	_____
Christine C. Dykstra	Brenda Griffin
Associate Professor	Associate Clinical Professor
Pathobiology	Scott-Richey Research Center

_____

Stephen L. McFarland
Acting Dean
Graduate School

SEROPREVALENCE AND ATTEMPTED TRANSMISSION TIMES OF *ANAPLASMA PHAGOCYTOPHILUM* AND/OR *BORRELIA BURGDORFERI* FROM NATURALLY INFECTED TICKS TO FELINES

Sarah Arnao Billeter

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SEROPREVALENCE AND ATTEMPTED TRANSMISSION OF *ANAPLASMA PHAGOCYTOPHILUM* AND/OR *BORRELIA BURGDORFERI* FROM NATURALLY INFECTED TICKS TO FELINES

Sarah Arnao Billeter

_____
Signature of Author

_____
Date

VITA

Sarah Arnao Billeter, daughter of Eugene and Georgia Jene Arnao, was born September 28, 1979 in Castle AFB, California. She graduated from Highland High School in Palmdale, California in 1998 with honors. She attended the University of Arizona and graduated May 2003 with a Bachelor of Science degree in Veterinary Sciences. She married Jason Donald Billeter, son of Donald Billeter and Wendy Bates-Niles, on May 16, 2003. She entered Graduate School, Auburn University, in June 2003 and upon completion of her Master's, she will be continuing with her education at North Carolina State University in Raleigh, North Carolina to earn her Doctoral degree.

THESIS ABSTRACT

SEROPREVALENCE AND ATTEMPTED TRANSMISSION OF *ANAPLASMA PHAGOCYTOPHILUM* AND/OR *BORRELIA BURGDORFERI* FROM NATURALLY INFECTED TICKS TO FELINES

Sarah Arnao Billeter
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A. phagocytophilum and *B. burgdorferi* are among the leading tick-borne disease agents in the United States. Both agents are of veterinary and public health significance as dogs, cats, and human beings are known to be susceptible. *B. burgdorferi* and *A. phagocytophilum* are transmitted trans-stadially 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 prevalences of these agents 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). Samples that were positive by IFA were subjected to polymerase chain reaction (PCR) analysis to measure presence of bacterial DNA. In an attempt to determine the minimum transmission times of *A. phagocytophilum* and *B burgdorferi*, wild caught adult *I. scapularis* ticks were applied to

cats and removed at 8, 16, 24, and 36 hours after attachment. Blood was collected on days 42, 56, 72, 84 and 98 after tick removal and examined using both IFA and PCR. Biopsies were collected from sites of tick attachment and examined for *A. phagocytophilum* and *B. burgdorferi*-specific DNA by PCR procedures.

Two samples from Providence, Rhode Island were positive using an indirect immunofluorescence assay at a titer of 1:200; however, whole blood samples were not provided and positivity could not be corroborated by PCR. Eighteen other samples (12/90 from Florida, 3/45 from Michigan, and 3/23 from California) appeared positive at a titer of 1:50, however bacterial DNA was not detected using PCR procedures. The remaining samples were seronegative as determined by IFA. All ticks were negative by PCR for *A. phagocytophilum*, however, *B. burgdorferi* was detected in two of eight ticks attached to the 8 hour cat and five of seven attached to the 16 hour cat. Fourteen of 31 pooled unattached ticks were positive for *B. burgdorferi* and one was positive for *B. miyamotoi*. Cats remained antibody negative for both *A. phagocytophilum* and *B. burgdorferi* throughout the transmission study. Biopsy sites from all cats were negative for both *A. phagocytophilum* and *B. burgdorferi*. These results demonstrate that natural infection of *A. phagocytophilum* in cats is uncommon. Methodologies used in the study to determine transmission times could be of value in determining times necessary for vector-mediated transmission of agents. However, several features of the model could be improved. These potential modifications are discussed below.

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I. INTRODUCTION

The importance of zoonotic vector-borne diseases has grown as humans and their pets encroach on wildlife habitats. *Borrelia burgdorferi* and *Anaplasma phagocytophilum* are the leading vector-borne agents transmitted in the United States and, more importantly, are transmitted by the same tick vector, *Ixodes scapularis*. *I. scapularis* will feed on an assortment of animals, including horses, cattle, dogs, cats, and humans. Secondary infections do occur with these agents and, if left untreated, can potentially cause death in immunocompromised individuals.

Vector:

I. scapularis, the black-legged or deer tick, and *I. pacificus*, the western black-legged tick, may transmit multiple pathogens, among them, *A. phagocytophilum*, *B. burgdorferi*, *Babesia microti*, and possibly *Bartonella henselae* (Chang et al., 2001; Eskow et al., 2001; Magnarelli et al., 1995; Mitchell et al., 1996). *I. scapularis* is distributed throughout the east and midwest, while *I. pacificus* is found on the western coastal states. It has been shown that the white-footed mouse, *Peromyscus leucopus*, is the main reservoir host for larval ticks and the white-tailed deer, *Odocoileus virginianus*, is the principal host for adult ticks (Little et al., 1998; Telford et al., 1996).

A. phagocytophilum and *B. burgdorferi* are distributed mainly in the northeast and midwest and this may be due to the feeding habits of the tick vector (Bakken et al., 1996). *I. scapularis* ticks feed on a wide range of mammalian hosts in the northeast and mid-western United States, while *Ixodes* ticks in the southeast feed on mammals, as well as lizards and birds (Keirans et al., 1996). A study has demonstrated that *I. scapularis* ticks from the southeast have the ability to transmit *B. burgdorferi*, however there are very few reported cases of tick-borne infections in this location (Jacobs et al., 2003). It was postulated that there are a variety of reasons for low transmission of tick-borne infections in the southeast: 1) underreporting of disease, 2) decreased infectivity and pathogenicity of strains, 3) differences in human populations from the northeast and southeast, 4) possibility that infected overwintered nymphs fail to feed on and infect new reservoir hosts, 5) lizards are a main source of food for nymphal ticks and are simply not competent reservoir hosts for infection, and 6) differences in feeding behavior of nymphal *I. scapularis* ticks (Jacobs et al., 2003; Oliver, 1996). It has also been demonstrated that *B. burgdorferi* is quite rare in southeastern nymphal ticks, while >25% of northern *I. scapularis* are infected (Piesman, 2002).

Prevalence of these agents may vary greatly from year to year in the same location. In a four year study performed in Bridgeport, Connecticut, the percentage of *A. phagocytophilum* positive adult ticks varied from 7% in 1996 to 19% in 1998 (Massung et al., 2002). Ticks collected from Rhode Island were more variable in their positive status: 46% in 1997 to 10% in 1999 (Massung et al., 2002). In another prevalence survey carried out in Erie County, Pennsylvania over a two year period, none of the ticks were positive for *A. phagocytophilum* and 43.1% were positive for *B. burgdorferi* in

2000, however in 2001, 2.5% were positive for *A. phagocytophilum* and 67.7% were positive for *B. burgdorferi* (Courtney et al., 2003). The annual variations in infectivity are most likely due to low host prevalence or climatic changes that inhibit tick feeding.

Anaplasma phagocytophilum:

As previously mentioned, *I. scapularis* ticks have the ability to transmit *Anaplasma phagocytophilum. A. phagocytophilum* is a small, gram-negative intracellular bacterial organism that infects granulocytes in humans, horses, ruminants, dogs, cats, and a variety of other animals (Chen et al., 1994; Lewis, 1976). 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). Based on molecular findings, these species have been synonymized and the complex is now referred to as *Anaplasma phagocytophilum* (Dumler et al., 2001).

The first human *A. phagocytophilum* infections were reported in 1994 in twelve patients from Wisconsin and Minnesota. Patients complained of fever, chills/rigors, fatigue, myalgias/arthralgias, headaches, stiff neck, nausea, cough, and diarrhea within one week of a tick bite (Aguero-Rosenfeld et al., 1996; Bakken et al., 1996; Petrovec et al., 1997). Laboratory findings included thrombocytopenia, leucopenia, lymphopenia, elevations in lactate dehydrogenase, and elevations in mild hepatocellular enzymes (Aguero-Rosenfeld et al., 1996; Bakken et al., 1996). Anemia and abnormalities of renal function were also reported but were not as common (Aguero-Rosenfeld et al., 1996; Bakken et al., 1996). In each instance, the infection responded to multiple doses of doxycycline.

Antibody responses to *A. phagocytophilum* infection usually occur within ten days to one month after exposure (Horowitz, 1997). A cell-mediated immune response has been shown in horses and sheep and protective immunity remains for up to one year after recovery from acute anaplasmosis (Barlough et al., 1995; Corstvet et al., 1993; Gribble, 1969; Nyindo et al., 1978; Stamp et al., 1950). Deaths have occurred because of secondary opportunistic infections but do not generally occur in non-immunocompromised individuals. Sheep can develop infections including listeriosis, pasteurellosis, chlamydiosis (Foggie, 1951); both humoral and cellular immunodeficiencies have been found for up to six weeks after infection in sheep (Larsen et al., 1994).

Borrelia burgdorferi:

Borrelia burgdorferi, the causative agent of Lyme disease, is the principal vector-transmitted disease agent present in the United States (Dennis, 2002). *B. burgdorferi* sensu lato contains three clinically relevant genospecies: *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* (Liveris et al., 2002). However, *B. burgdorferi* sensu stricto is the only organism of the three genospecies found within the United States. These organisms are motile, corkscrew-shaped bacteria, or spirochetes. They also have the ability to infect a wide variety of hosts, many of which are also infected with *A. phagocytophilum* (Steere et al., 2004).

Three to 32 days after initial tick bite, one generally sees a slowly expanding rash from the area of the bite, or erythema migrans (Steere et al., 2004). The same general clinical symptoms exhibited with patients infected with *A. phagocytophilum* are also seen in *B. burgdorferi* patients—fatigue, headache, arthralgias, myalgias, and fever (Steere et

al., 1983). Spirochetes are able to disseminate throughout the body and can cause secondary annular skin lesions, acute lymphocytic meningitis, cranial neuropathy, radiculoneuritis, atrioventricular nodal block, and migratory musculosketal pain in joints, bursae, tendon, muscle, or bone (Steere et al., 2004). If left untreated, patients will experience arthritis in the large joints, primarily in the knee, and possible chronic erosive disease (Steere et al., 1987).

Complement-mediated lysis of the spirochetes usually occurs at the site of the tick bite (Breitner-Ruddock et al., 1997). A recruitment of lymphocytes, macrophages, dendritic cells, and a small number of plasma cells occurs during the innate immune response (Mullegger et al., 2000). A T cell-dependent immune response will occur and within several weeks to months, the antibody response usually can control dissemination of spirochetes. As spirochetes circulate, the organisms are able to change or minimize antigenic expression on their surface and, thereby, evade certain host immune responses (Hefty et al., 2002; Liang et al., 2002). The organisms may survive for several years, however, and approximately 60% of patients will experience intermittent recurrences of arthritis (Steere et al., 1987).

Small mammals as reservoir hosts:

The white-footed mouse, *Peromyscus leucopus,* is a competent reservoir for *A. phagocytophilum* (Telford et al., 1996). It was demonstrated that white-footed mice that were caught in the wild were capable of transmitting *A. phagocytophilum* to laboratory-reared *I. scapularis* (Telford et al., 1996). These ticks were then able to infect C3H/HeJ mice (Telford et al., 1996). It is now known that the larval stages prefer to feed on these hosts. Raccoons (*Procyon lotor*) and gray squirrels (*Sciurus carolinensis*) are also

capable reservoirs, however, Virginia opossums (*Didelphis virginiana*) and striped skunks (*Mephitis mephitis*) are not competent vectors (Levin et al., 2002). Opossums and striped skunks were shown to not harbor infected larvae, only infected adults (Levin et al., 2002). The authors speculated that adult ticks that had fed on the opossums and striped skunks were probably infected after feeding on a previous host. Other *Peromyscus* species and woodrats, *Neotoma* sp., have also been shown to be infected with the human granulocytic ehrlichiosis agent, but attempts to determine if these animals were competent reservoir hosts were unsuccessful (Nicholson et al., 1998).

B. burgdorferi has been isolated from the blood, kidney, eyes, spleen, and liver of the white-footed mouse (Anderson et al., 1985). In an endemic area of Massachusetts, it was demonstrated that 90% of caught *P. leucopus* were infected with *Borrelia* spirochetes (Mather et al., 1989). Chipmunks (*Tamias striatus*), red backed voles (*Clethrionomys gapperi*), woodrats, brush rabbits (*Sylvilagus bachmani*) and other small mammals also serve in the maintenance of *B. burgdorferi* (Bey et al., 1995; Mather et al., 1989; Peavy et al., 1997). In a study performed in 1990, larval ticks collected from raccoons, opossums, and striped skunks were allowed to molt and then dissected for presence of *Borrelia*. Positive ticks were found on raccoons and striped skunks, however, ticks collected from opossums did not survive long enough to be examined for spirochetes (Fish and Daniels, 1990).

Deer as sentinels for disease:

I. scapularis adults feed primarily on larger animals, such as the white-tailed deer, while larvae and nymphs feed on smaller animals. Many serologic surveys of *A. phagocytophilum* have confirmed infection in the white-tailed deer throughout Wisconsin

and Maryland (Belongia et al., 1997; Walls et al., 1998). A prevalence study in Missouri using polymerase chain reaction (PCR), to test for the 16S rRNA gene of *A. phagocytophilum* from blood samples collected from wild deer showed that 214/217 deer were positive for the agent (Arens et al., 2003). In three counties of Minnesota, tests performed on serum collected from white-tailed deer (317 samples) verified that 80% were infected with *B. burgdorferi* (Gill et al., 1994). These experiments demonstrated that white-tailed deer are natural reservoirs and the use of these animals as sentinels could determine the frequency of *A. phagocytophilum* and *B. burgdorferi* infection.

Avian reservoirs:

Birds are also possible reservoir hosts for both *A. phagocytophilum* and *B. burgdorferi*. This is of interest to researchers because these birds may carry the agents great distances during flight. In one article, 5/25 larval pools from a veery, *Catharus fuscescens*, and two pools from a robin, *Turdus migratorius*, were positive for *A. phagocytophilum* (Daniels et al., 2002). Another study demonstrated that stress can cause reactivation of *Borrelia* infection in birds which may serve as a new mechanism of spreading the disease (Gylfe et al., 2000).

Disease in canines:

The more common *Ehrlichia* sp. infecting dogs are *Ehrlichia canis* (monocytic ehrlichiosis) and *Ehrlichia ewingii* (canine granulocytic ehrlichiosis). However, it has also been shown that canines can be host to *Anaplasma phagocytophilum*. In one report, two Northern California dogs (two separate cases) presented to the University of California College of Veterinary Medicine with fever, anorexia, and depression. Cytoplasmic inclusions were present in neutrophils on blood smears from these cases

(Madewell and Gribble, 1982). Twenty milliliters of blood from one of the dogs was used to inoculate two ponies. After ten days, mild febrile disease and neutrophilic inclusions were present in both horses (Madewell and Gribble, 1982).

Table one summarizes *Anaplasma* and *Ehrlichia* sp. known to infect dogs, ruminants, humans, and other hosts.

Table 1. Taxonomy of *Ehrlichia* and *Ehrlichia*-like organisms

Genotype	Infected cell (canine)	Principal host	Vector
Genotype I			
Ehrlichia canis	Mononuclear cell	Dog	*Rhipicephalus sanguineus*
E. chaffeensis	Mononuclear cell	Human	*Amblyomma americanum, Dermacentor variabilis (?)*
E. ewingii	Granulocytic cell	Dog	*A. americanum, D. variabilis, R. sanguineus*
Genotype II			
A. phagocytophilum	Granulocytic cell	Horses, humans, ruminants, etc.	*Ixodes* spp.
A. platys	Platelet	Dog	*R. sanguineus*

In a clinical study performed in Minnesota and Wisconsin, seventeen dogs presented with fever and lethargy. The most frequent laboratory findings were lymphopenia, thrombocytopenia, elevated activities of serum alkaline phosphatase and amylase, and hypoalbuminemia (Greig et al., 1996). All dogs were positive to *A. phagocytophilum* antibodies using indirect immunofluorescence testing. Sequence

analysis revealed the agent to be identical to the agent of Human Granulocytic Ehrlichiosis (Greig et al., 1996). The authors of this study believed that it was possible that dogs contributed to the enzootic cycle of *Anaplasma phagocytophilum* and human infection (Greig et al., 1996). Wild canids have also been indicted in maintenance of the parasite. Serologic and molecular evidence has determined that coyotes (*Canis latrans*) in California (Pusterla et al., 2000) and jackals (*Canis aureus syriacus*) in Israel (Waner et al., 1999) can also been infected with *A. phagocytophilum*. However, it is unknown whether clinical disease observed in dogs is also produced in wild canids.

It has been suggested that dogs can be used as sentinels for human infection with *B. burgdorferi* (Duncan et al., 2004). Dogs present with the same clinical symptoms as humans, often the same number of human and dog infections are seen in a general location each year (Duncan et al., 2004). Prevalence studies performed in Alabama demonstrated that *Borrelia* in dogs is quite low. This was thought to be due to the different feeding habits of the vector in this location (Wright et al., 1997). Cases of borreliosis in dogs predominate in the northeast and mid-Atlantic states.

Disease in horses and ruminants:

Clinical signs that have been observed in dogs are also seen in horses, sheep, and other ruminants infected with *A. phagocytophilum*. In horses, the organisms cause a mild to moderate disease. The organism occurs predominantly in neutrophils of both naturally and experimentally infected horses (Lewis, 1976). At necropsy, infected horses displayed petechial and ecchymotic hemorrhages, edema, jaundice, and orchitis in mature males (Gribble, 1969). Mild inflammatory vascular lesions of the brain, heart, lungs, and kidneys and inflammation of small arteries and veins were also observed (Gribble, 1969).

In lambs experimentally infected with a less pathogenic strain of *A. phagocytophilum*, eight out of nine presented with hyperthermia and demonstrated intracytoplasmic inclusions in neutrophils (Garcia-Perez et al., 2000). Clinical signs noted were apathy, decreased food and water intake and decreased weight gain (Garcia-Perez et al., 2000). The intracytoplasmic inclusions were first observed four days post infection and remained in the neutrophils until day ten (Garcia-Perez et al., 2000). One lamb that did not present with clinical signs nor was *A. phagocytophilum* detected in leucocytes (Garcia-Perez et al., 2000).

In 1987, a cow presented with arthritis, myocarditis, glomerulonephritis, and pneumonitis and was determined to be infected with *B. burgdorferi* (Burgess et al., 1987). Spirochetes were isolated from the liver and lung by immunofluorescence staining and the carpal, stifle, and tarsal joints had marked synovial proliferation (Burgess et al., 1987). Infected horses often exhibit signs of single or recurring bouts of lameness (Cohen et al., 1988; Magnarelli et al., 1988). In epidemiological surveys performed in New Jersey in 1983-1985, it was demonstrated that 10% of the horses surveyed had demonstrable antibody to *B. burgdorferi* (Cohen et al., 1988). However, on one farm alone 60% of the mares and yearlings had antibodies to *B. burgdorferi*; the farm was located in a highly endemic area (Cohen et al., 1988). In addition to swelling of joints and arthritis, cows and horses also present with stiffness, laminitis, abortions, and fever and clinical signs are often seen one month after emergence of adult ticks (Burgess, 1988). Not all animals will exhibit clinical symptoms (Burgess, 1988).

Disease in cats:

Research involving *Anaplasma phagocytophilum* and cats is limited. The first report of experimental transmission was in 1975; cats were inoculated using infected equine and canine blood (Lewis et al., 1975). Seven to eight days after inoculation morulae were observed; 1 of 3 cats infected with equine blood and 1 of 2 cats infected with canine blood demonstrated cytoplasmic inclusions within eosinophils (Lewis et al., 1975). The first reported case of natural infection occurred in Sweden; a fourteen month old shorthaired cat presented with fever, dehydration, and tick infestation (Bjoersdorff et al., 1999). Laboratory findings included neutrophila, lymphopenia, and hyperglycemia (Bjoersdorff et al., 1999). Immunofluorescence and PCR, using the same primers that were used in the present study, determined that the cat was infected with *Anaplasma phagocytophilum* (Bjoersdorff et al., 1999). Since that first report, feline infections have been documented in Denmark and Ireland, with possible infections in Sweden, Brazil, and Italy (Lappin et al., 2004). In the latter study, morulae were found within neutrophils. Studies were not conducted to identify the causative *Ehrlichia* species (Lappin et al., 2004).

Within the United States, PCR and immunofluorescence have been used to demonstrate disease in five domestic cats in Massachusetts (n=4) and in Connecticut (n=1) (Lappin et al., 2004). As with the cat in Sweden, these cats presented with fever, anorexia, and lethargy. Symptoms resolved twenty-four to forty-eight hours after treatment with doxycycline or tetracycline (Lappin et al., 2004). Mountain lions (*Felis concolor*) are also susceptible to *Anaplasma phagocytophilum* (Foley et al., 1999). Of forty-seven mountain lions from California, 17% were positive for *A. phagocytophilum*

by immunofluorescence and 16% were positive by PCR. Eight were PCR positive and seronegative, one was PCR and IFA positive, and eight were PCR-negative and IFA positive (Foley et al., 1999). Experimental studies demonstrated that felines can remain seropositive for up to one year (Madigan, 1999).

Cats are also possible hosts to *B. burgdorferi*. In a prevalence study performed in 1990, *B. burgdorferi* antibodies were detected in felines ranging from 8.8% (n = 34 May through July) to 33.3% (n = 12 in February through April) depending upon the time of year blood was taken in Connecticut (Magnarelli et al., 1990). Nymphs molt to adults in late fall to early winter exposing cats to a large population of infected adult ticks. Over a two year period in 1988 and 1989, four of five feral cats tested on Cape Hatteras island, North Carolina, had positive titers for *B. burgdorferi*: the average in 1988 was 1:508 and in 1989 1:2,560 (Oliver et al., 1999).

Most cats infected with *B. burgdorferi* appear to be asymptomatic (Burgess, 1992). In one study, however, cats inoculated with *B. burgdorferi* presented with front and hind limb lameness and hyperemia in all joints (Gibson et al., 1993). At necropsy, the cats exhibited hepatic degeneration, hyperplasia of the spleen, plasmacytosis of regional lymph nodes, and occasionally pneumonitis (Gibson et al., 1993). Seroconversion is generally observed in felines three to four weeks after experimental infection (Gibson, 1995). Cyclic changes in blood cell populations, characterized by a decrease in percentage of neutrophils accompanied by an increase in percentage of lymphocytes and eosinophils, were observed eleven weeks after initial exposure to the agent and at 2-4 week intervals there after (Gibson, 1995).

Diagnosis of disease:

There are a variety of methods used to successfully diagnose *A. phagocytophilum* and *B. burgdorferi* infection. Most laboratories rely on commercially available tests that detect antibodies in serum. Enzyme immunoassays and immunofluorescence assays are the most commonly used. Although these tests are highly sensitive, they generally lack specificity (Tugwell et al., 1997). Cross-reactivity with similar *Ehrlichia* species is often observed in serologic tests used to detect infection with *A. phagocytophilum* (Horowitz and Wormser, 1997). Furthermore, there is little standardization among commercially available kits and there is often a poor correlation between results of reference and in-veterinary laboratories (Fritz and Kjemtrup, 2003; Reed, 2002). In addition, detection of antibodies is not definitive evidence of active or early infection as serologically detectable antibodies may persist for months or years (Fritz and Kjemtrup, 2003). One study found that the prevalences of serum antibody against *B. burgdorferi* did not differ between healthy dogs (89.6% seropositive) and those with joint or limb disorders compatible with Lyme borreliosis (92.9% seropositive) (Magnarelli et al., 1990). This method of diagnosis should be reserved for animals with a history and clinical presentation that is highly suggestive of active infection.

The most precise method of diagnosis is by polymerase chain reaction (PCR) because infections can be detected prior to seroconversion (Horowitz and Wormser, 1997). The 16S rRNA nested *A. phagocytophilum* PCR utilized in this study is capable of detecting an equivalent of 0.25 infected cells and does not cross react with other rickettsial agents, such as *Ehrlichia chaffeensis*, *Rickettsia rickettsii*, or *Bartonella henselae* (Massung and Slater, 2003). Most laboratories, however, will not use PCR as

primers are not standardized, reagents are expensive, and the technique is time consuming (Horowitz, 1997). Direct cultivation for *A. phagocytophilum* has been used as a means of diagnosis but is also expensive and it takes time as cultures generally become positive between three and nine days of co-cultivation (Horowitz, 1997). Due to the fastidious growth requirements of *B. burgdorferi* and difficulties of maintenance, direct cultivation of this organism from blood or other tissues is not generally performed in culture (Fritz and Kjemtrup, 2003).

II. LITERATURE CITED

1. Aguero-Rosenfeld, M.E., Horowitz, H.W., Wormser, G.P., McKenna, D.F., Nowakowski, J., Munoz, J., Dumler, J.S., 1996. Human granulocytic ehrlichiosis: a case series from a medical center in New York State. Ann Intern Med 125, 904-908.
2. Anderson, J.F., Johnson, R.C., Magnarelli, L.A., Hyde, F.W., 1985. Identification of endemic foci of Lyme disease: isolation of *Borrelia burgdorferi* from feral rodents and ticks (*Dermacentor variabilis*). J Clin Microbiol 22, 36-38.
3. Arens, M.Q., Liddell, A.M., Buening, G., Gaudreault-Keener, M., Sumner, J.W., Comer, J.A., Buller, R.S., Storch, G.A., 2003. Detection of *Ehrlichia* spp. in the blood of wild white-tailed deer in Missouri by PCR assay and serologic analysis. J Clin Microbiol 41, 1263-1265.
4. Bakken, J.S., Krueth, J., Wilson-Nordskog, C., Tilden, R.L., Asanovich, K., Dumler, J.S., 1996. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA 275, 199-205.
5. Barlough, J.E., Madigan, J.E., DeRock, E., Dumler, J.S., Bakken, J.S., 1995. Protection against *Ehrlichia equi* is conferred by prior infection with the human granulocytotropic *Ehrlichia* (HGE agent). J Clin Microbiol 33, 3333-3334.

6. Belongia, E.A., Reed, K.D., Mitchell, P.D., Kolbert, C.P., Persing, D.H., Gill, J.S., Kazmierczak, J.J., 1997. Prevalence of granulocytic *Ehrlichia* infection among white-tailed deer in Wisconsin. J Clin Microbiol 35, 1465-1468.
7. Bey, R.F., Loken, K.I., Wu, C.C., Lin, T.L., 1995. Experimental infection of the red-backed vole (*Clethrionomys gapperi*) with *Borrelia burgdorferi*. J Wildl Dis 31, 428-431.
8. Bjoersdorff, A., Svendenius, L., Owens, J.H., Massung, R.F., 1999. Feline granulocytic ehrlichiosis--a report of a new clinical entity and characterisation of the infectious agent. J Small Anim Pract 40, 20-24.
9. Breitner-Ruddock, S., Wurzner, R., Schulze, J., Brade, V., 1997. Heterogeneity in the complement-dependent bacteriolysis within the species of *Borrelia burgdorferi*. Med Microbiol Immunol (Berl) 185, 253-260.
10. Burgess, E.C., 1988. *Borrelia burgdorferi* infection in Wisconsin horses and cows. Ann N Y Acad Sci 539, 235-243.
11. Burgess, E.C., 1992. Experimentally induced infection of cats with *Borrelia burgdorferi*. Am J Vet Res 53, 1507-1511.
12. Burgess, E.C., Gendron-Fitzpatrick, A., Wright, W.O., 1987. Arthritis and systemic disease caused by *Borrelia burgdorferi* infection in a cow. J Am Vet Med Assoc 191, 1468-1470.
13. Chang, C.C., Chomel, B.B., Kasten, R.W., Romano, V., Tietze, N., 2001. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. J Clin Microbiol 39, 1221-1226.

14. Chen, S.M., Dumler, J.S., Bakken, J.S., Walker, D.H., 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J Clin Microbiol 32, 589-595.
15. Cohen, D., Bosler, E.M., Bernard, W., Meirs, D., 2nd, Eisner, R., Schulze, T.L., 1988. Epidemiologic studies of Lyme disease in horses and their public health significance. Ann N Y Acad Sci 539, 244-257.
16. Corstvet, R.E., Gaunt, S.D., Karns, P.A., McBride, J.W., Battistini, R.A., Mauterer, L.A., Austin, F.W., 1993. Detection of humoral antigen and antibody by enzyme-linked immunosorbent assay in horses with experimentally induced *Ehrlichia equi* infection. J Vet Diagn Invest 5, 37-39.
17. Courtney, J.W., Dryden, R.L., Montgomery, J., Schneider, B.S., Smith, G., Massung, R.F., 2003. Molecular characterization of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes scapularis* ticks from Pennsylvania. J Clin Microbiol 41, 1569-1573.
18. Daniels, T.J., Battaly, G.R., Liveris, D., Falco, R.C., Schwartz, I., 2002. Avian reservoirs of the agent of human granulocytic ehrlichiosis? Emerg Infect Dis 8, 1524-1525.
19. Dennis, D.T., Hayes, E. B., 2002. Epidemilogy of Lyme Borreliosis. CABI Publishing, Oxford, United Kingdom, pp. 251-280.
20. Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with

Neorickettsia, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol 51, 2145-2165.

21. Duncan, A.W., Correa, M.T., Levine, J.F., Breitschwerdt, E.B., 2004. The dog as a sentinel for human infection: prevalence of *Borrelia burgdorferi* C6 antibodies in dogs from southeastern and mid-Atlantic states. Vector Borne Zoonotic Dis 4, 221-229.
22. Eskow, E., Rao, R.V., Mordechai, E., 2001. Concurrent infection of the central nervous system by *Borrelia burgdorferi* and *Bartonella henselae*: evidence for a novel tick-borne disease complex. Arch Neurol 58, 1357-1363.
23. Fish, D., Daniels, T.J., 1990. The role of medium-sized mammals as reservoirs of *Borrelia burgdorferi* in southern New York. J Wildl Dis 26, 339-345.
24. Foggie, A., 1951. Studies on the infectious agent of tick-borne fever in sheep. J Pathol Bacteriol 63, 1-15.
25. Foley, J.E., Foley, P., Jecker, M., Swift, P.K., Madigan, J.E., 1999. Granulocytic ehrlichiosis and tick infestation in mountain lions in California. J Wildl Dis 35, 703-709.
26. Fritz, C.L., Kjemtrup, A.M., 2003. Lyme borreliosis. J Am Vet Med Assoc 223, 1261-1270.
27. Garcia-Perez, A.L., Mandaluniz, N., Barral, M., Juste, R.A., 2000. Microscopic and PCR findings in sheep after experimental infection with *Ehrlichia phagocytophila*. Small Rumin. Res. 37, 19-25.

28. Gibson, M., Young, CR, Omran, MT, Palma, K, Edwards JF, Rawlings, JA, Lewis, DH., 1995. Lyme disease in an experimental cat model. International Journal of Angiology 4, 155-159.

29. Gibson, M.D., Young, C.R., Omran, M.T., Edwards, J., Palma, K., Russell, L., Rawlings, J., 1993. *Borrelia burgdorferi* infection of cats. J Am Vet Med Assoc 202, 1786.

30. Gill, J.S., McLean, R.G., Shriner, R.B., Johnson, R.C., 1994. Serologic surveillance for the Lyme disease spirochete, *Borrelia burgdorferi*, in Minnesota by using white-tailed deer as sentinel animals. J Clin Microbiol 32, 444-451.

31. Greig, B., Asanovich, K.M., Armstrong, P.J., Dumler, J.S., 1996. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. J Clin Microbiol 34, 44-48.

32. Gribble, D.H., 1969. Equine ehrlichiosis. J Am Vet Med Assoc 155, 462-469.

33. Gylfe, A., Bergstrom, S., Lundstrom, J., Olsen, B., 2000. Reactivation of *Borrelia* infection in birds. Nature 403, 724-725.

34. Hefty, P.S., Jolliff, S.E., Caimano, M.J., Wikel, S.K., Akins, D.R., 2002. Changes in temporal and spatial patterns of outer surface lipoprotein expression generate population heterogeneity and antigenic diversity in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect Immun 70, 3468-3478.

35. Horowitz, H., Wormser, GP, 1997. Human Granulocytic Ehrlichiosis. Clinical Immunology Newsletters 17, 141-146.

36. Jacobs, M.B., Purcell, J.E., Philipp, M.T., 2003. *Ixodes scapularis* ticks (Acari: Ixodidae) from Louisiana are competent to transmit *Borrelia burgdorferi*, the agent of Lyme borreliosis. J Med Entomol 40, 964-967.
37. Keirans, J.E., Hutcheson, H.J., Durden, L.A., Klompen, J.S., 1996. *Ixodes scapularis* (Acari:Ixodidae): redescription of all active stages, distribution, hosts, geographical variation, and medical and veterinary importance. J Med Entomol 33, 297-318.
38. Lappin, M.R., Breitschwerdt, E.B., Jensen, W.A., Dunnigan, B., Rha, J.Y., Williams, C.R., Brewer, M., Fall, M., 2004. Molecular and serologic evidence of *Anaplasma phagocytophilum* infection in cats in North America. J Am Vet Med Assoc 225, 893-896, 879.
39. Larsen, H.J., Overnes, G., Waldeland, H., Johansen, G.M., 1994. Immunosuppression in sheep experimentally infected with *Ehrlichia phagocytophil*a. Res Vet Sci 56, 216-224.
40. Levin, M.L., Nicholson, W.L., Massung, R.F., Sumner, J.W., Fish, D., 2002. Comparison of the reservoir competence of medium-sized mammals and *Peromyscus leucopus* for *Anaplasma phagocytophilum* in Connecticut. Vector Borne Zoonotic Dis 2, 125-136.
41. Lewis, G., 1976. Equine ehrlichiosis: a comparison between *E. equi* and other pathogenic species of *Ehrlichia*. Veterinary Parasitology 2, 61-74.
42. Lewis, G.E., Jr., Huxsoll, D.L., Ristic, M., Johnson, A.J., 1975. Experimentally induced infection of dogs, cats, and nonhuman primates with *Ehrlichia equi*, etiologic agent of equine ehrlichiosis. Am J Vet Res 36, 85-88.

43. Liang, F.T., Nelson, F.K., Fikrig, E., 2002. Molecular adaptation of *Borrelia burgdorferi* in the murine host. J Exp Med 196, 275-280.

44. Little, S.E., Stallknecht, D.E., Lockhart, J.M., Dawson, J.E., Davidson, W.R., 1998. Natural coinfection of a white-tailed deer (*Odocoileus virginianus*) population with three *Ehrlichia* spp. J Parasitol 84, 897-901.

45. Liveris, D., Wang, G., Girao, G., Byrne, D.W., Nowakowski, J., McKenna, D., Nadelman, R., Wormser, G.P., Schwartz, I., 2002. Quantitative detection of *Borrelia burgdorferi* in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. J Clin Microbiol 40, 1249-1253.

46. Madewell, B.R., Gribble, D.H., 1982. Infection in two dogs with an agent resembling *Ehrlichia equi*. J Am Vet Med Assoc 180, 512-514.

47. Madigan, J.E., Foley, JE., 1999. Unpublished data. University of California, Davis.

48. Magnarelli, L.A., Anderson, J.F., Levine, H.R., Levy, S.A., 1990. Tick parasitism and antibodies to *Borrelia burgdorferi* in cats. J Am Vet Med Assoc 197, 63-66.

49. Magnarelli, L.A., Anderson, J.F., Schreier, A.B., 1990. Persistence of antibodies to *Borrelia burgdorferi* in dogs of New York and Connecticut. J Am Vet Med Assoc 196, 1064-1068.

50. Magnarelli, L.A., Anderson, J.F., Shaw, E., Post, J.E., Palka, F.C., 1988. Borreliosis in equids in northeastern United States. Am J Vet Res 49, 359-362.

51. Magnarelli, L.A., Dumler, J.S., Anderson, J.F., Johnson, R.C., Fikrig, E., 1995. Coexistence of antibodies to tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in human sera. J Clin Microbiol 33, 3054-3057.

52. Massung, R.F., Mauel, M.J., Owens, J.H., Allan, N., Courtney, J.W., Stafford, K.C., 3rd, Mather, T.N., 2002. Genetic variants of *Ehrlichia phagocytophila*, Rhode Island and Connecticut. Emerg Infect Dis 8, 467-472.

53. Massung, R.F., Slater, K.G., 2003. Comparison of PCR assays for detection of the agent of human granulocytic ehrlichiosis, *Anaplasma phagocytophilum*. J Clin Microbiol 41, 717-722.

54. Mather, T.N., Wilson, M.L., Moore, S.I., Ribeiro, J.M., Spielman, A., 1989. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete (*Borrelia burgdorferi*). Am J Epidemiol 130, 143-150.

55. Mitchell, P.D., Reed, K.D., Hofkes, J.M., 1996. Immunoserologic evidence of coinfection with *Borrelia burgdorferi*, *Babesia microti*, and human granulocytic Ehrlichia species in residents of Wisconsin and Minnesota. J Clin Microbiol 34, 724-727.

56. Mullegger, R.R., McHugh, G., Ruthazer, R., Binder, B., Kerl, H., Steere, A.C., 2000. Differential expression of cytokine mRNA in skin specimens from patients with erythema migrans or acrodermatitis chronica atrophicans. J Invest Dermatol 115, 1115-1123.

57. Nicholson, W.L., Muir, S., Sumner, J.W., Childs, J.E., 1998. Serologic evidence of infection with *Ehrlichia* spp. in wild rodents (Muridae: Sigmodontinae) in the United States. J Clin Microbiol 36, 695-700.

58. Nyindo, M.B., Ristic, M., Lewis, G.E., Jr., Huxsoll, D.L., Stephenson, E.H., 1978. Immune response of ponies to experimental infection with *Ehrlichia equi*. Am J Vet Res 39, 15-18.

59. Oliver, J.H., Jr., 1996. Lyme borreliosis in the southern United States: a review. J Parasitol 82, 926-935.

60. Oliver, J.H., Jr., Magnarelli, L.A., Hutcheson, H.J., Anderson, J.F., 1999. Ticks and antibodies to *Borrelia burgdorferi* from mammals at Cape Hatteras, NC and Assateague Island, MD and VA. J Med Entomol 36, 578-587.

61. Peavy, C.A., Lane, R.S., Kleinjan, J.E., 1997. Role of small mammals in the ecology of *Borrelia burgdorferi* in a peri-urban park in north coastal California. Exp Appl Acarol 21, 569-584.

62. Petrovec, M., Lotric Furlan, S., Zupanc, T.A., Strle, F., Brouqui, P., Roux, V., Dumler, J.S., 1997. Human disease in Europe caused by a granulocytic Ehrlichia species. J Clin Microbiol 35, 1556-1559.

63. Piesman, J., 2002. Ecology of *Borrelia burgdorferi* sensu lato in North America. CABI publishing, Wallingford, Oxon, United Kingdom, 223-249 pp.

64. Pusterla, N., Chang, C.C., Chomel, B.B., Chae, J.S., Foley, J.E., DeRock, E., Kramer, V.L., Lutz, H., Madigan, J.E., 2000. Serologic and molecular evidence of *Ehrlichia* spp. in coyotes in California. J Wildl Dis 36, 494-499.

65. Reed, K.D., 2002. Laboratory testing for Lyme disease: possibilities and practicalities. J Clin Microbiol 40, 319-324.

66. Stamp, J.T., Watt, J.A., Jamieson, S., 1950. Tick-borne fever as a cause of abortion in sheep. Vet Rec 62, 465-470.

67. Steere, A.C., Bartenhagen, N.H., Craft, J.E., Hutchinson, G.J., Newman, J.H., Rahn, D.W., Sigal, L.H., Spieler, P.N., Stenn, K.S., Malawista, S.E., 1983. The early clinical manifestations of Lyme disease. Ann Intern Med 99, 76-82.

68. Steere, A.C., Coburn, J., Glickstein, L., 2004. The emergence of Lyme disease. J Clin Invest 113, 1093-1101.

69. Steere, A.C., Schoen, R.T., Taylor, E., 1987. The clinical evolution of Lyme arthritis. Ann Intern Med 107, 725-731.

70. Telford, S.R., 3rd, Dawson, J.E., Katavolos, P., Warner, C.K., Kolbert, C.P., Persing, D.H., 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc Natl Acad Sci U S A 93, 6209-6214.

71. Tugwell, P., Dennis, D.T., Weinstein, A., Wells, G., Shea, B., Nichol, G., Hayward, R., Lightfoot, R., Baker, P., Steere, A.C., 1997. Laboratory evaluation in the diagnosis of Lyme disease. Ann Intern Med 127, 1109-1123.

72. Walls, J.J., Asanovich, K.M., Bakken, J.S., Dumler, J.S., 1998. Serologic evidence of a natural infection of white-tailed deer with the agent of human granulocytic ehrlichiosis in Wisconsin and Maryland. Clin Diagn Lab Immunol 5, 762-765.

73. Waner, T., Baneth, G., Strenger, C., Keysary, A., King, R., Harrus, S., 1999. Antibodies reactive with *Ehrlichia canis*, *Ehrlichia phagocytophila* genogroup antigens and the spotted fever group rickettsial antigens, in free-ranging jackals (Canis aureus syriacus) from Israel. Vet Parasitol 82, 121-128.

74. Wright, J.C., Chambers, M., Mullen, G.R., Swango, L.J., D'Andrea, G.H., Boyce, A.J., 1997. Seroprevalence of *Borrelia burgdorferi* in dogs in Alabama, USA. Prev Vet Med 31, 127-131.

III. SEROPREVALENCE AND ATTEMPTED TRANSMISSION OF *ANAPLASMA PHAGOCYTOPHILUM* AND/OR *BORRELIA BURGDORFERI* FROM NATURALLY INFECTED TICKS TO FELINES

Abstract**:** *A. phagocytophilum* and *B. burgdorferi* are among the leading tick-borne disease agents in the United States. Both agents are of veterinary and public health significance as dogs, cats, and human beings are known to be susceptible. *B. burgdorferi* and *A. phagocytophilum* are transmitted trans-stadially 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 prevalences of these agents 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). Samples that were positive by IFA were subjected to polymerase chain reaction (PCR) analysis to measure presence of bacterial DNA. In an attempt to determine the minimum transmission times of *A. phagocytophilum* and *B burgdorferi*, wild caught adult *I. scapularis* ticks were applied to cats and removed at 8, 16, 24, and 36 hours after attachment. Blood was collected on days 42, 56, 72, 84 and 98 after tick removal and examined using both IFA and PCR. Biopsies were collected from sites of tick attachment and examined for *A. phagocytophilum* and *B. burgdorferi*-specific DNA by PCR procedures.

Two samples from Providence, Rhode Island were positive using an indirect immunofluorescence assay at a titer of 1:200; however, whole blood samples were not provided and positivity could not be corroborated by PCR. Eighteen other samples (12/90 from Florida, 3/45 from Michigan, and 3/23 from California) appeared positive at a titer of 1:50, however bacterial DNA was not detected using PCR procedures. The remaining samples were seronegative as determined by IFA. All ticks were negative by PCR for *A. phagocytophilum*, however, *B. burgdorferi* was detected in two of eight ticks attached to the 8 hour cat and five of seven attached to the 16 hour cat. Fourteen of 31 pooled unattached ticks were positive for *B. burgdorferi* and one was positive for *B. miyamotoi*. Cats remained antibody negative for both *A. phagocytophilum* and *B. burgdorferi* throughout the transmission study. Biopsy sites from all cats were negative for both *A. phagocytophilum* and *B. burgdorferi*. These results demonstrate that natural infection of *A. phagocytophilum* in cats is uncommon. Methodologies used in the study to determine transmission times could be of value in determining times necessary for vector-mediated transmission of agents. However, several features of the model could be improved. These potential modifications are discussed below.

1. Introduction

Ixodes scapularis, the deer/black-legged tick, and *I. pacificus*, the western black-legged tick, may transmit multiple pathogens, including *A. phagocytophilum, B. burgdorferi*, *Babesia microti*, and possibly *Bartonella henselae* (Chang et al., 2001; Eskow et al., 2001; Magnarelli et al., 1995; Mitchell et al., 1996). *Ixodes scapularis* is distributed throughout the east and Midwest; *I. pacificus* is found principally in western coastal states. Ticks in the northeast feed primarily on mammals, while *Ixodes* in the

southeast feed on mammals, birds, and lizards (Keirans et al., 1996). Larval *Ixodes* ticks prefer to feed on the white-footed mouse, *Peromyscus leucopus*, or other small rodents (Mather et al., 1989; Telford et al., 1996). 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, humans and others.

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 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).

B. burgdorferi sensu lato contains three clinically relevant genospecies: *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* (Liveris et al., 2002). Of the three agents, *B. burgdorferi* sensu stricto is only found within the United States. *Borrelia* is a corkscrew motile bacterium, or spirochete, that causes a slowly expanding rash around the tick bite (Steere et al., 2004).

The same general clinical symptoms exhibited with patients infected with *A. phagocytophilum* are also seen in *B. burgdorferi* patients—fatigue, headache, arthralgias, myalgias, and fever (Aguero-Rosenfeld et al., 1996; Bakken et al., 1996; Petrovec et al., 1997; Steere et al., 1983). Spirochetes will disseminate throughout the body and can cause arthritis of the large joints, as well as secondary annular skin lesions, acute lymphocytic meningitis, cranial neuropathy, radiculoneuritis, atrioventricular nodal block, and migratory musculosketal pain in joints, bursaes, tendons, muscles, or bones

(Steere et al., 2004). Secondary infections may occur, however, these agents are easily treated with doxycycline (Foggie, 1951). These infections may be particularly severe in immunocompromised individuals. Identifying of animals that may serve as reservoir hosts is of key importance in the treatment and control of these diseases.

Research reports involving felines with vector-borne diseases is limited. A serologic prevalence test detecting antibodies to *B. burgdorferi* in felines was performed in Connecticut and results ranged from 8.8% (n = 34 May through July) to 33.3% (n = 12 in February through April) (Magnarelli et al., 1990). Although most felines remain asymptomatic, those that are clinically ill exhibit fever, lethargy, lameness, and decreased appetite (Magnarelli et al., 1990; Stubbs et al., 2000). The purpose of this study was to examine the seroprevalence of *A. phagocytophilum* in cats from different regions of the United States and to attempt to determine the minimum time necessary for transmission of *A. phagocytophilum* and *B. burgdorferi* from naturally infected ticks to cats.

2. Materials and Methods

2.1 Seroprevalence of A. phagocytophilum in felines

2.1.1 Source of serum

Feline serum samples were obtained from collaborators in Auburn, Alabama (n = 175), Gainesville, Florida (n = 90), San Diego, California (n = 23), Verona and Oregon, Wisconsin (n = 96), East Lansing, Michigan (n= 45), and Providence, Rhode Island (n = 31). Collaborators were required to collect serum and 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 the tick

vector. The sex, indoor/outdoor status of each cat, and the location of the cat within the geographical area were included with each sample.

Plasma/serum was separated from whole blood prior to shipment. Samples 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 were performed.

2.1.2 Indirect immunofluorescence assay for seroprevalence survey

Indirect immunofluorescence assays (IFA) were performed on the feline samples using *A. phagocytophilum* IFA substrate slides obtained from a commercial source (VMRD, Inc., Pullman, Washington) A goat anti-cat IgG FITC conjugate was used as the secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland). The recommended staining procedure from VMRD, Inc. was followed except that the conjugate was diluted at 1:50 (20 microliters of conjugate, 63 microliters of Evans blue, and 917 microliters of PBS). Sera were screened at a dilution of 1:50 for *A. phagocytophilum*. Mounting fluid was added (glycerol/FA rinse buffer) and slides were viewed with a fluorescence microscope at 400X. Samples were evaluated concurrently with positive and negative controls.

The positive status of 18 samples was questionable at 1:50, therefore, the cut off for positivity using the indirect immunofluorescence assay was determined to be at 1:100.

2.1.3 DNA extraction of feline whole blood

DNA was extracted from feline whole blood as previously described (Sambrook et al., 1989). Only samples that were positive at the 1:50 dilution using the indirect immunofluorescence assay were subjected to PCR analysis.

2.1.4 Polymerase chain reaction for detection of A. phagocytophilum in whole blood

Polymerase chain reaction (PCR) was performed on feline whole blood samples to determine the presence of *A. phagocytophilum* specific DNA. A total volume of 50 µL was used to carry out DNA amplification: one time PCR buffer, 2.0 mM $MgCl_2$, 0.2 nM of each primer, 0.25 mM of each deoxynucleotide triphosphate, and 1 unit of *Taq* polymerase. To amplify the 16S rRNA gene of *A. phagocytophilum*, a nested PCR was performed using the methods of Massung et al. (1998). 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 of DNA from each sample was used in the PCR. All reactions were performed in either an Eppendorf thermocycler or a Strategene RoboCycler Gradient 96 machine and final products were analyzed by agarose gel electrophoresis.

2.2 Attempted determination of transmission times of A. phagocytophilum and B. burgdorferi from ticks to cats

2.2.1 Source of ticks for transmission study

Adult male and female *I. scapularis* were collected by dragging naturally infested habitats in Bridgeport, Connecticut (Fairfield County). Ticks were placed in plastic vials (ten males and ten females) and secured with air-permeable fabric covers. The ticks were sent to Auburn, AL overnight and then placed in a secure humidified glass enclosure for a week before being placed on cats for the transmission study.

2.2.2 Source of cats for transmission study

Four neutered male class B cats, approximately three to four years in age, were used in this study. Cats were obtained from Auburn University's Department of Laboratory Animal Health and were housed as groups in a Biosafety Level 2 facility. The study was reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (AUIACUC PRN #2004-0764). All cats were antibody tested for *A. phagocytophilum* and *B. burgdorferi* prior to selection.

2.2.3 Experimental transmission

To determine the time required for naturally infected ticks to transmit *A. phagocytophilum* and *B. burgdorferi* to cats, 160 ticks were placed in plastic enclosures (N = 20 within 8 enclosures) and applied to shaved areas on the lateral flanks of each of four cats (two enclosures per cat). Plastic enclosures, measuring 2'' by 3'' and opened at one end to allow for attachment, were bound to cats with Elastikon tape. The enclosures containing ticks were removed from each cat at either 8, 16, 24, or 36 hours after attachment. Due to the difficulty of assessing an exact time of tick attachment, the times were based upon the number of hours cassettes remained on the cats. Sites of tick attachment were identified and marked with a permanent marker. Blood specimens were collected from all cats at 42, 56, 72, 84, and 98 days after removal of ticks. Serum was separated from whole blood and assayed for *A. phagocytophilum* and *B. burgdorferi*-specific antibodies using the immunofluorescence assay described previously.

Dermal biopsy sites were taken adjacent to tick attachment sites on study day 98. Cats were anesthetized using Domitor (1.0 mg/square meter of body surface administered intramuscularly) (Pfizer, New York, NY). Closure of biopsy sites was accomplished

using two to three non-absorbable sutures and the anesthesia was reversed using Antisedan (dosed at the same volume as Domitor) (Pfizer, New York, NY).

2.2.4 DNA extraction of ticks from transmission study

DNA was extracted from ticks using a method similar to Whitlock et al. (2000). Ticks were ground using a sterile mortal and pestle or were macerated using a razor blade. Ground up ticks were placed in 1.5 mL tubes and incubated at 65ºC for thirty minutes with 100-200 µL of CTAB buffer (100 mM Tris HCl, pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2 % (wt:vol) CTAB, and 0.2% (vol:vol) 2-mercaptoethanol). After removal from the water bath, 200 µL of chloroform, 200 µL of STE, and 50 µL of phenol were added to each sample and vortexed. The tubes were centrifuged for five minutes at 13,000 rpm in a microfuge. The supernatant was removed and 100 µL of chloroform was added. After vortexing, the tubes were centrifuged as above. The supernatant was removed and a 3:1 volume of 95% ethanol was added to the tubes. After cooling at -80ºC for approximately 20 minutes, the tubes were centrifuged for 15 minutes at 13000 rpm and the supernatant was decanted. The resulting pelleted DNA was washed with cold 70% ethanol, allowed to air dry and resuspended in 50 µL of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Samples were incubated at 65ºC for 30 minutes to dissolve the DNA. Five microliters was examined by electrophoresis through a 1% agarose gel to evaluate the quality of the high molecular weight DNA.

Tissues from site biopsies were cut into small pieces using a razor blade and DNA was extracted using methods of Sambrook *et al*. (1989).

2.2.5 IFA for transmission study

IFAs were conducted on plasma collected from each time interval described above. Assays were performed as described except *B. burgdorferi* samples were diluted 1:64 following the VMRD recommended protocol.

2.2.6 Polymerase chain reaction of A. phagocytophilum and B. burgdorferi for transmission study

PCR was performed on *I. scapularis* ticks and site biopsies for *A. phagocytophilum* as described previously. For *B. burgdorferi*, a nested PCR was performed to amplify the 16S-23S ribosomal DNA spacer region. A modification of a PCR described by Liveris et al. (1999) was utilized: extension of 55ºC for 30 sec. Primers used in the primary amplification were Pa (5' GGTATGTTTAGTGAGGG) and P95 (5' GGTTAGAGCGCAGGTCTG). Nested primers were Pb (5' CGTACTGGAAAGTGCGGCTG) and P97 (5' GATGTTCAACTCATCCTGGTCCC).

Attached ticks were examined individually by PCR for *A. phagocytophilum* and/or *B. burgdorferi*. Ticks that did not attach during the allotted time period were removed and pooled into groups of five. The pooled ticks also were examined by PCR.

3. Results

3.1 Serologic prevalence of A. phagocytophilum in cats

Table 2 summarizes the location, number of samples provided, and the number of samples appearing positive at a titer of 1:50 using an indirect immunofluorescence assay.

Table 2. Results of seroprevalence survey in cats

Location	Number of samples provided	Number positive (% positive*)
Gainesville, Florida	90	12 (13.3)
Auburn, Alabama	175	0 (0)
San Diego, California	23	3 (13)
Providence, Rhode Island	31	2 (6.5)**
East Lansing, Michigan	45	3 (6.7)
Verona and Oregon, Wisconsin	96	0 (0)
Total:	460	20 (4.3)

*Samples were evaluated at a dilution of 1:50.
**These samples remained positive at reciprocal titers of up to 1:200

The samples collected from cats in Florida, Michigan, and California that appeared positive by IFA were negative for bacterial DNA by PCR. Two of 31 samples from Rhode Island were positive by IFA. These samples remained positive at reciprocal titers of up to 1:200, however, whole blood was not provided for PCR analysis.

3.2 Transmission study serology

All cats remained serologically negative for *A. phagocytophilum* and *B. burgdorferi* throughout the entire study.

3.3 PCR results for transmission study

None of the *Ixodes scapularis* ticks tested positive for *Anaplasma phagocytophilum* by PCR. However, 14 of 31 pooled ticks were positive for *Borrelia burgdorferi.* Of the eight ticks (1 male, 7 females) attached to the 8 hour transmission study cat, two females tested positive by PCR for *B. burgdorferi* and five of the seven females attached to the 16 hour transmission study cat also tested positive by PCR. Neither the 24 nor 36 hour transmission study cats had PCR-positive ticks attached. Sixteen ticks attached to the 24 hour transmission study cat (1 male, 15 females) and 17 female ticks attached to the 36 hour transmission study cat.

During PCR analysis of the pooled ticks, a 500 bp amplicon from one pool was obtained that did not correspond to the 940-bp amplicon of *B. burgdorferi* sensu stricto. The sample was subsequently cloned using a TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA) and the resulting clone was submitted for sequence analysis. The sequence was most closely related to *Borrelia miyamotoi*, an agent of relapsing fever. Although this agent is observed most often in Japan, in a survey of ticks from Connecticut, Rhode Island, New York, and New Jersey, 1.9-2.5% of 712 nymphs were positive for this agent (Scoles et al., 2001).

The sixteen hour transmission study cat was removed from the study on day 73 due to declining health unrelated to the study. A biopsy was taken at this time and was negative for *B. burgdorferi* and *A. phagocytophilum*. None of the biopsies of tick attachment sites removed at day 98 from the remaining cats were positive for either *A. phagocytophilum* or *B. burgdorferi*.

4. Discussion

Results of the seroprevalence survey suggest that cats are not commonly infected with *A. phagocytophilum* and are unlikely to serve as maintenance hosts for this parasite. Approximately 0.43% of samples tested by immunofluorescence assay were positive. Of four hundred and sixty serum samples, two from Providence, Rhode Island appeared positive at a dilution of 1:200. There were 18 questionable samples at 1:50 that tested negative by PCR.

Due to lower survey response rates, fewer samples were received from the northeastern United States, a region where *A. phagocytophilum* is naturally more common (Horowitz and Wormser, 1997). 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. Consequently, results of the serosurvey in cats must be interpreted with some caution at this point.

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 is possible that the samples were positive by serology and negative by PCR due to low levels of parasitemia. It is also possible that the eighteen samples positive on IFA, in this study, at a titer of 1:50 were cross-reactive with similar species or that back ground fluorescence was present.

Though ticks from the Connecticut area were positive for *A. phagocytophilum* the previous year (Blagburn et al., 2004), there were no positive ticks identified in the present transmission study based on similar PCR procedures. Annual variations in infected tick populations are seen within the same counties or states (Courtney et al., 2003; Massung et al., 2002). This may in part be due to seasonal climatic changes that affect the ability of nymphs and adults to attach to infected hosts, or simply that reservoir hosts are not infected at as high a rate as previous years.

The absence of *A. phagocytophilum* in ticks during the transmission study based on our PCR results is consistent with the results seen in the seroprevalence survey. Low levels of organisms in the tick population, the decreased likelihood that cats will visit tick endemic areas, the likelihood for ticks to attach to other hosts, and the ability of cats to groom ticks off are all variables that might explain the low prevalence of *A. phagocytophilum* in our study.

Our research suggests that transmission of *B. burgdorferi* did not occur at the eight or 16 hour intervals because cats remained antibody negative throughout the study and were negative on biopsy. Previous research has shown that *B. burgdorferi* spirochetes are not transmitted efficiently to the host until 24 to 48 hours after tick attachment (des Vignes et al., 2001; Piesman et al., 1987; Piesman et al., 2002;). In a study performed by des Vignes et al. (2001), *Borrelia* infected nymphs were fed on mice and transmission did not occur within the first 24 hours. However, transmission occurred more frequently between 48 and 72 hours after attachment. Given that positive *Borrelia* ticks were not attached to the 24 or 36 hour transmission study cats in the present study, we cannot compare results obtained in our feline model with those in previous studies.

These results are corroborated in part by results obtained in a serosurvey of 200 cats conducted at Cornell University. Cats surveyed were from Lyme borreliosis endemic areas; many were infested with ticks at the time that samples were obtained. Of the 200 samples examined using an indirect immunofluorescence assay, two were positive at titers of 1:2,048 and 1:1,024. The cat with the higher titer presented with signs of Borreliosis and was successfully treated (Margaret B. Pough, Cornell University, 2005, personal communication). Results of the Cornell study and those of the present study suggest that transmission of *Borrelia* from infected ticks to cats does not occur as commonly as is observed in dogs. We cannot speculate at this point whether the low transmission rates in cats are due to unique features of host susceptibility, vector biology, or both.

Changes in research methods in future studies could enhance the likelihood of successful transmission. Such changes could include induced infections in laboratory animals on which ticks could feed. This would likely result in higher rates of infection in ticks and a greater likelihood of experimental transmission. It is not known whether restricting ticks to feeding enclosures such as those used in the present study may have inhibited attachment, feeding, and subsequent transmission of agents. Caging cats and allowing ticks to attach voluntarily may be an alternative method to using feeding enclosures. In the present study, cats were allowed to roam freely in an attempt to reduce stress placed on the cats. Also, entrapment of ticks allowed us to locate, enumerate, and determine attachment sites of ticks.

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References

Aguero-Rosenfeld, M.E., Horowitz, H.W., Wormser, G.P., McKenna, D.F., Nowakowski, J., Munoz, J., Dumler, J.S., 1996. Human granulocytic ehrlichiosis: a case series from a medical center in New York State. Ann Intern Med 125, 904-908.

Aguirre, E., Tesouro, M.A., Amusategui, I., Rodriguez-Franco, F., Sainz, A., 2004. Assessment of feline ehrlichiosis in central Spain using serology and a polymerase chain reaction technique. Ann N Y Acad Sci 1026, 103-105.

Bakken, J.S., Krueth, J., Wilson-Nordskog, C., Tilden, R.L., Asanovich, K., Dumler, J.S., 1996. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA 275, 199-205.

Blagburn, B.L., Spencer, J.A., Billeter, S.A., Drazenovich, N.L., Butler, J.M., Land, T.M., Dykstra, C.C., Stafford, K.C., Pough, M.B., Levy, S.A., Bledsoe, D.L., 2004. Use of imidacloprid-permethrin to prevent transmission of *Anaplasma phagocytophilum* from naturally infected *Ixodes scapularis* ticks to dogs. Vet Ther 5, 212-217.

Chang, C.C., Chomel, B.B., Kasten, R.W., Romano, V., Tietze, N., 2001. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. J Clin Microbiol 39, 1221-1226.

Courtney, J.W., Dryden, R.L., Montgomery, J., Schneider, B.S., Smith, G., Massung, R.F., 2003. Molecular characterization of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes scapularis* ticks from Pennsylvania. J Clin Microbiol 41, 1569-1573.

des Vignes F.P., Piesman J., Heffernan R., Schulze T.L., Stafford K.C., Durland F., 2001. Effect of tick removal on transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* nymphs. J Inf Dis. 183, 773-778.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol 51, 2145-2165.

Eskow, E., Rao, R.V., Mordechai, E., 2001. Concurrent infection of the central nervous system by *Borrelia burgdorferi* and *Bartonella henselae*: evidence for a novel tick-borne disease complex. Arch Neurol 58, 1357-1363.

Foggie, A., 1951. Studies on the infectious agent of tick-borne fever in sheep. J Pathol Bacteriol 63, 1-15.

Horowitz, H., Wormser, GP, 1997. Human Granulocytic Ehrlichiosis. Clinical Immunology Newsletters 17, 141-146.

Keirans, J.E., Hutcheson, H.J., Durden, L.A., Klompen, J.S., 1996. *Ixodes scapularis* (Acari:Ixodidae): redescription of all active stages, distribution, hosts, geographical variation, and medical and veterinary importance. J Med Entomol 33, 297-318.

Liveris, D., Varde, S., Iyer, R., Koenig, S., Bittker, S., Cooper, D. McKenna, D., Nowakowski, J., Nadelman, R.B., Wormser, G.P., Schwartz, I., 1999. Genetic diversity of *Borrelia burgdorferi* in Lyme disease patients as determined by culture versus direct PCR with clinical specimens. J Clin Micro 37, 565-569.

Magnarelli, L.A., Anderson, J.F., Levine, H.R., Levy, S.A., 1990. Tick parasitism and antibodies to *Borrelia burgdorferi* in cats. J Am Vet Med Assoc 197, 63-66.

Magnarelli, L.A., Dumler, J.S., Anderson, J.F., Johnson, R.C., Fikrig, E., 1995. Coexistence of antibodies to tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in human sera. J Clin Microbiol 33, 3054-3057.

Massung, R.F., Mauel, M.J., Owens, J.H., Allan, N., Courtney, J.W., Stafford, K.C., 3rd, Mather, T.N., 2002. Genetic variants of *Ehrlichia phagocytophila*, Rhode Island and Connecticut. Emerg Infect Dis 8, 467-472.

Massung, R.F., Slater, K., Owens, J.H., Nicholson, W.L., Mather, T.N., Solberg, V.B., Olson, J.G., 1998. Nested PCR assay for detection of granulocytic ehrlichiae. J Clin Microbiol 36, 1090-1095.

Mather, T.N., Wilson, M.L., Moore, S.I., Ribeiro, J.M., Spielman, A., 1989. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete (*Borrelia burgdorferi*). Am J Epidemiol 130, 143-150.

Mitchell, P.D., Reed, K.D., Hofkes, J.M., 1996. Immunoserologic evidence of coinfection with *Borrelia burgdorferi*, *Babesia microti*, and human granulocytic *Ehrlichia* species in residents of Wisconsin and Minnesota. J Clin Microbiol 34, 724-727.

Petrovec, M., Lotric Furlan, S., Zupanc, T.A., Strle, F., Brouqui, P., Roux, V., Dumler, J.S., 1997. Human disease in Europe caused by a granulocytic *Ehrlichia* species. J Clin Microbiol 35, 1556-1559.

Piesman J., Mather, T.N., Sinsky, R.J., Spielman, A., 1987. Duration of tick attachment and *Borrelia burgdorferi* transmission. J Clin Microbiol 25, 557-558.

Piesman J, Dolan M.C.., 2002. Protection against Lyme disease spirochete transmission provided by prompt removal of nymphal *Ixodes scapularis* (Acari: Ixodidae). J Med Entomol 39, 509-512.

Sambrook, J., Fritsch, EF, Maniatis, T, 1989. Molecular cloning. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.

Scoles GA, P.M., Beati L, Fish D., 2001. A Relapsing Fever Group Spirochete Transmitted by *Ixodes scapularis* ticks. Vector Borne Zoonotic Dis 3, 21-34.

Steere, A.C., Bartenhagen, N.H., Craft, J.E., Hutchinson, G.J., Newman, J.H., Rahn, D.W., Sigal, L.H., Spieler, P.N., Stenn, K.S., Malawista, S.E., 1983. The early clinical manifestations of Lyme disease. Ann Intern Med 99, 76-82.

Steere, A.C., Coburn, J., Glickstein, L., 2004. The emergence of Lyme disease. J Clin Invest 113, 1093-1101.

Stubbs, C.J., Reif, J.S., Bruns, C., Lappin, M.R., Holland, C.J., Wheller, S., 2000. Feline Ehrlichiosis. Compendium 22, 307-317.

Telford, S.R., 3rd, Dawson, J.E., Katavolos, P., Warner, C.K., Kolbert, C.P., Persing, D.H., 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc Natl Acad Sci U S A 93, 6209-6214.

Whitlock, J.E., Fang, Q.Q., Durden, L.A., Oliver, J.H., 2000. Prevalence of *Ehrlichia chaffeensis* (Rickettsiales: Rickettsiaceae) in *Amblyomma americanum* (Acari: Ixodidae) from the Georgia coast and barrier islands. J Med Entomol 37, 276-280.