WHITE-TAILED DEER AND RACCOONS AS HOSTS
FOR SELECTED ZOONOTIC TICK-BORNE RICKETTSIAE

by

VIVIEN GRACE DUGAN

(Under the Direction of William R. Davidson)

ABSTRACT

The central aim of this research was to develop a more complete epidemiologic understanding of Anaplasma phagocytophilum, Ehrlichia chaffeensis, Ehrlichia canis, and Ehrlichia ewingii in the southeastern United States. These bacterial cause clinical infection in humans, dogs and/or horses, are transmitted by three different genera of ticks, and involve wildlife in their epidemiology.

Raccoons (Procyon lotor) and opossums (Didelphis virginianus) from Georgia were examined for their role in the maintenance of these ehrlichial and anaplasmal species. Raccoons were positive for antibodies to E. chaffeensis, E. canis, and to a lesser extent, A. phagocytophilum and blood from one raccoon was PCR positive for E. canis. All raccoons were PCR negative for E. chaffeensis, E. ewingii, and A. phagocytophilum; however, a novel Ehrlichia-like gene fragment was amplified from over half of the raccoons. In contrast, opossums were negative by all assays. These findings suggest that raccoons, but not opossums, may be involved in the epidemiology of one or more of these pathogens.

White-tailed deer (WTD; Odocoileus virginianus) were evaluated for their suitability as natural sentinels for discerning the geographic distribution of A. phagocytophilum across 19
states. This goal was accomplished by testing 2,666 WTD from 507 populations for *A. phagocytophilum*-reactive antibodies, with confirmatory PCR testing and *in vivo* xenodiagnostic cultures. WTD met crucial standards as sentinels and evaluation of field data indicated that WTD antibodies reflect infection, small sample sizes were adequate for accurate surveillance, and *A. phagocytophilum* infection in WTD was correlated with the presence of *I. scapularis.*

Serologic WTD data were used in two different modeling analyses to predict the distribution of *A. phagocytophilum* across 19 states. Kriging and logistic regression model analyses disclosed moderately high (> 64%) accuracy, sensitivity, and specificity and the projected distribution conformed to human case data available for this region. Additionally, analyses determined significant ecologic variables associated with the presence of *A. phagocytophilum.* This study demonstrated that WTD would be an effective surveillance species and represent a feasible alternative to human surveillance in predicting the geographic distribution of locations where there is a risk of *A. phagocytophilum* infection.

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by

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DEDICATION

For KWS
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CHAPTER 1
INTRODUCTION

Overall, the central aim of this research project is to develop a more comprehensive understanding of the ecology, natural history, and epidemiology of ehrlichial and anaplasmal tick-borne organisms among selected species of southeastern wildlife. Tick-borne pathogens are responsible for causing some of the most important emerging diseases in the world today. Many of these zoonotic pathogens are rickettsial organisms from the genera *Ehrlichia* and *Anaplasma*, which are obligately intracellular bacteria of human and veterinary health importance. Members of the *Ehrlichia* and *Anaplasma* groups are found in membrane-lined vacuoles separated by a trilaminar cell wall within the cytoplasm of infected cells. Organisms enter the host cell by induced phagocytosis, replicate by binary fission forming clusters of organisms termed morulae, and are released to infect other cells when the host cell ruptures (Harkess 1991; McDade, 1990).

*Anaplasma phagocytophilum* (human granulocytotropic anaplasmosis; HGA), *E. chaffeensis* (human monocytotropic ehrlichiosis; HME), *E. canis* (canine monocytotropic ehrlichiosis; CME), and *E. ewingii* (human and canine ehrlichiosis; EWE, CE) are the most significant tick transmitted organisms in this group. These organisms cause clinical disease by invasion of either monocytes or granulocytes of humans, dogs, and/or horses. Infection with *Ehrlichia* and/or *Anaplasma* spp. typically presents as a non-specific, acute, febrile illness, with
clinical symptoms ranging from asymptomatic to severe and even fatal (Rikihisa et al., 1999). Laboratory findings may also include detection of leuokopenia, thrombocytopenia, and elevated serum hepatic transaminases (Eng et al., 1990).

The first case of infection with *E. canis* was documented in dogs over 65 years ago (Donatien and Lestoquard, 1935), and in 1954, *Rickettsia sennetsu* was identified as the first ehrlichial pathogen of humans (Misao and Kobayashi, 1954). However, it was not until the mid 1980’s/early 1990’s that the significant ehrlichial/anaplasmal pathogens of both humans and animals were isolated, identified, and fully described. Since their discoveries, approximately 1220, 2000, and 20 cases of HGA, HME, and EWE, respectively, have been reported to the Centers for Disease Control and Prevention (Childs and Paddock, 2003; Gardner et al., 2003).

These four bacterial species are transmitted by the bite of three different genera of ticks, and they all are known to involve wildlife in their epidemiology to some degree. Wild rodents and *Ixodes scapularis/pacificus* are believed to be important reservoirs and vectors of *A. phagocytophilum* (Hodzic et al., 1998; Ogden et al., 1998), which causes clinical disease in horses, ruminants, and humans. *Ehrlichia chaffeensis* is maintained in a cycle involving white-tailed deer (*Odocoileus virginianus*) and *Amblyomma americanum* as principle reservoir host and vector, respectively (Ewing et al., 1995; Lockhart et al., 1997a, 1997b). *Ehrlichia canis* and *E. ewingii* primarily are pathogens of dogs; however, coyotes can be infected with *E. canis* via *Rhipicephalus sanguineus* and *E. chaffeensis* via the bite of *A. americanum* (Groves et al., 1975; Lewis et al., 1977). Additionally, deer and humans can be infected with either *E. chaffeensis* and/or *E. ewingii* via the bite of *A. americanum*, the vector for both of these pathogens (Ewing et al; Lockhart et al., 1997a, 1997b; Anziani et al., 1990; Murphy et al., 1998; Steiert et al., 2002; Yabsley et al., 2002).
Although the life cycles of *E. chaffeensis* and *E. canis* are relatively well understood, the natural history and epidemiology of *E. ewingii* and *A. phagocytophilum* are currently incomplete. Previous research has identified *E. chaffeensis*-reactive antibodies in deer and other wild mammals, including raccoons (*Procyon lotor*), red foxes (*Vulpes vulpes*), gray foxes (*Urocyon cinereoargenteus*), opossums (*Didelphis virginianus*), and rabbits (*Sylvilagus* sp.) (Comer et al., 2000; Lockhart 1996, 1997a). Recent research has identified *A. phagocytophilum* infection, suggestive of reservoir competency, of eastern gray squirrels, eastern cottontail rabbits, and raccoons in the New England region (Levin et al., 2002; Goethert and Telford 2003). Because the Piedmont physiographic region of Georgia is an area of *E. chaffeensis* endemicity and because raccoons are parasitized by ticks known to harbor and transmit various zoonotic *Ehrlichia* and *Anaplasma* spp. (Lockhart et al., 1997a; Pung et al., 1994), we proposed to survey raccoons for evidence of infection with these pathogens to provide important epidemiologic information about the role of raccoons in the natural maintenance of this pathogen.

Previous research found serologic and/or molecular evidence of *A. phagocytophilum* infection in white-tailed deer (WTD), from various areas of the United States including Connecticut, Georgia, Indiana, Maryland, Missouri, South Carolina, and Wisconsin (Arens et al., 2003; Belongia et al., 1997; Yeh et al., 1997; Little et al., 1998; Magnarelli et al., 1999a; 2004; Walls et al., 1998). While these data have been confirmed with either DNA sequencing or western blot analysis, clinical disease due to infection with *A. phagocytophilum* has never been reported in WTD. Thus, the role of deer in the life cycle of *A. phagocytophilum* is currently unclear. Because WTD 1) are implicated as potential reservoir hosts, 2) are the principle host for adult *I. scapularis* and thus are routinely exposed to *A. phagocytophilum*, 3) are widespread throughout the United States, 4) have no prior history of antibiotic or acaracide treatment, 5)
have limited home ranges and long life spans, and 6) exist in natural habitats and in habitats close to humans, we proposed to evaluate WTD as natural sentinels to ascertain the distribution and potential risk of infection by *A. phagocytophilum*.

Overall, the major objective of this research was to further understand the epidemiology of multiple anaplasmal and ehrlichial tick-transmitted organisms within southeastern wildlife. Specific objectives of this study include:

1. Identify the species of *Ehrlichia* and *Anaplasma* sp. which occur naturally in a local raccoon population in Georgia
2. Determine the geographic distribution and prevalence of *A. phagocytophilum* infection in WTD from the southeastern United States and evaluate WTD as a natural sentinel system
3. Analyze the landscape epidemiology of *A. phagocytophilum* in the southeastern United States using GIS and spatial analyses

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CHAPTER 2
LITERATURE REVIEW

Background

Members of the genera *Anaplasma* and *Ehrlichia* are obligately intracellular, pleomorphic, gram-negative bacteria, found in membrane-lined vacuoles within the cytoplasm of an infected host cell. Organisms can be observed individually or in clusters, called morulae, which measure approximately 0.5 μm and 4.0 μm in size, respectively (Harkess, 1991; McDade et al., 1990). *Anaplasma* and *Ehrlichia* spp. are generally classified by the host cell in which they infect and replicate; *Anaplasma* spp. tend to invade neutrophils or granulocytes, and *Ehrlichia* spp. infect monocytes or macrophages. *Rickettia sennetsu* was the first ehrlichial pathogen of humans to be described in Japan in 1954 (Misao and Kobayashi, 1954), and the first case of ehrlichiosis in the United States was documented in 1986 (Maeda et al., 1987).

Human Granulocytotropic Anaplasmosis (HGA)

In 1994, an ehrlichial organism infecting circulating granulocytes was described from patients in Minnesota and Wisconsin (Chen et al., 1994). Termed the ‘HGE-agent’, this organism infecting humans was considered to be separate from but closely related to *E. equi*, the etiologic agent of equine granulocytotropic ehrlichiosis, and *E. phagocytophila*, the causative...
agent of tick-borne fever in European ruminants. After much scrutiny, debate, and multiple phylogenetic analyses of these nearly identical organisms, the accepted taxonomic classification of these three pathogens was revised, synonymizing all three species under the specific epithet *phagocytophilum* and re-assigning these agents to the genus *Anaplasma*, despite differences in strains, host preference, clinical symptoms of disease, and geographical prevalence (Dumler et al., 2001).

Since 1994, over 1200 US cases of HGA from 21 states have been reported to the Centers for Disease Control and Prevention in Atlanta, Georgia (Childs and Paddock, 2003; Gardner et al., 2003). Although most HGA infections occur in the north central and northeastern regions of the United States, some have recently been reported from southeastern states including Arkansas, Florida, Georgia, Maryland, Missouri, North Carolina, Oklahoma, Tennessee, and Virginia (Comer et al., 1999; Gardner et al., 2003). In 1998, the Centers for Disease Control and Prevention in collaboration with the Council of State and Territorial Epidemiologists (CSTE) recommended that human ehrlichioses, including HGA, be a nationally reportable disease and to date, 39 of 50 states report the occurrence of these diseases (Gardner et al., 2003; McQuiston et al., 1999).

HGA typically presents as an acute, non-specific, febrile illness, with symptoms ranging from subclinical (general malaise), to moderate (myalgia, anorexia), and even fatal, most notably in immunocompromised individuals. Key laboratory findings often accompanying these clinical symptoms usually include thrombocytopenia, leukopenia, and increases in serum hepatic enzymes (Eng 1990). Although patients tend to have a history of a tick bite, a cluster of HGA
cases in Minnesota were linked to direct contact with PCR positive blood from deer (Bakken et al., 1996). Additionally, human infection with either *A. phagocytophilum* or *E. chaffeensis* is clinically indistinguishable (Park et al., 2003).

In addition to recommending human ehrlichioses be nationally reportable diseases, CSTE also established case definitions for clinical practitioners in the diagnosis of HGA and HME. This definition included the presence of an acute illness accompanied by myalgia, malaise, rigor, and headache with at least one of the three following positive laboratory criteria: 1) a minimum four-fold increase of antibody titer, 2) a positive PCR assay, and/or 3) observation of morulae within blood, bone marrow, or cerebral spinal fluid leukocytes in conjunction with a positive indirect fluorescent antibody assay test greater or equal to 64 (Centers for Disease Control and Prevention, 1997).

Potential methods for diagnosing HGA include examination of peripheral blood smears, immunocytology, immunohistology, culture of *A. phagocytophilum*, serology, and PCR assays. The most rudimentary diagnostic technique for HGA diagnosis is the examination of Romanovsky-stained blood smears; however, serologic methods tend to be favored due to the extreme difficulty in detecting the presence of morulae. The indirect fluorescent antibody (IFA) assay is commonly used to diagnose HGA, however, the enzyme-linked immunosorbent assay (ELISA) is reported to be more sensitive (Ravyn et al., 1998). Additionally, serologic cross-reactivity between *E. chaffeensis* and *A. phagocytophilum* has been reported from human cases (Comer et al., 1999) thus making the use of confirmatory tests such as western blotting and immunoblotting important.

While cell culture isolation techniques are the ‘gold standards’ for diagnosing infections with most ehrlichial and anaplasmal organisms, culture systems can be difficult to maintain, are
not readily available to clinicians, and rickettsial organisms are difficult to propagate given the inability to supplement with antibiotics. *Anaplasma phagocytophilum* was first propagated in the HL60 human leukemia suspension cell line by the direct addition of whole blood (Goodman et al., 1996), however, this organism can also be grown in adherent *Ixodes scapularis* embryonic cells (ISE6; Munderloh et al., 1996).

PCR assays can provide rapid and specific diagnosis of *A. phagocytophilum* using multiple gene targets. The gene target first explored for use in a PCR assay was the 16S rRNA (rDNA) gene (Chen et al., 1994) using a nested PCR protocol that has since become one of the standard PCR assays utilized. Recently, other gene targets have produced more sensitive and specific PCR assays including the *groESL* heat shock operon gene (Sumner et al, 1997) that amplifies both *A. phagocytophilum* and *E. chaffeensis* in a nested protocol, and the *epank1, p44, gltA, rpoB,* and *ftsZ* genes (Walls et al., 2000; Zeidner et al., 2000; Inokuma et al., 2001; Taillardat-Bisch et al., 2003; Lee et al., 2003). While these PCR assays are practical in clinical settings where human patients typically have infection with a single organism, use of these primers for detection of *A. phagocytophilum* in tick vectors and wildlife commonly infected with multiple ehrlichial and anaplasmal species can be problematic.

While the 16S rRNA PCR assay for *A. phagocytophilum* is sequence specific, it is not agent specific and has been shown to amplify other anaplasmal and ehrlichial organisms from wildlife (Little et al., 1997; Dugan et al., 2005). To address the issue of sensitivity and specificity of the aforementioned PCR assays for clinical diagnostic use in humans, Massung and Slater (2003) conducted a comparison of 13 different primer sets using serial dilutions of *A. phagocytophilum* DNA extracted from the blood of human patients, cell culture isolates, as well as other known *Ehrlichia* and *Anaplasma* spp. The result of this study concluded that for *A.
Phagocytophilum detection, the original nested 16S gene PCR protocol and the standard p44 PCR assays were the most sensitive and specific for the detection of A. phagocytophilum in humans. Given that the p44 gene is species-specific to A. phagocytophilum, the p44 PCR assay currently appears to be the most reliable protocol for use in wildlife and tick vectors.

The recommended treatment for confirmed or suspected HGA is doxycycline therapy, as well as supportive care, which have been proven effective by both in vitro and in vivo testing and clinical observations (Centers for Disease Control, 1995, 2001, 2002). Alternative pharmacologic therapies may include rifamycins and quinolones (Klein et al., 1997), however the best way to prevent acquisition of tick-borne diseases is to avoid exposure to ticks. Wearing light colored clothing, long pants, and long-sleeved shirts discourage tick attachment and can be combined with the use of topical acaracides and thorough self-examination after tick exposure.

Since the description of HGA in 1994, considerable research has aimed at identifying the key elements in the natural maintenance of A. phagocytophilum. Although less information is known about larger mammalian hosts of this pathogen, early studies have confirmed I. scapularis, the black-legged tick, and Peromyscus leucopus, the white-footed mouse (WFM), as the cardinal vector and reservoir in the northeastern portions of the United States (Telford et al., 1996; Des Vignes and Fish, 1997).

In northeastern and north central states where HGA infection is endemic, serologic and PCR assays, culture isolation, and/or sequence evidence of A. phagocytophilum have been found in WFM and/or I. scapularis from Connecticut, Delaware, Maryland, Massachusetts, Minnesota, New York, Rhode Island, and Wisconsin (Telford et al., 1996; Daniels et al., 1997; Magnarelli et al., 1997, 1999a; Walls et al., 1997; Yeh et al., 1997; Levin et al., 1999; Curran et al., 2000; Layfield et al., 2002). Along the Pacific coast where granulocytotropic ehrlichiosis of horses is
well described, serologic, molecular, and sequence evidence of *A. phagocytophilum* has been identified in rodents and/or *I. pacificus* from California (Barlough et al., 1997; Magnarelli et al., 1997; Nicholson et al., 1998, 1999; Kramer et al., 1999; Lane et al., 2001; Foley et al., 2002).

Because WTD are parasitized by adult stages of *Ixodes* ticks likely carrying viable *A. phagocytophilum*, prior research has attempted to determine the exposure and/or infection status of deer in locations where *A. phagocytophilum* infection is endemic in WFM and ticks. Seropositive deer have been found in Connecticut, Georgia, Indiana, Maryland, Missouri, and Wisconsin. PCR positives results from deer, with some infections confirmed by western blot or products confirmed by sequencing, have been detected in animals from Connecticut, Georgia, Indiana, and Wisconsin (Belongia et al., 1997; Little et al., 1998; Walls et al., 1998; Magnarelli et al., 1999a; Arens et al., 2003).

In addition to sampling WFM and WTD, various projects have sampled alternative rodent species and small mammals from *A. phagocytophilum*-endemic areas in the northeastern United States to gain further epidemiologic information. Serologic and/or molecular evidence of infection has been demonstrated in New England rodents including *P. boylii, P. maniculatus,* and *P. gossypinus* (Nicholson et al., 1997, 1998; Magnarelli et al., 1999b). A survey of small mammals in Minnesota for *A. phagocytophilum* infection revealed that about 10% of WFM, eastern chipmunks (*Tamias striatus*), southern red-backed voles (*Clethrionomys gapperi*), and shrews (*Blarina brevicauda* and *Sorex cinereus*) were positive by either IFA or PCR (Walls et al., 1997).

More recently, small and medium sized mammals and *I. scapularis* ticks from Connecticut were tested via PCR assay for the presence of *A. phagocytophilum* DNA. PCR products were amplified from the blood of >10% of raccoons as well as 23% of ticks that fed on
raccoons and 5-7% of ticks that fed on skunks (*Mephitis mephitis*) and opossums. Ticks that fed upon raccoons and gray squirrels (*Sciurus carolinensis*) were able to transmit *A. phagocytophilum* to laboratory mice, suggesting that these animals may be competent reservoirs for *A. phagocytophilum* (Levin et al., 2002). In addition, a retrospective survey of Pennsylvania black bears (*Ursus americanus*) found that 56% of animals tested possessed antibodies reactive to HGA antigen (Schultz et al., 2002). A study of eastern cottontail rabbits (*S. floridanus*) discovered 66% seroprevalence to the agent of HGA with 23% of rabbits positive by either microscopy and/or PCR assay (Goethert and Telford, 2003).

In the Pacific Northwest, many studies have focused on both rodent and small/medium sized mammals for evidence of exposure and/or infection with *A. phagocytophilum*. In California and Colorado, several species of woodrats (*N. fuscipes, N. lepida, N. albigula*, and *N. mexicana*) had antibodies that reacted with the HGA agent. An additional survey of California dusky-footed wood rats (*N. fuscipes*) discovered serologic, molecular, and DNA sequencing evidence of natural *A. phagocytophilum* infection, in addition to xenodiagnostic passage of infection to naïve wood rats (Foley et al., 2002; Nicholson et al., 1998, 1999; Castro et al., 2001).

Testing of California mountain lions (*Puma concolor*) found 17% seroprevalence against *E. equi* antigen (now *A. phagocytophilum*) and 16% PCR-prevalence of granulocytic *Ehrlichia* DNA obtained from blood. Additionally, 19% of engorged *I. pacificus* and *Dermacentor variabilis* tick pools collected from these animals were PCR positive (Foley et al., 1999). Testing of California coyotes (*Canis latrans*) found 46% seroprevalence against *E. equi* antigen, with one sequence confirmed HGA/*E. equi* PCR positive animal (Pusterla et al., 2000). A survey of California cervids yielded serologic and molecular evidence of *E. equi* infection from
black-tailed deer (*O. hemionus columbianus*) and elk (*Cervus elaphus nannodes*), and isolated *E. equi* in tissue culture from 2 elk samples, confirmed by sequence analysis (Foley et al., 1998).

A similar situation exists in Europe: in 2001, Norwegian case reports documented *A. phagocytophilum* infections in a roe deer (*Capreolus capreolus*) and a moose calf (*Alces alces*) confirmed by PCR assay and/or sequence confirmation and visualization of morula (Jenkins et al., 2001; Stuen et al., 2001). A year later, serosurveillance of moose, red deer and roe deer from *I. ricinis* infested counties in Norway yielded *A. phagocytophilum* antibody prevalences of 43%, 55%, and 96%, respectively (Stuen et al., 2002). Additional serologic and molecular surveys of red and roe deer, other wild mammals, and birds in Europe for *A. phagocytophilum* have been performed, all of which report high prevalences (equal to or greater than 50%) of infection (Alberti et al., 2000; Hulinska et al., 2002; Liz et al., 2002; Petrovec et al., 2002).

While successful *A. phagocytophilum* experimental infections have been performed with horses and laboratory mice via both needle-inoculation and feeding of *I. scapularis* (Madigan et al., 1995; Reubel et al., 1998; Pusterla et al., 1998; Kim et al., 2002; Telford et al., 1996; Sun et al., 1997; Levin and Fish, 2000; Levin et al., 2004), infectivity of WTD with this pathogen was not explored until recently, despite evidence of naturally occurring infection in WTD. In 2005, Tate and others experimentally infected four naïve WTD with the HGE-1 human strain of *A. phagocytophilum*. The results of this study determined that WTD seroconverted and remained seropositive through the end of the 66-day study. Deer were also RT-PCR positive for 17 days post infection despite lack of clinical symptoms of disease. Conclusions suggest that WTD can be infected with a human strain of *A. phagocytophilum* and mount a detectable, long-term serologic response. In addition, WTD may be suitable as sentinel species for human risk of exposure to *A. phagocytophilum*; however, WTD are most likely an insignificant source of
infection for *I. scapularis* ticks because WTD are the primary hosts for the adult stage of this tick (Tate et al., 2005).

The hypothesis that the WTD strain of *A. phagocytophilum* is not infectious to humans and exists in a separate infection cycle within WTD and *I. scapularis* has been proposed (Massung et al., 2002, 2003a, 2003b, de la Fuente et al., 2005). A 2 base pair difference in the 16S gene found in all known *A. phagocytophilum* sequences amplified from WTD has been described as a ‘marker’ for host infectivity. This strain, termed the AP-1 variant, has been unsuccessful in establishing infection in laboratory mice further supporting a separate epizootic cycle of host infectivity for this variant (Massung et al., 2003a, 2003b). Successful *A. phagocytophilum* infection trials with WTD using a human strain of *A. phagocytophilum* are contradictory to this idea and future research may resolve issues related to this theory.

**Human Monocytotropic Ehrlichiosis (HME)**

The first US case of human ehrlichiosis was documented in 1986 in a febrile patient with a history of a tick bite (Maeda et al., 1987). Although the disease was termed human monocytotropic ehrlichiosis, the etiologic agent, *E. chaffeensis*, was not isolated and identified until 1991 (Dawson et al., 1991). Human monocytotropic ehrlichiosis is a non-specific, acute, febrile illness with clinical symptoms ranging from asymptomatic to fatal. Laboratory findings include thrombocytopenia, leukopenia, and mild hepatitis (Eng et al., 1990). Since 1986, more than 1000 cases of HME have been reported to the Centers for Disease Control and Prevention in Atlanta, Georgia (Childs and Paddock, 2003) with most occurring in the southeastern and southcentral United States.

Several studies indicate that *E. chaffeensis* is maintained in a system involving WTD and *A. americanum* (LST). Serologic evidence of *E. chaffeensis* infection of WTD in the eastern
United States discovered \textit{E. chaffeensis}-reactive antibodies in 43\% of WTD sampled (Dawson et al., 1994a). In 1995, a temporal association between \textit{E. chaffeensis}-reactive antibodies and \textit{A. americanum} in Georgia was demonstrated. When no ticks were observed on WTD, no antibodies were detected; however, as LST prevalence increased and populations became established over a 13-year time span, \textit{E. chaffeensis}-reactive antibodies were detected in most WTD sampled (Lockhart et al., 1995). Additionally, a spatial association was established for \textit{E. chaffeensis} and LST in 1996 when antibodies were detected in deer from areas with known LST infestations, but not in deer from \textit{A. americanum} negative locations (Lockhart et al., 1996).

Molecular evidence of naturally occurring \textit{E. chaffeensis} infection in deer has been reported and this pathogen has been isolated from naturally infected WTD (Little et al., 1997; Lockhart et al., 1997a, 1997b, Yabsley et al., 2002). Additionally, WTD are susceptible to experimental infection with \textit{E. chaffeensis} but not \textit{E. canis} (Dawson et al., 1994b; Ewing et al., 1995; Davidson et al., 2001). Molecular evidence of \textit{E. chaffeensis} infection in LST has been documented via survey, and experimental transmission studies verified vector competence (Ewing et al., 1995; Lockhart et al., 1997b; Whitlock et al., 2000).

Further research has focused on the role of non-ungulate vertebrate reservoirs in the epidemiology of \textit{E. chaffeensis}. Experimental infection of dogs showed that organisms could persist and replicate for extensive periods of time (Dawson et al., 1992). Serologic and/or molecular evidence of infection with one or more ehrlichial species has implicated dogs as potential reservoirs for \textit{E. chaffeensis} (Dawson et al., 1996b; Murphy et al., 1998; Breitschwert et al., 1998; Kordick et al., 1999) despite failed attempts to infect ticks from experimentally infected animals (Ewing et al., 1995).
Although *E. chaffeensis*-reactive antibodies have been detected in wild mice from southeastern and northeastern US states where this pathogen is endemic in deer and humans, rodents do not appear to play an important role in the natural maintenance of *E. chaffeensis* because molecular and cell culture evidence of *E. chaffeensis* infection has not documented from these rodent species (Magnarelli et al., 1997; Lockhart et al., 1998). However, successful experimental infections of immunocompromised mice with *E. chaffeensis* suggest that C3H/HeJ and/or SCID mice might provide a model for studying host infection dynamics (Telford and Dawson, 1996; Winslow et al., 1998; Lockhart et al., 1999).

In 1997, Lockhart et al. (1997a) tested mammalian sera obtained from a location in the Piedmont physiographic region of Georgia, where *E. chaffeensis* infection is endemic. *Ehrlichia chaffeensis*-reactive antibodies were identified in 8%, 21%, and 92% of opossums, raccoons, and WTD respectively. Red foxes have since been demonstrated as competent potential reservoir hosts but gray foxes appeared refractory to infection (Davidson et al., 1999). Recently, 71% of Oklahoma coyotes tested *E. chaffeensis* PCR positive, documenting natural infection (Kocan et al., 2000), and serologic, molecular, and cell culture isolation evidence demonstrated natural infection of domestic goats with *E. chaffeensis* (Dugan et al., 2000). Furthermore, a southeastern retrospective study of raccoon sera spanning a 20-year period determined approximately 20% of animals tested possessed *E. chaffeensis*-reactive antibodies, strongly suggesting exposure to an ehrlichial species (Comer et al., 2000). The latest finding has been molecular and tissue culture evidence *E. chaffeensis* infection in ring-tailed lemurs (*Lemur catta*) and ruffed lemurs (*Varecia variegata*) from North Carolina (Williams et al., 2002).

Most recently, a broad-scale, collaborative study evaluating WTD as sentinels in a prototypic surveillance system for *E. chaffeensis* was performed. The use of WTD as
surveillance animals was assessed using WTD serologic results, confirmed by PCR and cell culture assay techniques, and relevant considerations to the applicability of a surveillance system. This study determined that almost half of WTD from 17 of 18 southeastern and south central states possessed *E. chaffeensis*-reactive antibodies and 47% of seropositive WTD populations were confirmed by either PCR and culture assays. The system was successful in detecting the stability and spread of *E. chaffeensis* in relation to the tick vector, *A. americanum*, and a significant association of seropositive WTD with the presence of *A. americanum* was also confirmed. In addition, the system discerned that small WTD sample sizes are effective for accurate surveillance, that deer of any age are appropriate for surveillance testing, and concluded that WTD are effective sentinel animals that can be used in a surveillance system for *E. chaffeensis* (Yabsley et al., 2003).

**Canine Monocytotropic Ehrlichiosis (CME)**

*Ehrlichia canis*, the etiologic agent of tropical canine pancytopenia or CME, was first described from dogs in Algeria in 1935 (Donatien and Lestoquard, 1935) and recognized in the US in 1962 (Ewing 1963; Rikihisa et al., 1992). Before the isolation of *E. chaffeensis* in 1991 (Dawson et al., 1991), it was believed that *E. canis* was the causative agent of human ehrlichiosis based on serologic cross reactivity between these two organisms. Today, CME is the most important cause of ehrlichial infection in dogs worldwide. Acute clinical signs are comprised of depression, fever, anorexia, and lymphadenopathy while chronic disease is characterized by edema, emaciation, shock, and ultimately death. Typical of most ehrlichial infections, *E. canis*-infected dogs exhibit abnormal decreases in platelets and leukocytes.

*Ehrlichia canis* is vectored by the brown dog tick, *R. sanguineus* (Groves et al., 1975), which feeds on dogs in all life stages, yet will incidentally bite humans (Lewis et al., 1977;
Unver et al., 2001). Recently, a new strain of *E. canis*, referred to as Venezuelan human *Ehrlichia* (VHE), was isolated from a human patient in Venezuela (Perez et al., 1996). Molecular and antigenic characterization of this VHE isolate suggested that this subspecies might cause persistent infections in South American individuals and that dogs and *R. sanguineus* might serve as the reservoir and vector, respectively (Unver et al., 2001).

In 1973, an experimental infection of red and gray foxes with *E. canis* suggested potential for reservoir status (Amyx and Huxsoll, 1973), however the only additional evidence of natural infection has been seropositive results documented in red foxes from Switzerland (Pusterla et al., 1999).

Kenyan jackals (*C. mesomelas*) were thought to have potential reservoir status when *E. canis* was isolated in cell culture and used to successfully experimentally infect naïve pups (Price et al., 1980). More recent serologic studies on free-ranging jackals (*C. aureus*) in Israel have shown high prevalence (35.8% to 54.3%) of *E. canis*-reactive antibodies (Waner et al., 1999; Shamir et al., 2001) in conjunction with 26.4% and 26.0% seroprevalence to *E. chaffeensis* and *A. phagocytophilum*, respectively.

**Human and Canine Granulocytotropic Ehrlichiosis (HE and CE)**

*Ehrlichia ewingii*, most closely related to *E. chaffeensis*, is the causative agent of granulocytotropic ehrlichiosis in humans and dogs. To date, *E. ewingii* infection in humans has been reported from Missouri, Oklahoma, and Tennessee (Buller et al., 1999; Paddock et al., 2001) and infection in dogs has been reported from North Carolina, Oklahoma, and Virginia with a prevalence range of 6.2%-15.8% (Anderson et al., 1992; Dawson et al, 1996; Goldman et al., 1998; Murphy et al., 1998; Kordick et al., 1999). Clinical symptoms of disease include fever,
malaise, and headache with laboratory findings of thrombocytopenia at times accompanied by leukopenia (Buller et al., 1999; McQuiston et al., 1999; Paddock et al., 2001).

Little is known about the natural history and epidemiology of *E. ewingii* and serologic assays are not currently available because there are no sources of cell culture antigen for IFA tests (Yabsley et al., 2002). In nature, *E. ewingii* is believed to be vectored by *A. americanum*. In experimental infection trials, LST were shown to be competent vectors of this organism (Anziani et al., 1990). However, molecular evidence of *E. ewingii* infection has been obtained from other ticks including *R. sanguineus* and *D. variabilis* in Oklahoma (Murphy et al., 1998) and Missouri (Steiert et al., 2002).

Recently, both the geographic and host range of this pathogen were expanded with PCR evidence that 7.3% of WTD from Georgia, Kentucky, and South Carolina are naturally infected with *E. ewingii* (Yabsley et al., 2002). Additionally, 44 of 217 (20%) Missouri deer were PCR positive for *E. ewingii*, although products were not sequence confirmed (Arens et al., 2003). Furthermore, the ability of the organism to replicate within WTD was confirmed by the experimental infection of two naïve fawns inoculated with *E. ewingii* PCR positive blood from field collected deer (Yabsley et al., 2002).

**Geographic Information Systems Analyses**

A geographic information system (GIS) is defined as a system that is used to input, store, retrieve, manipulate, analyze, and output geographically referenced data or geospatial data. GIS systems were first used in support of decision making for planning and management of land use, natural resources, and the environment. Recently, GIS analyses have been used in conjunction with spatial statistics to allow epidemiologists to thoroughly address the spatial aspects of disease transmission. A GIS has a number of important aspects that make it a valuable tool.
These models can 1) determine the predicted distribution of a disease, 2) discern if specific attributes are related to the occurrence of a disease, and 3) predict the occurrence of disease for unknown areas using a predictive model based on significant ecologic variables.

Implementing predictive modeling into a GIS can be performed by logistic regression analysis. Landscape attributes and climactic variables that are identified as significantly associated with disease occurrence through logistic regression can be analyzed to produce a predictive model of disease distribution and in turn, construct a map of this distribution.

While logistic regression using a GIS is an important tool, geospatial analyses including kriging can be used to generate an alternative predictive model for disease distribution based on spatial relationships. Kriging models are based on the assumptions that predictions for unsampled locations can be calculated by weighted averages of data for sampled locations in close proximity, and that the distance between observations is directly related to the spatial variability of the data.

Both of these techniques have been used to generate predictive models for a number of tick borne pathogens including Lyme disease (LD), *E. chaffeensis*, and HGA. In 1996, Nicholson and Mather developed a GIS model using spatial autocorrelation that indicated a correlation between the abundance of *I. scapularis* nymphs with the incidence of LD, and the prevalence of *B. burgdorferi* infection in ticks. Through geospatial analyses, increasing latitude was positively associated with decreased tick prevalence, predicting a decreased incidence of LD. Guerra et al., 2001 implemented a surveillance system using dogs as sentinels for assessing the risk of LD. Serologic test results along with tick exposure and vaccine, travel, and veterinary
medical histories were spatially correlated and integrated using a GIS, and determined that in Wisconsin and Illinois, seropositive dogs were associated with living in forested, urban, and sandy soiled areas.

Chaput et al., 2002 focused on a 12-town area near Lyme, Connecticut to determine the risk factors associated with HGA incidence. Using human case data, spatial analyses detected a decrease of HGA incidence in relation to increased distance from the coast, and determined the spatial distribution of *A. phagocytophilum* and a patterned distribution of HGA case distribution. Most recently, geospatial analyses were used to map the endemnicity probability of *E. chaffeensis* across a 19-state region (Yabsley et al., 2005). Using both kriging and logistic regression modeling techniques based on the serostatus of WTD in this region, the predictive models generated had good concordance with HME case data and accurately discerned the occurrence of *E. chaffeensis*. This study also determined ecologic variables associated with high risk for HME that corresponded to the known distribution of the vector, *A. americanum*.

**Factors influencing *Ixodes scapularis* distribution**

Prior studies have determining the spatial distribution of LD and as a result, variables affecting the prevalence of *I. scapularis* have been elucidated. Positive associations have been made between WTD and *I. scapularis* abundance suggesting that WTD density strongly influences the life cycle of this tick (Rand et al., 2003; Wilson, 1988, 1998).

Temperature, relative humidity, and habitat type have also been shown to affect the distribution, developmental cycles, and mortality rates of *I. scapularis* (Rand et al., 2003; Bunnell et al., 2003). The presence of wooded and forest habitats are positively associated with *I. scapularis* abundance while wetlands, increased elevation, and increased distances to water have negative associations with this tick (Bunnell et al., 2003). Extreme temperatures also may
play a significant role in the maintenance of *I. scapularis* populations based on the effect for
habitat suitability. Relative humidity, cooler winter seasons, and dry summer seasons have direct
positive associations with *I. scapularis*, and it has been suggested that extreme low temperatures
increase the rate of tick mortality by raising the life cycle development period (Ogden et al.,
2005).

Overall, a multitude of ecologic attributes appear important for *I. scapularis* maintenance.
Because the adult life stage of this tick primarily feeds on WTD, and WTD can be infected with
*A. phagocytophilum, I. scapularis* are important to the establishment and spread of this pathogen
(Rand et al., 2003). As determined by prior studies with LD, it is likely that ecologic factors
affecting *I. scapularis* distributions may also be significant with prevalence of *A.
\[\text{phagocytophilum}.\]

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CHAPTER 3

DETECTION OF *EHRlichia* SPP. IN RACCOONS (*PROcyon lotor*) FROM GEORGIA¹

ABSTRACT

Raccoons (Procyonis lotor) and opossums (Didelphis virginianus) acquired from six contiguous counties in the Piedmont physiographic region of Georgia were investigated for their potential role in the epidemiology of ehrlichial and anaplasmal species. Serum was tested by indirect fluorescent antibody (IFA) assay for the presence of antibodies reactive to Ehrlichia chaffeensis, E. canis, and Anaplasma phagocytophilum (HGA agent). Nested polymerase chain reaction (PCR) assay was used to test whole blood or white blood cell preparations for the presence of Ehrlichia and Anaplasma spp. 16S rRNA (rDNA) gene fragments. In addition, ticks were collected from these animals and identified. Twenty-three of 60 raccoons (38.3%) had E. chaffeensis-reactive antibodies (>1:64), 13 of 60 raccoons (21.7%) had E. canis-reactive antibodies, and 1 of 60 raccoons (1.7%) had A. phagocytophilum-reactive antibodies. A sequence confirmed E. canis product was obtained from one of 60 raccoons and a novel Ehrlichia-like 16S rDNA sequence was detected in 32 of 60 raccoons. This novel sequence was most closely related to an Ehrlichia-like organism identified from Ixodes ticks and rodents in Asia and Europe. Raccoons were PCR negative for E. chaffeensis and E. ewingii DNA. Five tick species including Dermacentor variabilis, Amblyomma americanum, Ixodes texanus, I. cookei, and I. scapularis were identified from raccoons and represent potential vectors for the ehrlichiae detected. Opossums (n=17) were free of ticks and negative on all IFA and PCR assays. This study suggests that raccoons are potentially involved in the epidemiology of multiple ehrlichial organisms with known or potential public health and veterinary implications.
INTRODUCTION

Members of the family Anaplasmataceae are obligately intracellular bacteria, usually found in membrane-lined vacuoles within the cytoplasm of infected host cells. *Ehrlichia chaffeensis, E. canis, E. ewingii,* and *Anaplasma phagocytophilum* are significant tick-transmitted pathogens within this family that are the cause of emerging diseases of human and veterinary health importance. These species are generally categorized by the cells which they infect and are separated into mononocytotropic and granulocytotropic groups. *Ehrlichia chaffeensis* and *E. canis* invade monocytes and macrophages; infection with *E. chaffeensis* causes human monocytotropic ehrlichiosis (HME) and one strain of *E. canis* is thought to cause asymptomatic human infections in South America (Perez et al. 1996; Unver et al. 2001). *Anaplasma phagocytophilum* and *E. ewingii* infect neutrophils and less commonly eosinophils, causing human granulocytic anaplasmosis (HGA) and human granulocytic ehrlichiosis (HGE), respectively. All four species also are canine pathogens (Greig et al. 1996; Breitschwerdt et al. 1998; Kordick et al. 1999) and *A. phagocytophilum* additionally causes clinical disease in horses and ruminants (Madigan et al. 1996; Pusterla et al. 2002; Garcia-Perez et al. 2000, 2003; Ogden et al. 1998, 2002). Though fatal human cases have been reported, clinical infection with one or more of these microbes typically presents as a non-specific, acute, febrile illness with signs ranging from asymptomatic to severe (McQuiston et al. 2003).

*Ehrlichia chaffeensis, E. canis, E. ewingii,* and *A. phagocytophilum* are transmitted by three different genera of ticks and are known to involve various wild mammals in their epidemiology. While the epidemiology of *E. chaffeensis* and *E. canis* is fairly well characterized, less is known about the natural history of *A. phagocytophilum* and *E. ewingii. *Ehrlichia chaffeensis* is maintained in nature primarily through a cycle involving the white-tailed
deer (WTD; *Odocoileus virginianus*) and *Amblyomma americanum* as principle vertebrate reservoir and vector, respectively (Ewing et al. 1995; Lockhart et al. 1997a, 1997b). *Ehrlichia canis* principally cycles between canid species and the brown dog tick, *Rhipicephalus sanguineus* (Groves et al. 1975; Lewis et al. 1977). The white-footed mouse (*Peromyscus leucopus*) and other wild rodents along with *Ixodes scapularis* and *I. pacificus* are believed to be vertebrate reservoirs and vectors of *A. phagocytophilum* (Hodzic et al. 1998; Ogden et al. 1998). *Ehrlichia ewingii* has recently been detected in southeastern WTD (Yabsley et al. 2002) and *A. americanum* appears to be an important vector (Anziani et al. 1990), although *R. sanguineus* and *Dermacentor variabilis* are suspected to be involved in transmission in dogs (Murphy et al. 1998; Steiert and Gilfoy 2002).

Previous evidence of *Ehrlichia* and *Anaplasma* infection has been detected in other wild mammals within the United States. *Ehrlichia chaffeensis* DNA has been detected in coyotes (*Canis latrans*) (Kocan et al. 2000) and *E. chaffeensis*-reactive antibodies have been identified in gray foxes (*Urocyon cinereoargenteus*), opossums (*Didelphis virginianus*), rabbits (*Sylvilagus* sp.), red foxes (*Vulpes vulpes*), and most notably in raccoons (*Procyon lotor*) (Lockhart et al. 1996, 1997a; Comer et al. 2000). Naturally occurring *E. chaffeensis* infections also have been observed in domestic goats and both ring-tailed and ruffed lemurs (*Lemur catta, Varecia variegata*) (Dugan et al. 2000; Williams et al. 2002). In addition to *Peromyscus* spp., serologic or molecular evidence of *A. phagocytophilum* has been detected in woodrats (*Neotoma* spp.), eastern chipmunks (*Tamias striatus*), southern red-backed voles (*Clethrionomys gapperi*), shrews (*Blarina* and *Sorex* spp) (Foley et al. 2002; Castro et al. 2001; Nicholson et al. 1998, 1999; Walls et al. 1997) as well as opossums, striped skunks (*Mephitis mephitis*), coyotes (*Canis latrans*), mountain lions (*Puma concolor*), and black bears (*Ursus americanus*) (Levin et al.
2002; Pusterla et al. 2000; Foley et al. 1999; Schultz et al. 2002). While these mammals are suspected reservoirs, recent xenodiagnostic assays have confirmed *A. phagocytophilum* infection in eastern gray squirrels (*Sciurus carolinensis*), raccoons, and eastern cottontail rabbits (*S. floridanus*) indicating that these mammals are competent reservoirs for this pathogen in the New England region (Levin et al. 2002; Goethert and Telford 2003).

The Piedmont physiographic region of Georgia is a location where multiple *Ehrlichia* and *Anaplasma* species are enzootic (Lockhart et al. 1997a, 1997b, Little et al. 1998, 1999; Yabsley et al. 2002) and tick species known to harbor and transmit these organisms commonly parasitize both opossums and raccoons in this area (Pung et al. 1994). Thus, the objective of this study was to utilize serologic and molecular tools to identify the species of *Ehrlichia* and *Anaplasma* that occur in local raccoon and opossum populations in northeastern Georgia in order to assess the potential role of these animals in the maintenance of these pathogens.

**MATERIALS AND METHODS**

**Animal and tick collections**

Raccoons and opossums were caught in live traps set overnight in Clarke, Elbert, Lincoln, Madison, Oconee, and Wilkes counties, GA, between February and June of both 1999 and 2000. After capture, animals were sedated via intramuscular injection with xylazine (4 mg/kg body weight; Mobay Corp., Animal Health Division, Shawnee, KS, USA) mixed with ketamine hydrochloride (20 mg/kg; Aveco Co. Inc., Fort Dodge, IA, USA) and humanely euthanitized via intracardiac injection of sodium pentobarbital solution (1 ml/kg; Fatal Plus, J.A. Webster, Inc., Sterling, MA, USA). Whole blood and serum samples from sedated animals were collected in Vacutainer® EDTA tubes and serum tubes. All animals were examined for ectoparasites and if present, ticks were collected, stored in 70% ethanol, and submitted to the
National Veterinary Services Laboratory, APHIS, USDA, Ames, IA for identification. All animals were collected and handled as permitted by the Georgia Department of Natural Resources and as approved by the Animal Care and Use Committee at the University Of Georgia College Of Veterinary Medicine.

Serology

Serum samples were tested for antibodies reactive to *E. chaffeensis*, *E. canis*, and *A. phagocytophilum* by indirect fluorescent antibody (IFA) assay as previously described (Dawson et al. 1991). Commercially manufactured IFA slides were available for *E. chaffeensis* (Arkansas strain) and *A. phagocytophilum* (human strain) (Focus Technologies, Cypress, CA, USA). *Ehrlichia canis* IFA slides were produced in our laboratory using a canine strain obtained from the Centers for Disease Control and Prevention, Atlanta, grown in the DH82 continuous cell culture line. Fluorescein isothiocyanate (FITC)-labeled anti-raccoon IgG antibodies (Kirkegaard and Perry laboratories, Gaithersburg, Maryland) or FITC-labeled or anti-opossum IgG antibodies (obtained from the CDC, Atlanta, Georgia) were diluted 1:50 in phosphate buffered saline and used as a conjugate. For *A. phagocytophilum* IFA assays, a 1:50 dilution of eriochrome black T counter stain (Sigma-Aldrich, St. Louis, MO) was used in the final wash step of the assay. All samples were screened at initial dilutions of 1:64 and 1:128, with positive samples further tested in twofold serial dilutions to an endpoint, and the geometric mean titer (GMT) calculated (Villegas 1998).

DNA preparation and PCR amplification

For 26 raccoons and 17 opossums collected in 1999, white blood cells (WBC) were isolated from 10 ml of whole blood, resuspended in PBS as previously described (Little et al. 1997) and used to extract genomic DNA, whereas for 34 raccoons collected in 2000, whole
blood was used directly for DNA extraction. DNA was extracted from 100 µl of either WBC or whole blood using the GFX Genomic Blood Purification Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) following the manufacturer’s protocol for fast DNA extraction from either 100 µl of WBC or whole blood. Each DNA pellet was suspended in 50 µl of molecular biology grade water. PCR amplification was performed in a PTC-100 MJ Research thermalcycler (MJ Research Inc., Waltham, MA) in 25 µl reactions. Five microliters of extracted DNA was tested by nested PCR assay for the presence of 16S rDNA using 0.8 uM each of genus-wide primers ECC and ECB in a 25 µl reaction containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.4 mM MgCl₂, 0.2 mM each dNTP, and 2.5 units Taq DNA polymerase (Promega US, Madison, Wisconsin) (Dawson et al. 1994a). One microliter of primary product was used as a template in a 25µl nested reaction containing the same PCR reagents with the substitution of species specific inside primers producing products for E. chaffeensis (HE1/HE3, 389 bp) (Anderson et al. 1992a), E. canis (HE1/ECA, 390 bp) (Wen et al. 1997), A. phagocytophilum (GE9F/GA1UR, 411 bp) (Chen et al. 1994; Little et al. 1998), and E. ewingii (HE3/EE72, 407 bp) (Dawson et al. 1996b). Products were separated by gel electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination.

Positive control material used in DNA extraction and PCR was DH82 canine macrophage cell cultures infected with E. chaffeensis (Arkansas strain) or E. canis (canine strain), I. scapularis ISE6 tick cell cultures infected with a human A. phagocytophilum isolate (kindly provided by Dr. Ulrike G. Munderloh, University of Minnesota, St. Paul), or E. coli transformed with a plasmid containing a segment of 16S rRNA of E. ewingii (kindly provided by John Sumner, CDC, Atlanta). Non-template negative controls were included in DNA extraction,
primary, and secondary PCR amplification steps. In order to minimize contamination, all steps were performed in designated PCR cabinets located in separate laboratories.

**Sequencing and sequence analysis**

Select PCR products were purified using the Qiagen Gel Purification Kit (Qiagen Inc., Valencia, California) and were sequenced in both the 3’ and 5’ directions with a Perkin Elmer ABI Prism 3700 automated DNA sequencer at the Molecular Genetics Instrumentation Facility at the University of Georgia. The sequences were assembled and edited with the Sequencher software package version 4.0.5 (Gene Codes Corp., Ann Arbor, MI), and a nucleotide-nucleotide BLAST (blastn) search was performed to determine the most similar GenBank sequences to include in phylogenetic analysis (National Center for Biotechnology Information site [http://www.ncbi.nlm.nih.gov/BLAST/]). The ClustalX Multiple Sequence Alignment Program version 1.83 (Feb 2003) was used to align edited sequences, and phylogenetic analyses were conducted using MEGA version 2.1 (Kumar et al. 2001).

**Nucleotide sequence accession numbers**

The GenBank nucleotide sequence accession numbers for the 16S rRNA’s used for comparison in this study are as follows: *E. chaffeensis*, AF416764; *E. canis*, M73221; *E. ewingii*, M73227; *E. muris*, U15527; *A. phagocytophilum*, U02521; Anaplasma-like deer organism, U27103; *E. ruminantium*, X62432; *R. rickettsii*, M21293; *Ehrlichia* sp. IS58, AB074460; *Ehrlichia*-like sp. ‘Schotti’, AF104680; *Ehrlichia* sp. ‘Rattus’, AY135531; *Ehrlichia*-like sp. TK44556, AB084582.

The 16S rRNA gene sequence reported in this study has been deposited into GenBank, where it received accession number AY781777.
**Statistical Analysis**

Chi-squared tests were used to assess whether the occurrence of antibodies to *E. chaffeensis*, *E. canis*, and *A. phagocytophilum* were independent of each other. Chi square analyses were also used to assess independence between IFA antibodies and PCR data.

**RESULTS**

**Animal and tick collections**

Sixty raccoons and 17 opossums were sampled from 6 contiguous counties in northeast Georgia. Ticks were collected from 38 of 60 raccoons, the number of ticks collected per animal ranged from zero to 72, and five tick species were identified. Twenty-eight of 38 infested raccoons (73.7%) harbored *D. variabilis*, 17 of 38 (44.7%) had *A. americanum*, 17 of 38 (44.7%) had *I. texanus*, 11 of 38 (28.9%) had *I. cookei*, and 2 of 38 (5.3%) had *I. scapularis*. Ticks were not observed on the 17 opossums.

**Serology**

Twenty-three of 60 raccoons (38.3%) had antibodies reactive to *E. chaffeensis* at a titer ≥ 1:64, with a maximum titer of 1:512 and a GMT of 227. Thirteen (21.7%) had antibodies reactive to *E. canis* at a titer ≥ 1:64, with a maximum titer of 1:1024 and a GMT of 186. One (1.7%) had antibodies reactive to *A. phagocytophilum* at a titer of 1:256 (Table 1). None of the opossums had detectable antibodies to *E. chaffeensis*, *E. canis*, or *A. phagocytophilum* at the cutoff dilution of 1:64 (Table 1).
PCR Assay and Sequence Analysis

All 60 raccoons were PCR negative for *E. chaffeensis* and *E. ewingii*; however, one raccoon was PCR positive for *E. canis* (Table 1). Sequence analysis of this PCR product confirmed 100% identity with the registered *E. canis* 16S gene sequence in GenBank (Accession number M73226).

Products approximating the expected 411 bp size were produced when 32 of 60 raccoons (53.3%) were tested with *A. phagocytophilum* 16S rDNA PCR primers (Table 1). Viable nucleotide sequences were obtained for 29 PCR products. Each of these products possessed 100% identity to each other, but contained 21 nucleotide differences in comparison with published *A. phagocytophilum* 16S rDNA sequences (94.5% identity). Comparison with the DNA sequences in the GenBank revealed that this 16S rRNA gene sequence grouped with but differed from all other published *Ehrlichia* and *Anaplasma* 16S gene sequences. For this reason, we designate this sequence as the ‘*Ehrlichia*-like’ organism of raccoons. All opossums tested negative by PCR assay for *E. chaffeensis*, *E. canis*, *A. phagocytophilum*, and *E. ewingii*.

Phylogenetics/comparisons

The neighbor joining, maximum parsimony, and minimum evolution methods used to construct phylogenetic trees produced similar results. Tree stability was estimated with bootstrap analysis using 100 replications within the same program. The *Ehrlichia*-like organism sequence of the 16S rRNA gene clustered within the phylogenetic tree with other *Ehrlichia*-like isolates, in a clade separate from described monocytotropic and granulocytotropic *Ehrlichia* and *Anaplasma* species (Fig. 1). Our sequences were most similar (98-99%) but not identical to the published sequences of *Ehrlichia*-like sp. ‘Schotti variant’of Dutch *I. ricinus* ticks (Schouls et al. 1999), *Ehrlichia* sp. IS58 gene for 16S rRNA and TK4456 gene for 16S rRNA found in Japanese
Ixodes ticks and wild rats, and Ehrlichia sp. ‘Rattus strain’ of Chinese rodents (Rattus spp.) (Pan et al. 2003). Our sequences were 95.0% identical to E. chaffeensis, 94.7% identical to E. ewingii, 94.7% identical to E. ruminantium, 94.5% identical to A. phagocytophilum, 94.2% identical to E. muris, and 93.9% identical E. canis.

Statistical Analyses

All chi-square tests indicated independence between E. chaffeensis, E. canis, and A. phagocytophilum IFA results ($\chi^2 < 3.84$; df=1; $\alpha=.05$). Similarly, E. canis and Ehrlichia-like organism PCR results were independent of E. chaffeensis, E. canis, and A. phagocytophilum serologic status ($\chi^2 < 3.84$; df=1; $\alpha=.05$).

DISCUSSION

This field survey of two common meso-mammals from the Piedmont physiographic region of Georgia found evidence of naturally occurring infections by multiple Ehrlichia and Anaplasma species in raccoons but not in opossums. The stronger evidence for involvement of raccoons is consistent with previous serologic surveys for E. chaffeensis antibodies (Lockhart et al 1997a; Comer et al. 2000) and with serologic, molecular, and xenodiagnostic surveys for A. phagocytophilum (Levin et al. 2002). In contrast to raccoons, previous studies have produced limited evidence that opossums are involved in the natural history of E. chaffeensis (Anderson et al. 1992a; Lockhart et al. 1997a) or A. phagocytophilum (Levin et al. 2002). While the current study concurs with these findings, the epidemiologic role of opossums can not be defined solely on this evidence given the small sample size (n=17) tested and the absence of ticks observed on these animals.

Prior investigations have disclosed E. chaffeensis seroprevalences of 42-100% in WTD, domestic goats, and dogs, but a lower seroprevalence (approximately 20%) in raccoons from
enzootic areas (Arens et al. 2003; Steiner et al. 1999; Irving et al. 2000; Lockhart et al. 1995, 1996, 1997a, 1997b, Dugan et al. 2000; Dawson et al. 1994b; Comer et al. 2000). In many of the studies involving WTD, goats, or dogs, positive *E. chaffeensis* PCR results were detected in 16-75% of the animals positive from seropositive populations (Arens et al. 2003; Lockhart et al. 1997a, 1997b; Dugan et al. 2000, 2004; Dawson et al. 1996b; Kordick et al. 1999; Breitschwerdt et al. 1998). Similarly, experimental *E. chaffeensis* infection trials in WTD, dogs, and red foxes have resulted in both seroconversion and positive PCR results from blood (Dawson et al. 1994a; Ewing et al. 1995; Zhang et al. 2003; Davidson et al. 1999). Although the detection of *E. chaffeensis*-reactive antibodies is consistent with prior findings in raccoons, our inability to confirm infection within this seropositive raccoon population was unexpected and differed from both field and experimental studies of several other wild and domestic species. This finding could result from at least two scenarios. First, infection dynamics in raccoons may differ from those of other species, with either no or very transient rickettsemia. Alternatively, the antibodies detected may represent cross-reactivity, which is well documented among certain ehrlichiae/anaplasmal organisms (Maeda et al. 1987; Dawson et al. 1996a). The second scenario is intriguing because over half of the seropositive raccoons also were PCR positive for a novel *Ehrlichia*-like organism. Our findings indicate that *E. chaffeensis* seroreactions among raccoons should be interpreted with caution and that experimental infection studies likely will be necessary to resolve the role of raccoons as natural hosts for *E. chaffeensis*.

Recent findings demonstrate that *A. phagocytophilum* exists in an enzootic cycle within both rodents and meso-mammals in New England where the tick vector, *I. scapularis*, is common. Studies of Connecticut raccoons (Levin et al. 2002) and Nantucket Island cottontail rabbits (Goethert and Telford 2003) established a 90% prevalence of *A. phagocytophilum*—
reactive antibodies with approximately 20-46% of animals testing PCR positive as confirmed by nucleotide sequencing. *Anaplasma phagocytophilum* infection rates in *Ixodes* ticks collected directly from animals and in ticks allowed to molt after removal from animals were as high as 40% and 23%, respectively. Additionally, *I. scapularis* obtained from raccoons transmitted *A. phagocytophilum* infection to white-footed mice (Levin et al. 2002). In contrast, our survey of Georgia raccoons found limited evidence of *A. phagocytophilum* infection with only one animal possessing *A. phagocytophilum*-reactive antibodies. This raccoon was also seropositive for *E. canis* and PCR positive for the *Ehrlichia*-like organism. *Ixodes scapularis*, the only proven vector for *A. phagocytophilum*, was observed infrequently (3%) on the raccoons examined. Thus, the apparent lower prevalence of *A. phagocytophilum* infection in this Georgia raccoon population may reflect a marked difference in vector abundance compared to Connecticut (Levin et al. 2002).

To our knowledge, this is the first report of serologic and molecular evidence of *E. canis* in raccoons. Approximately one fifth of raccoons were seropositive for *E. canis*-reactive antibodies with four animals possessing maximum titers of 1024; however, cross reactivity is known to occur between *E. canis* and both *E. chaffeensis* and *E. ewingii* which complicates interpretation of serologic data alone (Maeda et al. 1987; Anderson et al. 1991, 1992b; Dawson et al. 1996a). The positive PCR assay from a seropositive animal and the nearly significant association ($\chi^2 = 3.68, p \leq 0.10$) between PCR positivity and serology confirm that at least some of the antibody titers represent *E. canis* infection. Furthermore, the two proven *E. canis* vectors, *R. sanguineus* and *D. variabilis* (Groves et al. 1975; Lewis et al. 1977; Johnson et al. 1998), are known to parasitize dogs and medium-sized wild mammals (James and Harwood 1969; Kollars, 1993) allowing for inter-species transmission in suburban settings where many of the raccoons in
this study were obtained. Collectively, these results suggest that raccoons may play a limited role as a vertebrate reservoir for this pathogen.

Ascertaining *E. ewingii* infection status is difficult because PCR is the only diagnostic tool available. Despite this limitation, PCR surveys have detected *E. ewingii* in up to 28% of WTD from the southeastern United States (Yabsley et al. 2002; Arens et al. 2003) and up to 23% of domestic dogs from Oklahoma and Missouri (Murphy et al. 1998; Liddell et al. 2003). The absence of molecular evidence of *E. ewingii* among raccoons suggests that the role of these animals in the natural maintenance of this organism may be negligible compared to that of WTD in this region of Georgia.

Our data demonstrate that a new *Ehrlichia*-like organism is prevalent among raccoons in the Piedmont physiographic region of Georgia. This organism appears to be most closely related to four other uncharacterized *Ehrlichia*-like organisms including the *Ehrlichia*-like sp. ‘Schotti variant’ (Schouls et al. 1999), *Ehrlichia* sp. ‘Rattus strain’ (Pan et al. 2003), *Ehrlichia* sp. IS58 and *Ehrlichia*-like sp. TK44556 (Kawahara et al. 2004), each of which occurs outside of the United States. Recently, ultrastructure and phylogenetic analyses of these and other unclassified Anaplasmataceae has lead to the proposal of a novel candidate genus and species, *Candidatus ‘Neoehrlichia mikurensis’*, incorporating the four *Ehrlichia*-like sequences to which our sequence from raccoons was very closely related, along with two additional novel sequences (Kawahara et al. 2004). From the perspective of epidemiologic surveys, the presence of this novel *Ehrlichia*-like organism is important because of its ability to confound 16S PCR assays for *A. phagocytophilum* and potentially cause serologic cross reactions with *E. chaffeensis* or other species. Future work to characterize this raccoon *Ehrlichia*-like organism should include
expanded gene sequencing and phylogenetic assessment, experimental infection studies, isolation attempts, and development of specific diagnostic assays.

ACKNOWLEDGMENTS

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TABLE 3.1. SEROLOGIC AND MOLECULAR EVIDENCE OF *EHRLICHIA* AND *ANAPLASMA* INFECTION DETECTED AMONG 60 RACCOONS FROM NORTHEASTERN GEORGIA

<table>
<thead>
<tr>
<th>Species</th>
<th>IFA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. chaffeensis</em></td>
<td>23/60 (38.3%)</td>
<td>0/60 (0%)</td>
</tr>
<tr>
<td></td>
<td>1:64 8/60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:128 5/60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:256 7/60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:512 3/60</td>
<td></td>
</tr>
<tr>
<td><em>E. canis</em></td>
<td>13/60 (21.7%)</td>
<td>1/60 (1.7%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1:64 7/60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:128 1/60</td>
<td></td>
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<tr>
<td></td>
<td>1:256 0/60</td>
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<tr>
<td></td>
<td>1:512 1/60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1024 4/60</td>
<td></td>
</tr>
<tr>
<td><em>E. ewingii</em></td>
<td>NA</td>
<td>0/60 (0%)</td>
</tr>
<tr>
<td><em>A. phagocytophilum</em></td>
<td>1/60 (1.7%)</td>
<td>0/60 (0%)</td>
</tr>
<tr>
<td></td>
<td>1:256</td>
<td></td>
</tr>
<tr>
<td><em>Ehrlichia</em>-like GA</td>
<td>NA</td>
<td>32/60 (53.3%)</td>
</tr>
<tr>
<td>raccoon variant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Titers ≥ 1:64 considered positive for IFA assays.

<sup>b</sup> Sequence confirmed 100% identical with *E. canis* GenBank Accession M73226.

NA, not available
FIG. 3.1. Phylogenetic tree comparing 380 bp of 16S rDNA sequences from related organisms using neighbor joining model; bootstrap values represent neighbor joining/minimum evolutionmaximum parsimony models. A sequence from this study.
CHAPTER 4

EVALUATION OF WHITE-TAILED DEER (ODOCOILEUS VIRGINIANUS) AS NATURAL SENTINELS FOR ANAPLASMA PHAGOCYTOPHILUM

ABSTRACT

*Anaplasma phagocytophilum*, the causative agent of human granulocytotropic anaplasmosis, can infect white-tailed deer (WTD; *Odocoileus virginianus*), and this species represents a host for adult *Ixodes scapularis*, the primary vector of *A. phagocytophilum*. The goal of this study was to determine the geographic distribution of *A. phagocytophilum* among WTD across a 19 state region and to evaluate the utility of WTD as natural sentinels. Serologic testing using the indirect fluorescent antibody (IFA) assay was conducted on WTD serum samples and molecular and xenodiagnostic tests were performed to confirm serologic results. The surveillance system was assessed through examination of vital attributes including WTD age and gender associations with serologic status, sample size adequacy for accurate infection status classification, and presence of the vector, *I. scapularis*. Six hundred thirty-three of 2,666 (24%) WTD in 17 states tested positive for antibodies (≥ 128) when tested by indirect fluorescent antibody assay. PCR testing for p44 and/or 16S rRNA gene targets identified 73 (16%) of 458 positive WTD, all of which originated from 88 seropositive populations. Attempts to culture *A. phagocytophilum* isolates from WTD were unsuccessful; however, xenodiagnostic mice inoculated with wild WTD blood became infected. Deer age and gender were not associated with seroprevalence; however, WTD ≤ 0.75 years old had higher PCR positivity. Using seroprevalence data, a sample size of 6-9 animals per population was projected to accurately identify seropositive populations. The presence of *I. scapularis* was significantly associated with *A. phagocytophilum*-reactive antibodies in WTD. Collectively, the results of this study demonstrate that WTD would be suitable natural sentinels this emerging zoonotic pathogen.
INTRODUCTION

Anaplasma phagocytophilum, formerly known as Ehrlichia equi, E. phagocytophila and the “human granulocytotropic ehrlichiosis (HGE) agent” (Dumler et al., 2001) is currently recognized as the cause of human granulocytotropic anaplasmosis (HGA). Individuals with HGA typically have a history of a tick bite and clinically present with an acute, non-specific febrile illness though symptoms may range from subclinical to fatal (McQuiston et al., 1999). Since the initial description of HGA in 1994, over 1,220 cases have been reported in the United States with the majority diagnosed in northeastern and north central regions (Chen et al., 1994; Paddock and Childs, 2003). While HGA is a nationally notifiable disease, only 20 states currently require reporting of cases to state health departments (Gardner et al., 2003). This organism is also of veterinary medical importance, causing clinical disease in horses and dogs in the United States and in sheep, goats, and cattle in Europe (Dumler et al., 2001).

Anaplasma phagocytophilum is vectored in the northeastern and north central United States by Ixodes scapularis, the black-legged tick (Telford et al., 1996; Curran et al., 2000; Layfield et al., 2002) and by I. pacificus in northwestern states (Barlough et al., 1997; Kramer et al., 1999; Lane et al., 2001). While many small rodents and medium-sized mammals are both naturally infected and competent hosts of A. phagocytophilum (Walls et al., 1997; Nicholson et al., 1998, 1999; Magnarelli et al., 1999b; Levin et al., 2002; Goethert and Telford, 2003), the white-footed mouse (Peromyscus leucopus) is believed to be the primary reservoir host in the northeastern and north central United States (Telford et al., 1996; DesVignes and Fish, 1997; Magnarelli et al., 1997).

White-tailed deer (WTD; Odocoileus virginianus) have been identified as a host for A. phagocytophilum (Belongia et al., 1997; Little et al., 1998; Bakken and Dumler, 2000; Arens et
al., 2003; McQuiston et al., 2003). Because WTD are principally parasitized by adult *I. scapularis* (Mount et al., 1997), they are thought to be consistently exposed to *A. phagocytophilum* in nature. Evidence of *A. phagocytophilum* infection has been determined in clinically normal WTD from seven states in the Atlantic coastal region, midwest, and north central United States. Seropositive and PCR positive WTD, confirmed by either western blot or sequence analyses, respectively, have been identified in Connecticut, Georgia, Indiana, Maryland, Missouri, South Carolina, and Wisconsin (Belongia et al., 1997; Little et al., 1998; Walls et al., 1998; Magnarelli et al 1999a, 2004; Arens et al., 2003). Most recently, 4 WTD experimentally infected with a human strain of *A. phagocytophilum* developed reactive antibodies as early as 14 days post inoculation (dpi) and remained seropositive through 49 to 66 dpi. These data support the contention that wild WTD exposed to *A. phagocytophilum* mount a detectable, long-term immunologic response, an important characteristic for sentinel surveillance species (Tate et al., 2005a). Collectively, these results suggest that WTD are involved in enzootic *A. phagocytophilum* transmission, possibly serving a minor role as a reservoir host, and that WTD could be utilized as a sentinel species for human risk of *A. phagocytophilum* (Belongia et al., 1997; Little et al., 1998; Walls et al., 1998; Magnarelli et al., 1999a, 2004; Tate et al., 2005a).

Recent investigations on *Ehrlichia chaffeensis*, the causative agent of human monocytotropic ehrlichiosis (HME), (Dawson et al., 1994; Lockhart et al., 1997a, 1997b; Mueller-Anneling et al., 2000) lead to the development of a prototype WTD sentinel surveillance system for *E. chaffeensis* and the projected distribution of *E. chaffeensis* across a 19 state region (Yabsley et al., 2003; 2005). White-tailed deer have specific advantages as a surveillance species for certain tick-transmitted zoonoses such as HME (Yabsley et al., 2003) and HGA.
These include 1) broad distribution rendering them suitable for surveillance at national, regional, state-wide, and/or county levels, 2) high population densities in rural, urban, and suburban locations, 3) limited home range, 4) high rate of exposure to multiple tick species, 5) long average life expectancy, 6) lack of exposure to antibiotics and acaracides, and 7) state-wide regular hunting seasons which facilitate consistent, cost efficient sample collection.

The central aim of this study was to evaluate the utility of WTD as natural sentinels for *A. phagocytophilum* in a broad surveillance system across a multi-state region. The specific objectives for accomplishing this overall goal included: 1) compilation of an extensive regional *A. phagocytophilum* serologic database for WTD, 2) application of PCR assays and sequence analysis to confirm *A. phagocytophilum* serologic results among WTD populations, 3) employment of xenodiagnostic and tissue culture techniques in attempts to isolate *A. phagocytophilum* from naturally infected WTD, 4) determination of age and/or gender relationships to serologic and infection status among WTD, and 5) estimation of sufficient sample sizes required to accurately classify infected and uninfected WTD populations.

**MATERIALS AND METHODS**

**Sample Collections**

Serum samples from a total of 2,666 WTD from 507 populations in 18 states (AL, AR, FL, GA, KS, KY, LA, MD, MO, MS, NC, NJ, OK, SC, TN, TX, VA, and WV) were utilized in this study. The samples were collected either from hunter harvested WTD or during Southeastern Cooperative Wildlife Disease Study (SCWDS) WTD population health evaluation activities, or for other SCWDS research projects. Whole blood was collected in untreated or EDTA treated tubes either from the heart, jugular vein, or from the body cavity from the majority of WTD; however, blood samples from 12 WTD from MO and 5 from NJ were collected using
Nobuto strips and later eluted in phosphate-buffered saline (pH 7.4) as previously described (Mueller-Anneling et al., 2000). Additionally, data from 5 WTD from Ossabaw Island, GA previously tested by Little et al. (1998) were included in this study. Serum, plasma, or eluted blood were frozen at –20° C until serologic testing was performed.

Whole blood samples were collected and stored at –20° C for PCR testing of 333 WTD from 88 seropositive populations and 120 from 20 seronegative populations. Fresh whole blood collected from 49 WTD from 10 populations in August and September of 2001-2003 was overnight shipped on ice and used to inoculate laboratory mice in attempts to isolate *A. phagocytophilum*. Ticks were collected from a third subset of 871 WTD representing 143 populations and submitted to the National Veterinary Services Laboratory, APHIS, USDA, Ames, IA for identification. All sampling procedures were approved by the Institutional Animal Care and Use Committee, College of Veterinary Medicine, The University of Georgia.

**Serology**

Serum, plasma, or eluted blood samples were tested by indirect fluorescent antibody (IFA) assay for the presence of *A. phagocytophilum*-reactive antibodies as previously described (Yeh et al., 1997). *Anaplasma phagocytophilum* antigen slides (human strain; Focus Technologies, Cypress, CA) were used to screen samples at a 1:128 dilution, a 1:50 dilution of fluorescein isothiocyanate (FITC) labeled rabbit anti-deer immunoglobulin G (Kirkegaard and Perry Laboratories, Gaithersberg, MD) was used as a conjugate, and a 1:50 dilution of eriochrome black T counterstain (Sigma-Aldrich, St. Louis, MO) was used in the final wash step. One hundred and eighty-five seropositive WTD samples were further tested in two-fold serum dilutions to an endpoint and a geometric mean titer (GMT) was calculated (Villegas, 1998).
Molecular assays

Multiple PCR assays were evaluated as potential means of molecular confirmation of *A. phagocytophilum* in wild WTD because the routinely used nested 16S rRNA PCR assay (primers ECC/ECB, Dawson et al., 1994; GE9F/GA1UR, Little et al., 1998) amplifies both *A. phagocytophilum* and an *Anaplasma* sp. of WTD (Little et al., 1997; Munderloh et al., 2003). Prior comparisons of this 16S rRNA PCR assay with assays for other gene targets determined the *p44* gene amplifying primers MSP3F/MSP3R (Zeidner et al., 2000) to more specific and equally as sensitive as the 16S nested primers (Massung and Slater, 2003). We also performed comparisons between these assays and the *groESL* gene primers HS1/HS6 and HS43/HSVR (Sumner et al., 1997; Lotric-Furlan et al., 1998) to test the sensitivity and specificity with regard to amplification of *A. phagocytophilum*. To evaluate specificity, PCR was performed on cell culture extracted DNA from for *E. chaffeensis*, *E. canis*, *A. phagocytophilum*, *Anaplasma* sp. of WTD, an *E. coli* transformed plasmid containing 16S rRNA of *E. ewingii*, and *A. phagocytophilum* sequence positive blood from experimental and wild WTD. To evaluate sensitivity, 10-fold serial dilutions of *A. phagocytophilum* DNA extracted from cell culture and WTD blood were tested using each primer set.

The *p44* assay was confirmed as more sensitive and specific than the *groESL* PCR assay and as sensitive as the 16S rRNA PCR assay (data not shown) as previously described (Massung and Slater, 2003; Magnarelli et al., 1999b) and was utilized as the initial validation assay; 16S gene PCR was also performed on all *p44* positive samples as previously described (Little et al., 1998).

DNA was extracted from 300 µl of whole blood from 453 WTD using the GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NY) following the
manufacturer’s protocol. For the $p44$ gene assay, reactions were subjected to the following cycling parameters: 94°C (1 min), 55°C (45 sec), and 72°C (1 min) for 40 cycles total. For the $groESL$ gene assay, both primary and secondary reactions were initially denatured at 95°C for 5 min. Primary PCR reactions were subjected to 40 cycles at 95°C (30 s), 52°C (30 s), 72°C (60 sec); secondary reactions were subjected to 30 cycles at 95°C (30 s), 55°C (30 s), 72°C (60 sec).

For all assays, PCR amplification was performed in 25 µl reactions using a PTC-100 MJ Research thermalcycler (MJ Research Inc., Waltham, MA). Five microliters of extracted DNA was tested by either standard single step $p44$ gene PCR or primary 16S/$groESL$ PCR using 0.8 uM each of respective primers in a reaction containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.4 mM Mg Cl$_2$, 0.2 mM each dNTP, and 2.5 units Taq DNA polymerase (Promega US, Madison WI). For the nested 16S and $groESL$ PCR assays, one microliter of primary product was used as a template in a secondary 25 µl reaction containing the same PCR reagents and specific primers.

Products were separated by gel electrophoresis in either a 1% or 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. Non-template negative controls were included in each step of the assay (DNA extraction, primary, and if applicable secondary amplifications) and an $A. phagocytophilum$ DNA control was included with each set of PCR reactions. All phases were performed in separate, designated PCR cabinets located in separate laboratories to minimize sample contamination.

**Sequencing and Nucleotide Accession Numbers**

A subset of $p44$ and 16S gene PCR positive WTD samples ($n = 22$) from seropositive populations were purified via the Qiagen Gel Purification kit (Qiagen, Inc., Valencia, CA), and sequenced in the forward and reverse directions with either a Perkin Elmer ABI Prism 3700
Xenodiagnostic Isolation

Previous DH82 and ISE6 cell culture attempts for *Ehrlichia/Anaplasma* using WTD blood have been difficult and often unsuccessful due to the presence of *Trypanosoma cervi*, a commonly found flagellated parasite specific to WTD (Lockhart et al., 1997a; 1997b; Munderloh et al., 2003). To avoid culture contamination with trypanosomes in this study, WTD blood was cultured indirectly for *A. phagocytophilum* using a xenodiagnostic mouse model. Previous research validated this xenodiagnostic system as being suitable for infection of laboratory mice using cell culture grown *A. phagocytophilum* (HGE-1 human strain) (Tate et al., 2005a). For these xenodiagnostic assays, 300-400 µl of EDTA whole WTD blood was each injected intraperitoneally (IP) into 1-3 C3H/He3 4-6 week old mice (Harlan Laboratories, Indianapolis, IN). Due to logistic constraints, WTD blood was PCR tested using the assays described above only after mouse inoculation.
Mice were sedated via subcutaneous injection with xylazine (100 mg/kg body weight; Mobay Corp., Animal Health Division, Shawnee, KS) mixed with ketamine hydrochloride (10 mg/kg; Aveco Co., Inc., Fort Dodge, IA) for sample collections. Whole blood, sera, and blood smears were obtained from each mouse before inoculation and every 3-7 days thereafter. Approximately 200-300 ul of whole blood was collected from inoculated mice via retro-orbital bleeding using heparinized hermaocrit tubes at each sampling date. Blood was spun @ 2500 rpm for 10 min to separate sera, and the remaining portion was used immediately for DNA extraction and 16S/p44 PCR. Sera was frozen and stored at –70°C for IFA assays and blood smears were Giemsa-stained (Karyomax, GIBCO; Grand Island, NY) and examined by light microscopy for morulae.

IFA assays were performed on all mice inoculated with sequence confirmed *A. phagocytophilum* and these samples were initially screened at 1:64 and 1:128 dilutions with positive samples further tested in twofold dilutions to an endpoint. A 1:50 dilution of FITC-labeled goat anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Gaithersberg, MD) was used as a conjugate.

HL-60 culture attempts as previously described (Goodman et al., 1996) were performed at both the University of Georgia and the University of Minnesota on blood samples from xenodiagnostic mice at 6, 10, 16, 18, 24, and 28 dpi. Portions of cell culture samples obtained during culture feeding were tested by PCR and used to produce Giemsa-stained cytospins, as previously described (Tate et al., 2005a). Blood from mice testing PCR positive was used to inoculate HL-60 cell cultures and intraperitoneally inoculated into naïve mice. Mice inoculated with *A. phagocytophilum* PCR negative WTD blood were euthanized with CO₂ at approximately
16 dpi; mice inoculated with *A. phagocytophilum* PCR positive WTD blood were euthanized at 24-28 dpi and exsanguinated by cardiac puncture.

**Data Analysis**

Serologic data from this study (*n* = 2666) were combined with data from a previous study conducted by Little et al., 1998 (*n* = 5) to assemble a map of the distribution of *A. phagocytophilum* seropositive WTD (titers ≥ 1:128) in the southeastern and south central United States. Serostatus of WTD populations were categorized by parish or county. If one or more WTD possessed *A. phagocytophilum*-reactive antibodies, that WTD population and its corresponding county or parish was classified as seropositive; if all WTD tested seronegative, the population was classified as seronegative. Sample sizes of *n* ≥ 5 were used for assigning a negative serostatus except for Texas where *n* ≥ 2 was used because of limited samples. Statistical confidence intervals (CI) were calculated using guidelines set by Newcombe 1998; chi-square analysis was used to determine if differences existed between age classes and gender for seroprevalence.

PCR assays and sequence analyses were used to confirm serologic data for 333 WTD from 88 seropositive populations and 120 WTD from 20 seronegative populations. Chi-square analysis was used to test for differences in PCR prevalence between seropositive and seronegative populations. Prevalence of PCR positivity among different age and gender classes were determined for WTD in seropositive populations. Chi-square analyses were used to test for differences in PCR positivity among age and gender classes.

The minimal sample size required to accurately categorize the serostatus of a WTD population was evaluated. To determine the minimum number of WTD samples needed to detect the presence of seropositive WTD in a population, the formula \( n = (1 - (1-a)^{1/D})(N - (D - \)
1)/2) was used as previously described (Thrusfield, 1995). To accurately classify seronegative populations, which are more difficult to confidently classify, two testing methods were employed; for the first, larger numbers of WTD \( (n = 8 - 39) \) were tested from 18 seronegative counties/parishes to enhance the detection of potentially low prevalences within those populations. Secondly, testing was performed on 67 WTD samples collected over several years that were located in 4 *I. scapularis* free locations. Additionally, chi-square analysis was used to assess if an association existed between the presence of *I. scapularis* and the *A. phagocytophilum* serostatus of WTD at the population level.

Chi-square analyses were used to assess the association between presence or absence of *I. scapularis* and *A. phagocytophilum* serologic status at the population level using tick data collected during this study. However, the majority of WTD populations studied were evaluated in the summer months when adult *I. scapularis* are rare or absent on WTD (Soneshine, 1993); and consequently we an additional alternative approach for comparison of serologic data with the distribution of *I. scapularis*. *Ixodes scapularis* population data obtained from Dennis et al. (1998) was used to increase the number of counties represented by serologic testing in this study to 315. In addition, this association was further evaluated through chi-square analysis comparing the seroprevalence between a geographic region where contiguous *I. scapularis* populations predominated and a region where *I. scapularis* was uniformly absent (158 total counties) based on Dennis et al. (1998). The first region (established *I. scapularis* populations) encompassed 1-3 tiers of counties along the Atlantic coastal region including SC, GA, all of FL, SW corner of AL, majority of MS, all of LA, western two-thirds of AR, and all of TX. The second region (*I. scapularis* populations absent) included the majority of WV, the western and southwestern 2-3
tiers of counties in VA and NC, extreme NW and NE GA, and the eastern quarter of TN and the eastern third of KY. These areas were separated by a broad buffer zone that was not analyzed in this approach.

**RESULTS**

**Regional serologic database for WTD**

Of the 2666 WTD serologically tested, 633 (23.74 %; CI95% = 22.2%, 25.4%) animals possessed *A. phagocytophilum*-reactive antibodies at titers ≥ 128 (Table 4.1.). For seropositive populations, the mean prevalence of antibodies reactive to *A. phagocytophilum* was 47.22% (SD = 26.9%; range 6.6 - 100%). One hundred eighty five seropositive WTD possessed a modal antibody titer of 128, a maximum titer of 4096, and a GMT of 409.

Seropositive WTD were detected in every state with the exception of West Virginia but the overall distribution of seropositive populations was patchy and uneven across this multistate region (Fig 4.1). Seropositive populations predominated within the coastal plain regions of the Atlantic coastal states and in a broad area centered around the lower Mississippi Valley. A large cluster of negative populations occurred in the Appalachian Mountain region extending from western Maryland and West Virginia to northern Georgia and Alabama. A western boundary was also apparent in Kansas, Oklahoma, and Texas and an irregular northern boundary extended across Kansas, Missouri, and Kentucky.

**Molecular, xenodiagnostic, and cell culture validation of *A. phagocytophilum***

Of the total 458 WTD PCR tested, including 5 WTD from Chatham Co., GA that only were tested by 16S PCR (Little et al., 1998), 73 (16%) were positive with either *p44* or 16S PCR assay. Seventy of 333 (21%) WTD from seropositive populations were PCR positive for *A. phagocytophilum* *p44* gene fragments; all *p44* PCR positive WTD also were positive by 16S
gene PCR assay. In contrast to seropositive populations, p44 and 16S PCR assays on 120 WTD from 19 seronegative populations were uniformly negative. The presence of PCR positive WTD was strongly associated with seropositive populations ($\chi^2 = 66.2; \text{df}=1, p \leq 0.001$).

Sequence analyses of A. phagocytophilum p44 PCR products from 22 WTD in 11 seropositive populations (Chatham Co., Liberty Co., McIntosh Co., GA; Bossier Par. and Webster Par., LA; Dare Co., NC; Muskogee Co., OK; Georgetown Co., SC; Albermarle Co., Prince William Co., VA, Stafford Co., VA) produced 4 different DNA sequences. Seventeen of 22 sequences were 100% identical to each other (hereafter referred to as p44 WTD variant 1) and were obtained from one or more WTD in each of these populations except for Chatham Co., GA. Sequences from two WTD, one from Chatham Co., GA and one from Albermarle Co., VA, possessed 100% identity to each other (WTD variant 2), 2 sequences (one from Chatham Co., GA and one from Georgetown Co., SC) possessed 100% identity to each other (WTD variant 3), and a single WTD from Georgetown Co., SC produced an additional sequence (WTD variant 4). A complete alignment of these four variants showed differences at 3 positions within the 339 consensus sequence: variant 2 possessed C/T at consensus position 140, variant 3 possessed A/T at position 282, and variant 4 possessed an A/T at base position 282 and C/T at position 302. In comparison to the published p44 gene sequences of North American A. phagocytophilum strains available in GenBank, the p44 WTD variant 1 was most similar to (99.7%) the p44-12 gene of the HZ-13 isolate from humans (Zhi et al., 1999). Variant 1 also had a high degree of similarity (97.3%-98.8%) with other North American strains of A. phagocytophilum (Zhi et al., 2002; Murphy et al., 1998; Barbet et al., 2003; Ijdo et al., 1998; Ravyn et al., 2001; Lodes et al., 2001;
Lin et al., 2004). Variants 2-4 also were most similar (99.3-99.4%) to the $p44-12$ gene of the HZ-13 human isolate and were 97.6 - 99.6% similar to other North American isolates of *A. phagocytophilum*.

Products amplified by 16S PCR produced 22 *A. phagocytophilum* DNA sequences from 10 seropositive WTD populations (Chatham Co., and McIntosh Co., GA; Bossier Par., LA; Washington Co., and Frederick Co., MD; Dare Co., NC; Georgetown Co., SC; Albermarle Co., Prince William Co., and Stafford Co., VA). Fifteen sequences possessed 100% identity to each other and with the full length *A. phagocytophilum* sequence from WTD/*I. scapularis* as reported by Massung et al. (1998); 1 sequence possessed a single base difference from the *A. phagocytophilum* sequence of WTD/*I. scapularis* at consensus position 135. The full-length sequences of six *A. phagocytophilum* products could not clearly be determined due to the occurrence of a secondary DNA sequence within the initial 76-84 consensus region of the 16S rRNA gene.

Of 49 PCR positive WTD blood samples inoculated into mice, 8 injected into 16 mice (2 per WTD) contained sequence confirmed *A. phagocytophilum* DNA. Of these mice, one tested 16S and $p44$ PCR positive on 6 and 10 dpi and two other mice (inoculated with blood from 1 WTD) became 16S and $p44$ PCR positive at 16 dpi. Sequence analysis of 16S PCR products obtained on dpi 6 and 10 for one mouse were confirmed to be *A. phagocytophilum* and were 100% identical to the sequence obtained from the WTD blood inoculum. Nine of the 16 mice seroconverted as early as 9 dpi and remained seropositive ($\geq 64$) when last tested at 24 dpi. Antibody titers ranged from 64 to 128 for 8 mice but one mouse had a maximum titer of 256 at 22 dpi.
All HL60 culture attempts using blood from xenodiagnostic mice receiving PCR positive WTD blood were unsuccessful in isolating *A. phagocytophilum* in both laboratories. *Anaplasma phagocytophilum* morulae were never detected on Giemsa stained blood smears from experimental mice. Mice inoculated with PCR positive mouse blood were IFA, PCR, and cell culture negative throughout the course of study.

**Relationships of age and gender to antibody prevalence and PCR detection**

Age data was available for 2,230 WTD tested by IFA assay and gender data was available for 2,109 WTD. The six classes used to classify WTD age included: 1) ≤ 0.75 years, 2) 0.76 – 1.5 years, 3) 1.6 – 2.5 years, 4) 2.6 – 3.5 years, 5) 3.6 – 4.5 years, 6) > 4.5 years. Chi-square analysis indicated no significant differences in serologic status within 6 age classes of WTD ($\chi^2 < 11.07; \text{df}=5; \alpha=.05, p\leq 1$) (Fig 2). Nor were differences detected in seroprevalence between male and female WTD (51.3% male vs 48.7% female; $\chi^2 < 3.84; \text{df}=1; \alpha=.05, p\leq 0.1$).

Age and gender were available for 272 WTD from seropositive populations that were tested by PCR. Chi-square analysis indicated no difference in PCR positivity between male and female animals ($\chi^2 < 3.84; \text{df}=1; \alpha=.05, p\leq 1$); however, the youngest age class (≤ 0.75 years) had significantly higher proportions of PCR positive animals (28.6%) than the 1.6 – 2.5 age class (9.5%; $\chi^2 < 5.98; \text{df}=1, p\leq 0.025$). All other age-class comparisons produced non-significant results. Because of small sample sizes for the three oldest age classes (2.6 – ≥ 4.5 years), PCR results for these age classes were pooled with the 1.6 – 2.5 age class and reanalyzed relative to ≤0.75 and 0.76 – 1.5 year old animals. With this analysis, PCR positivity again was significantly lower in the 1.6 – ≥ 4.5 year-old animals in comparison with WTD ≤ 0.75 years old ($\chi^2 = 4.27; \text{df}=1, p\leq 0.05$). The mean age of PCR positive WTD was 2.1 years, and PCR positive animals were 0.25 – 6.5 years old.
Evaluation of sample size adequacy

Utilizing the mean of 47% seroprevalence for seropositive populations, post-hoc statistical analyses indicated that testing of 6 to 9 WTD per population would meet 95% and 99% probabilities of detecting at least one seropositive animal within populations (Thrusfield, 1995). Testing 5 WTD per population, the predominant sample size in this study, carried a 93% probability of detecting at least one seropositive animal. Serologic testing of larger numbers of WTD from several seronegative populations and repeated testing of 4 populations over time produced consistent results, suggesting that these were correctly classified as seronegative populations. Application of the sample size strategy involved testing 221 WTD from 18 seronegative populations (range 6 - 37) and produced negative results from a mean of 12.3 WTD per population. The sequential testing strategy involved 67 WTD from 4 populations from which *I. scapularis* was never detected collected across a span of 2-6 years and produced uniformly negative data for *A. phagocytophilum*-reactive antibodies. Confirmation that a population is truly negative is very problematic (Thrusfield, 1995); however, the strategies employed here have been used previously when evaluating WTD for *E. chaffeensis*-reactive antibodies (Yabsley et al., 2003). The overall mean number of WTD tested per population in this study was 5.27 (SD = 3.25; range 1-37) and sample sizes less than 5 WTD were used only if a seropositive animal was detected with the exception of Texas where as few as 2 seronegative samples were used because of small sample sizes per county.

Association between *I. scapularis* and WTD serologic status

*Ixodes scapularis* was infrequently observed parasitizing WTD in this study. Of 143 WTD populations examined for ticks, only 13 (9.1%) were confirmed to be parasitized by *I. scapularis*. Ten of these 13 (77%) populations contained at least one seropositive animal. When
evaluated by the broader geographic approach based on the previously published distribution of *I. scapularis* (Dennis et al., 1998), chi-square analysis of WTD serologic data yielded a significant association between *A. phagocytophilum* antibodies and the presence or absence of *I. scapularis* ($\chi^2 = 31.1$, df=1; $p \leq 0.001$). Furthermore, chi-square analysis of data large geographic areas where *I. scapularis* was either absent or abundant also disclosed a significant association between the presence of the vector and *A. phagocytophilum*-reactive antibodies in WTD ($\chi^2 = 17.2$, df=1; $p \leq 0.001$).

**DISCUSSION**

The overall goal of this study was to evaluate the utility of WTD as natural sentinels for *A. phagocytophilum* across a broad geographic region, as was recently described for *E. chaffeensis* (Yabsley et al., 2003). Specifically, our chief findings relative to this goal include: 1) *A. phagocytophilum*-reactive antibodies in WTD reflect infection with this pathogen, 2) relatively small sample sizes of WTD are suitable for surveillance monitoring, 3) effective surveillance can be achieved by testing any WTD, especially animals $\geq 0.75$ months of age, 4) serologic and molecular evidence of *A. phagocytophilum* infection in WTD was significantly correlated with the presence of *I. scapularis*, the primary tick vector. These findings closely parallel a recent companion study that evaluated WTD as sentinels for *E. chaffeensis* (Yabsley et al., 2003), which lends credence for the utility of WTD as sentinels for *A. phagocytophilum*. Furthermore, the geographic distribution of *A. phagocytophilum* seropositive WTD populations conformed to the projected risk of *Borrelia burgdorferi* infection (Centers for Disease Control and Prevention, 1999), a zoonotic pathogen also vectored by *I. scapularis*.

While a number of studies have detected *A. phagocytophilum* infected WTD populations within several states, this study provides a more comprehensive *A. phagocytophilum* distribution
by using WTD distributed throughout the entire southeastern and south central regions of the United States. Prior evaluations of WTD for evidence of *A. phagocytophilum* exposure have primarily focused on locations where risk of HGA infection is recognized as being higher than the geographic locations tested in this study and consequently have encountered seroprevalences as high as 64% and PCR positivity rates as high as 37% (Magnarelli et al., 1999a; 2004; Walls et al., 1998; Massung et al., 1998; Belongia et al., 1997). In areas of probable lower risk, studies of South Carolina and Missouri WTD previously detected *A. phagocytophilum*-reactive antibodies in 2 and 25% of WTD, respectively (Magnarelli et al., 2004; Arens et al., 2003). Similar to *E. chaffeensis*, seropositive populations were detected in every state sampled except for West Virginia, and *A. phagocytophilum* seronegative WTD were concentrated in the Appalachian Mountain region of the southeastern United States, where WTD are notably free of ticks (Yabsley et al., 2003). Additionally, a western boundary for *A. phagocytophilum* seropositive populations was observed across Kansas, Oklahoma, and Texas and a northern boundary extended from Kansas, through Missouri, and Kentucky. Large clusters of seropositive populations also extended throughout the Atlantic coastal states and the lower Mississippi Valley region. This distribution corresponded well with the reported distribution of *I. scapularis* (Dennis et al., 1998; Centers for Disease Control and Prevention, 1999).

As stated previously, the reliability of a pathogen surveillance system is highly dependent on the accuracy of the diagnostic assays utilized to determine infection status (Yabsley et al., 2003). The IFA serologic assay utilized in this study appeared to be reliable in its ability to identify *A. phagocytophilum* infected WTD populations. PCR assays confirmed *A. phagocytophilum* infection in over 20% of seropositive WTD populations and DNA sequencing supplemented by *in vivo* assays provided additional supportive evidence of *A. phagocytophilum*
infection in WTD. Additionally, seronegative WTD populations were consistently PCR and culture negative. These findings tend to validate the use of serologic assays specifically, IFA assay testing as being effective in detecting *A. phagocytophilum* infection in WTD and further substantiate the use of WTD as sentinel animals for this pathogen.

Potentially important considerations for the use of WTD as surveillance animals include serologic cross-reactivity and non-specific PCR amplification that can occur between ehrlichial and anaplasmal species (Maeda et al., 1987; Dawson et al., 1996; Little et al., 1997; Dugan et al., 2005). Deer from this region are commonly infected with multiple organisms including *E. chaffeensis*, *Anaplasma* sp. of WTD, and *E. ewingii* (Dawson et al., 1996; Lockhart et al., 1997a, 1997b; Little et al., 1997, 1998; Yabsley et al., 2002; Arens et al., 2003; Munderloh et al., 2003; Tate et al., 2005b) and cross-reactions have been reported between *E. chaffeensis* and *A. phagocytophilum*-reactive antibodies in human sera (Comer et al., 1999) and between *E. chaffeensis* and *E. ewingii*-reactive antibodies in human and dog sera (Murphy et al., 1998). A recent study demonstrated that serum from WTD experimentally infected with *Anaplasma* sp. of WTD or *A. marginale* were seronegative when tested against *A. phagocytophilum* by IFA assay (Tate et al., 2005b); however, serum from WTD infected with *E. chaffeensis* (1024 titer) had limited (64 titer) seroreactivity to *A. phagocytophilum* antigens (Tate et al., 2005b). The cross-reactivity with *E. chaffeensis* reported by Tate et al. (2005b) was below the screening dilution of 1:128 used to test deer sera in the current study. Although many seropositive WTD from the present study also had *E. chaffeensis*-reactive antibodies (Yabsley et al., 2003), numerous WTD possessed monospecific antibodies to either organism. No data are currently available regarding *E. ewingii* cross-reactivity in WTD sera, and *A. marginale* can further be discounted because this organism is not present in southeastern WTD (Keel et al., 1995). Collectively, data from these
studies suggest that meaningful cross-reactivity issues do not exist between A. phagocytophilum and related organisms with respect to antibodies in WTD.

Many of the 16S PCR products amplified from WTD possessed dual sequences of both A. phagocytophilum and the Anaplasma sp. of WTD, and the majority of these 16S gene products were determined to be Anaplasma sp. of WTD. This issue of non-specific agent amplification using 16S gene primers was addressed by the employment of an additional, species-specific gene target for PCR assay that amplified p44 gene fragments of A. phagocytophilum. The application of sensitive and specific molecular diagnostic assays to confirm many seropositive populations, together with data from prior studies indicating the specificity of A. phagocytophilum-reactive antibodies in experimental WTD provide strong evidence that the A. phagocytophilum-reactive antibodies detected are specific, and do not represent antibodies to E. chaffeensis, Anaplasma sp. of WTD, or related organisms.

Important considerations of a WTD surveillance system would include the distribution of reactive antibodies and PCR positivity with respect to WTD age and gender (Yabsley et al., 2003) and to our knowledge, previous studies have not investigated either of these relationships. In this study, neither WTD age nor gender was significantly correlated to the presence of A. phagocytophilum-reactive antibodies, suggesting that any WTD, especially animals ≥ 0.75 years of age, are appropriate for use in an A. phagocytophilum sentinel system. Seropositive results were consistently detected in all age classes suggesting that multiple exposures in the wild likely result in increased antibody titers with age and/or that antibodies in WTD are long lasting, both of which are consistent with WTD serologic data generated from an experimental infection trial in WTD with A. phagocytophilum (Tate et al., 2005a). In contrast to the stability of antibody prevalence among WTD age classes, the prevalence of PCR positivity declined with age. This
decrease was notable in all age classes over 0.75 years. These findings are consistent to our earlier *E. chaffeensis* study where antibodies were stable across age classes while rickettsemia, measured by PCR positivity, declined with age (Yabsley et al., 2003). Thus, serologic assays appear to be more effective tools when surveying wild WTD for evidence of both *A. phagocytophilum* and *E. chaffeensis*.

Previous serologic and molecular surveys of WTD for *A. phagocytophilum* have not evaluated the minimum number of WTD needed to correctly classify the infection status of a population (Walls et al., 1998; Arens et al., 2003; Belongia et al., 1997; Magnarelli et al., 1999a, 2004). Infected WTD populations in the southeastern and south central United States in this study had a mean seroprevalence that exceeded 40% and therefore a sample size of 6 or more WTD per population should reliably detect most seropositive populations. This sample size would be logistically feasible in an operational use of a WTD sentinel surveillance system. While this study may not have detected low seroprevalences within populations based on our most frequent sample sizes of 5 WTD, many populations were consistently classified as seronegative based on repeated testing over time and testing of larger sample sizes. In comparison, *E. chaffeensis* surveillance determined that testing an average of 5 WTD per population was sufficient for detection of seropositive WTD; however, the mean seroprevalence of this pathogen is almost 30% higher than *A. phagocytophilum* among WTD in the same region (Yabsley et al., 2003).

The distribution of seropositive and seronegative WTD populations closely paralleled the distribution of *I. scapularis* (Dennis et al., 1998) across the 19 state region, thereby conforming to the expected distribution based on the competence of *I. scapularis* as the critical vector for this pathogen. The distribution of *I. scapularis* within the southeastern and south central United
States (Dennis et al., 1998) had a strong correlation with evidence of *A. phagocytophilum* infection in WTD; seropositive and PCR positive populations were consistently detected in WTD from areas where *I. scapularis* occurs. In contrast, all diagnostic assays of WTD from locations where *I. scapularis* is absent produced negative results. Although detecting the stability and spread of *A. phagocytophilum* among WTD was beyond the scope of this study, the distribution of *A. phagocytophilum* among WTD populations likely is related to various ecologic variables that influence *I. scapularis* such as soil moistures, elevation, wooded wetlands, cropland, and proportion of forest/savannah as described for *E. chaffeensis* and its vector, *Amblyomma americanum* (Yabsley et al., 2005).

Infection dynamics and the reservoir status of WTD with regard to *A. phagocytophilum* remain largely unclear. Recently, Tate et al. (2005a) demonstrated that WTD experimentally infected with *A. phagocytophilum* seroconverted and maintained detectable antibody titers for up to 2 months. Conversely, experimentally infected WTD rarely had PCR positive blood after 17 dpi suggesting humoral mediated clearance of the bacteria. However, PCR detection of *A. phagocytophilum* in bone marrow at 66 dpi indicated a potential for release of organisms sequestered in tissues (Tate et al., 2005a). Given that primarily adult *I. scapularis* parasitize WTD (Lane et al., 1991; Dennis et al., 1998; Rand et al., 2003) and that *A. phagocytophilum* has not been documented to be maintained transovarially, Tate et al. (2005a) contended that WTD would be useful sentinels but that they likely do not play a significant epidemiologic role in the infection of immature *I. scapularis* (Tate et al., 2005a). Our demonstrations of stable seroprevalence but decreasing PCR positivity among older WTD are consistent with the hypothesis that WTD clear circulating *A. phagocytophilum* but retain detectable antibodies. In the field, wild WTD would be repeatedly exposed to *A. phagocytophilum*, potentially resulting in
increased and/or longer lasting serologic titers and effective clearance (Tate et al., 2005a). Collectively, results from these studies demonstrate that while WTD are suitable sentinels for delineating *A. phagocytophilum* distribution they most likely are not epidemiologically important reservoir hosts.

Considerable research has focused on variant typing and comparisons of 16S rRNA *A. phagocytophilum* sequences obtained from mammalian hosts (Massung et al., 1998; Belongia et al., 1997) and has lead to the hypothesis that the strain of *A. phagocytophilum* in WTD is not infectious to humans and exists in a separate epizootic cycle within WTD and *I. scapularis* (Massung et al., 2002, 2003a, 2003b; de la Fuente et al., 2005). Only one *A. phagocytophilum* 16S rRNA sequence amplified from WTD is presently available in GenBank; however, all *A. phagocytophilum* 16S rRNA sequences amplified from WTD are reportedly identical and differ from the human template 16S rRNA sequence of *A. phagocytophilum* at nucleotide consensus positions 76 and 84 (Belongia et al., 1997; Massung et al., 1998, 2002, 2003a, 2003b; de la Fuente et al., 2005). This 2 base pair difference has been described as characteristic of the WTD “strain” of *A. phagocytophilum* (termed AP-1 variant), and furthermore, AP-1 variants from WTD have been proposed to have infectivity for WTD but not humans (Massung et al., 2002, 2003a, 2003b; de la Fuente et al., 2005). Additionally, experiments transmitting the AP-1 variant from Rhode Island *I. scapularis* ticks to *P. leucopus* and *Mus musculus* laboratory mice were unsuccessful (Massung et al., 2003a; 2003b). Because strains from humans (AP-ha variant) readily infect mice, seroconversion and amplification of *A. phagocytophilum* gene fragments (AP-1 variant) from the blood of xenodiagnostic mice in this study provides evidence that this *A. phagocytophilum* genotype may indeed be capable of infecting mice. Of additional interest with
regard to this issue, WTD recently were shown to be susceptible to infection with a human isolate of *A. phagocytophilum* (Tate et al., 2005a).

Our data demonstrate that WTD meet critical biological criteria for use as *A. phagocytophilum* sentinels and that testing WTD represents a logistically feasible surveillance system. A WTD sentinel system would have considerable advantages based on its potential for broad-scale application and economic efficiency using hunter collected samples. In a companion study, Yabsley et al. (2003) were able to detect the spread of *E. chaffeensis*, which also is an important attribute of a surveillance system. As with *E. chaffeensis*, a WTD surveillance system for *A. phagocytophilum* could be applied in the public health sector (Mueller-Annealing et al., 2000; Yabsley et al., 2003, 2005). Issues related to use of WTD as sentinels that should be evaluated further include: 1) resolution of the AP-1 variant hypothesis, 2) assessment of the ability to detect spread of *A. phagocytophilum*, and 3) further comparisons with HGA case data or other measures of human exposure (e.g. serologic surveys). In addition, comprehensive landscape modeling analyses (Yabsley et al., 2005) would be desirable in order to better elucidate locations where there is a risk for HGA.

ACKNOWLEDGMENTS

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FIG. 4.1. Indirect fluorescent antibody assay results for *Anaplasma phagocytophilum* in white-tailed deer. Black counties represent seropositive populations; light gray counties represent seronegative populations for *A. phagocytophilum*-reactive antibodies (titer $\geq 128$).
<table>
<thead>
<tr>
<th>State</th>
<th>Years</th>
<th>Counties tested by IFA assay</th>
<th>Number IFA positive/ no. tested (%)</th>
<th>Number PCR positive/ no. tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alabama</td>
<td>1981-2001</td>
<td>34</td>
<td>34/170 (20)</td>
<td>NT</td>
</tr>
<tr>
<td>Arkansas</td>
<td>1982-2002</td>
<td>37</td>
<td>54/220 (25)</td>
<td>1/32 (3)</td>
</tr>
<tr>
<td>Georgia</td>
<td>1973-2002</td>
<td>56</td>
<td>96/330 (29)</td>
<td>19/80 (24)</td>
</tr>
<tr>
<td>Kansas</td>
<td>1998-2002</td>
<td>23</td>
<td>14/63 (22)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>Kentucky</td>
<td>1983-2001</td>
<td>31</td>
<td>6/134 (5)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>Maryland</td>
<td>1988-2002</td>
<td>8</td>
<td>22/40 (55)</td>
<td>14/16 (88)</td>
</tr>
<tr>
<td>Missouri</td>
<td>1991-2002</td>
<td>19</td>
<td>26/96 (27)</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Mississippi</td>
<td>1986-2001</td>
<td>35</td>
<td>43/175 (25)</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>North Carolina</td>
<td>1982-2003</td>
<td>24</td>
<td>27/142 (19)</td>
<td>8/51 (16)</td>
</tr>
<tr>
<td>New Jersey</td>
<td>2001</td>
<td>2</td>
<td>8/10 (80)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Oklahoma</td>
<td>1982-2001</td>
<td>12</td>
<td>19/50 (38)</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>Tennessee</td>
<td>1981-2001</td>
<td>23</td>
<td>37/119 (31)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Texas</td>
<td>1992-2002</td>
<td>58</td>
<td>91/288 (32)</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>Virginia</td>
<td>1983-2002</td>
<td>39</td>
<td>59/189 (31)</td>
<td>14/41 (34)</td>
</tr>
<tr>
<td>West Virginia</td>
<td>1977-2001</td>
<td>20</td>
<td>0/141 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>507</td>
<td>633/2666 (24)</td>
<td>75/458 (16)</td>
</tr>
</tbody>
</table>

*a PCR data from p44 and 16S gene assays; includes results for 5 WTD previously tested (Little et al., 1998); NT not tested
FIG. 4.2. (A) Prevalence of *A. phagocytophilum*-reactive antibodies among age classes of WTD. (B) Prevalence of *p44* gene PCR positives among age classes of WTD; numbers represent number of deer tested. Bars with the same alphabetic identities were not significantly different (p > 0.05). Analysis of the prevalence of PCR positive animals for the combined four older age classes (indicated by *) was significantly lower (p > 0.05) than the < 0.75 year age class.
CHAPTER 5

THE PREDICTED DISTRIBUTION OF *ANAPLASMA PHAGOCYTOPHILUM*, ETIOLOGIC AGENT OF HUMAN GRANULOCYTOTROPIC ANAPLASMOSIS, USING GEOGRAPHIC INFORMATION SYSTEMS ANALYSES

1


To be submitted to *American Journal of Tropical Medicine and Hygiene*. 
ABSTRACT

Human granulocytotropic anaplasmosis (HGA), caused by *Anaplasma phagocytophilum*, is an emerging zoonotic disease transmitted by *Ixodes scapularis*. Using two different predictive models, the endemnicity probabilities of *A. phagocytophilum*, and by implication identification of locations with HGA risk, were mapped based on data obtained from a surveillance system utilizing white-tailed deer (WTD) as sentinels. This study is novel in estimating the broad geographic distribution of *A. phagocytophilum* using spatial analyses and parallels a recent companion study on *Ehrlichia chaffeensis* in this region. The *A. phagocytophilum* serologic status of WTD from 562 counties in 19 southeastern and south central United States was used for kriging and logistic regression analyses. Both analyses produced reasonably accurate distributions of *A. phagocytophilum* endemnicity and the predicted distribution of *A. phagocytophilum* was in agreement with existing HGA case data. The use of both purely geospatial modeling (kriging) and logistic regression modeling, incorporating ecologic predictor variables, allows differing inferences regarding the successful use of WTD as sentinels in combination with modeling in a GIS system and produced *A. phagocytophilum* distribution maps that should be useful within the public health sector.

INTRODUCTION

Human granulocytotropic anaplasmosis (HGA), caused by infection with *Anaplasma phagocytophilum*, was first described in 1994 from Minnesota and Wisconsin patients and is currently recognized as an emerging tick-borne zoonosis (Chen et al., 1994; Bakken et al., 1996). Clinically, the disease is characterized as an acute illness characterized by fever, headache, myalgia, and laboratory findings of elevated liver enzymes, leukopenia and thrombocytopenia (Walker and Dumler, 1996). Approximately 1,220 cases of HGA from 21 states have been
reported to the Centers for Disease Control and Prevention (Childs and Paddock, 2002; Gardner et al., 2003), but the geographic foci of disease are concentrated in the northeastern and north central United States (Gardner et al., 2003). Currently, eleven states do not report HGA cases despite recommendations from the CDC and the Council of State and Territorial Epidemiologists that human ehrlichioses, including HGA, be nationally reportable diseases (McQuiston et al., 1999, 2003; Gardner et al., 2003).

Infection with *A. phagocytophilum* occurs through the bite of infected ticks in the genus *Ixodes*: *Ixodes pacificus* vectors the organism in the western states and *I. scapularis* is the primary vector in the eastern and Midwestern United States (Telford et al., 1996; Barlough et al., 1997; Kramer et al., 1999). Although various wild mammals have evidence of infection (Levin et al., 2002; Goethert and Telford, 2003), *A. phagocytophilum* is believed to be maintained in nature through a cycle similar to *Borrelia burgdorferi*, the agent of Lyme disease (LD), with small rodents including *Peromyscus leucopus* as competent reservoir hosts (Telford et al., 1996; DesVignes and Fish, 1997). White-tailed deer (WTD; *Odocoileus virginianus*) are major hosts for adult *I. scapularis* and suitable hosts for *A. phagocytophilum* but are not considered important vertebrate reservoirs (Massung et al. 2003a, 2003b; Dugan et al., 2005; Tate et al., 2005).

There are many complex ecological relationships that define how and where zoonotic vector-borne agents are maintained in nature. To better ascertain these relationships, current epidemiologic studies of ticks and tick-borne diseases have incorporated spatial analyses and geographic informational systems (GIS) to identify the environmental and landscape characteristics associated with human exposure and risk of disease. Recently, the distribution of *Ehrlichia chaffeensis*, causative agent of human monocytotropic ehrlichiosis (HME), was determined using a novel prototype WTD surveillance system (Yabsley et al., 2003). Using data
from this WTD surveillance system, kriging and logistic regression GIS models were utilized to create *E. chaffeensis* distribution maps based on the serologic status of WTD for a 19-state region (Yabsley et al., 2005). The projected *E. chaffeensis* distribution accurately identified counties reporting HME cases.

Extensive analyses mapping and predicting the distribution and frequency of *Ixodes* ticks and the risk of LD have been produced and implemented on both small and large scales within the public health infrastructure (Nicholson and Mather, 1996; Mount et al., 1997; Centers for Disease Control and Prevention, 1999; Glavanakov et al., 2001). Because WTD serve as the primary reproductive stage host for *I. scapularis* in eastern and central regions of the United States (Spielman et al., 1985; Mount et al., 1997; Rand et al., 2003), prior studies have determined a positive relationship between WTD density and the abundance of *I. scapularis* in areas where LD cases occur (Lastavica et al., 1989; Rand et al., 2003). Additionally, a small-scale spatial analysis based on reported HGA cases in Connecticut disclosed that the distribution of disease within this endemic region had a distinct geospatial pattern (Chaput et al., 2002).

Recently, the distribution of *A. phagocytophilum* was determined across a 19-state region using WTD as a sentinel species (Dugan et al., 2005). The goal of the current study was to use GIS analyses to create and evaluate contiguous, fine-scale maps of predicted status of *A. phagocytophilum* across the southeastern and south central United States based on data from this WTD sentinel system. Analyses utilized to accomplish this goal include 1) geostatistical modeling (kriging) using WTD serostatus to spatially interpolate between sampled counties and unsampled counties and 2) logistic regression modeling to identify ecologic variables associated
with *A. phagocytophilum* distribution. In addition, the projected *A. phagocytophilum* distributions were compared with HGA case data reported to the Centers for Disease Control and Prevention, as reported by Gardner et al. (2003).

**MATERIALS AND METHODS**

*Anaplasma phagocytophilum* database

The database utilized in this study was derived by serotesting of 562 WTD populations (Dugan et al., 2005; Little et al., 1998). Serum samples were tested by indirect fluorescent antibody assay for the presence of *A. phagocytophilum*-reactive antibodies, as previously described (Yeh et al., 1997; Dugan et al., 2005). Serologic results for each deer population were categorized by county or parish: if one or more seropositive deer was detected in a population, the county or parish was classified as positive; if all deer within a population (≥ 5 except for ≥ 2 in Texas) tested negative for *A. phagocytophilum*-reactive antibodies, the county or parish was classified as negative. This data was linked in a GIS to a county boundary map using Federal Information Processing Standard (FIPS) codes. Geospatial analyses were patterned after those previously described by Yabsley et al., 2005.

**Geostatistical modeling using kriging**

County-level serostatus was classified as an indicator variable with 1 representing presence and 0 representing absence of *A. phagocytophilum*-reactive antibodies in WTD. Indicator kriging (Isaaks and Srivastava., 1989) was used to interpolate the spatial distribution of *A. phagocytophilum* based on the assumptions that: 1) an association exists between the spatial variability of *A. phagocytophilum* serostatus and the distance between counties and, 2) the status of sampled counties can be used to predict the status of nearby unsampled counties. Using data from sampled locations (n = 562), an empirical semivariogram was calculated over twelve 50-km
lag intervals. The spatial location of each county was represented by a centroid and an exponential model was fitted to the semivariogram resulting in a kriging probability map of *A. phagocytophilum* presence or absence. Using the kriging probabilities generated, accuracy, sensitivity, and specificity of the kriging model were assessed as previously described (Yabsley et al., 2005). Kriged counties were classified as positive for *A. phagocytophilum* if the probability was > 0.5, and negative if the probability was < 0.5. Semivariogram analysis, kriging, and validation were conducted using ArcGIS 9.0 (Environmental Systems Research Institute, Redlands, CA).

**Logistic regression modeling**

**Explanatory Variables**

Spatial predictor variables used to build the logistic regression models were obtained as GIS datasets from a number of sources, as previously described (Yabsley et al., 2005). Climate variables for 1980-1997 were obtained as 1-km grids generated using the DayMet model (Thornton et al., 1997), land cover data were obtained from the Global Land Cover Characteristics Database and derived from 1-km Advanced Very High Resolution Radiometer data collected from April 1992 to March 1993 (Loveland et al., 2000), elevation data was obtained as a 1-km digital elevation model, a soil drainage index was computed for each county using the State Soil Geographic (STATCO) database (1:250,000), and 1999 deer density data was obtained as a paper map from the Quality Deer Management Association (Watkinsville, GA). The specific variables included in modeling were: average elevation (range 1-1501 m); ruggedness defined as the difference between maximum and minimum elevation (range 1-1843 m); soil drainage status (range 1.9-80.7); deer density population estimates (range 0≥ 45 deer/km²); percent urban landcover (range 0-100); percent cropland (dryland, grassland,
woodland, pasture, irrigated cropland, savannah, range 0-100); percent forest (deciduous
broadleaf, evergreen needleleaf, and/or mixed forest, range 0-100); percent wooded wetlands (0-
63); percent ‘total landcover’ consisting of urban landcover, cropland, total forest, and wooded
wetlands; annual, seasonal, and monthly Celsius temperatures for 1980-1997 (annual range 2.3-
29.6, monthly range 9.9-29.6); average precipitation in cm for 1980-1997 (annual range 23.5-
189.6, monthly range 0.6-24.6); percent average relative humidity for 1980-1997 (annual range
35.7-85.8, monthly range 20.6-131.1); and average number of frost days for 1980-1997 (range
0.4-164.5). Seasons were classified as summer (June, July, August), fall (September, October,
November), winter (December, January, February) and spring (March, April, May).

Relative humidity was calculated as previously described (Thornton et al., 2000) using
water vapor pressure and minimum and maximum temperatures. Low soil drainage values
characterized frequently saturated soils, whereas high values characterized well-drained soils and
a composite index for each mapped soil polygon was calculated based on the area-weighted
average of drainage indices of the associated soil series. Deer density data were mapped as
polygons with five density levels: 1) deer absent, rare, or urban with unknown populations, 2) <
15 deer/km², 3) 15 – 30 deer/km², 4) 31 – 45 deer/km², 5) > 45 deer/km².

The 19 states included in this study were digitized, georeferenced, and converted to 1-km
grids, the variable data sets were overlaid on the county boundaries, and summary values for
each county were computed. Mean values for each county were computed for climate variables,
soil drainage, and elevation; land cover was calculated as a percentage of each county occupied
by different land cover classes; ruggedness index was calculated by subtracting the lowest
elevation from the highest elevation per county, and the deer density class covering the majority
of each county was used to represent the entire county.
Logistic regression modeling

The SAS 8.2 program (SAS Institute, Inc., Cary, NC) was used to perform logistic regression analyses for the entire 19-state region and for three separate subregions divided into western, central, and eastern states within the 19-state region. The western subregion contained Kansas, Oklahoma, and Texas (n = 109 WTD populations/ sampled counties), the central subregion contained Alabama, Arkansas, Louisiana, Mississippi, Missouri (n = 180), and the eastern subregion consisted of Delaware, Florida, Georgia, Kentucky, New Jersey, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia (n = 273). The *A. phagocytophilum* serologic status of white-tailed deer populations was used as the dependent variable in stepwise regression analyses with a $P < 0.05$ cutoff to select a more accurate subset of important ecologic variables with the highest predictive power.

The logistic regression models were applied in ArcView 3.2 (Environmental Systems Research Institute) using spatial predictor variables from all of the counties in the 19-state region to create a map of the probability of *A. phagocytophilum* occurrence (hereafter termed endemnicity probability). Similar to geostatistical modeling, the overall fit of the models was determined by comparing the observed values with the predicted values (*A. phagocytophilum* presence > 0.5, absence if < 0.5) as well as evaluating model accuracy, sensitivity, and specificity as previously described (Yabsley et al., 2005). Additionally, the area under the receiving operator characteristic curve (AUC ROC) and the pseudo-$R^2$ values were computed as overall indices of model fit (Fielding and Bell, 1997; Nagelkerke, 1991).

Comparison of predicted *A. phagocytophilum* distribution with HGA case data

Human granulocytotrophic anaplasmosis incidence data as reported to the Centers for Disease Control and Prevention for 1997-2001 was obtained from Gardner et al. (2003). Mean
estimates of annual incidence were calculated using the corresponding yearly census estimate, and the incidence of HGA per million population was classified into 4 levels: low (< 1.9), low-medium (2.0 – 6.2), medium-high (6.3 – 17.0), and high (≥17.1; max 1135.0) (Gardner et al., 2003) and compared on a county level with the endemnicity probability maps generated by geostatistical and logistic regression models.

RESULTS

Geostatistical modeling using kriging

The fitted semivariogram had a range of 249.3 km, identifying the maximum distance at which spatial autocorrelation was detected among the sampled counties. The nugget variance was 0.13816, quantifying the minimum variability at a lag distance of zero, and a partial sill of 0.099474 was determined. The total sill of 0.237634 quantified the maximum variability among spatially independent variables. A high nugget to total sill ratio of 0.58 implied that a significant amount of detail (< 50 km) was not accounted for in the spatial variability of *A. phagocytophilum* distribution based on the kriging model. Using a threshold of 0.5, the accuracy, sensitivity, and specificity of the kriging model was 72.8%, 72.0%%, and 73.8%, respectively (Table 5.1).

Throughout the entire study region, large and small clusters of positive and negative geographic areas were observed (Figure 5.1). Three large areas were predicted to be positive: 1) in the northeast, an intensely endemic area extending from New Jersey southward to coastal North Carolina, 2) a generally contiguous arc extending from northern Florida and coastal South Carolina extending westward across southern Alabama and northward encompassing most of Mississippi and central Tennessee, and 3) a broad irregular band of positive counties in Texas and extending northeastward into southern Missouri. Southern Florida also possessed a smaller positive area outside these locations that was also predicted to be positive. Negative counties
were distributed unevenly throughout the region; however, two main areas were predicted to be negative: 1) a large area in the Appalachian Mountain region incorporating West Virginia, Kentucky, western Virginia, western North Carolina, northwestern portions of South Carolina and Georgia, northern Alabama, and eastern Tennessee, and 2) a large cluster of negative populations in northwestern Texas, extending northward to Oklahoma, Kansas, and Missouri. Smaller negative areas also were observed in the Gulf coastal regions of Alabama extending westward through Louisiana, in extreme south central and southwestern Georgia, and a cluster of largely negative populations in the northern half of the Mississippi Valley region. A total of 44 locations observed to be negative were predicted as positive; 28 locations observed as positive were predicted as negative counties. This misclassification of county status often occurred with isolated individual locations within a region or at the interface of positive and negative locations.

**Logistic regression modeling**

Three ecologic variables were significantly associated with *A. phagocytophilum* WTD serostatus for the 19-state regional model (Table 5.2, Figure 5.2). *Anaplasma phagocytophilum* endemnicity decreased with elevation, and increased with deer density and summer maximum temperature. Using a cutoff value of 0.5, this model accurately predicted the *A. phagocytophilum* serostatus of 408 (72.6%) of 562 counties (Table 5.2) but predicted negative counties with only 66.1% sensitivity and positive counties with 64.2% specificity. The endemnicity probability ranged from 0.0 to 0.8 in this regional model.

The regional logistic regression model predicted the large negative area centered along the Appalachian Mountain region and generally predicted western and northern boundaries of distribution. However, the regional logistic regression model failed to predict the intensely enzootic area extending from New Jersey to coastal Virginia.
Four variables were significantly associated with *A. phagocytophilum* serostatus in the eastern subregion (Table 5.2, Figure 5.3). The *A. phagocytophilum* predicted distribution decreased with elevation and cropland/grassland landcover, but increased with ruggedness and spring relative humidity. In the central subregion, three variables were significantly related with *A. phagocytophilum* serostatus in WTD. The predicted distribution increased with deer density and deciduous forest landcover, but decreased with summer precipitation (Table 5.2, Figure 5.3). In the western subregion model, predicted *A. phagocytophilum* counties increased with both deer density and winter precipitation (Table 5.2, Figure 5.3). The endemicity probability ranged from 0.0-1.0 using these models.

The eastern subregion model had a lower specificity (57.4% vs 64.2%), no appreciable change in the accuracy, but a higher sensitivity compared to the regional model (17.5%). The western subregion model had increased accuracy, sensitivity, and specificity by 5.4%, 7.9%, and 12.5%, respectively when compared with the region-wide model. However, the central subregion model had lower accuracy and sensitivity, and only slightly higher specificity (77.0% vs 73.8%). Additionally, all three subregion models predicted a high-end endemicity level of 1.0, whereas the maximum endemicity level in the region-wide model was 0.8-0.9, and the subregional models produced a better fit of the data with higher max-rescaled $R^2$ values and AUC ROC (Table 5.1).

The three subregional models had a better overall fit in comparison with the 19-state regional model and generally predicted distributions that conformed better with field data. For example, the eastern subregional model predicted the intensely enzootic area in New Jersey, Maryland, and coastal Virginia and the large negative area in the Appalachian Mountain region but it underestimated the distribution across central Georgia. Similarly, the central and western
subregional models predicted distributions that conformed better with field data from the coastal areas of Alabama through Louisiana, areas in the northern Mississippi River flood plain, and areas at the western and northern boundaries.

**Comparison with HGA case data**

In the 19-state region, 90 counties in 11 states reported HGA cases during the years of 1997-2001 (Gardner et al., 2003). High correlations with human case data were observed with the projected *A. phagocytophilum* distributions produced by all models; both the 19-state region wide and subregional models classified 78 of the 90 counties (86.7%) with HGA cases positive and the kriging model predicted 74 (82.2%) of the counties as positive (Figure 5.5).

**DISCUSSION**

Two different types of modeling analyses were performed to project the distribution of *A. phagocytophilum* across a 19-state region based on the *A. phagocytophilum* serologic status of 562 WTD populations (Dugan et al., 2005) and thereby identified locations with risk for HGA. This study, and its companion study on *E. chaffeensis* (Yabsley et al., 2005), are novel in their use of WTD as natural sentinels, in their concurrent application of two distinctly different modeling analyses, and in their geographic scope.

Kriged models are based on the assumptions that predictions for unsampled locations can be calculated by weighted averages of sampled locations in close proximity, and the distance between observations is related to the spatial variability of the data. In contrast, logistic regression develops a predictive equation based on multiple predictor variables potentially associated with the variable of interest, and applies the equation to predict status of unsampled locations. Thus, kriging predictions are based on spatial patterns of *A. phagocytophilum* serostatus across the region whereas logistic regression predictions are based on the relationship
of *A. phagocytophilum* serostatus with ecologic variables from the same location. Although both kriged and logistic regression models generally performed well in predicting the distribution of *A. phagocytophilum* within the 19-state region, the kriged model is probably the best estimate of current *A. phagocytophilum* distribution and would be more useful in monitoring spread. In contrast, the incorporation of ecologic attributes in logistic regression models should render it more suitable in identifying locations where spread of *A. phagocytophilum* is more likely to occur in the future. Yabsley et al. (2005) drew similar conclusions regarding the utility of kriging and logistic regression modeling of *E. chaffeensis* across this same region, and aptly noted that alternative analyses that essentially combine attributes of purely spatial and purely ecological models into a unified modeling framework likely would be superior.

To relate our analyses with HGA occurrence, model outputs were compared with previously published 1997-2002 HGA case data reported to the Centers for Disease Control and Prevention. The endemicity maps generated by both models identified most counties with documented occurrence of HGA in this region (Gardner et al., 2003).

Important issues for modeling include the accuracy of input data, fitness between predicted and observed data, and the sampling scope and scale (Matthews, 1990; Yabsley et al., 2005). The WTD serology data used encompassed over one-third of the total counties in the 19-state region and had previously been shown to be diagnostically accurate, to be comprised of statistically sufficient sample sizes, and to be spatially associated with *I. scapularis*, the vector of *A. phagocytophilum* (Dugan et al., 2005). Accuracy, sensitivity, and specificity of kriging and logistic regression analyses for *A. phagocytophilum* were relatively comparable, although somewhat less similar to calculations for *E. chaffeensis* modeling that exceeded 83.6% and were as high as 93.1% (Yabsley et al., 2005). Generally, the predicted models for *A.
*phagocytophilum* endemnicity adequately fit field data and performed as well as similar studies with other tick-transmitted pathogens (Chaput et al., 2002; Glavanakov et al., 2001; Yabsley et al., 2005; Centers for Disease Control, 1999).

The large nugget: sill ratio of the kriging semivariogram indicated that uncertainty existed within small groupings of counties with high *A. phagocytophilum* variability. Because kriging relies on a threshold sample size to predict unsampled populations and is based on data from nearest neighbors, the model was incapable of detecting fine scale (≤ 50 km) variability and thus incorrectly predicted both the presence and absence of *A. phagocytophilum* in locales with a mixture of seropositive and seronegative populations in close proximity. This was most evident at the interfaces of seropositive and seronegative clusters but also occurred when one or more populations of one serostatus were embedded within area of populations with the opposite serostatus. This misclassification was most evident within Texas and at the Louisiana and Mississippi state borders where *A. phagocytophilum* prevalence was over-predicted. A similar situation existed with *E. chaffeensis* where the kriged model over-predicted the presence of *E. chaffeensis* at interfaces of positive and negative populations (Yabsley et al., 2005).

The region-wide logistic regression model misclassified many locations but dividing the 19-state region into 3 subregions produced a distribution with a better fit with field data. All subregion models demonstrated the full range of endemnicity probabilities, and exhibited better ability to predict both positive and negative locations. This suggests that the influence of predictor variables is not uniform across such a wide geographic region and that these variables may be replaced by or interact with other variables in distant and ecologically different locations.

Similar to the companion *E. chaffeensis* study (Yabsley et al., 2005), the kriging and logistic regression modeling can be directly compared due to the lack of specific account of
spatial autocorrelation within the logistic regression models. Future work based on this study might incorporate additional alternative statistical kriging methods including autologistic and geographically weighted regressions (Augustin et al., 1996; Goovaerts, 1997; Fotheringham et al., 2002) that combine both ecologic predictor variables and spatial analyses into one model.

The distribution of *A. phagocytophilum* is directly dependent on the presence on *I. scapularis* as a vector, and thus indirectly dependent on ecologic and climatic variables that limit the distribution of this tick. Extensive studies have determined that climatic and environmental extremes including temperature, relative humidity, habitat type, distance to water, and host density are all-important factors affecting the distribution, development cycles, and mortality rates of *I. scapularis* (Rand et al., 2003; Bunnell et al., 2003). In relation to ecologic variables, the increased abundance of *I. scapularis* has been shown to be positively correlated with the presence of wooded habitats and forest habitats consisting of deciduous, evergreen and mixed forest types (Guerra et al., 2001; Stafford and Magnarelli, 1993; Nicholson and Mather, 1996; Maupin et al., 1991; Bunnell et al., 2003); however, the presence of wetlands, high elevation, and increased distance to coastal waters has been negatively associated (Bunnell et al., 2003). The significant ecologic variables in our models corresponded to these critical environmental attributes necessary for suitable *I. scapularis* habitats: deciduous forests and ruggedness (steepness or slope) were significantly associated with the presence of *A. phagocytophilum*, while a negative correlation was observed with increased elevation and crop-grassland landcover (Figure 5.4). While this study did not specifically measure distance to coastal water as an ecologic factor, highly endemic counties distributed along the Atlantic and Gulf Coastal states were predicted by all modeling analyses.
For climatic variables, prior laboratory and field studies have determined that minimum, mean, and maximum monthly temperatures play a significant role in sustaining \textit{I. scapularis} populations, and vapor pressure and climatic extremes are major indicators of tick habitat suitability (Brownstein et al., 2003). More specifically, the prevalence of \textit{I. scapularis} has been shown as directly dependent on increased relative humidity, cooler winters, and drier summers (Nicholson and Mather, 1996). Conversely, extreme low temperatures increase the time of life cycle development, thus increasing the rate of tick mortality (Ogden et al., 2005). In concordance with these factors, logistic regression modeling determined a positive relationship between the presence of \textit{A. phagocytophilum} with summer maximum temperature, winter precipitation, and spring relative humidity. In contrast, a negative correlation with summer precipitation was noted in the central subregion model, suggestive of wet soils and/or wetlands that exist in much of this region. Wetlands have been demonstrated as unfavorable microhabitats for both \textit{I. scapularis} (Bunnell et al., 2003) and \textit{A. americanum} was also negatively associated with wooded wetlands (Yabsley et al., 2005).

Because WTD are the primary vertebrate host for adult \textit{I. scapularis} ticks and therefore are important to the establishment and spread of \textit{A. phagocytophilum} (Mount et al., 1997; Rand et al., 2003), selection of WTD density as a significant predictor variable in three of four logistic regression models is consistent with the epidemiologic role of WTD. A large number of studies have discerned positive, direct associations between WTD density and \textit{I. scapularis} abundance suggesting that WTD density strongly influences the reproduction and abundance of this tick within its range (Rand et al., 2003; Wilson, 1988, 1998; Deblinger et al., 1993; Lastavica et al., 1989). Additionally, high WTD densities in North America have been proposed to explain the increase in the regional abundance of \textit{I. scapularis} (Wilson, 1998; Cronon, 1983; Spielman,
1994) and proposed management strategies for decreasing *I. scapularis* populations involved decreasing WTD densities (Mount et al., 1997). In contrast, WTD density usually was not identified as a significant variable for predicting *E. chaffeensis* endemnicity (Yabsley et al., 2005), even though WTD are important to the maintenance of high densities of *Amblyomma americanum*, the vector of this pathogen (Mount et al., 1993).

Comparing the predicted county-level *A. phagocytophilum* endemnicity of all models with the corresponding HGA case data from 90 southeastern and south central counties exhibited >82% agreement. In addition, over 90% percent of counties with high HGA incidence were classified as positive with both kriging and logistic regression analyses. The ability of the WTD based models, especially the kriged model, to perform well in identifying locations (counties) reporting HGA cases within this region strongly indicates that WTD surveillance can be used to identify locations with HGA risks.

Comparison of the kriged *A. phagocytophilum* map with the National Lyme disease risk map (Centers for Disease Control, 1999) reveals a similar geographic pattern and further supports our analysis given that LD is a zoonoses also vectored by *I. scapularis*. The predictive *A. phagocytophilum* distribution maps presented herein are preliminary instruments that can be used as groundwork for future research to more fully discern the risk of HGA infection within this geographic region. It is encouraging that analyses predicting both *E. chaffeensis* (Yabsley et al., 2005) and *A. phagocytophilum* endemnicities using WTD as natural sentinels both appeared to give positive, concurring results in regard to reports of HME and HGA, respectively. Future work in this area of research might incorporate unified geospatial modeling procedures, acquisition of widespread *I. scapularis* prevalence data, and more extensive sampling of larger numbers of WTD for *A. phagocytophilum*-reactive antibodies to improve reliability of such
modeling. Of particular relevance would be comparison of predicted *A. phagocytophilum* distributions with an enhanced human infection database, such as a regional survey of humans for *A. phagocytophilum* antibodies. This could lead to an effective risk map for integration into the public health infrastructure.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


TABLE 5.1. Comparison of geospatial model (kriging) and four logistic regression models for predicting the occurrence of \textit{Anaplasma phagocytophilum} distribution based on a white-tailed deer serologic survey across 19 states*.

<table>
<thead>
<tr>
<th></th>
<th>Accuracy (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Maximum-rescaled R²</th>
<th>AUC ROC</th>
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<tr>
<td><strong>Geospatial Model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Kriging</td>
<td>72.8</td>
<td>72.0</td>
<td>73.8</td>
<td>NA</td>
<td>NA</td>
</tr>
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<td><strong>Logistic Regression Models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19-State region</td>
<td>72.6</td>
<td>66.1</td>
<td>64.2</td>
<td>0.1273</td>
<td>0.664</td>
</tr>
<tr>
<td>Eastern subregion</td>
<td>73.3</td>
<td>83.6</td>
<td>57.4</td>
<td>0.3903</td>
<td>0.778</td>
</tr>
<tr>
<td>Central subregion</td>
<td>69.4</td>
<td>62.4</td>
<td>77.0</td>
<td>0.2048</td>
<td>0.741</td>
</tr>
<tr>
<td>Western subregion</td>
<td>78.0</td>
<td>73.1</td>
<td>76.7</td>
<td>0.3579</td>
<td>0.811</td>
</tr>
</tbody>
</table>

* NA = not applicable; AUC ROC = area under the receiving operator characteristic curve
TABLE 5.2. Comparison of logistic regression models for predicting the distribution of *Anaplasma phagocytophilum*.

<table>
<thead>
<tr>
<th>Region</th>
<th>Variable</th>
<th>Coefficient</th>
<th>$X^2$</th>
<th>P</th>
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<tr>
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<td>Intercept</td>
<td>-10.1006</td>
<td>18.7080</td>
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<td></td>
<td>Elevation</td>
<td>-0.0498</td>
<td>12.8949</td>
<td>0.0003</td>
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<td></td>
<td>Deer density</td>
<td>0.2516</td>
<td>7.8387</td>
<td>0.0051</td>
</tr>
<tr>
<td></td>
<td>Summer maximum temp</td>
<td>0.2974</td>
<td>19.0582</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Eastern subregion</td>
<td>Intercept</td>
<td>-14.6789</td>
<td>9.8674</td>
<td>0.0017</td>
</tr>
<tr>
<td></td>
<td>Elevation</td>
<td>-0.0111</td>
<td>25.6789</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Ruggedness</td>
<td>0.00398</td>
<td>8.0331</td>
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</tr>
<tr>
<td></td>
<td>Crop/grassland</td>
<td>-1.4029</td>
<td>5.4447</td>
<td>0.0196</td>
</tr>
<tr>
<td></td>
<td>Spring relative humidity</td>
<td>0.2345</td>
<td>11.6042</td>
<td>0.0007</td>
</tr>
<tr>
<td>Central subregion</td>
<td>Intercept</td>
<td>-0.0343</td>
<td>0.0011</td>
<td>0.9734</td>
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<td></td>
<td>Deer density</td>
<td>0.7688</td>
<td>15.0964</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>Deciduous forest</td>
<td>0.2202</td>
<td>5.0292</td>
<td>0.0249</td>
</tr>
<tr>
<td></td>
<td>Summer precipitation</td>
<td>-0.2099</td>
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<td>0.0088</td>
</tr>
<tr>
<td>Western subregion</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Deer density</td>
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<td>8.7063</td>
<td>0.0032</td>
</tr>
<tr>
<td></td>
<td>Winter precipitation</td>
<td>0.2244</td>
<td>6.1296</td>
<td>0.0133</td>
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FIGURE 5.1. Map of *Anaplasma phagocytophilum* endemicity probabilities predicted by kriging the 19-state region. White circles represent serologic negative data and black circles represent serologic positive data obtained from a previous serologic survey of white-tailed deer for *A. phagocytophilum*-reactive antibodies (Dugan et al., 2005).
FIGURE 5.2. Map of *Anaplasma phagocytophilum* endemicity probabilities predicted by a logistic regression model derived for the 19-state region. White circles represent serologic positive data and black circles represent serologic negative data obtained from a previous serologic survey of white-tailed deer for *A. phagocytophilum*-reactive antibodies (Dugan et al., 2005).
FIGURE 5.3. Maps of *Anaplasma phagocytophilum* endemnicity probabilities predicted by separate subregional logistic regression models. White circles represent serologic positive data and black circles represent serologic negative data obtained from a previous serologic survey of white-tailed deer for *A. phagocytophilum*-reactive antibodies (Dugan et al., 2005).
FIGURE 5.4. Geographic information systems (GIS) overlay maps of selected variables used in logistic regression analyses. **A.** Ruggedness; **B.** Deciduous forest; **C.** Deer density, 1999; **D.** Elevation.
FIGURE 5.5. Comparison of human granulocytotropic anaplasmosis (HGA) case data (from Gardner et al., 2003) with the predicted presence of *Anaplasma phagocytophilum* generated by kriging and logistic regression models.
CHAPTER 6
CONCLUSIONS

This research was conducted to acquire a more comprehensive understanding of the ecology, natural history, and epidemiology of ehrlichial and anaplasmal tick-borne organisms among wild mammals, specifically white-tailed deer (WTD; Odocoileus virginianus), raccoons (Procyon lotor), and opossums (Didelphis virginiana) in the southeastern and south central United States. Specific objectives completed in this study include: 1) identification of the species of Ehrlichia and Anaplasma sp. which occur naturally in a local raccoon population in Georgia, 2) determination of the geographic distribution and prevalence of A. phagocytophilum infection in WTD from the southeastern and south central United States and the evaluation of WTD as a natural sentinel system, and 3) mapping of the distribution of A. phagocytophilum at a landscape scale across the southeastern and south central United States using GIS and spatial analyses.

Study 1 (Chapter 3)

This study investigated raccoons (Procyonis lotor) and opossums (Didelphis virginiana) collected from the Piedmont physiographic region of Georgia to better determine their potential role in the epidemiology of ehrlichial and anaplasmal species. Reactive antibodies in raccoons
were detected frequently for *E. chaffeensis* and *E. canis*, but rarely for *A. phagocytophilum*. Five species of ticks were collected from these animals, and *Dermacentor variabilis* was the predominant ectoparasite. The first sequence confirmed evidence of *E. canis* infection was documented from one raccoon, although all raccoons tested PCR negative for *E. chaffeensis*, *E. ewingii*, and *A. phagocytophilum*. Interestingly, a novel 16S rDNA sequence was amplified from over half of the raccoons tested; this sequence was most similar to an *Ehrlichia*-like organism from *Ixodes* ticks and rodents in Asia and Europe. Additionally, all opossums were seronegative, PCR negative, and free of ticks. The results of this study suggest that Georgia raccoons may be involved in the epidemiology of ehrlichial organisms and may potentially harbor a novel organism with potential public health and/or veterinary health implications.

**Study 2 (Chapter 4)**

The primary objective of this study was to evaluate the utility of WTD as sentinels for determining the geographic distribution of *A. phagocytophilum* throughout a 19-state region in the southeastern and south central United States. Specifically, the principal findings included 1) *A. phagocytophilum*-reactive antibodies in WTD reflect infection, 2) small sample sizes are suitable for surveillance monitoring, 3) any WTD > 0.75 months of age can be used for surveillance, and 4) evidence of *A. phagocytophilum* infection in WTD was significantly correlated with the presence of *I. scapularis*, the primary tick vector. These findings are novel in their use of WTD as sentinels and their geographic scope and compliment a prior companion study for *E. chaffeensis* (Yabsley et al., 2003). This system should be advantageous based on broad-scale applications, economic efficiency of sample collection, and along with the *E. chaffeensis* companion study, collectively confirm the suitability of WTD as sentinel animals for monitoring the distribution of these tick-borne zoonotic pathogens.
Study 3 (Chapter 5)

The major objective of this goal was to utilize two different modeling techniques to predict the geographic distribution of *A. phagocytophilum* across a 19-state region and by implication, to identify locales with HGA risk in the southeastern and south central United States. Analyses were performed using WTD serologic data generated from the use of WTD for *A. phagocytophilum* (Dugan et al., 2005). This study was a companion study to previous research on *E. chaffeensis* (Yabsley et al., 2005) and utilized similar kriging and logistic regression analyses. Kriging analyses are purely spatial and used known WTD serostatus to predict status at unknown locations; logistic regression utilized ecologic variables associated with WTD serostatus to predict unknown locales and identified several climatic and ecologic attributes that are useful predictors of *A. phagocytophilum* occurrence. Both analyses proved to be generally accurate in predicting the distribution of *A. phagocytophilum* and produced results that mirrored the distribution of *I. scapularis*, the tick vector of this organism (Dennis et al., 1998). Additionally, HGA case data obtained from the Centers for Disease Control and Prevention, compiled by Gardner and others (2003) as well as the predicted risk of Lyme disease (LD) for this region (Centers for Disease Control and Prevention, 1998) were used to compare and validate the geospatial analyses with disease occurrence and risk. These comparisons exhibited agreement among HGA case data, LD risk, and the predicted *A. phagocytophilum* endemnicities produced by kriging and logistic regression models.


