COURSE OF INFECTION, PATHOLOGY, AND BIOLOGY

OF *ANAPLASMA PHAGOCYTOPHILUM* AND

A PREVIOUSLY UNDESCRIBED *ANAPLASMA* SP. IN WHITE-TAILED DEER

by

CYNTHIA M. TATE

(Under the Direction of William R. Davidson)

ABSTRACT

The goals of this research were to better understand the course of infection, pathology, and biology of *Anaplasma* organisms harbored by white-tailed deer (WTD; *Odocoileus virginianus*). The study comprised two objectives: 1) to evaluate the ability of WTD to serve as hosts of and as sentinels for *A. phagocytophilum*, and 2) to characterize an undescribed *Anaplasma* sp. of WTD. Four WTD were inoculated with a human isolate of *A. phagocytophilum* and two served as negative controls. All 4 deer developed antibody titers ≥ 64, three circulated organisms in blood as detected by polymerase chain reaction (PCR) for over two weeks, one had PCR-positive bone marrow at 66 days, but none appeared clinically ill. These data confirm that WTD are susceptible to infection with a human isolate of *A. phagocytophilum* and suggest that WTD may maintain circulating organisms for a sufficient time to infect ticks. Their susceptibility and immunologic response render WTD suitable candidates as natural sentinels. To accomplish the second goal, six WTD were inoculated with either a culture isolate of the *Anaplasma* sp. or with infected deer blood. All six became persistently infected, as

determined by PCR of blood, but none appeared clinically ill. Light microscopy revealed tiny, dark, spherical structures in platelets of infected deer and electron microscopy demonstrated membrane-bound, *Anaplasma*-like organisms. Immunohistochemistry and *in situ* hybridization localized to *Anaplasma* sp. organisms in platelets and *Anaplasma* sp. DNA was amplified from purified platelets. Five deer developed antibodies reactive to *Anaplasma* sp. antigen and remained seroreactive for ten or more weeks. Slight antigenic cross-reactivity occurred among this *Anaplasma* sp., *A. phagocytophilum*, and *A. marginale*. Phylogenetic analysis demonstrated that a partial *gltA* gene sequence amplified from this *Anaplasma* sp. was closest to *A. platys gltA* sequence, which is concordant with previous analyses of the *Anaplasma* sp. 16S and *GroESL* genes. Two attempts to transmit *Anaplasma* sp. to deer via *Amblyomma americanum*, a suspected tick vector, were unsuccessful. The ultrastructural, antigenic, and phyogenetic characteristics of this organism confirm that it is a distinct species of *Anaplasma* which persistently infects WTD platelets. The name *Anaplasma odocoilei* is proposed for this new species.

INDEX WORDS: *Amblyomma americanum*, *Anaplasma odocoilei*, *Anaplasma phagocytophilum*, Experimental infection, Lone star tick, *Odocoileus virginianus*, Platelet, Sentinel, White-tailed deer

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DEDICATION

For Farimae and Andy, two exceptional individuals whom I am so fortunate to know as Mom and Dad.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The research presented in this dissertation was conducted to further the understanding of the role of wild white-tailed deer (WTD; *Odocoileus virginianus*) in the maintenance of zoonotic tick-borne pathogens. Specifically, the goal of this work was to characterize infection of WTD with two *Anaplasma* species: *A. phagocytophilum* and an undescribed *Anaplasma* sp. recently isolated from WTD (Munderloh et al., 2003).

Within the last two decades, wild WTD have been implicated in the epidemiology of several zoonotic tick-borne diseases emerging in the United States, including those caused by bacteria in the family Anaplasmataceae: *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* are the causative agents of human granulocytotropic anaplasmosis (HGA) and human monocytotropic ehrlichiosis (HME), respectively. *Ehrlichia ewingii* also causes granulocytotropic disease in humans in rare cases in the United States (Childs and Paddock, 2003). Through 2001, approximately 1150 cases of HME and 1220 cases of HGA were reported to the Centers for Disease Control (Childs and Paddock, 2003).

White-tailed deer populations in the southeastern United States are known to harbor *E. chaffeensis*, *A. phagocytophilum*, and *E. ewingii* (Lockhart et al., 1997; Little et al., 1997; Yabsley et al., 2002). Previous studies have demonstrated the importance of WTD to the enzootic maintenance of *E. chaffeensis*; deer serve as principal hosts to all three life-stages of the vector, the lone-star tick (LST; *Amblyomma americanum*), and serve as persistently rickettsemic reservoirs (Davidson et al., 2001).

The importance of WTD as reservoirs of *E. ewingii* and *A. phagocytophilum* is not established. Because *Ixodes scapularis* is the primary North American vector of *A. phagocytophilum* east of the Rocky Mountains (Telford et al., 1996), and because WTD are the primary host for the adult stage of the tick (Lane et al., 1991), WTD predictably are exposed to

A. phagocytophilum. Their ability to maintain rickettsemia with *A. phagocytophilum* of sufficient duration to serve as a source of the bacteria for ticks is unknown.

White-tailed deer populations in the southeastern United States host a fourth organism, referred to as "WTD-agent", in the family Anaplasmataceae (Dawson et al., 1996). Phylogenetically, this organism falls in a clade with *Anaplasma* spp. (Dumler et al., 2001). Previous studies suggest that it is transmitted by ticks, possibly the LST (Brandsma et al., 1999), along with other rickettsiae, including *E. chaffeensis*, and *E. ewingii* (Ewing et al., 1995; Little et al., 1998; Yabsley et al., 2002). This *Anaplasma* sp. has generated interest in the human medical community because it has the potential to confound serologic and molecular diagnostic assays for *E. chaffeensis* and *A. phagocytophilum* (Little et al., 1997). With the recent isolation of this *Anaplasma* sp. in tick cell culture (Munderloh et al., 2003), biological material is now available for diagnostic assays.

Specific objectives of this dissertation were as follows:

1. Experimentally assess the susceptibility of and response by WTD to infection with a human isolate of *A. phagocytophilum.*
2. Establish captive "donor deer" for the undescribed *Anaplasma* sp. via experimental inoculation of field-collected deer blood; describe course of infection; visualize and characterize WTD-agent in deer tissues.
3. Develop indirect fluorescent antibody test for detection of the *Anaplasma* sp. and subsequently determine degree of antigenic cross-reactivity among this *Anaplasma* sp., *A. phagocytophilum*, *A. marginale*, and *E. chaffeensis* in deer.
4. Investigate competence of *Amblyomma americanum* ticks to transmit the Anaplasma sp.

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Review of *Anaplasma phagocytophilum*

Phylogeny of *A. phagocytophilum* in relation to other Anaplasmataceae members

The genera *Anaplasma* and *Ehrlichia* were established in the first half of the 20th century in response to the discovery of these intracellular bacteria of veterinary importance. The zoonotic potential of several of these organisms was not recognized until later. The genus *Anaplasma* was established in 1910 with Arnold Theiler's description of the economically important erythrocyte pathogen, *A. marginale*, from domestic cattle in South Africa. The genus *Ehrlichia* was established in 1945, in honor of the German microbiologist, Paul Ehrlich (Moshkovski, 1945; Silverstein, 1998). The type species is the veterinary pathogen now known as *E. canis*, described from dogs in Algeria by Donatien and Lesquotard in 1935. The mouse granulocyte pathogen, *Cytoecetes microti*, originally described by Tyzzer in 1938, was renamed *E. phagocytophila* in 1940 and is presumed to have constituted the first description of the organism now known as *A. phagocytophilum*. Tyzzer's original materials no longer exist, however, so this assumption cannot be verified (Ristic and Huxsoll, 1984). In Europe, tick-borne fever of domestic ruminants was described from sheep and was attributed to infection with *E. phagocytophila* by Gordon et al., in 1932. In the United States, in 1969, *E. equi* was described from clinically ill horses (Gribble, 1969; Stannard et al., 1969).

The first human pathogen discovered in the family Anaplasmataceae, *E. sennetsu* (=*Neorickettsia sennetsu*), was described by Misao and Kobayashi in 1955 in Japan. In the United States, human disease attributed to a new species, *E. chaffeensis*, was described by Anderson et al. in 1991. The disease was characterized by bacterial clusters, known as morulae, in monocytes; thus the disease was referred to as human monocytic ehrlichiosis (HME). The

human disease characterized as "granulocytic ehrlichiosis" was described from patients in the upper midwestern United States in 1994 (Bakken et al., 1994; Chen et al., 1994).

In 2001, after extensive phylogenetic analysis by Dumler et al., the description of the family Anaplasmataceae was emended so as to encompass all species in the genera *Ehrlichia*, *Cowdria, Wolbachia* and *Neorickettsia*, as well as to retain species in the genera *Anaplasma* and *Aegyptianella*. Thus, the currently accepted description of Anaplasmataceae specifies that all members reside within cytoplasmic vacuoles of eukaryotic host cells, including erythrocytes, endothelial cells, reticuloendothelial cells, bone marrow- derived phagocytic cells, and cells of insect, helminth, and arthropod reproductive tissues (Dumler et al., 2001).

Furthermore, the "agent of HGE" (aoHGE) and *E. equi* were synonymized with the historically precedent *E. phagocytophila*, and the organism was re-assigned to the genus *Anaplasma*, resulting in the currently accepted designation *A. phagocytophilum* (Dumler et al., 2001). *Anaplasma phagocytophilum* is widespread throughout North America and Europe and is responsible for highly variable disease in a diversity of mammals, including domestic ruminants, horses, domestic dogs and cats, and humans (Dumler et al., 2001; Lappin et al., 2004). It is hypothesized that different strains or variants of *A. phagocytophilum* are responsible for the marked differences that exist in host preference, clinical manifestations and geographical distribution (Rikihisa et al., 1991; Pusterla et al, 1998 and 1999c). This review focuses primarily on North American strains of *A. phagocytophilum*.

Human Infection with *A. phagocytophilum*

In 1994, an "ehrlichia-like" disease was described in 12 patients from Wisconsin and Minnesota, an area where the lone-star tick, the vector of HME, is not present. In these patients, morulae were observed in neutrophils (as opposed to monocytes), hence the name "human

granulocytic ehrlichiosis" (HGE) was adopted. The patients never developed antibodies to *E. chaffeensis*, but most had antibodies reactive to one of two ehrlichial species known to infect granulocytes of horses and ruminants, *E. equi* and *E. phagocytophilum*, respectively (Bakken et al., 1994). DNA sequence analysis of 16S ribosomal DNA products amplified from peripheral blood specimens of the human patients revealed that the products were identical to each other, and nearly identical to both *E. equi* (differing by three nucleotides) and *E. phagocytophila* (differing by two nucleotides). This molecular analysis confirmed that a new human disease agent had been identified (Chen et al., 1994). Shortly thereafter, "HGE" was diagnosed in patients from New York and Massachusetts (Centers for Disease Control, 1995; Telford et al., 1995). Through 2001, approximately 1220 cases of "HGE" were diagnosed in the United States, and reported to the Centers for Disease Control (Childs and Paddock, 2003). In Europe, 65 cases of "HGE" have been reported through March 2003 (Strle et al., 2004).

With the adoption of the new designation *A. phagocytophilum* in 2001, scientific literature began to refer to the human disease caused by this organism as either "Human Anaplasmosis" (HA) or the more descriptive "Human Granulocytic Anaplasmosis" (HGA). Henceforth, the acronym HGA will be used in this publication.

HGA: Clinical Illness and Pathology

The clinical presentation of human infection with *A. phagocytophilum* is nearly indistinguishable from that of *E. chaffeensis*, with the exception that human central nervous system (CNS) infection with *A. phagocytophilum* has never been convincingly demonstrated, whereas approximately 20% of *E. chaffeensis* infections include CNS infection (Park et al., 2003). Infection of human leukocytes with either *A. phagocytophilum* or *E. chaffeensis* may result in an acute, febrile, systemic illness. Signs and symptoms reported for HGA include fever (94-100%), malaise (98%), myalgia (78-98%), headache (61-85%), nausea (39%), vomiting

(34%), diarrhea (22%), cough (29%), arthralgias (27-78%), rash (2-11%), stiff neck (22%), and confusion (17%) (Walker and Dumler, 1997). The clinical manifestations of HGA are nonspecific, and are present in a variety of illnesses, including viral infections that may not require specific pharmacotherapy. Fortunately, laboratory findings such as leukopenia, thrombocytopenia, and elevation of serum hepatic enzymes often provide important clues in the diagnosis of HGA (Walker and Dumler, 1997).

Reported complications of HGA include severe pancytopenia, pneumonitis, acute renal failure and opportunistic infections (Walker and Dumler, 1997; Modi et al., 1999; Lepidi et al., 2000; Remy et al., 2003). Risk factors for severe illness with *A. phagocytophilum* include advanced age, prior immunosuppression, lower neutrophil or higher lymphocyte differential leukocyte counts, anemia and the presence of morulae in peripheral blood neutrophils (Aguero-Rosenfeld et al., 1996; Bakken et al., 1996; Lepidi et al., 2000). In one prospective analysis of HGA cases, 28-56% of patients required hospitalization and 5-7% had illness severe enough to result in admission to an intensive care unit (Bakken et al., 1996). HGA mortality rates of up to 5% have been reported in the United States (Bakken et al., 1996); however, severe cases are probably overrepresented. A case fatality rate of less than 1% has been reported by Walker and Dumler (1997). Most HGA fatalities result from opportunistic infection associated with immunosuppression or pre-existing disease (Bakken et al., 1994 and 1996; Jahangir et al., 1998).

The pathogenesis of *A. phagocytophilum* infection is poorly understood. In comparing tissues from fatal and non-fatal HGA cases with tissues of sheep and horses experimentally infected with *A. phagocytophilum*, Lepidi et al. (2000) concluded that pathogenesis may result from host-mediated injury and immunosuppression, once the process was initiated by infected cells. Histopathologic findings of 4 fatal cases of HGA included lymphoid depletion in the

spleen (4/4) and lymph nodes (3/4), lymphohistiocytic lobular hepatitis (4/4); and inflammatory lung involvement (1/4) (Lepidi et al., 2000).

HGA Diagnosis

HGA is most easily diagnosed following clinical presentation of an acute, febrile illness and a patient history of tick bite in an area where HGA is endemic. Bakken et al. (1994) reported that 92% of HGA patients recalled a tick bite within ten days of onset of illness. Nonetheless, the lack of such a history should not preclude clinical suspicion of HGA; lack of tick bites in the patient history is a well documented phenomenon in studies of other tick-borne diseases such as Lyme disease and Rocky Mountain spotted fever (Comer et al., 1999). Similarly, laboratory values of leukopenia, thrombocytopenia and elevated liver enzymes are helpful clues to HGA diagnosis; however, HGA should not be excluded from the differential list based on normal blood parameters (Bakken et al., 2001).

In September 1996, the Council of State and Territorial Epidemiologists (CSTE) established a case definition for ehrlichiosis (both HME and HGA): acute illness with headache, myalgias, rigors and/or malaise, accompanied by at least one of three laboratory criteria, 1) seroconversion (four-fold or greater rise in IFA titer), 2) positive PCR, or 3) the presence of morulae in blood, bone marrow, or CSF leukocytes, and an antibody titer greater than or equal to 64 (Centers for Diseases Control, 1997). In December 2000, the diagnostic criteria list was expanded to include immunohistochemistry and cell culture isolation. At the same time, CSTE created a third reporting category for cases caused by unspecified or novel *Ehrlichia* species, including *E. ewingii* (Centers for Disease Control, 2001).

Examination of Romanovsky-stained blood smears via light microscopy is the most basic of techniques for diagnosis of HGA. Morulae (clumps of membrane-bound intracytoplasmic

organisms) in granulocytes have been reported in approximately 25% of HGA patients (Bakken et al., 1996; Aguero-Rosenfeld et al., 1996). However, this diagnostic technique is useful only during the acute, febrile phase of infection when the number of organisms in circulation is highest (Bakken et al., 1996; Aguero-Rosenfeld et al., 1996). Furthermore, both the specificity and sensitivity of this test entirely depend on the skill and diligence of the examiner (Bakken and Dumler, 2000; Blanco and Oteo, 2002; Walker and Dumler, 1997). False positive results may result from overlying platelets, Dohle bodies and toxic granulations. False negative results may result from failing to examine an appropriate number of granulocytes. Bakken et al. (1994) reported examining at least 800 granulocytes per slide in order to find morulae.

Serology is the most commonly used technique for HGA diagnosis. Serologic testing for HGA has been available to state health departments through CDC since August 1995 (CDC, 1995). Most culture-confirmed HGA patients (>95%) develop antibodies, typically within two weeks of onset of illness. Peak titers occur during the first month, and titers remain detectable in about half of the patients at 1 year after onset of illness (Aguero-Rosenfeld et al., 2000). Before *A. phagocytophilum* was isolated in cell culture from human patients, antigen slides for indirect fluorescent antibody (IFA) testing were produced from neutrophils of experimentally-infected horses and cows (Bakken et al. 1994).

In 1996, the causative agent of "HGE" was cultivated from the blood of three human patients in a human promyelocytic leukemia (HL60) cell line (Goodman et al, 1996). This achievement allowed for the development of a specific serologic test for "HGE", by production of antigen slides using the newly isolated organism as substrate (Goodman et al., 1996; Nicholson et al., 1997).

Nicholson et al. (1997) reported that the *A. phagocytophilum* antigen generated in horses gave results comparable to those in cell culture-derived HGA antigen when the same human serum samples were tested with both antigens. In the same study, however, the authors reported extensive dual reactivity of human serum samples *to A. phagocytophilum* and *E. chaffeensis*. Whether these results reflect actual exposure of the patient to both organisms, or simply reflect existence of cross-reactive antigens is not clear. Other researchers have reported *A. phagocytophilum*/*E. chaffeensis* antigenic cross-reactivity (Comer et al., 1999; Rikihisa et al., 1997; Walls et al., 1999; Wong et al., 1997) as well as antigenic cross-reactivity between *A. phagocytophilum* and *Borrelia burgdorferi* (Wormser et al., 1996; Wormser et al., 1997). These cross-reactivities have been attributed, at least in part, to heat shock proteins (IJdo et al., 1998; Unver et al., 2001; Wong et al., 1997)

In light of the cross-reactivity of sera evaluated by whole cell antigen testing, researchers began looking for specific immunodominant proteins for use in HGA diagnostic assays. Dumler et al. (1995) reported that HGA patients develop antibodies reactive to a 44 kilodalton (kDa) immunodominant protein antigen prepared from neutrophils harvested from *A. phagocytophilum*-infected horses. Once isolation and propagation of *A. phagocytophilum* was possible, antigen from various human isolates was purified and analyzed by immunoblotting. IJdo et al. (1997) were the first to use a human *A. phagocytophilum* isolate to identify immunogenic proteins. Using sera from 18 HGA patients to probe lysates of *A. phagocytophilum*-infected HL60 cells, they found that the 44kDa protein was the most commonly detected protein. Importantly, sera from HME patients did not react to this protein. They also proposed that the detection of the 44-kDa protein by both IgM and IgG, or by IgG alone plus one or more of the other characteristic bands identified in the study should be considered diagnostic of HGA infection.

Further experimentation with different strains of *A. phagocytophilum* and various infection sera confirmed the antigenic dominance of proteins in the size range of 40- to 49-kDa (Asanovich et al., 1997; Kim and Rikihisa, 1998; Zhi et al., 1997). This complex of antigens, collectively referred to as P44s, are outer membrane proteins (Zhi et al., 1997) and are encoded by a polymorphic multi-gene family with homology to the *msp-2* genes of *A. marginale* (Murphy et al., 1998), consisting of more than 20 different paralogous genes dispersed throughout the genome (Zhi et al., 1998; Zhi et al., 1999). Furthermore, characterization of the expression profiles of *p44* paralogs of *A. phagocytophilum* in cell culture and blood of animal models as well as human patients revealed that the genes are differentially expressed in different host environments (Zhi et al., 1999; Jauron et al., 2001; Zhi et al., 2002; Lin et al., 2002; Barbet et al., 2003).

The characterization of immunodiagnostic antigens of *A. phagocytophilum* enabled the development of more efficient serodiagnostic tools, such as enzyme-linked immunosorbent assay (ELISA). ELISAs are inexpensive to perform, easily standardized, can be automated, and allow for simultaneous testing of multiple samples. Ravyn et al. (1998) reported that the 42- to 45-kD antigens were early, persistent and specific markers of HGA, as employed in an ELISA. The ELISA was reported to be more sensitive than IFA, nonetheless, Ravyn et al. recommended results be confirmed with western blot.

Genes encoding the 44-kDa antigen complex have been cloned from several different *A. phagocytophilum* strains (IJdo et al., 1998; Murphy et al., 1998; Zhi et al., 1998) and this work facilitated production of recombinant versions of the protein for diagnostic application, in the form of rP44-based ELISAs (IJdo et al., 1999; Tajima et al., 2000; Magnarelli et al., 2001) that are increasingly used by medical practitioners.

Considering the abundance of P44 variants expressed by *A. phagocytophilum*, some researchers have cautioned against reliance upon this antigen complex in diagnostic assays (Barbet et al., 2003). Although most of the focus on characterization of *A. phagocytophilum* diagnostic antigens has been on the immunodominant 44-kDa protein, some work has focused on identifying alternative diagnostically significant antigens, with the intent of improving the sensitivity of P44-based assays, particularly for acute-phase sera. Several non-P44 immunoreactive proteins have been identified and characterized, such as the 80 kDa HSP-70 homologue (IJdo et al., 1998), groEL (Kolbert et al., 1997), and others (Storey et al., 1998; Lodes et al., 2001). Lodes et al. tested seven different recombinant antigens, including two derived from *ankA* genes and demonstrated that the use of combinations of non-MSP recombinant proteins can increase sensitivity of P44-based assays without decreasing specificity.

Immunohistochemistry (IHC), employing stained tissue sections, was first used for HGA diagnosis by Bakken et al. (1994) as one test in a battery of diagnostic techniques in describing the first fatal cases. This technique was added to the expanded 'ehrlichiosis' diagnostic criteria list by the CDC in 2000. Undoubtedly, due to the necessity of obtaining biopsy or postmortem samples, IHC is more useful in *A. phagocytophilum* research than in HGA diagnosis because the majority of HGA cases are non-fatal.

Molecular detection of *A. phagocytophilum* is widely employed by researchers and PCR assays for diagnosis of HGA are increasingly used, although they are not commonly available to most physicians (McQuiston et al., 2003). Chen et al. (1994) reported use of the 16S rRNA gene target in a nested polymerase chain reaction (PCR) for confirmation of the first cases of HGA. Primer sets and protocols for PCR amplification of numerous *A. phagocytophilum* gene targets have been published, including those for *gro*ESL (Sumner et al., 1997), *epank1* (Walls et al.,

2000), *msp* (Zeidner et al., 2000), *gltA* (Inokuma et al., 2001), *rpoB* (Taillardat-Bisch et al., 2003), and *ftsZ* (Lee et al., 2003) genes. Sensitivity and specificity vary widely, however, as reported by Massung and Slater (2003), who conducted a comparison study of 13 primer sets using sequential dilutions of *A. phagocytophilum* template DNA. Although molecular detection is a powerful tool, its usefulness for HGA diagnosis is limited by its ability to detect only active infections. Rickettsemia decreases rapidly after the first week of human infection with *A. phagocytophilum* (Bakken and Dumler, 2000).

The gold standard of HGA diagnostic techniques is cell culture. Positive HL60 blood cultures exhibit rapid development of cytopathic effect (CPE) and allow visualization of intracellular *A. phagocytophilum* in Giemsa-stained cytospins as few as five days after inoculation of the flask (Goodman et al., 1996). Alternatively, *A. phagocytophilum* can be isolated and propagated in an *Ixodes scapularis* tick cell line (Munderloh et al., 1996) and in endothelial cell lines (Munderloh et al., 2004). In a prospective, year-long study of a series of 23 confirmed and probable HGA cases, 8 (34.8%) were culture positive (Horowitz et al., 1998b). Although few diagnostic laboratories are equipped and staffed to attempt culture of *A. phagocytophilum*, Kalantarpour et al. (2000) demonstrated that *A. phagocytophilum* remains viable for up to 18 days in refrigerated blood, indicating that shipping of suspect samples to reference laboratories is a feasible option.

Treatment of HGA

Empiric therapy with doxycycline is recommended if HGA is suspected because delayed treatment while awaiting laboratory confirmation may increase the risk of adverse outcome (CDC, 1995). In HGA patients for whom doxycycline is contraindicated (such as the case for

children under twelve years of age, individuals allergic to doxycycline, and pregnant women) alternative drugs such as rifamycins and quinolones may be effective (Klein et al., 1997).

Epidemiology of HGA in the United States

Although CSTE and CDC recommended in 1998 that HME and HGA be added to the list of nationally notifiable diseases (CDC, 2001), only 36 states required reporting of these diseases through December 2000 (CDC, 2002). Presumably, this results in underreporting of both diseases in the United States.

A national surveillance study conducted from 1997 to 2001 documented that the states with the highest average annual incidence of HGA were CT, MN, RI and NY, in ranked order, with NY having the highest absolute number of cases. Most cases occurred from states in the northeast and upper midwest (Gardner et al., 2003). Onset of HGA cases typically occurs April through December, with a strong summer peak in June and July (Comer et al., 1999). This seasonal peak in HGA cases coincides with the peak in host-seeking activity of *I. scapularis* nymphs in the northeastern U.S. (Comer et al., 1999; Stafford et al., 1993), supporting the hypothesis that nymphs are the most important life stage of *I. scapularis* in the transmission of HGA (Bakken et al., 1996; Comer et al., 1999). The fewer numbers of autumn and early winter HGA cases coincide with the onset of adult *I. scapularis* activity in the northeast and upper midwest (Comer et al., 1999).

HGA patients range in age from < 5 to >90 years, with a reported median age of 51 years (Garder et al., 2003). There are few reports of HGA in children, leading to speculations that children under the age of 19 years may be less susceptible to *A. phagocytophilum* infection. A study of children in Westchester County, NY indicated a 5% *A. phagocytophilum* seropositivity rate regardless of presence of antibodies to *B. burgdorferi*, a disease agent transmitted by the

same tick vector. In the same study, adult subjects with *B. burgdorferi* antibodies demonstrated a higher *A. phagocytophilum* seropositivity rate (36%) than adults without evidence of *B. burdorferi* antibodies (11%) (Aguerro-Rosenfeld et al., 2002).

Serosurveys of healthy adults in *A. phagocytophilum*-endemic areas indicate that exposure may frequently result in asymptomatic seroconversion. These studies indicate seroprevalences of approximately 11-15% (Bakken et al., 1998; Aguerro-Rosenfeld et al., 2002**).** Although there is evidence of partial protective humoral immunity against *A. phagoctyophilum* in animals (Barlough et al., 1995; Levin et al., 2004; Sun et al., 1997; Pusterla et al., 1999c), parallel evidence in humans is lacking, and one case report of HGA re-infection exists (Horowitz et al., 1998c). This suggests that a single exposure to *A. phagocytophilum* does not induce long-lasting protective immunity in humans.

Transmission of *Anaplasma phagocytophilum*

Transmission of *A. phagoctyophilum* by tick bite has been demonstrated experimentally for *I. scapularis* (Telford et al., 1996), *I. pacificus* (Richter et al., 1996), and *I. ricinus* (MacLeod and Gordon, 1933). Molecular evidence indicates that in North America, east of the Rocky Mountains, *I. scapularis* is the principal vector of *A. phagocytophilum* (Daniels et al., 1997; Des Vignes and Fish, 1997; Magnarelli et al., 1995; Telford et al., 1996), whereas along the west coast, *I. pacificus* is the probable principal vector (Barlough et al., 1997; Kramer et al., 1999). Similarly, throughout Europe, the principal vector of *A. phagocytophilum* is thought to be *I. ricinus* (Cinco et al., 1997; MacLeod and Gordon, 1933; Parola et al., 1998; Petrovec et al., 1999; Pusterla et al., 1999a; von Stedingk et al., 1997).

When HGA was first detected, the disease was suspected to be tick-borne, because of its similarity to HME, and because Bakken et al. (1994) reported that 92% of HGA patients recalled

an arthropod bite within 10 days of onset of illness. Furthermore, 67% of the patients had ticks removed from them that were identified as either *I. scapularis* (=*I. dammini*) or *Dermacentor variabilis*. In 1995, Pancholi et al. amplified "ehrlichial DNA" from an engorged female *I. scapularis* (=*I. dammini*) found on an HGA patient, as well as from 7 of 68 wild-caught *I. scapularis* ticks. Reed et al. (1995) amplified "*Ehrlichia* DNA" from the blood of a patient with symptoms of HGA, as well as from a female *I. scapularis* (=*I. dammini*) tick removed from the patient.

Telford et al. (1996) demonstrated vector competence of *I. scapularis* for *A. phagocytophilum* among experimentally-infected laboratory mice. Furthermore, they proved reservoir competence of the white-footed mouse (WFM; *Peromyscus leucopus*) via acquisition-feeding larval ticks on wild WFM from a field site endemic for *A. phagocytophilum* (=agent of HGE). Concurrently, Richter et al. (1996) demonstrated vector competence of *I. pacificus* for *A. phagocytophilum* (= *E. equi*) among horses. Katavolos et al. (1998) determined the duration of tick attachment required for transmission of *A. phagocytophilum* by removing feeding nymphs from laboratory mice at various time points. As with other tick-borne pathogens, few mice became infected with *A. phagocytophilum* when nymphs were removed prior to 36 hours.

Although tick-bite is by far the predominant mode of *A. phagocytophilum* transmission, cases of HGA have been reported after solid organ transplantation (Adachi et al, 1997; Trofe et al., 2001; Vannorsdall et al., 2002) and one case of perinatal HGA transmission has been reported (Horowitz et al., 1998a). The survival of *A. phagocytophilum* in refrigerated blood specimens for up to 18 days suggests that HGA could be transmitted through blood transfusion (Kalantaarpour et al., 2000). One HGA case has been attributed to transfusion of 30-day old packed red blood cells collected from an asymptomatic donor (Eastlund et al., 1999).

Nonetheless, human blood products are not screened for evidence of *A. phagocytophilum* at this time (McQuiston et al., 2000).

Natural Infection of Horses with *A. phagocytophilum*

Natural infection of horses with *A. phagocytophilum* was first reported from the foothills of northern California in the late 1960's (Gribble,1969; Stannard et al., 1969). At this time, the disease agent was known as *E. equi*, and the disease was referred to as equine granulocytic ehrlichiosis (EGE).

Reviewing 49 cases of EGE in northern California, Madigan and Gribble (1987) found that cytoplasmic inclusions were observed within peripheral blood neutrophils of infected horses at an infection rate of 1 to 30%. Clinical manifestations included fever, lethargy, reluctance to move, partial inappetance, limb edema, mild petechiation, icterus and ataxia. Hematologic abnormalities included anemia, thrombocytopenia, and leukopenia. The disease was normally self-limiting with a course of 10-14 days in untreated horses. Fatalities were attributed to secondary injuries or infections (Madigan and Gribble, 1987). Subclinical infection may be common in endemic areas. A 1990 survey by Madigan et al. demonstrated *E. equi* seropositivity in 50% of 64 healthy horses at two California ranches.

While *A. phagocytophilum* infection of horses is predominantly observed in California, cases have been diagnosed with increasing frequency in other states including Colorado, Connecticut, Florida, Illinois, Minnesota, New Jersey, Oregon, Washington and Wisconsin. Clinical illness of equines with *A. phagocytophilum* has also been reported in Canada, Brazil, and throughout Europe (Madigan and Pusterla, 2000).

Experimental Infection of Horses with North American Isolates of *A. phagocytophilum*

Historically, because the newly described "agent of HGE" was very similar in cell tropism and microscopic appearance to *E. equi* and because subsequent molecular analysis of the two pathogens revealed nucleotide sequence similarity approaching identity at the level of the 16s ribosomal RNA gene, Madigan et al. (1995) set out to investigate the biologic similarities of these two organisms. Using whole blood from a human acutely ill with granulocytic ehrlichiosis as inoculum, they produced disease in horses indistinguishable from that caused by *E. equi* and concluded that aoHGE and *E. equi* were potentially conspecific.

After this ground-breaking experiment, Chang et al. (1998) inoculated two specific pathogen free (SPF) ponies with blood from two human patients acutely infected with HGE, with the intent of harvesting infected equine neutrophils for development of an indirect immunofluorescence assay (IFA) for the diagnosis of HGE. Both ponies developed typical signs of equine granulocytic ehrlichiosis (EGE), and although they were no longer rickettsemic as determined by PCR at termination of the study on 37 and 38 DPI, 16S rDNA of the pathogen was detected in several tissue samples. This result suggested that *A. phagocytophilum* either persisted in the bloodstream below the level of detection or was sequested in tissues.

Reubel et al. (1998) followed shortly thereafter with a tick transmission study, exposing three horses to naturally infected adult *I. pacificus*. In Reubel's study, two of the horses showed clinical signs typical of EGE and seroconverted. Transient rickettsemia, lasting from 3 to 16 days, was detected by 16S PCR in all three horses.

Pusterla et al. (1999b) used 16S quantitative real-time PCR in the first study of equine *A. phagocytophilum* infection comparing outcome of transmission via syringe and via ticks. Four horses were inoculated with human strains of *A. phagocytophilum* (BDS and Webster) by each route and were monitored for 18 days. While all horses developed transient rickettsemia, the

tick-infected horses demonstrated a longer incubation and later seroconversion. Titer ranges were similar between the two groups. Furthermore, while the initial ehrlichial load was lower, the mean load from 7-9 dpi was higher, and the detection period of rickettsemia was longer in the tick-infected horses.

Kim et al. (2002) conducted a similar study, involving a different human strain (HZ) of *A. phagocytophilum*, using horses experimentally infected by syringe and tick feeding. While the stated purpose of the study was to characterize pathogenic and immune mechanisms of HGE, its basic design also served to illustrate the similar outcomes of the two infection routes. Both the syringe- and tick-inoculated horses developed transient rickettsemia lasting from 4 to 20 DPI, as detected by nested PCR of the *p44* gene target, although only the syringe-infected horses developed clinical signs typical of *A. phagocytophilum* infection.

Experimental Infection Studies of North American Isolates of *A. phagocytophilum* in Cattle

Because of the long-standing existence of sheep and cattle-infective strains of *A. phagocytophilum* in Europe, and the lack of reported disease in North American domestic ruminants despite the established presence of equine- and human-infective strains of *A. phagocytophilum* in the North America, veterinary researchers sought to evaluate the susceptibility of cattle to North American strains of *A. phagocytophilum* isolated from horses and humans. Pusterla et al. (2001) demonstrated that cattle were not susceptible to either an equine strain (MRK) or a human-infective strain (Webster) of *A. phagocytophilum* by intravenous (IV) inoculation. *Anaplasma phagocytophilum*-inoculated cattle demonstrated no rickettsemia, nor did they exhibit signs of clinical illness. The cattle seroconverted at 10 to 12 DPI, and maximum reciprocal titers of 80 to 120 were observed at 30 DPI. Positive control horses developed rickettsemia and clinical illness.

In 2002, Magnarelli et al. reported having surveyed 339 healthy cattle in Connecticut for antibodies to *A. phagocytophilum*, using both IFA and a recombinant HGE-44-based ELISA. By IFA and ELISA, 4% and 12% of samples were positive, respectively. Antibody titers were low, with the majority of positive samples ranging from 80-160 and 160-320 for IFA and ELISA, respectively. To determine banding patterns and for comparison with IFA and ELISA results, six ELISA-positive samples were subjected to western blotting. Few bands were observed; however, five samples showed distinct reactivity to the 44-kDa protein and the remaining sample showed very weak reactivity to this protein.

Murphy et al. (1998) cloned three homologous genes of *A. phagocytophilum* and reported that the P44 outer membrane protein is one of a group of antigens encoded by a multi-gene family with homology to the *msp-2* genes of the closely related cattle pathogen *A. marginale*.

Experimental infection studies of *A. phagocytophilum* in mice

The laboratory mouse is the most widely employed animal model for HGA (Borjesson and Barthold, 2002). In 1996, Telford et al. inoculated *Mus musculus* strains CD-1 and C3H/HeJ via intraperitoneal (IP) injection of various blood products from a Nantucket woman acutely ill with HGA. Five days after inoculation, all four mice exhibited morulae in granulocytes. Furthermore, peripheral blood samples were 16S PCR-positive. Thereafter, the organism (referred to as strain NCH-1) was efficiently maintained in the laboratory via serial blood passage among mice. *Mus musculus* strains CD-1, C3H/HeJ, and C3H/HeN, as well as *P. leucopus* and hamsters all responded with transient, low-level rickettsemia lasting about 10 days, as determined by observation of infected neutrophils and 16S PCR. None of the rodents exhibited clinical signs of illness.

Sub-inoculation of naive C3H mice with blood from C3H mice that appeared to have lost infection (ie. in which rickettsemia was no longer detectable) resulted in infection of the naive mice. Furthermore, splenectomy of CD-1 mice that appeared to have lost infection resulted in recrudescence of rickettsemia. Immunodeficient strains of *M. musculus*, such as SCID and DBA maintained rickettsemia for greater than 6 weeks.

In 1997, after *A. phagocytophilum* isolates became available for study, Sun et al. designed a set of experiments to evaluate the immunology of HGA. Injecting the NCH-1 isolate into C3H mice, they found that untreated mice resolve *A. phagocytophilum* infection within 60 days. The first experiment involved vaccination of nine naive C3H mice with heat-killed *A. phagocytophilum* lysate, followed by two bi-monthly boosters. The mice were then infested with *A. phagocytophilum*-infected *I. scapularis*. When the mice were killed at 14 DPI, only 5 of 9 mice were PCR-positive and all were culture-negative for *A. phagocytophilum*, whereas 9 of 9 sham-vaccinated control mice were PCR-positive and culture-positive for the organism. In a second experiment, 12 mice were infected with *A. phagocytophilum* antisera and then challenged either with *A. phagocytophilum*-infected ticks or via IP inoculation of the organism. When the mice were killed at 14 DPI, only 4 of 12 were PCR-positive and 1 of 12 was culture-positive for *A. phagocytophilum*, whereas 12 of 12 control mice were PCR- and culture-positive for the organism. These data led the authors to conclude that humoral immunity to *A. phagocytophilum* is partially protective.

In 2000, Levin and Fish used a strain of *A. phagocytophilum* isolated from *I. scapularis* nymphs collected in Westchester County, New York to further investigate protective immunity in WFM. Mice were infected by allowing *A. phagocytophilum*-infected laboratory *I. scapularis* nymphs to feed upon them. The mice were then challenged either 12 or 16 weeks later by the

same method. Only 30% of "immunized" mice became infected, compared to 100% of control mice. Furthermore, an average of 6.3% of xenodiagnostic *I. scapularis* larvae acquired *A. phagocytophilum* from "immunized mice" when fed one week after challenge, compared to an 82.5% rate of acquisition from control mice. The authors concluded that immunity to *A. phagocytophilum* acquired by mice during *I. scapularis* nymphal activity in early summer may preclude a large proportion of the same mouse population from maintaining the organism during the period of larval activity later in the summer. These findings led the authors to speculate that host species of *I. scapularis* other than WFM might be involved in the maintenance of *A. phagocytophilum* in nature.

In 2004, Levin et al. used quantitative real-time PCR to assess humoral immunity in mice infected with *A. phagocytophilum* via tick feeding. They found that the presence of antibodies did not protect mice from re-infection with either the same or a sympatric strain of *A. phagocytophilum*. However, the second infection was milder and of shorter duration than the first infection with either isolate. Furthermore, the proportion of infected mice was smaller, the level of rickettsemia was lower, and the prevalence of infection in xenodiagnostic larvae was lower in mice exposed to *A. phagocytophilum* for the second time than in groups of control mice exposed for the first time.

Anaplasma phagocytophilum in white-tailed deer (*Odocoileus virginianus*)

White-tailed deer are the principal host for adult *I. scapularis* (Lane et al., 1991) and are thus likely to be routinely exposed to *A. phagocytophilum*. Seropositive WTD have been reported in Connecticut, Georgia, Maryland, Missouri, South Carolina, and Wisconsin (Arens et al., 2003; Belongia et al., 1997; Little et al., 1998; Magnarelli et al., 2004; Magnarelli et al., 1999; Walls et al., 1998). In IFA serosurveys involving large (>100) sample sizes, prevalence of

antibodies to *A. phagocytophilum* in wild WTD were 2% in Missouri, 43% in Connecticut, and from 8-60% in Wisconsin (Arens et al., 2003; Magnarelli et al., 2004; Belongia et al. 1997; Walls et al., 1998). The majority of the serologic data has been determined via IFA, however Magnarelli et al. (2004) reported that recombinant HGE-44-based ELISA results showed good agreement with IFA results in determining *A. phagocytophilum* antibody prevalence in 238 WTD sera from Connecticut and South Carolina.

Molecular evidence of *A. phagocytophilum* infection among WTD has been reported from Connecticut, Georgia, and Wisconsin (Belongia et al., 1997; Little et al, 1998; Magnarelli et al., 1999). Belongia et al. (1997) reported a 15% (27/181) prevalence of *A. phagocytophilum* 16S rDNA in blood samples from Wisconsin WTD. Little et al. (1998) reported that 3/5 blood samples from WTD on a Georgia barrier island were positive for *A. phagocytophilu*m by 16S PCR. Magnarelli et al. (1999) reported prevalences of *A. phagocytophilum* DNA in Connecticut WTD blood as 18% (11/63) and 37% (23/63) via 16S and *msp* PCR, respectively. *Anaplasma phagocytophilum* has not yet been isolated from WTD.

Massung et al. (2002) have hypothesized that biologically significant genovariants of *A. phagocytophilum* cycle independently of human-infective strains of *A. phagocytophilum* (AP-ha). One variant (AP-var1), detected in WTD in Maryland and Wisconsin and *I. scapularis* in Rhode Island (Massung et al., 1998; Belongia et al., 1997), is identified by a 2 base pair difference in the 16S rRNA sequence from the sequences of the original human cases, as well as the majority of extant human cases (Chen et al., 1994; Petrovec et al., 1997).

To investigate the significance of AP-var1, Massung et al. (2003) collected questing *I. scapularis* nymphs from Rhode Island. Using 16S PCR, they determined the prevalences of AP-ha and AP-var1 in a subset of the nymphs, and allowed the remainder of the nymphs to feed

upon three species of laboratory mice: DBA/2 mice, WFM, and SCID mice. Infection of mice was monitored by 16S PCR of blood. Of 12 mice exposed to AP-ha, 9 became infected with AP-ha. In contrast, of the 22 mice exposed to AP-var1, none became infected. These findings led the authors to hypothesize that AP-var1 is non-infectious to mice and that AP-var1 has an alternative reservoir in nature, possibly WTD.

Few surveys of North American wild cervids other than WTD have been conducted; however, one study in northern California reported black-tailed deer (*O. hemionus columbianus*) and elk (*Cervus elaphus nannodes*) to be 16S PCR-positive for *A. phagocytophilum*. Moreover, sequence-confirmed *A. phagocytophilum* was isolated in HL60 cell culture from two of the elk blood samples (Foley et al., 1998).

Although this review focuses primarily on North American strains of *A. phagocytophilum*, it is interesting to note that a parallel situation exists in European cervids. In Slovenia, roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*), which are primary hosts for adult *I. ricinus* ticks, are reported to have a high prevalence of *A. phagocytophilum* infection (86%) as detected by 16S PCR (Petrovec 2002). In Norway, *A. phagocytophilum* antibody prevalences in moose (*Alces alces*), red deer, and roe deer were 43%, 55%, and 96% respectively (Stuen et al., 2002). Inoculation of three red deer calves with *A. phagocytophilum*-infected sheep blood resulted in rickettsemia, as determined by 16S PCR of peripheral blood or observation of infected granulocytes. Infected neutrophils were observed in all three calves between 4 and 10 DPI and in one calf 13 weeks after inoculation. Clinical or pathologic abnormalities attributable to infection with *A. phagocytophilum* were not apparent (Stuen et al., 2001).

Review of WTD-agent in White-tailed Deer

In 1996, a novel "*Ehrlichia*-like" 16S rRNA gene fragment was amplified by PCR from the blood of wild WTD from Georgia and Oklahoma. DNA sequence analysis of the PCR products revealed that they were similar to, but distinct from members of what was then known as the "*E. phagocytophila* genogroup", having approximately 94% sequence identity with *E. phagocytophila*, *E. equi*, aoHGE, and *E. platys* (Dawson et al., 1996). Use of a PCR protocol developed specifically for detection of this organism (Little et al., 1997), which came to be known as "WTD-agent", demonstrated its prevalence in WTD populations in Alabama, Arkansas, Florida, Kentucky, Louisiana, Maryland, Missouri, North Carolina, South Carolina, Tennessee and Virginia (Little et al, 1997; Lockhart et al., 1997b; Brandsma et al., 1999). Only those deer populations infested by the lone star tick were infected (Little et al., 1997; Brandsma et al., 1999), suggesting that LST might be a vector of this organism (Brandsma et al., 1999). Furthermore, DNA sequences of 16S PCR-products amplified from a questing adult LST collected in Georgia were identical to sequences amplified from WTD blood (Lockhart et al., 1997b). A small sampling of California cervids (21 mule deer and black-tailed deer) revealed that 16S PCR prevalence of this *Anaplasma* sp. was 38% (Foley et al., 1998). This finding is important, because the suspected tick vector of this *Anaplasma* sp. in the southeastern U.S., the lone star tick, does not exist in California.

The *Anaplasma* sp. was isolated in tick cell culture by Munderloh et al. in 2003. Isolation was achieved via repeated culture attempts of buffy coat cells from two WTD fawns experimentally infected with the *Anaplasma* sp. via field-collected WTD blood. A second gene target for detection and phylogenetic analysis of the *Anaplasma* sp. was obtained by Sumner et al. in 2003. Using platelet-rich plasma from one experimentally infected fawn, the authors amplified *gro*ESL heat-shock operon sequences of the *Anaplasma* sp. and demonstrated that the

DNA sequence was most similar to *gro*ESL sequences from *A. platys* (83%) and *A. phagocytophilum* (80-83%), in concordance with previous results of 16S sequence comparisons.

Review of other members of Anaplasmataceae in White-tailed Deer

Ehrlichia chaffeensis in White-tailed Deer

White-tailed deer are the principal reservoir host of *E. chaffeensis*. Seroreactivity of deer to *E. chaffeensis* is common in deer populations throughout the southeastern and south-central United States; a recent survey of 3275 WTD from 17 states revealed 47% (1549) seropositivity, as determined by IFA testing (Yabsley et al., 2003).

The course of *E. chaffeensis* infection in deer has been investigated experimentally. Inoculation of two WTD fawns with a human isolate (Arkansas strain) of *E. chaffeensis* resulted in several re-isolations of the organism, demonstrating host competency of WTD (Dawson et al, 1994). An experimental transmission trial by Ewing et al. (1995) demonstrated the competence of LST to transmit *E. chaffeensis* to WTD. Natural infection of WTD with *E. chaffeensis* was confirmed by PCR-positive blood samples from wild deer in Georgia, Missouri and South Carolina (Little et al., 1997; Lockhart et al., 1997b). Isolation of *E. chaffeensis* from blood cultures of five naturally infected WTD in Georgia validated the role of WTD as a reservoir host (Lockhart et al., 1997a).

An experimental investigation of the persistence of *E. chaffeensis* in WTD further emphasized the importance of the WTD as a reservoir host (Davidson et al., 2001). Four WTD were inoculated with a WTD-derived isolate of *E. chaffeensis* and monitored for 9 months via IFA, PCR, and culture. *Ehrlichia chaffeensis* was re-isolated on at least two and as many as 6 occasions from each of 3 deer, as late as 73-108 DPI. Postmortem tissue samples at 278 DPI yielded positive PCR results from a rumen lymph node of one deer and a femoral bone marrow

sample of another deer. In concordance with the role of WTD as a reservoir host of *E. chaffeensis*, there are no reports of WTD having clinical signs associated with experimental or natural infection.

Ehrlichia ewingii in White-tailed Deer

WTD are naturally infected with *E. ewingii* (Yabsley et al., 2002). In that study, 110 wild deer blood samples from eight states revealed that 5.5% were PCR-positive for *E. ewingii*. States with PCR-positive WTD blood samples included Arkansas, Georgia, Kentucky, North Carolina and South Carolina. Furthermore, 20% of 217 deer blood samples from Missouri were PCR-positive for *E. ewingii* (Arens et al., 2003). Host competence was demonstrated by experimental infection of two captive WTD fawns with field-collected deer blood (Yabsley et al., 2002). *Ehrlichia ewingii* has not yet been isolated in cell culture.

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CHAPTER 2

EXPERIMENTAL INFECTION OF WHITE-TAILED DEER WITH *ANAPLASMA PHAGOCYTOPHILUM*, ETIOLOGIC AGENT OF HUMAN GRANULOCYTIC ANAPLASMOSIS[1]

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ABSTRACT

Serologic and molecular evidence of *Anaplasma phagocytophilum* has been demonstrated in white-tailed deer (WTD; *Odocoileus virginianus*) and deer are an important host for the tick vector, *Ixodes scapularis*. In this study we describe experimental infection of WTD with *A. phagocytophilum*. We inoculated four WTD with a human isolate of *A. phagocytophilum* propagated in tick cells. Two additional deer served as negative controls. All inoculated deer developed antibodies (titers ≥ 64) to *A. phagocytophilum*, as determined by indirect fluorescent antibody test, between 14 and 24 days post-infection (DPI), and two deer maintained reciprocal titers ≥ 64 through the end of the 66-day study. Although morulae were not observed in granulocytes and *A. phagocytophilum* was not re-isolated via tick cell culture of blood, 16S RT-nPCR results indicated that *A. phagocytophilum* circulated in peripheral blood of three deer through at least 17 DPI and was present in two deer at 38 DPI. Femoral bone marrow from one deer was RT-nPCR positive for *A. phagocytophilum* at 66 DPI. There was no indication of clinical disease. These data confirm that WTD are susceptible to infection with a human isolate of *A. phagocytophilum* and verify that WTD produce detectable antibodies upon exposure to the organism. Because adults are the predominant life stage of *I. scapularis* found on deer and because adult *I. scapularis* do not transmit *A. phagocytophilum* transovarially, it is unlikely that WTD are a significant source of *A. phagocytophilum* for immature ticks even though deer have a high probability of natural infection. However, the susceptibility and immunologic response of WTD to *A. phagocytophilum* render them suitable candidates as natural sentinels for this zoonotic tick-borne organism.

INTRODUCTION

Human granulocytic anaplasmosis is an acute, febrile disease that may be accompanied by headache, myalgia, pancytopenia and elevated serum aminotransferase levels (13). Although the disease is often mild, delayed treatment, misdiagnosis, and/or immunosuppression may result in severe or fatal outcome (28, 31). Approximately 1,220 cases of human granulocytic anaplasmosis (HGA) have been diagnosed in United States since 1994, when the disease was first described (13, 14). Sporadic HGA cases have been reported in Europe (10).

Previous to its recognition as a human pathogen, the organism was known in veterinary medicine as *Ehrlichia equi* (causing equine granulocytic ehrlichiosis) and *E. phagocytophila* (causing tickborne fever in sheep, goats, and cattle in several European countries). Based on recent phylogenetic analysis, the etiologic agent of HGA and *E. equi* were synonomized with *E. phagocytophila*. Furthermore, the analysis indicated that this organism should be re-assigned to the genus *Anaplasma*, thus resulting in the currently accepted designation, *A. phagocytophilum* (20). The marked differences in host preference, clinical manifestations and geographical distribution are attributed to the existence of multiple variant strains of *A. phagocytophilum* (20).

North American strains of *A. phagocytophilum* cause clinical disease in domestic animals, notably horses and dogs (20). Experimentally, both equine and human *A. phagocytophilu*m strains are pathogenic to horses (37) and cross-protective (6). Disease due to *A. phagoctyophilum* in domestic cattle has not been reported in North America. Furthermore, two steers were not susceptible to experimental infection with human and equine North American strains; although the steers seroconverted, blood was negative for *A. phagocytophilum* by 16S real-time PCR at all sampling dates (51). *Anaplasma phagocytophilum* was first isolated

from human patients using a human promyelocytic leukemia (HL60) cell line (24) and from horses and dogs using *Ixodes scapularis* cell lines (45, 46).

Knowledge of the natural history of *A. phagocytophilum* remains incomplete. In the eastern and midwestern United States, the white-footed mouse (*Peromyscus leucopus*) and the black-legged tick (*I. scapularis*) are a competent reservoir host and principal vector, respectively (54, 19). Although field studies indicate that numerous species of wild rodents and other wild mammals may be naturally infected with *A. phagocytophilum* (22, 23, 32, 40, 48, 59), the relative importance of these hosts as sources of *A. phagocytophilum* for ticks has not been determined.

White-tailed deer (WTD; *Odocoileus virginianus*) are the principal host for adult *I. scapularis* (30) and therefore predictably are exposed to *A. phagocytophilum*. Sequence-confirmed 16S rDNA identical to that of *A. phagocytophilum* and/or *A. phagocytophilum*-reactive antibodies have been demonstrated in wild WTD from Connecticut, Georgia, Indiana, Maryland, Missouri, South Carolina and Wisconsin (3, 8, 33, 38, 39, 60)**;** however, clinical disease due to *A. phagocytophilum* has not been reported in WTD. Thus, WTD have been identified as both a potential sentinel species for *A. phagocytophilum* (8, 33, 40, 39, 60) as well as a potential reservoir host of *A. phagocytophilum* (3, 5, 6, 8, 43); however, these potential roles have not been analyzed because data on susceptibility to and course of infection with *A. phagocytophilu*m in WTD have previously been unavailable. Therefore, we proposed to analyze these potential roles by experimentally infecting WTD with *A. phagocytophilum*.

MATERIALS AND METHODS

Animals and experimental design. Six WTD fawns (three females and three males, orphaned in the state of Georgia) were hand-raised and housed in tick-free facilities at the College of Veterinary Medicine, University of Georgia. Prior to initiation of the experiment, at

approximately 7 months of age, the deer were screened for various hematotropic micro-organisms known to infect wild WTD in the southeastern United States. The deer were determined to be seronegative by indirect fluorescence antibody (IFA) test for *A. phagocytophilum* and *E. chaffeensis* (8, 17) and PCR-negative for *A. phagocytophilum*, *E. chaffeensis*, *E. ewingii* and an undescribed *Anaplasma* sp. of WTD, as previously described (33, 44). All deer were determined to be culture-negative for *Trypanosoma cervi*, a protozoan known to interfere with tissue culture of deer blood (35, 36, 44). Two deer (WTD132 and WTD133) were diagnosed with *Theileria cervi* infection by observation of intra-erythrocytic protozoans in whole blood smears.

For all procedures, deer were anesthetized as described previously (16). Deer blood samples were collected via aseptic jugular venipuncture at 0, 6, 10, 13, 17, 24, 31, 38, 45, 54, and 66 DPI. Approximately 9 ml of whole blood was collected for culture, reverse transcriptase nested polymerase chain reaction (RT-nPCR) and clinical hematology in three separate EDTA Vacutainer tubes (Beckton Dickinson, Rutherford, NJ) and for serology in a tube containing no additive. On all sampling dates, Giemsa-stained blood smears were prepared and whole blood was submitted to the Clinical Pathology Laboratory, College of Veterinary Medicine, The University of Georgia, for analysis of the following parameters: hematocrit, erythrocyte count, hemoglobin levels, platelet count, total and differential leukocyte counts, and fibrinogen. Sera and duplicate samples of whole blood were stored at -20°C.

Deer were observed twice daily for visible signs of clinical illness, including decreased feed intake, depression and reluctance to move. Complete physical examination (11) of each deer was performed at each blood collection date. The two control deer were removed from the study

at 31 DPI. All deer were euthanized via intravenous sodium pentobarbital overdose and subjected to complete necropsies.

Preparatory to and concurrently with the experimental deer trial, female C3H-HeN mice (Harlan; Indianapolis, IN), approximately six weeks old, were used to test animal infectivity of the *A. phagocytophilum* isolate propagated in tick cells (46). Mice were anesthetized for all procedures via subcutaneous injection of 10 mg/kg xylazine (Phoenix Scientific, Inc., St. Joseph, MO) and 100 mg/kg ketamine HCl (Ft. Dodge Labs, Inc., Fort Dodge, IA). Samples of 6 DPI blood for RT-nPCR were collected retro-orbitally from all mice. After euthanasia of mice, blood samples were collected for culture by intracardiac puncture, and spleen samples were taken for RT-nPCR. All animals involved in this study were cared for and used in accordance with guidelines established by the Institutional Animal Care and Use Committee of the University of Georgia.

***Anaplasma phagocytophilum* inoculum.** An *I. scapularis* tick cell line (ISE6) was used to propagate a human isolate (HGE-1) of *A. phagocytophilum* (46). Stock ISE6 cultures were maintained as described previously (47). The inoculum consisted of five 12.5 cm^2 Falcon culture flasks (Beckton Dickenson; Franklin Lakes, NJ) of *A. phagocytophilum*-infected tick cells, in which approximately 35% of the cells were infected. Monolayers were resuspended in existing media, and sterile water (Sigma; St. Louis, MO) was added to bring the volume of each flask to 8.0 ml. Then contents of all five flasks were combined for a total volume of 40 ml, and the mixture was divided into 2.0 ml aliquots for deer and 0.5 ml aliquots for mice. Negative control injection material was prepared in a similar manner, using uninfected stock ISE6 cultures.

Each of four deer (WTD131, 132, 133, 134) was injected with 2.0 ml of the *A. phagocytophilum* inoculum by each of four routes: intradermal (ID), subcutaneous (SC),

intravenous (IV) and intraperitoneal (IP) for a total of 8.0 ml of inoculum per deer. Concurrently with the deer inoculation, each of nine mice was injected IP with 0.5 ml of the *A. phagocytophilum* inoculum. Two negative control deer (WTD127 and WTD139) and three negative control mice were injected in a similar manner with uninfected ISE6 cells. Remaining fractions of the *A. phagocytophilum* inoculum and uninfected tick cells were used to prepare Giemsa-stained cytospins and were tested by RT-nPCR and DNA sequencing (see below).

Serology. The IFA test was performed as described previously (8). In brief, sera were screened at a dilution of 1:64 in 0.01 M phosphate-buffered saline (PBS) on commercially prepared HGE substrate slides (Focus Technologies, formerly MRL Diagnostics; Cypress, CA). Fluorescein isothiocyanate-labelled rabbit anti-deer immunoglobulin G (Kirkegaard & Perry Laboratories; Gaithersburg, MD), diluted 1:50 in PBS, was used as a conjugate. When distinct fluorescent staining of organisms was observed at a 1:64 dilution, serial two-fold dilutions were performed. Serologic results are reported as reciprocals of the highest dilution at which specific fluorescence was observed.

Cell culture. Re-isolation attempts of *A. phagocytophilum* from mouse and deer blood were performed as described (45, 44) except that resuspended ISE6 stock cell monolayers and washed WTD buffy coat cells were mixed, pelleted at 720 x g for 20 minutes, and then allowed to stand at room temperature for 30 minutes before the pellet was resuspended in "ehrlichia medium" (46) and divided into two flasks. Duplicate flasks were monitored and maintained separately using different sets of reagents as a precaution against contamination; antibiotics were not used at any time. Cultures were monitored by visual observation for cytopathic effect (CPE) (46), light microscopy of Giemsa-stained cell spreads, and RT-nPCR of cell culture supernatant. Monolayers were examined daily for development and progression of CPE. Periodically,

samples were prepared from all cultures by centrifugation (720 x g for 20 minutes) of the entire volume of spent medium removed during feeding of the culture, followed by resuspension of pelleted cells in approximately 1.0 ml of spent medium. For cell spreads, 100 ul of each sample was placed in a Cytofuge® filter concentrator (StatSpin®, Iris Co.; Norwood, MA) and centrifuged at 27 x g for 4 minutes using a Cytofuge® 2 cytocentrifuge (StatSpin®). Slides were air-dried, fixed in 100% methanol and stained in a 4% solution of Giemsa (Karyomax, GIBCO; Grand Island, NY) in Sorensen buffer, pH 6.5, for 30 minutes in a 37°C water bath. Stained cells were examined microscopically (400-1000x) for intracytoplasmic organisms. Our cell culture protocol specified a 60-day monitoring period for CPE, followed by RT-nPCR testing.

RNA extraction, RT-nPCR and nucleotide sequence analysis. Total RNA was extracted from fresh deer and mouse blood samples with the RNA Blood Minikit (QIAGEN, Inc., Valencia, CA) and from cell culture aliquots and post mortem tissue stored at -70°C with the QiAmp® Viral RNA Extraction Kit and the RNEasy Minikit (QIAGEN, Inc.), respectively. All extractions were performed according to the manufacturer's instructions, and in an RNAase-free environment. RT-nPCR was performed on RNA extracted from deer blood, cell culture, and tissues in the following manner. Reverse transcription of 16S r-RNA to cDNA and subsequent primary amplification using primers ECC and ECB were carried out in a single-tube reaction, followed by secondary amplification as described previously (44), except that secondary primers GE9F and GA1UR were used to generate an internal 411 bp fragment (13, 34).

Two additional gene targets (p44 and *groESL*) for detection of *A. phagocytophilum* RNA were used. For detection of p44 RNA, RT-PCR was performed using primers MSP3F and MSP3R (65). Reaction mixtures were subjected to 45°C for 20 minutes, followed by 39 cycles of the following profile: 94°C for 60 seconds (s), 55°C for 45 s, 72°C for 60 s, using a PTC-

100TM Thermal Cycler. For detection of groESL RNA, RT-nPCR was performed using primers APF1 (5'TAGTGATGAAGGAGAGTGAC) and APR1 (5'CCAGGIGCCTTIACAGCWGCAAC) in a primary reaction and primers APF10 (5'TATGCTACGGTTGTTTGTTC) and APR11 (5'GGCGAAAGATATCCGCGA) in a secondary reaction to generate a 652 bp product (primers were generously provided by Dr. John Sumner, Centers for Disease Control). Primary reaction mixtures were subjected to 43°C for 15 minutes (m), followed by 95°C for 5 m, and 39 cycles of the following profile: 95°C for 30 seconds (s), 52°C for 30 s, 72°C for 60 s, and a final step of 72°C for 5 m. Secondary reaction mixtures were subjected to 95°C for 5 m, 29 cycles of the following profile: 95°C for 30 seconds (s), 52°C for 30 s, 72°C for 60 s, and a final step of 72°C for 5 m.

All RT-PCR reactions were carried out in a PTC-100TM Thermal Cycler. All reagent concentrations were identical to those used for *A. phagocytophilum* 16S rRNA RTn-PCR. Amplification products were separated by electrophoresis in a 2% agarose gel, stained in ethidium bromide and visualized using ultraviolet transillumination.

Quality control measures included negative controls (water) that were extracted and amplified in parallel with all specimens. In order to minimize the potential for DNA contamination, three separate, designated areas were used for extraction of RNA and preparation of primary and secondary PCR reactions. Additionally, two thermal cyclers were used, designated for either primary or secondary amplification.

RT-nPCR products from the original inoculum, all deer blood samples, selected mouse blood samples and cell culture aliquots, and one post-mortem tissue sample were subjected to DNA sequencing. Gene fragments were purified by gel electrophoresis, and bands were extracted and purified using the QIAquick gel extraction kit (QIAGEN, Inc.). DNA was

sequenced in forward and reverse directions at the Molecular Genetics Instrumentation Facility, The University of Georgia, with an ABI 3100 automated sequencer (Applied Biosystems, Perkin Elmer Corp, Foster City, CA). The sequences were assembled and edited using the Sequencher software package version 4.1.4 (Gene Codes Corp., Ann Arbor, MI). A nucleotide-nucleotide BLAST (blastn) search was performed to determine the most similar sequences of the target genes published in GenBank (http://www.ncbi.nlm.nih.gov).

Due to incidental molecular detection of *Bartonella* sp. in deer blood cultures during the course of the experiment, cell culture samples were subjected to RT-PCR for the *gltA* gene of *Bartonella* sp. using primers CS140f and BhCS1137n (9). In order to increase sensitivity, a hemi-nested RT-PCR was developed for the detection of *Bartonella* sp. directly from peripheral blood, using primer set CS140f and BhCS1137n in the primary reaction, and Bh731p and BhCS1137n (49) in the secondary reaction. Reagent concentrations and reaction conditions were identical to those used for 16S rRNA *A. phagocytophilum* RT-nPCR.

Pathology. Tissues collected for RT-nPCR included spleen, prescapular and prefemoral lymph nodes, bone marrow from the sternum and femoral head, and lung. The aforementioned tissues as well as heart, liver, kidney, adrenal gland, brain, bladder, haired skin, reproductive organs and gastrointestinal tract also were collected in 10% neutral buffered formalin for histopathologic examination.

RESULTS

Animal infectivity of *A. phagocytophilum* inoculum. The animal infectivity of the *A. phagocytophilum* inoculum was confirmed via RT-nPCR and DNA sequencing of a subset of products obtained from DPI 6 blood of 7 of 9 inoculated mice using both the 16S and p44 gene targets (data not shown). Furthermore, rare morulae were observed in granulocytes of 6 DPI

blood from RT-nPCR-positive mice. Although postmortem spleen samples for the previously mentioned 7 mice were positive for *A. phagocytophilum* by 16S RT-nPCR, the organism was not re-isolated in cell culture from terminal blood pooled from these mice at 11, 15 and 20 DPI**.** Negative RT-nPCR results of blood and spleen samples were obtained for mice inoculated with uninfected tick cells.

Serology. All experimental deer developed reciprocal antibody titers ≥ 64 to *A. phagocytophilum* between 14 and 24 DPI (Table 1). Two experimental deer (WTD133 and WTD134) seroconverted by 17 DPI and remained seropositive throughout the 66-day study. The peak reciprocal titer of 2,048 was detected in one of these deer (WTD134) on 38 DPI. The other two experimental deer (WTD131 and WTD132) had peak reciprocal titers of 128 and were seropositive only through 38 DPI. The geometric mean (57) of all titers ≥ 64 was 115. *Anaplasma phagocytophilum* IFA results for the control deer were negative (< 64) on all sample dates.

RT-nPCR and cell culture. Blood from all 4 experimental deer was positive for *A. phagocytophilum* by 16S RT-nPCR on one to five occasions between 6 and 38 DPI **(Table 2.1)**. All 16S rDNA amplicons from deer blood were sequenced and found to be identical to the sequence of the original inoculum and 99.5% similar to a published sequence of *A. phagocytophilum* (GenBank accession number U02521). Blood from WTD 132 and WTD 133 was positive for *A. phagocytophilum* by groESL RT-nPCR on 13 and 17 DPI. Sequenced groESL products were 100% identical to published sequences of *A. phagocytophilum* (GenBank accession numbers AY219849 and U96728). Use of the p44 RT-PCR did not yield *A. phagocytophilum* amplicons from deer blood**.** Blood from the two negative control deer

(WTD127 and 139) was negative for *A. phagocytophilum* by 16S and groESL RT-nPCR, and by p44 RT-PCR at 6, 10, 13, 17, 24 and 31 DPI.

Anaplasma phagocytophilum was not re-isolated in any of 36 culture attempts from experimental deer (Table 1). All DPI 10 and DPI 45 cultures failed when tick cells did not reform a monolayer in the flask after the deer blood culture procedure was completed. Of 29 remaining culture attempts of *A. phagocytophilum*-inoculated deer, 23 cultures exhibited CPE, and intracellular bacteria were observed in Giemsa-stained cytospins. Although RT-nPCR of these cell culture samples, using primers ECC/ECB and GE9F/GA1UR, yielded products of the expected size, DNA sequencing revealed that they were not 16S rRNA gene fragments of *A. phagocytophilum*, but rather of *Bartonella* sp. (see below).

Control deer blood cultures and experimental deer blood cultures not exhibiting CPE from 6, 17, 24, 31 and 38 DPI were lost due to a single bacterial contamination event involving use of a commercially purchased component of the tick cell media at 45, 34, 27, 20 and 13 days in culture, respectively. For the same reason, the 3 mouse blood cultures from 11, 15 and 20 DPI were lost at 40, 36 and 31 days in culture, respectively.

Clinical, hematologic and post-mortem findings. Clinical signs attributable to infection with *A. phagocytophilum* were not apparent in any deer throughout the 66-day study. Morulae were not observed in granulocytes on Giemsa-stained deer blood smears. Although results of complete blood counts were not always within normal limits for all fawns on all sampling dates, no consistent pattern of hematologic abnormalities attributable to infection with *A. phagocytophilum* was apparent.

At four sampling dates between 30 and 55 DPI, WTD133 exhibited low platelet counts (48 to 168 x 10^3/uL) relative to its own values before and after that time period and relative to all

platelet counts of the other experimental deer and the negative control deer (mean = 685 $x10^3$/uL). During this time period, WTD133 showed no other signs consistent with thrombocytopenia.

Gross and histopathologic lesions attributable to infection with *A. phagocytophilum* were not apparent in any deer. RT-nPCR assays of post-mortem samples of femoral and sternal bone marrow, prescapular and prefemoral lymph node, spleen and lung of experimental and control deer were negative for *A. phagocytophilum*, with the exception of a sequence-confirmed 16S rDNA amplicon from femoral bone marrow of WTD133.

Detection of incidental *Bartonella* sp. infections. Sequencing of 16S rDNA products amplified with primer sets ECC/ECB and GE9F/GA1UR from a randomly chosen subset (7 of 24) of 16S RT-nPCR positive cell culture samples revealed that the products were 98.6% similar to a sequence of *B. schoenbuchensis* (GenBank accession number AJ278190.1), an intra-erythrocytic bacteria first isolated from the blood of wild roe deer (*Capreolus capreolus*) in Germany (18). Subsequently, cell culture samples were subjected to RT-nPCR for the citrate synthase (*gltA*) gene of *Bartonella* spp., and 21/24 yielded products of the expected size. Furthermore, RT-hemi-nested gltA PCR for *Bartonella* spp. developed in this study for screening whole blood yielded products of the expected size from pre-inoculation blood of two deer, WTD134 and WTD127. DNA sequencing and alignment indicated that these two gltA fragments had 98.6% and 94.6% sequence similarity, respectively, to a sequence of *B. schoenbuchensis* (GenBank accession number AJ564633) recently isolated from the midgut of a deer ked (*Lipoptena cervi*). One 16S rDNA sequence of the *Bartonella* sp. amplified from tick cell culture and the gltA DNA sequences of the *Bartonella* sp. amplified from the blood of

WTD134 and WTD127 are available under GenBank accession numbers AY805111, AY805109, and AY805110, respectively.

DISCUSSION

We demonstrated that WTD can support infection with a human-infective strain of *A. phagocytophilum* and that infection of WTD with *A. phagocytophilum* is accurately reflected by seroconversion. Circulation of *A. phagocytophilum* in peripheral blood of experimental deer was transient, similar to mice and horses experimentally infected with various North American isolates of the organism (2, 27, 51, 50). *Anaplasma phagocytophilum* RNA was infrequently detectable in peripheral blood of experimental deer after development of antibodies, suggesting that the humoral immune system may have played a role in bacterial clearance. Although the function of humoral immunity in host elimination of rickettsiae is not well understood, Winslow et al. (61) demonstrated that antibodies can affect the course of active infection of *E. chaffensis* in SCID mice. Administration of immune sera 10 and 17 days after infection resulted in partial clearance of *E. chaffeensis* from infected mice; however, the organism eventually re-colonized the liver. This indicated that antibodies failed to mediate complete bacterial clearance. Subsequently, the authors hypothesized that *E. chaffeensis* may have persisted in low levels in the liver or emigrated from tissues that were inaccessible to the antibodies.

In the present study, *A. phagocytophilum* was detected by RT-nPCR in peripheral blood of one deer (WTD131) on 38 DPI, following a 3-week period of negative RT-nPCR results. The occurrence of recrudescent rickettsemia has been documented in a 9-month study of WTD experimentally infected with *E. chaffensis* (16). Recrudescent rickettsemia may result from release of organisms sequestered in tissue, but this hypothesis requires further evaluation with regard to *A. phagocytophilum* in WTD. The detection of *A. phagocytophilum* RNA in femoral bone marrow from WTD133 on 66 DPI suggests a potential site of latent infection.

While *A. phagocytophilum* was not visualized in granulocytes of experimental deer, the difficulty of finding and definitively identifying morulae in blood smears of humans with confirmed *A. phagocytophilum* infections is well known (58, 5), particularly in afebrile patients (4, 1). Furthermore, studies demonstrate that blood of experimentally-infected laboratory mice, although morula-negative, was still infectious to naïve mice, and sometimes PCR positive and culture positive as well (54, 26, 53). Thus, our negative light microscopy results for the deer are consistent with those of several previous experimental infection studies in mice.

Demonstration of gene transcription by the use of RT-nPCR is suggestive of *A. phagocytophilum* survival and replication within the deer (21). Therefore, our 16S RT-nPCR data imply that viable *A. phagocytophilum* circulated until at least 17 DPI in three of the four deer. Although limited, this time period is of sufficient duration hypothetically to infect ticks. Nonetheless, we contend it is unlikely that WTD play an epidemiologically significant role as a source of *A. phagocytophilum* for ticks. This conclusion is based on the fact that WTD are parasitized primarily by the adult forms of *I. scapularis* (30) and therefore are most likely to be exposed to *A. phagocytophilum* at the end of the tick life cycle. Furthermore, *A. phagocytophilum* is not known to be maintained transovarially in the tick.

Anaplasma phagocytophilum infection initiated by a single needle inoculation may differ substantially from infection naturally acquired by the bite of one or more infected ticks over the course of a season. In addition to the likelihood that wild WTD experience multiple exposures to *A. phagocytophilum*, various immunologically active components of tick saliva may be important factors influencing the outcome of natural exposures of WTD to *A. phagocytophilum* (25).

We succeeded in re-isolating *A. phagocytophilum* from a mouse inoculated with tick cell culture in the development phase of our research (unpublished data); however, we were unsuccessful in three attempts to re-isolate *A. phagoctyophilum* from mice inoculated concurrently with deer. Relatively few studies have attempted culture of blood from laboratory mice; some also report discrepancies between cell culture and PCR results (26, 53).

With regard to our attempts to re-isolate *A. phagocytophilum* from experimental deer, we encountered significant difficulties related to three separate issues. First, on two culture days (10 and 45 DPI), tick cells failed to reattach after the monolayer was disrupted and the cells were admixed with deer buffy coat cells. On these days, cellular debris created during resuspension may have resulted in cytotoxicity to the tick cells, as reported previously (64). Alternatively, the deer buffy coat cells may have killed the tick cells (Munderloh, unpublished). Second, although the experimental design specified monitoring all cultures not developing CPE for 60 days, bacterial contamination of a commercially purchased component of the tick cell media resulted in the loss of many cultures before the end of 60 days. Because all "negative" cultures initiated previous to the contamination event on 51 DPI were destroyed, "negative" culture results for WTD 127, 132, 133 and 139 are equivocal. Third, for at least 21 isolation attempts, the presence of *Bartonella* sp. confounded cell culture. For example, our best opportunities to re-isolate *A. phagocytophilum* would have been days when deer were RT-nPCR positive, as was the case on 6 and 17 DPI (WTD131, 132, 133) and on 38 DPI (WTD131 and 134); however, of these 8 culture opportunities, *Bartonella* sp. was isolated in all but two. *Bartonella* sp. replicated rapidly in deer blood cultures; CPE was apparent to the unaided eye as early as 7 days in culture (DIC) and was nearly 100% between 8 and 23 DIC. Perhaps the vigorous growth of *Bartonella* sp. in the tick cell media resulted in conditions unsuitable for the survival of *A. phagocytophilum*. If

this is the case, future attempts to culture *A. phagocytophilum* from the blood of deer co-infected with *Bartonella* sp. might be facilitated by the use of an antibiotic to which the former is resistant but to which the latter may be susceptible, such as erythromycin (7, 29).

In the present study, 16S RT-nPCR was the most sensitive assay for detection of *A. phagocytophilum* RNA in deer blood, followed by the groESL RT-nPCR assay. Because both of these assays are nested, we expected them to be more sensitive than the non-nested p44 RT-PCR. Massung et al. (41) demonstrated that specificity and sensitivity vary markedly among the numerous published PCR assays and primer pairs for *A. phagocytophilum*. With regard to specificity, our cell culture results illustrate that even in a controlled experimental setting, PCR-based methods of detection should be confirmed by use of alternative gene targets or by DNA sequencing. We were aware of the existence of an undescribed *Anaplasma* sp. of WTD that is amplified in 16S PCR assays using primers GE9F and GA1UR (34). Additionally, it has been reported that in blood samples containing a high concentration of *E. platys* DNA, *E. equi* primers have induced false priming (52). However, we were not aware of a *Bartonella* sp. infecting WTD in the southeastern U.S. until we sequenced RT-nPCR products amplified with primers GE9F/GA1UR from tick cell culture of deer blood. Of interest is the fact that although the deer were apparently coinfected with *A. phagocytophilum* and *Bartonella* sp., we never detected *Bartonella* sp. directly from deer blood using the aforementioned primers. Therefore, we believe that the copy number of *Bartonella* sp. circulating in peripheral blood of the deer was very low. In fact, in retrospective testing of all deer blood samples collected during this study, we were unable to detect *Bartonella* sp. directly in blood with a single-step gltA RT-PCR assay using primers cited in a standard PCR assay for amplification of DNA from cervid isolates of *Bartonella* spp. from Europe and the western U.S. (12, 18). Only after development of a hemi-

nested gltA RT-PCR were we able to detect RNA of *Bartonella* sp. in pre-inoculation blood samples from two of the deer.

Our serologic and molecular findings lend support to the premise that WTD should be suitable sentinels for human risk of exposure to *A. phagocytophilum* (8, 33, 40, 39, 60)**.** Because two experimental deer maintained detectable antibodies (titer ≥ 64) for at least 49 days, through the end of the 66-day study, we suggest that wild WTD repeatedly exposed to *A. phagocytophilum* may exhibit relatively long-lasting serologic response, a desirable trait in a potential sentinel species. Together with existing field data, our experimental findings, including use of a 1:64 dilution for serologic screening, lay the foundation for the development and validation of an *A. phagocytophilum* sentinel system using WTD. Although serologic cross-reactivity between *E. chaffeensis* and *A. phagocytophilum* in humans is reported (15, 55), recent work suggests that this phenomenon is not a significant limitation to use of WTD as sentinels for *A. phagoctyophilum* and *E. chaffeensis* when surveillance data sets are validated by confirmatory tests such as immunoblotting, PCR, and culture (60, 63).

Recently, Massung et al. (42) reported a genetic variant of *A. phagocytophilum* (AP-variant 1) from wild WTD in Wisconsin and Maryland and from *I. scapularis* in Rhode Island and Connecticut. Because this genovariant was not infectious for mice, the authors hypothesized that AP-variant 1 may be specific to WTD and may cycle independently of the human-infective strain of *A. phagocytophilum* (AP-ha) that is maintained in white-footed mice. If proven, this hypothesis would have implications for the use of WTD as *A. phagocytophilum* sentinels. Our serologic and RT-nPCR findings confirm that WTD are susceptible to a human-infective strain of *A. phagocytophilum* by needle inoculation, suggesting that both genetic variants, AP-ha and AP-variant 1, could be present in wild WTD. Future research related to *A. phagoctyophilum*

infection among WTD should include identifying the genovariants present in deer on a broad geographic scale and determining the infection dynamics of simultaneous or sequential *A. phagocytophilum* genovariants in WTD as recently reported for *E. chaffeensis* (56, 62).

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Table 2.1 Test results for experimental infection of white-tailed deer with *Anaplasma phagocytophilum*. Tests included cell culture (CC), 16S RT-nPCR (PCR) of peripheral blood with DNA sequence confirmation, and indirect fluorescent antibody (IFA) test, reported as reciprocal titers. [a]

DPI	WTD 131			WTD132			WTD133			WTD134		
	CC	PCR[b]	IFA	CC	PCR	IFA	CC	PCR	IFA	CC	PCR	IFA
0	ND	--	<64	ND	--	<64	ND	--	<64	ND	--	<64
6	--[c]	+	ND	--	+	ND	--[c]	+	ND	--[c]	--	ND
10	F	+	<64	F	+	<64	F	+	<64	ND	--	<64
13	ND	+	<64	ND	+[d]	<64	ND	+[d]	<64	--[c]	--	<64
17	--[c]	+	<64	--	+[d]	<64	--[c]	+[d]	64	--[c]	--	64
24	--[c]	--	128	--	--	128	--[c]	--	256	--[c]	--	1024
31	--[c]	--	<64	--	--	64	--[c]	--	64	--[c]	--	1024
38	--[c]	+	64	--	--	64	--	--	256	--[c]	+	2048
45	F	--	<64	F	--	<64	F	--	128	F	--	256
54	--	--	<64	--	--	<64	--[c]	--	128	--[c]	--	256
66	--[c]	--	<64	--[c]	--	<64	--[c]	--	64	--[c]	--	128
66 Nx	ND	--	NA	ND	--	NA	ND	+	NA	ND	--	NA

[a] Two control deer (WTD127 and WTD139) were negative on all tests through DPI 30 at which time they were removed from the study.

[b] PCR results are for whole blood except for last row. Entries for DPI 66 Nx provide results of 16S RT-nPCR of postmortem tissues; sequence-confirmed *A. phagocytophilum* DNA was amplified from femoral bone marrow of WTD133 only.

[c] Cultures negative for *A. phagocytophilum*, but *Bartonella* sp. isolated.

[d] GroESL RNA of *A. phagoctyophilum* amplified by RT-nPCR.

(F) Tick cells failed to re-attach after being admixed with WTD cells.

(NA) Not applicable.

(ND) Not done.

CHAPTER 3

ANAPLASMA ODOCOILEI SP. NOV., A PLATELET-INFECTING RICKETTSIA OF WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)[1]

[1]Tate, C. M., E. W. Howerth, A. I. Sahora, V. G. Dugan, M. P. Luttrell, D. G. Mead, U. G. Munderloh, and W. R. Davidson. To be submitted to *Journal of Clinical Microbiology*.

ABSTRACT

We used blood from white-tailed deer (WTD; *Odocoileus virginianus*) and an ISE6 tick cell culture isolate (UMUM76, Munderloh et al., 2003) to infect captive WTD with an undescribed *Anaplasma* sp., also known as "WTD-agent." Each of six inoculated deer became infected as determined by 16S reverse transcription nested polymerase chain reaction (RT-nPCR) testing of whole blood. All six deer remained infected for over 100 days and three deer remained infected for over 200 days. None of the deer showed evidence of clinical disease, but each of three deer evaluated had multiple episodes of transient thrombocytopenia. Light microscopy of Giemsa-stained thin-blood smears revealed tiny, dark, spherical structures in platelets of acutely infected deer. Transmission electron microscopy of infected platelets demonstrated cytoplasmic, membrane-bound, *Anaplasma*-like organisms. Immunohistochemistry and in-situ hybridization localized *Anaplasma* sp. organisms to platelets of experimentally infected deer. *Anaplasma* sp. DNA was amplified via 16S nPCR from platelet-rich plasma of experimental deer, and the PCR products were confirmed by DNA sequencing. Five of six deer developed antibodies reactive to *Anaplasma* sp. antigen, as detected by indirect fluorescent antibody testing, and remained seroreactive (antibodies detectable at a titer of ≥ 64) through 10 or more weeks. An RT-nPCR assay was developed and used to amplify the *gltA* gene of *Anaplasma* sp. and phylogenetic analysis demonstrated that the *gltA* gene sequence was most similar to *gltA* sequences of *A. platys*, a result that is concordant with previous analyses of the *Anaplasma* sp. 16S and *GroESL* genes. Two attempts to transmit *Anaplasma* sp. from donor deer to naïve deer via *Amblyomma americanum*, a suspected tick vector, were unsuccessful. The ultrastructural, antigenic, and phyogenetic characteristics of this organism confirm that it is a distinct new species of

Anaplasma which produces subclinical, persistent infection of platelets of WTD. The name *Anaplasma odocoilei* is proposed for this new species.

INTRODUCTION

White-tailed deer (WTD; *Odocoileus virginianus*) populations in the southeastern United States are known to host multiple tick-transmitted rickettsiae in the genera *Ehrlichia* and *Anaplasma* (25, 27, 48). Three species, *E. chaffeensis, E. ewing*ii, and *A. phagocytophilum* are zoonotic pathogens, and infect a wide range of mammalian species (14). A fourth organism, detected by PCR in blood of wild WTD, has been referred to as "WTD-agent" (11, 26) and was recently isolated in tick cell culture and identified as an *Anaplasma* sp. (29).

This *Anaplasma* sp. is common among deer populations in the southeastern United States (26, 28) and has been detected in mule deer (*O. hemionus hemionus*) and black-tailed deer (*O. hemionus columbianus*) from California (19). In the southeastern United States, it has significant temporal and spatial associations with lone star tick (LST; *Amblyomma americanum*) parasitism (4). Furthermore, it has been detected by 16S PCR in questing adult LST from Georgia (28). These previous studies suggest that this *Anaplasma* sp. is transmitted by LST, along with the zoonotic rickettsiae *E. chaffeensis* and *E. ewingii* (18, 25, 48). The significance of this organism as a pathogen of deer or as a potential zoonotic agent is not known. Molecular analysis of 16S and *GroESL* DNA sequences of WTD-agent disclosed that it is most closely related to *Anaplasma platys*, a canine rickettsia that infects platelets (11, 14, 42).

We experimentally infected deer with this *Anaplasma* sp. in order to (1) investigate infection dynamics and cellular tropism of this organism in vivo, (2) conduct a small scale, pilot LST transmission study, and (3) more fully characterize the molecular and antigenic relationships of this undescribed organism.

MATERIALS AND METHODS

Field Collection of Blood. In July of 2001, blood was obtained via intracardiac route using sterile technique from five wild deer (WTD #1-5) collected at Piedmont National Wildlife Refuge, Jones County, Georgia, a locale known to have a high prevalence of the *Anaplasma* sp. within the deer population. Blood was stored in tubes containing EDTA anticoagulant and refrigerated during transport from the field to the laboratory.

Experimental Fawns. Six laboratory-raised white-tailed deer fawns (WTD76, 77, 81, 86, 128 and 135) were housed in a tick-free building at the College of Veterinary Medicine, The University of Georgia, Athens, GA. Fawns were determined to be 16S polymerase chain reaction (PCR)-negative for *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum* and the *Anaplasma* sp., and antibody-negative via indirect fluorescent antibody (IFA) test for *E. chaffeensis* and *A. phagocytophilum*, as previously described (29). Deer were anesthetized for all procedures as described by Davidson et al., 2001. Whole blood was collected from fawns approximately every 7 - 10 days for PCR, serologic tests, and blood smears. Complete physical examination (3) of each deer was performed at each blood collection date. Periodically, whole blood anti-coagulated in EDTA from WTD76, WTD81, and WTD77 was submitted to the Clinical Pathology Laboratory, College of Veterinary Medicine, The University of Georgia, for analysis of the following parameters: hematocrit, erythrocyte count, hemoglobin levels, platelet count, total and differential leukocyte counts, and fibrinogen. All deer were euthanized via intravenous sodium pentobarbital overdose and subjected to complete necropsies.

Experimental Design:

I. Inoculation of Experimental Fawns with Field-collected Blood: Two 4-month old fawns (WTD76 and WTD81) were infected with the *Anaplasma* sp. via field-collected deer blood as previously described (29).

II. Blood Transfer from an Experimentally Infected to a Naïve Fawn (Subinoculation): 8.0 ml of whole blood in EDTA was collected from WTD76 on 177 DPI. A 10-month old deer (WTD77) was immediately injected with 2.0 ml of the inocula blood by each of 4 routes: intradermal, subcutaneous, intravenous and intraperitoneal (ID, SQ, IV, IP).

III. Cell Culture Inoculation of Naive Fawns: A thirteen-month old deer (WTD86) was inoculated with a tick cell culture isolate (passage 7) of the *Anaplasma* sp. (UMUM76) as previously described (29). A six-month old deer (WTD 135) was inoculated with the tick cell culture isolate (passage 14) in the same manner.

IV. Blood Transfer from a Cell Culture-Infected Fawn to a Naive Fawn:

A five month-old fawn (WTD128) was injected with 2.0 ml aliquots of 189 DPI whole blood from WTD86 by each of four routes (ID, SQ, IV, IP).

Laboratory Methods

I. Biomagnetic blood cell fractionation:

Blood Preparations: Whole blood in sodium citrate treated Vacutainer tubes (Beckton Dickinson, Rutherford, NJ) obtained from WTD76 and WTD81 was centrifuged at 50 x G for 20 minutes (min.). The platelet-rich plasma (PRP) was removed, centrifuged at 800 x G for 25 min., and the pellet was resuspended in Dulbecco's Phosphate Buffered Saline (DPBS; Sigma, St. Louis, MO). Platelet counts were performed by the Unopette System (Beckton Dickinson) and concentrations were adjusted with DPBS to 1 x 10^7cells/ml. The remainder of the blood was layered over Histopaque 1077 (Sigma) and centrifuged at 800 x G for 25 min. The mononuclear cells at the resulting interface were removed, pelleted by centrifugation and washed three times in DPBS plus heparin (Beckton Dickinson). Monocyte cell counts were performed using a hemacytometer and adjusted to a concentration of 1 x 10^7cells/ml with DPBS plus heparin.

Antibody coating of Dynabeads® (Dynal Biotech, Brown Deer, WI): Dynabeads® were prepared through a series of washes using DPBS, pH 7.4, with 0.1% bovine serum albumin (BSA) solution, using the Magnetic Particle Concentrator (MPC) magnet (Dynal) to sequester beads after each wash. Subsequently, the beads were coated with the appropriate antibody. For platelet fractionation, 1:25 dilution of P-selectin (CD62P) antibody (PharMingen, a division of BD Biosciences, San Diego, CA) in sheep anti-rabbit beads suspended in 0.1% BSA solution was used. For monocyte fractionation, M-M9 (CD14) antibody (VMRD, Inc., Pullman, WA) in mouse anti-human beads was used. Beads were incubated with the antibody overnight on a rocking plate at 4° C. Following incubation, beads were washed three times with 0.1% BSA solution. With each wash, beads were removed from suspension using the MPC magnet.

Fractionation: Platelet and monocyte preparations were purified via a double separation technique **(Table 3.1)** using the antibody-coated Dynabeads®. Briefly, two separate 24-hour incubations were performed at 4°C, each followed by exposure to the MPC magnet. The final pellet was washed three times with 0.1 % BSA and then resuspended in 0.1% BSA solution.

II. Propagation of *Anaplasma* sp. isolate UMUM76 in tick cells and re-isolation from deer blood. An isolate of the *Anaplasma* sp. from WTD76 (UMUM76) was maintained in ISE6 cells as previously described (29). Re-isolation in tick cells from blood of experimentally infected deer was performed as previously described for *A. phagocytophilum* (43).

III. Nucleic acid extraction, PCR assays, and nucleotide sequence analysis. Genomic DNA was extracted from fractionated deer blood platelet and monocyte preparations with the GFX Genomic Blood Purification Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) following the manufacturer's instructions. Total RNA was extracted from fresh whole blood samples with the RNA Blood Minikit (QIAGEN, Inc., Valencia, CA) and from cell culture

aliquots and ticks stored at -70°C with the QiAmp® Viral RNA Extraction Kit (QIAGEN). Extractions were performed according to the manufacturer's instructions and in an RNAase-free environment. For RNA liberation, individual ticks were frozen in liquid nitrogen and then macerated with glass beads in a Mini Beadbeater-8 (Biospec Products, Inc., Bartlesville, OK).

For molecular detection, two gene targets (16S rDNA, and *groESL*) and two different PCR assays were used. For detection in purified platelet and monocyte fractions, 16S PCR was conducted as previously described (26). For detection in deer blood, cell culture, and ticks, RT-nPCR assays for the 16S rDNA and *groESL* targets were performed as described previously (29, 43), except that for the *groESL* assay, secondary amplification was performed using primers EDF10 and EDR11 (primers were generously provided by Dr. John Sumner, Centers for Disease Control) **(Table 3.2)**.

For additional phylogenetic analysis, we designed an RT-nPCR assay for amplification of the citrate synthase (*gltA*) gene from *Anaplasma* sp.-infected cell cultures using degenerate primer pair F4b and HG-1085R, previously cited for amplification of partial *gltA* sequences of *A. platys* (21, 22). Reaction mixtures were subjected to 42°C for 15 minutes, followed by 32 cycles of the following profile: 94°C for 60 seconds (s), 42°C for 60 s, 72°C for 60 s.

As a control for the quality of the RNA preparations from frozen ticks, a random subset of samples that were negative for the *Anaplasma* sp. was assayed by RT-PCR for the presence of tick RNA using primers 16S+1 and 16S-1 to amplify a 450-bp fragment of the tick mitochondrial 16S RNA (34).

All assays were performed using a PTC-100TM Thermal Cycler. Amplification products were separated by electrophoresis in a 2% agarose gel, stained in ethidium bromide and visualized using ultraviolet transillumination. Quality control measures included negative

controls (water) that were extracted and amplified in parallel with all specimens. In order to minimize the potential for DNA contamination, three separate, designated areas were used for extraction of RNA and preparation of primary and secondary PCR reactions. Additionally, two thermal cyclers were used, designated for either primary or secondary amplification.

Products from 16S PCR of platelet fractions, and RT-nPCR products from selected deer blood samples and cell culture aliquots were subjected to DNA sequencing. Gene fragments were purified by gel electrophoresis, and bands were extracted and purified using the QIAquick gel extraction kit (QIAGEN, Inc.). DNA was sequenced in forward and reverse directions at the Molecular Genetics Instrumentation Facility, The University of Georgia, with an ABI 3100 automated sequencer. The sequences were assembled and edited using the Sequencher software package version 4.1.4 (Gene Codes Corp., Ann Arbor, MI). A nucleotide-nucleotide BLAST (blastn) search was performed to determine the most similar sequences of the target genes published in GenBank (http://www.ncbi.nlm.nih.gov). The ClustalX Multiple Sequence Alignment Program version 1.83 (Feb 2003) was used to generate an alignment with the most similar GenBank sequences for the *gltA* gene and percent identity and phylogenetic analyses were conducted using MEGA version 2.1 (24).

IV. Electron microscopy. Transmission electron microscopy (TEM) was completed on PRP collected from WTD76, WTD81, and WTD86. Platelet rich plasma was obtained from citrate anticoagulated blood as previously described. The platelets were pelleted and fixed in either 2% glutaraldehyde-2% (para)formaldehyde-0.2% picric acid in 0.1 M cacodylate-HCL buffer (pH 7.0 to 7.3) or 2% (para)formaldehyde-2% glutaraldehyde in phosphate buffer (pH 7.0). Pellets of fixed platelets were enrobed in agar, routinely embedded in Epon-Araldite (EMS, Hatfield, PA),

and ultrathin-sections were stained with Reynold's lead citrate and 5% methanolic uranyl acetate and viewed with an JEOL JSM-1210 transmission electron microscope.

V. Immunohistochemistry. Platelets from WTD135 were assayed by immunohistochemistry (IHC). Platelet-rich plasma was obtained as described above, pelleted by centrifugation, and the platelet pellet was fixed in 10% buffered formalin. Fixed platelet pellets were enrobed in agarose and routinely embedded in paraffin. Briefly, 3 um sections of formalin-fixed paraffin embedded tissue (PET) were deparaffinized and rehydrated. All subsequent steps were performed at room temperature. Sections were blocked with Universal Blocking Reagent (BioGenex, San Ramon, CA) for 7 min. Polyclonal primary antibody was prepared using precipitated pre- and post-inoculation (71 DPI) serum from WTD86. Sections were incubated with the primary antibody at a 1:100 dilution in DakoCytomation Antibody Diluent (DakoCytomation, Carpinteria, CA) for 60 min. and then incubated with 1:20 peroxidase-labeled rabbit anti-deer IgG (KPL, Gaithersburg, MD) for 30 min. Sections were then treated with supersensitive link biotinylated anti-rabbit immunoglobulins (BioGenex) for 20 min., followed by supersensitive label alkaline phosphatase conjugated streptavidin (BioGenex) and Fast Red (DAKO, Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin.

VI. Riboprobe design and labeling. The 16S rRNA riboprobe used in this study was generated from hemi-nested RT-PCR products amplified from the *Anaplasma* sp. by use of an interior reverse primer with an RNA polymerase promoter sequence. The primers were *A. phagocytophilum*- and *Anaplasma* sp.-specific primers GE9F and GA1UR in the primary reaction (7, 26), and DGA (26) and T7-398R in the secondary reaction. The primer T7-398R was complementary to 16S ribosomal RNA and had T7 promoter sequence at the 5' end (10). Template DNA, amplified from *Anaplasma* sp. isolate UMUM76 in tick cells, was purified as

previously described (10), and the digoxigenin-labeled RNA probe was generated as previously described (10).

VII. *In situ* hybridization assay. *In-situ* hybridization assays using the digoxigenin-labeled riboprobe and a biotin-streptavidin method were performed. Briefly, 3-um sections of formalin-fixed PET (platelets from WTD135) were placed on SuperFrost®/Plus slides (Fisher Scientific, Hampton, NH), and then deparaffinized and rehydrated as described above for IHC slides. Tissue proteases were digested in "PK Buffer" (10) for 30 min. Sections were then post-fixed with 4% paraformaldehyde (J. T. Baker Chemical Co., Phillipsburg, NJ) in Dulbecco's PBS (Sigma), acetylated with fresh 0.25% acetic anhydride (Sigma) in 0.1 M triethanolamine (J. T. Baker), and denatured with 70% deionized formamide (DFA; Sigma) in 2X saline sodium citrate (SSC; Roche, Switzerland). Slides were prehybridized with Dig Easy Hyb Buffer (Boehringer Mannheim, Germany) at 42°C for 30 minutes. The RNA probe was diluted to100 ng/ml in Dig Easy Hyb Buffer, denatured, and then applied to sections. Sections were covered with ApopTag^{TM} Plastic Coverslips (ONCOR, Gaithersburg, MD) and hybridized overnight at 42°C in a humidified chamber. Slides were then washed twice in 50% DFA/2X SSC at 52°C, followed by two washes with 2X SSC at room temperature. Sections were then blocked with 10% goat serum. Mouse anti-digoxigenin antibodies (Roche) diluted to 0.4 ug/ml were applied to the sections for 60 min. and detected by serial application of goat anti-mouse biotinylated immunoglobulins (Biogenex), streptavidin alkaline phosphatase (Biogenex), and naphthol fast red substrate (DAKO). Sections were counterstained in Mayer hematoxylin (DAKO) and mounted with Supermount Permanent Aqueous Mounting Adhesive (Biogenex). All steps were carried out at room temperature unless otherwise noted. All solutions were formulated with

diethylpyrocarbonate (DEPC; Sigma)-treated deionized water and autoclaved prior to use. All antibodies were diluted with DakoCytomation Antibody Diluent (DAKO).

VIII. Antigen slide preparation and indirect fluorescent antibody assays. Actively growing (3-4 days from last passage) ISE6 tick cells infected with *Anaplasma* sp. isolate UMUM76 were gently rinsed with and then resuspended in PBS (pH 7.2) to obtain a concentration of approximately 5 x 10^5 cells/ml. The suspension was pipetted onto 18-well (4 mm) HTC(R) Cel-Line slides (Erie Scientific Company, Portsmouth, NH). Slides were placed in a humidified chamber and incubated for 30 min. at 37°C, then dried overnight under a laminar flow hood, fixed twice in 50% methanol/acetone for 10 min. and immediately used.

Test sera were diluted for screening and determination of end-point titrations as previously described (12). *Anaplasma* sp. antigen slides were treated with 0.1 mg/ml Proteinase K (Sigma) for 2 min. and then rinsed twice in PBS for 5 min. Slides were incubated with test sera for 60 min. in humidified chambers at 37°C and then washed twice with PBS. Slides were then incubated as before with fluorescein isothiocyanate (FITC)-labeled, rabbit anti-deer immunoglobulin G (IgG) heavy and light chain conjugate (KPL) diluted 1:50 in PBS and then washed twice in PBS for 5 min. Slides were counterstained with 1.65% eriochrome Black T (Sigma) in deionized water for 5 min. and then coverslipped with Vectashield Anti-fade Mounting Medium (Vector Laboratories, Burlingame, CA). Slides were examined with a UV epifluorescence microscope at x200 and x400 magnification for specific intracellular fluorescence.

Antigenic cross-reactivity was evaluated with species of *Anaplasma* and *Ehrlichia* known to infect WTD and for which antigens and WTD antisera were available for IFA assays. *Anaplasma phagocytophilum* and *E. chaffeensis* IFA assays were conducted as previously

described (29) using commercially available antigen slides (Focus Technologies, Inc., Cypress, CA). *Anaplasma marginale* IFA assays were conducted with the St. Marie isolate propagated in ISE6 tick cells as antigen. The antigen slide preparation and IFA assay procedure was identical to that described above for the *Anaplasma* sp., except that a blocking step, using 10% non-fat dry milk in PBS, was added before primary antibody incubation. For the IFA cross-reactivity study, we used white-tailed deer antisera to *A. phagocytophilum*, *E. chaffeensis*, and *A. marginale* from previous experimental infection studies in our laboratory (13, 23, 43).

IX. Pilot tick transmission trials. Laboratory-reared LST nymphs were placed in tick containment chambers adhered with tissue glue to areas of shaved skin on donor and recipient deer. Briefly, two separate transmission trials were conducted in which LST nymphs were first acquisition-fed on *Anaplasma* sp. donor deer (on WTD76 at 119 DPI; on WTD135 at 41 DPI), allowed to molt, and then transmission-fed on naïve recipient deer (WTD82, WTD130, and WTD140). For each trial, 16S RT-nPCR was performed on multiple blood samples of recipient deer and on a subset of the adult ticks derived from acquisition-fed as nymphs.

RESULTS

Course of infection and clinical outcome. All six deer experimentally inoculated with the *Anaplasma* sp. became infected. All deer remained infected, as determined by RT-nPCR of blood, at every sampling date for at least 100 days until euthanized. Three deer remained infected for over 200 days at every sampling date until euthanized **(Table 3.3)**. Light microscopy of Giemsa-stained thin blood smears of acutely infected deer revealed tiny, dark, spherical structures in platelets. Clinical signs of illness were not apparent in any of the infected deer. All three deer for which platelet counts were performed experienced 2 - 4 episodes of transient thrombocytopenia (fewer than 100 x 10^3 platelets/ µl blood) during the time period

(duration 75–245 days) in which platelets were measured**.** Thrombocytopenia occurred as early as 13 DPI, as late as 187 DPI, and at intervals of 21–65 days. Mean platelet counts (excluding values < 100 x 10^3) were 619, 419, and 363 x 10^3/ul blood for WTD76, WTD77, and WTD81, respectively. For comparison, the mean platelet count of five WTD not infected with the *Anaplasma* sp., but that were housed, anesthetized, and sampled in a similar manner was 685 x 10^3/ul blood (43). Significant gross and histopathologic lesions of post-mortem tissues were not apparent in any of the deer.

Cellular tropism and ultrastructure. Antibody-coated magnetic bead purified-platelets from WTD76 and WTD81 were 16S PCR-positive for the *Anaplasma* sp. in 15 of 16 samples. Similarly prepared and assayed monocyte preparations were 16S PCR-positive in 14 of 16 samples. All PCR-positive samples yielded the expected 411 base pair (bp) product. Sequence analysis of the 411 bp products obtained from platelet and monocyte preparations from both deer yielded DNA sequences identical to the16S sequence of the *Anaplasma* sp. tick cell isolate UMUM76 (GenBank accession number AY208945). The sequences from the purified platelets of WTD76 and WTD81 were deposited in GenBank under accession numbers DQ007351 and DQ007352.

Immunohistochemistry demonstrated antigen reactive to the *Anaplasma* sp. immune sera in platelets of WTD135 **(Figure 3.1)**. Antigen appeared as a single, eccentrically located, densely stained red area within platelets. Positive reactivity of infected platelets was not apparent with nonimmune serum.

In-situ hybridization demonstrated RNA of the *Anaplasma* sp. in platelets of WTD135 **(Figure 3.1)**. Nucleic acid appeared as a single, eccentrically located, densely stained red area

within platelets. Positive reactivity of infected platelets was absent when DigEasyHyb without template RNA was used.

Ultrastructurally, platelets of deer infected via both blood (WTD76 and WTD81) and cell culture inoculum (WTD86) contained anaplasma-like organisms. Infected platelets contained one to several organisms in individual membrane-bound vacuoles or two to five organisms in a single large membrane-bound vacuole which was consistent with a morula **(Figure 3.2)**. The singlet organisms were spherical, ranged from 0.250-0.786 µm (n=8) in diameter, and usually tightly filled the vacuole **(Figure 3.3, A and B)**. They were surrounded by two smooth-contoured trilaminar membranes, one protoplasmic and one cell wall, with a very narrow intervening periplasmic space. Peripherally, organisms had a rim of electron-dense granular material and centrally were either relatively electron-lucent with dispersed fibrillar material or moderately electron-dense with uniformly dispersed fibrillar and granular material. Vacuoles containing multiple organisms (morulae) were irregularly spherical, approximately 1.000 µm to 1.150µm in greatest diameter (n=5), and contained loosely packed organisms that varied in size, shape, and protoplasmic characteristics **(Figure 3.3, C and D)**. These organisms were either spherical, 0.250-0.675 µm in diameter (n=8), or ovoid, 0.250-0.450 µm in width and 0.450-0.675 µm in length (n=4), and were surrounded by two relatively smooth-contoured trilaminar membranes with a small periplasmic space. Larger organisms had central moderately electron-dense protoplasm with evenly distributed fibrils and clumps of electron-dense densely granular material and a peripheral rim of more electron-dense granular material. As organisms decreased in size there was condensation of the fibrils and granular material making the protoplasm diffusely more electron-dense with the eventual formation of an eccentric electron-dense condensation. Overlying these condensations, the periplasmic space was

enlarged. The vacuolar matrix was generally electron-lucent but had a few small bits of moderately electron-dense fibrillar material.

Antibody responses of infected deer. The antiserum (WTD86; 71 DPI) selected as the standard *Anaplasma* sp. positive control had a titer of 1:128 to the UMUM76 antigen and consistently gave this endpoint titer through repeated testing. With this sample, individual organisms and clusters of organisms within tick cells fluoresced bright green **(Figure 3.1)**. The negative control antiserum (WTD128; pre-inoculation) showed no specific fluorescence at dilutions ≥ 1:32 on repeated testing. Neither pre-immune sera nor the *Anaplasma* sp. antisera showed reactivity with slides made from uninfected ISE6 tick cells. Five of six deer developed antibody titers ≥ 64 (seropositive cut-off) between 2 and 4 weeks post-inoculation, and two deer were still seropositive when euthanized at 36 weeks. Peak titer was 512 and gross mean titer (44) was 151. WTD128 did not seroconvert **(Figure 3.4).**

Immunologic cross-reactivity. One of two *Anaplasma* sp. antisera was slightly reactive with *A. marginale* antigen, but the titer of the *Anaplasma* sp. antiserum with *A. marginale* antigen was eight-fold lower than with the homologous antigen. *Anaplasma phagocytophilum* antisera reacted weakly with both the *Anaplasma* sp. antigen and with *A. marginale* antigen; however, titers of two *A. phagocytophilum* antisera samples with both of these heterologous antigens were four-fold and thirty two-fold lower than titers with the homologous antigen. *Ehrlichia chaffeensis* antisera reacted weakly with *A. phagocytophilum* antigen, but the titers of *E. chaffeensis* antisera against A. *phagocytophilum* antigen were sixteen-fold lower than titers with the homologous antigen. All other IFA results for specific antisera with heterologous antigens were negative **(Table 3.4).**

Phylogenetic analysis of partial *gltA* sequences. A 912 bp sequence of the citrate synthase (*gltA*) gene of the *Anaplasma* sp. (deposited in GenBank under accession number DQ020101) was most similar to published *gltA* sequences of *A. platys* (70.0 % identity) and differed more from *A. phagocytophilum* (64.0%), and *A. marginale* (60.3%) **(Figure 3.5).**

Pilot tick transmission trials. Attempts to transmit the *Anaplasma* sp. to naïve deer using LST nymphs acquisition-fed on infected deer were unsuccessful in two separate trials, as determined by RT-nPCR of blood samples on multiple days post-tick infestation **(Table 3.5).** In addition, 16S RT-nPCR results for individual unfed adult ticks acquisition-fed as nymphs were negative in both trials. Tick 16S mitochondrial RNA was amplified from all of a random subset of the samples, verifying the extraction of viable RNA from the previously frozen, macerated ticks.

DISCUSSION

Taken collectively, our findings demonstrate that this undescribed *Anaplasma* sp. (WTD-agent) exhibits a tropism for platelets as confirmed by light and electron microscopy, immunohistochemical staining, and in-situ hybridization assay. Most experimentally infected deer developed antibodies that remained detectable for weeks when measured by IFA assay. Antigenically, the organism exhibited slight cross-reactivity with *A. phagocytophilium* antisera; no reactivity was detected with *A. marginale* and *E. chaffeensis* antisera. Antisera against the *Anaplasma* sp. was slightly reactive when tested with *A. marginale* antigen but unreactive with *A. phagocytophilium* or *E. chaffeensis* antigens. All deer maintained persistent infections of months duration characterized by 2-4 episodes of transient thrombocytopenia; however, at no time did deer appear clinically ill. Phylogenetic analyses of multiple genes, including *gltA* described here, confirm that this *Anaplasma* sp. is most closely related to but genetically distinct from *A. platys*, a platelet-infecting organism of dogs (14). These findings, when combined with

the previous description of this organism cultivated in ISE6 tick cells (29), serve as the basis for its designation as *Anaplasma odocoilei* sp. nov., as described herein. The natural vector(s) of this organism remain unknown; a pilot tick transmission experiment with lone star ticks was unsuccessful but prior testing of field-caught ticks suggests that lone star ticks may be vectors (28).

The rationale for the blood fractionation experiment was to evaluate whether the *Anaplasma* sp. infected platelets, similar to its close congener *A. platys*. We were surprised that both platelet and monocyte preparations were PCR-positive because many members of the family Anaplasmataceae predominantly infect a single cell type *in vivo* and because *A. platys* is not known to infect cell types other than platelets (14). Our data suggest that this *Anaplasma* sp. may infect two cell types; however, alternative explanations for PCR positive monocyte preparations include contamination of the monocyte preparations with platelets, phagocytosis of infected platelets, or organisms freed from platelets during sample processing. The latter explanation seems plausible, since a previous study reported amplification of 16S rDNA of this *Anaplasma* sp. from numerous serum samples of wild WTD (4).

Ultrastructurally, the organisms and morulae in platelets are morphologically similar to various *Anaplasma* and *Ehrlichia* spp. described in previous studies. These include organisms seen in platelets of other mammals, including *A. platys* in dogs (1), an unidentified rickettsia-like organism in human platelets (2), and a ricksettsia-like organism seen in impala platelets (15). The fibrillar and granular material observed in the protoplasm of organisms is most likely DNA fibrils and ribosomes, respectively, as previously described for *Ehrlichia* (36) and the morphologic changes seen in morulae are consistent with the normal life cycle of related organisms (36). The singlet organisms and those with similar morphology in morulae resemble

the reticulated forms while the cells with electron-dense condensations resemble the dense forms of other *Anaplasma* and *Ehrlichia* spp. (31, 36, 37).

The organisms in platelets differ morphologically from those isolated and grown in tick cell culture (29). In tick culture, this *Anaplasma* sp. forms much larger morulae with numerous organisms, and the organisms are very pleomorphic and elongate rather than more spherical. This may be due to the cell type infected with the small size of the platelets perhaps conferring a limit on the size of a developing morula. Differing morphology between *in vivo* infected cells and various cell culture infected cells has been documented with related organisms. For example, *Anaplasma phagocytophilum* in *in vivo* infected neutrophils (7, 39) and in *in vitro* infected HL-60 cells (a human promyelocytic leukemia cell line) (38) tends to have small morulae with few organisms, whereas *A. phagocytophilum* in *in vitro* infected endothelium and tick cells has very large morulae containing numerous organisms (30, 31, 46).

The IFA assay devised using tick-cell grown organisms as antigen demonstrated that five of six experimentally infected deer developed *Anaplasma* sp.-reactive antibodies (≥ 1:64); however, these titers, as measured by this IFA assay, remained relatively low. Three of the five deer that seroconverted maintained detectable antibodies through the end of the study, but only one of these deer had detectable antibodies at every post-inoculation sampling date. Our serologic results must be interpreted cautiously, however, because the IFA assay has not been rigorously validated and because a single needle infection of deer is unlikely to accurately reflect natural exposure to this organism among wild deer. Nonetheless, development of a serologic test for this *Anaplasma* sp. is important for epidemiologic surveys for this organism. Antigenic cross-reactivity among *Anaplasma* and *Ehrlichia* spp. has been reported when testing polyclonal antibodies by immunofluorescence (8, 33, 38, 45, 47), and this issue should be further

investigated with regard to this *Anaplasma* sp. Our preliminary investigation of cross-reactivity among sera from deer with monospecific experimental infections with other *Anaplasma* and *Ehrlichia* demonstrated nonreactivity of the *Anaplasma* sp. antisera to both *A. phagocytophilum* and *E. chaffeensis* antigen and weak reactivity to some *A. marginale* antigen via IFA. Furthermore, *A. phagocytophilum* antisera reacted weakly with the *Anaplasma* sp. antigen.

Despite the IFA cross-reactivity with other *Anaplasma* and *Ehrlichia* spp., we believe that the antibodies detected in WTD experimentally infected with the *Anaplasma* sp. are homologous antibodies. Support for this conclusion includes (1) the fact that deer were free of known co-infecting *Anaplasma* and *Ehrlichia* spp. prior to inoculation and (2) demonstration of antibodies homologous to this *Anaplasma* sp. in sera of experimentally-infected deer, as confirmed by IHC using UMUM76-infected tick cells and infected platelets as antigen.

Via both immunocytohistochemistry and *in-situ* hybridization, the *Anaplasma* sp. organisms appeared as single, small, eccentrically located, red areas in platelets. This appearance corresponds to the single, small, eccentrically located, darkly-stained structures visualized within platelets in Giemsa-stained blood smears of acutely infected deer and strongly suggests that these structures were actually the *Anaplasma* sp.

Results of phylogenetic analysis using partial *gltA* sequences from this *Anaplasma* sp. concur with previous analyses using 16S and *GroESL* sequences (11, 42). Specifically, analyses with all three gene targets indicate that this *Anaplasma* sp. is most similar to but not identical with *A. platys*. For the *gltA*, *GroESL*, and 16S rDNA genes, sequence similarities between the *Anaplasma* sp. and *A. platys* were 70% (this study), 83% (42), and 94% (11), respectively.

It has been suggested that low-level persistent infection is a common feature of *Anaplasma* species (35), having been documented in cattle infected with *A. marginale* (17), goats

infected with *A. ovis* (32, 35), rodents, dogs, and sheep infected with *A. phagocytophilum* (5, 16, 41), and dogs infected with *A. platys* (16, 20). Our data demonstrating persistence of the *Anaplasma* sp. in all six experimentally infected deer further strengthens this argument. Similar to dogs infected with *A. platys* (20), infected deer demonstrated no apparent clinical signs of illness despite episodes of thrombocytopenia. Most notably, hemorrhages which are a risk during thrombocytopenia, were not apparent.

The natural mode(s) of transmission of this *Anaplasma* sp. remains unknown. Our limited transmission attempts using LST were unsuccessful; however, further investigation of the vector capacity of LST is warranted because our attempts involved few animals and relatively few ticks. Use of wild-caught LST from an area endemic for this *Anaplasma* sp. would be an alternative approach in future transmission studies.

Description of *Anaplasma odocoilei* sp. nov. *Anaplasma odocoileus* (odo coil'eus L. gen. n. *odocoileus*; the species was first detected in and isolated from white-tailed deer, *Odocoileus virginianus,* and has been reported from other species of *Odocoileus*). This organism can establish infections in white-tailed deer when whole blood of infected donor deer or infected cell culture preparations are needle-injected. Clinical signs of illness were not apparent in experimentally infected deer. Organisms were observed in platelets of acutely infected deer via light microscopy of Giemsa-stained thin-blood smears and these findings were confirmed by immunohistochemistry and *in-situ* hybridization. As determined by electron microscopy, organisms are small, pleomorphic cocci within membrane-lined vacuoles, and each organism is surrounded by two trilaminar membranes, one protoplasmic and one cell wall, with a narrow periplasmic space. Within platelets, organisms may occur singly or in clusters known as morulae. Singlet organisms are spherical (mean diameter 0.521 μm $\pm$ 0.200 μm) while clustered

organisms vary in shape and size (mean diameter 1.070 μm ± 0.057 μm with spherical organisms 0.423 μm ± 0.11μm in diameter and ovoid organisms 0.350μm ± 0.913 μm wide and 0.503 μm ± 0.823 μm long). This organism can be cultivated in ISE6 tick cells and in this cell culture system the organism has a different morphology than in platelets. In tick cells, the organism tends to form larger morulae with numerous organisms and is very pleomorphic and elongate, rather than spherical. The organism appears to be weakly cross-reactive with *A. phagocytophilum* antisera in whole organism indirect fluorescent antibody assays (IFA). Antisera from infected deer did not cross-react with *A. phagocytophilum or E. chaffeensis* antigens in IFA assays but in some cases did cross-react with *A. marginale* antigen. The DNA sequence of a 912 bp segment of the *gltA* gene of this organism is most similar to *gltA* sequences of *A. platys* (level of sequence similarity 70.0%), *A. phagoctyophilum* (64.0%), and *A. marginale* (60.3%). These results concur with previous comparisons of partial 16S and *GroESL* sequences of this *Anaplasma* sp. The type strain of *A. odocoileu*s is UMUM76.

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Table 3.1 Double separation technique for fractionation of platelets and monocytes[a].

Cell type	1st Filtration-cleansing	2nd Filtration-product
Platelets: 250 µl of sample at 1*10^7 cells/ml	6 µl of M-M9 beads	6 µl of CD62P beads
Monocytes: 250 µl of sample at 1*10^7 cells/ml	6 µl of CD62P beads	6 µl of M-M9 beads

[a]The first separation reduced contamination by non-target cell types. The second separation sequestered the desired cell target. For each step, 250 µl of sample with 6 µl of coated beads was used.

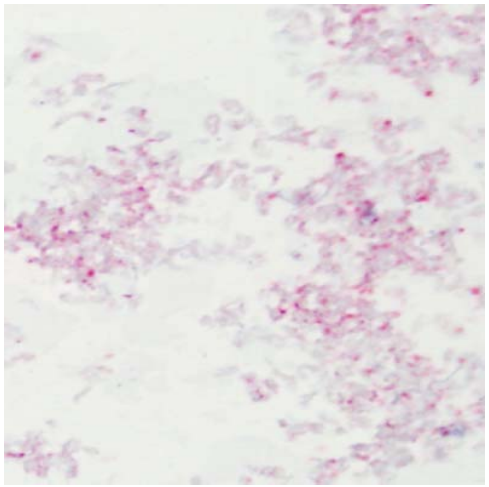
Table 3.2 Oligonucleotide sequences of primers used in this study

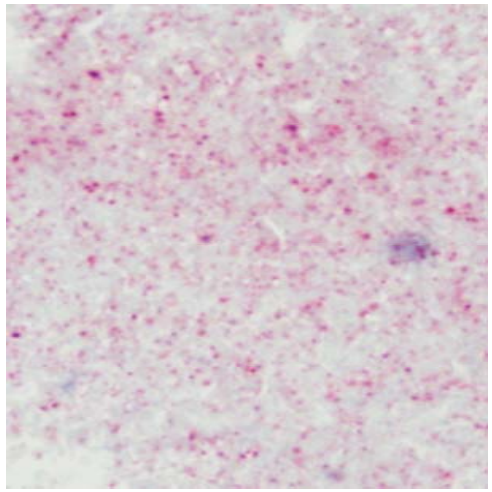
Primer Pair	Specificity and target gene	Nucleotide sequence (5'→ 3')	Product size (bp)	References
ECC ECB	*Anaplasma* and *Ehrlichia* spp. 16S rDNA	CGT-ATT-ACC-GCG-GCT-GCT-GGC-A AGA-ACG-AAC-GCT-GGC-GGC-AAG-CC	480	Dawson et al., 1994
GE9F GA1UR	*A. phagocytophilum* and WTD-agent 16S rDNA	AAC-GGA-TTA-TTC-TTT-ATA-GCT-TGC-T GAG-TTT-GCC-GGG-ACT-TCT-TCT	411	Chen et al., 1994 Little et al., 1997
DGA GA1UR	WTD-agent 16S rDNA	TTA-TCT-CTG-TAG-CTT-GCT-ACG GAG-TTT-GCC-GGG-ACT-TCT-TCT	411	Little et al., 1997
DGA T7-398R	WTD-agent 16S rDNA	TTA-TCT-CTG-TAG-CTT-GCT-ACG T7-GCA-TAG-CTG-GAT-CAG-GCT-TTC	325	Little et al., 1997 Dawson et al., 1994
APF1 APR1	*A. phagocytophilum* and WTD-agent *groESL*	TAG-TGA-TGA-AGG-AGA-GTG-AC CCA-GGI-GCC-TTI-ACA-GCW-GCA-AC	1,603	Sumner et al., 2003
EDF10 EDR11	WTD-agent *groESL*	GAT-TCT-CCG-GTT-TGT-TCT-GT GGA-GAA-AGA-TAA-CCC-CTG	650	Sumner et al., 2003
F4b HG1085R	*Anaplasma* and *Ehrlichia* spp. *gltA*	CCG-GGT-TTT-ATG-TCT-ACT-GC ACT-ATA-CCK-GAG-TAA-AAG-TC	935	Inokuma et al., 2001 and 2002
16S+1 16S-1	Tick 16S mitochondrial RNA	CCG-GTC-TGA-ACT-CAG-ATC-AAG-T CTG-CTC-AAT-GAT-TTT-TTA-AAT-TGC-TGT-GG	460	Norris et al., 1999

Table 3.3 Detection of the *Anaplasma* sp. in blood of experimentally infected deer by 16S RT-nPCR

WTD #	Source of infection	DPI first tested	DPI first PCR positive	# of times sampled	# of times PCR positive	DPI last tested
76	Wild deer blood	2	5	36	35	250
81	Wild deer blood	2	2	22	22	117
77	Blood from WTD76	10	10	13	13	252
86	Tick cell isolate (UMUM76)	12	12	8	8	214
128	Blood from WTD86	4	4	9	5	153
135	Tick cell isolate (UMUM76)	15	15	9	5	129

Figure 3.1 Results of immunohistochemistry (IHC), in situ hybridization (ISH), and indirect fluorescent antibody (IFA) assays. IHC (left) and ISH (middle) assays performed on platelets of WTD135 (129 DPI). Signal appears as red dots in platelets. IFA (right) assay performed on tick cell culture antigen slides using WTD86 (71 DPI) serum at a dilution of 1:64.





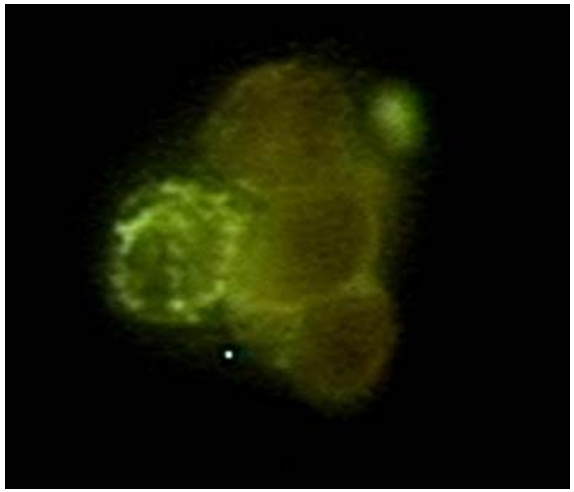


Figure 3.2 Transmission electron micrograph of white-tailed deer platelets from WTD81 at 20 DPI. One platelet contains a single organism (arrow) and one has a membrane-bound morula loosely filled with three organisms (1,2,3). Bar = 0.2 μm. Lead citrate/uranyl acetate.

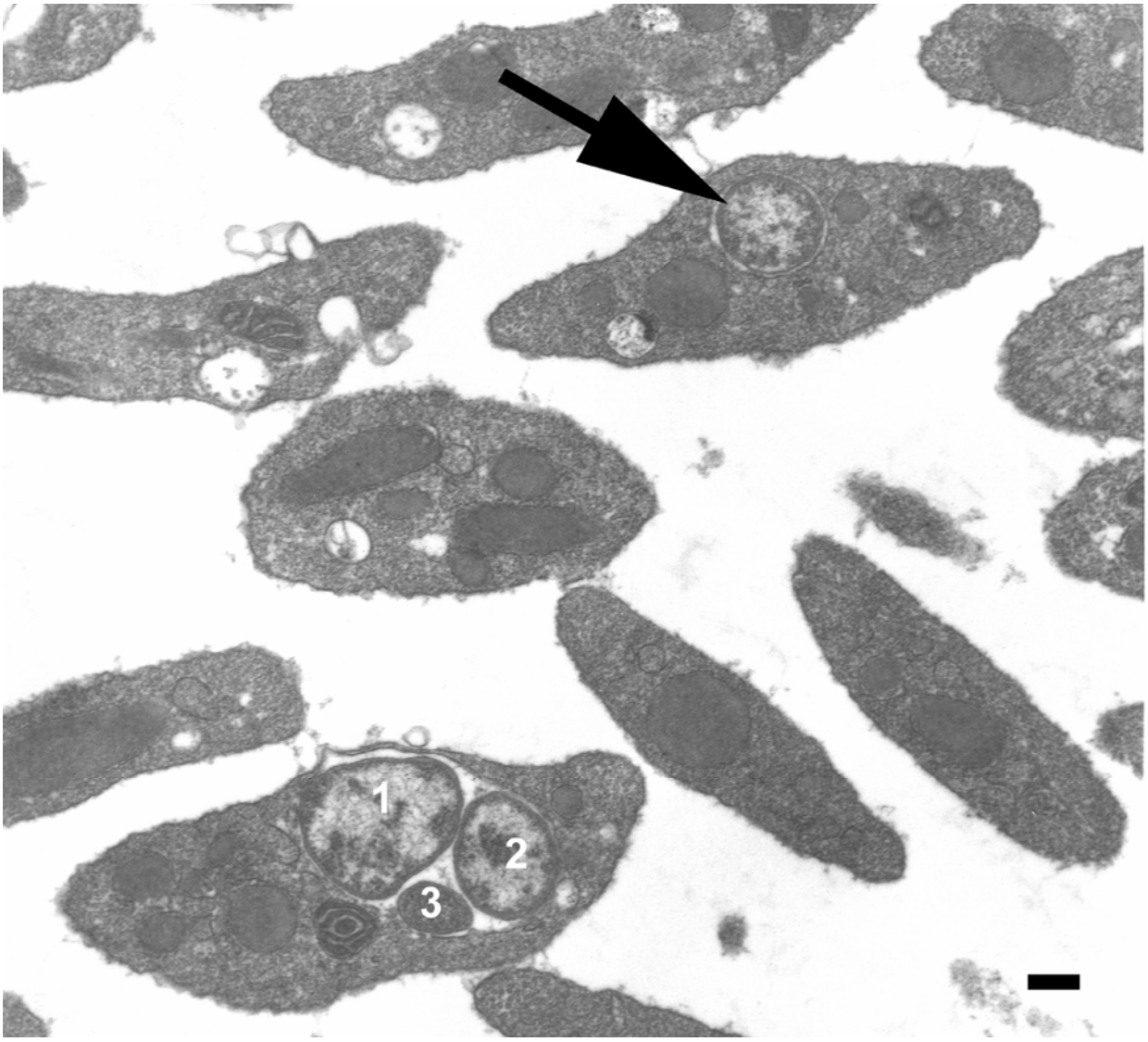

1
2
3

Figure 3.3 Transmission electron micrograph of white-tailed deer platelets. A) Platelet from WTD86 at 33 DPI containing single reticulated form of the organism (R) tightly filling a membrane-bound vacuole. Note electron-lucent center primarily containing a few DNA fibrils and peripheral granular layer of ribosomes. Inset is enlargement of square showing membrane of vacuole (long black arrow) and the two trilaminar membranes surrounding the organism (two short white arrows) separated by narrow periplasmic space (long white arrow). The platelet contains normal platelet alpha granules (A). Bar = 0.1 µm. B) Platelet from WTD81 at 20 DPI containing single reticulated form of the organism (R) tightly filling a membrane-bound vacuole. The center of this organism is denser due to the fairly evenly distributed DNA fibrils and clumps of ribosones. The platelet contains normal platelet alpha granules (A). Bar = 0.1 µm. C) Platelet from WTD76 at 20 DPI as seen on edge. A membrane-bound morula loosely filled with 3 organisms forms a bulge in the platelet (arrow). The organisms are surrounded by two membranes and have fairly evenly dispersed DNA fibrils and ribosomal aggregates. Bar = 0.2 µm. D) Platelet from WTD81 at 20 DPI containing a morula loosely filled with 5 organisms. The matrix of the membrane-bound vacuole is electron-lucent and contains a small amount of fibrillar material. Organisms are bound by two trilaminar membranes and there is an eccentric electron-dense condensation in three of the organisms. The periplasmic space is enlarged over the condensation (arrow). Bar = 0.1 µm. Lead citrate/uranyl acetate.

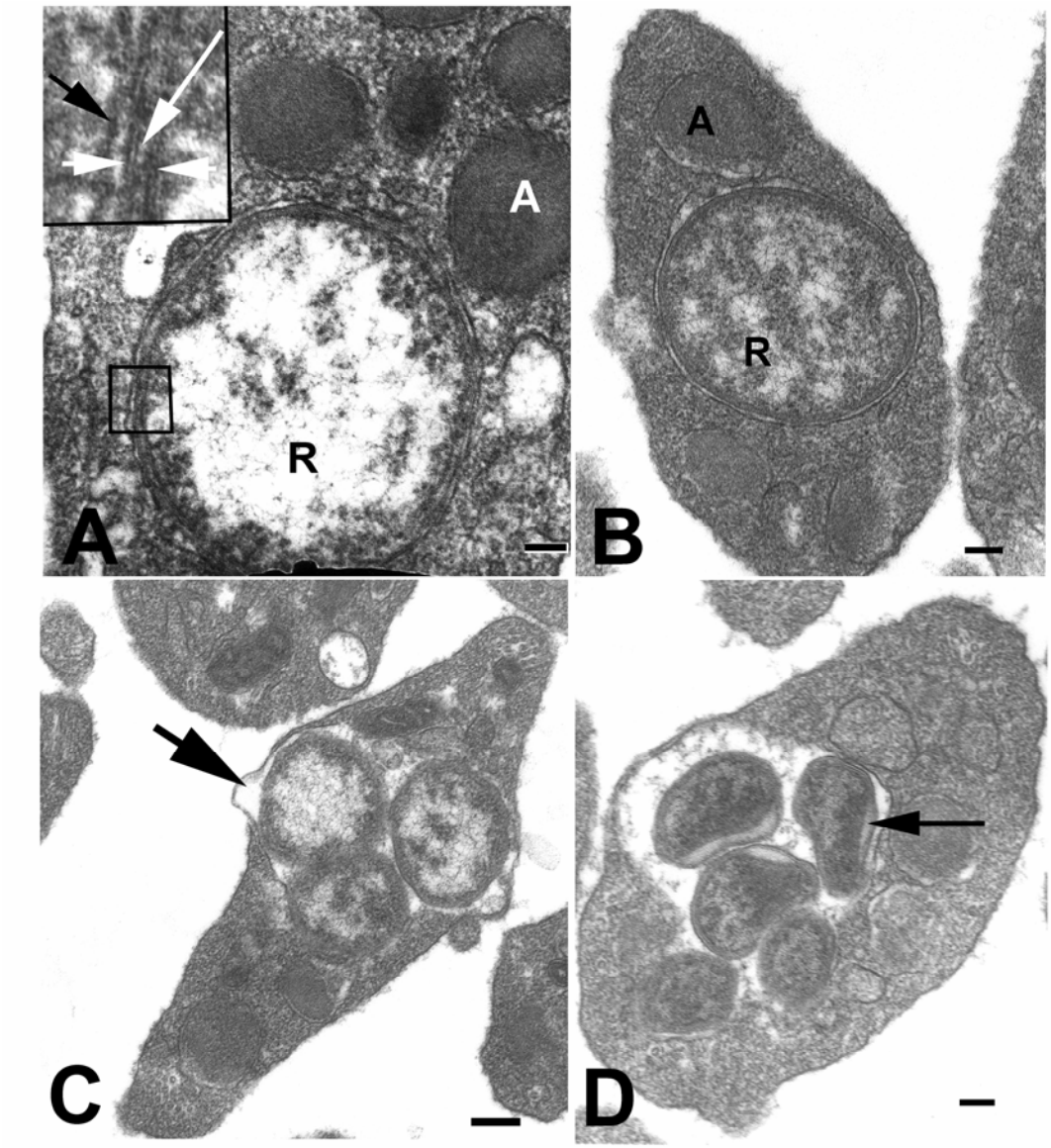

A
R
A
A
R
B
C
D

Figure 3.4 Antibody response of deer experimentally infected with the *Anaplasma* sp., as determined by IFA; (A) Animals inoculated with blood from wild deer, or with blood from animals inoculated with wild deer blood, (B) animals inoculated with tick cell isolate UMUM76, or with blood from animals inoculated with UMUM76.

Figure 3.4A

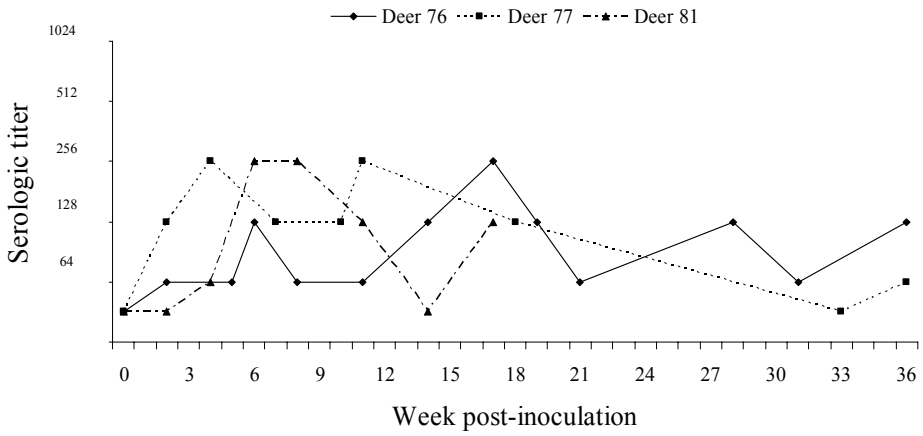


Figure 3.4B

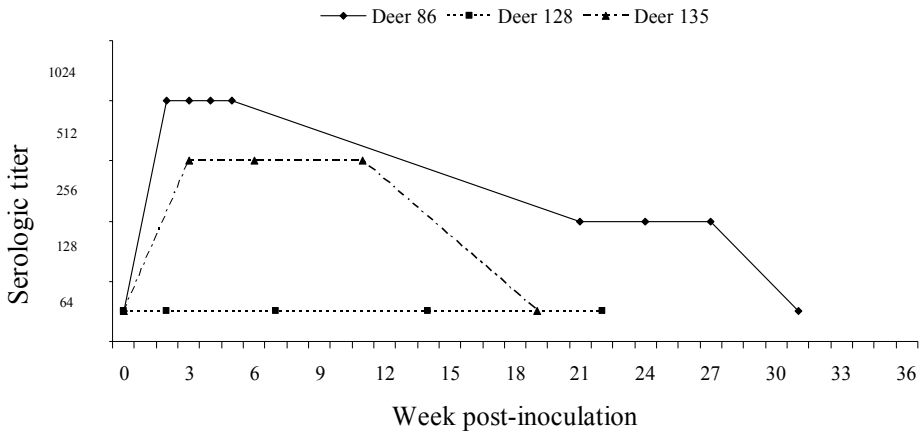


Table 3.4 Antigenic cross-reactivity of selected *Anaplasma* and *Ehrlichia* spp. as determined by indirect fluorescent antibody testing of sera from experimentally infected white-tailed deer

ANTISERA	**ANTIGEN**			
Deer ID / DPI	***Anaplasma* sp. in ISE6 cells**	***A. phagocytophilum* in HL60 cells**	***E. chaffeensis* In DH82 cells**	***A. marginale* in ISE6 cells**
***Anaplasma* sp.**[1]				
WTD 86 / 12	**512**	< 64	< 64	64
WTD 135 / 72	**256**	< 64	< 64	< 64
A. phagocytophilum[2]				
WTD 133 / 38	64	**256**	< 64	64
WTD 134 / 38	64	**2048**	< 64	64
E. chaffeensis[3]				
WTD 7 / 42	< 64	64	**1024**	< 64
WTD 9 / 35	< 64	64	**1024**	< 64
A. marginale[4]				
WTD 9564 / 9.30	< 128[a]	< 128	< 128	**512**
WTD 9569 / 4.20	< 128	< 128	< 128	**128**

Origin of antisera; [1] this study; [2] Tate et al., in press; [3] Dugan et al., 2004; [4] Keel et al., 1992.
[a]Because original IFA reactivity of the *A. marginale* antisera was determined at a dilution of 1:100, a negative cut-off value of 1:128 was used in this study for *A. marginale* antisera.

Figure 3.5 Alignment of the partial *gltA* sequence from the *Anaplasma* sp. isolate with partial *gltA* sequences of recognized *Anaplasma* spp. available in GenBank; identical bases are indicated by asterisks. Four sequences from *A. marginale* (AF304139, AF304140, CP000030, NC_004842) were aligned, determined identical, and represent the sequence labeled "*A._marginale*", 11 *A. phagocytophilum* sequences (AF304136-AF304138, AY339602, AY464132-AY464138) were used to arrive at the consensus sequence designated "*A._phagocyto*", and 4 *A. platys* sequences (AB058782, AF478130, AY077620, AY530807) were aligned and used to produce a consensus sequence shown in the line labeled "*A.__platys*".

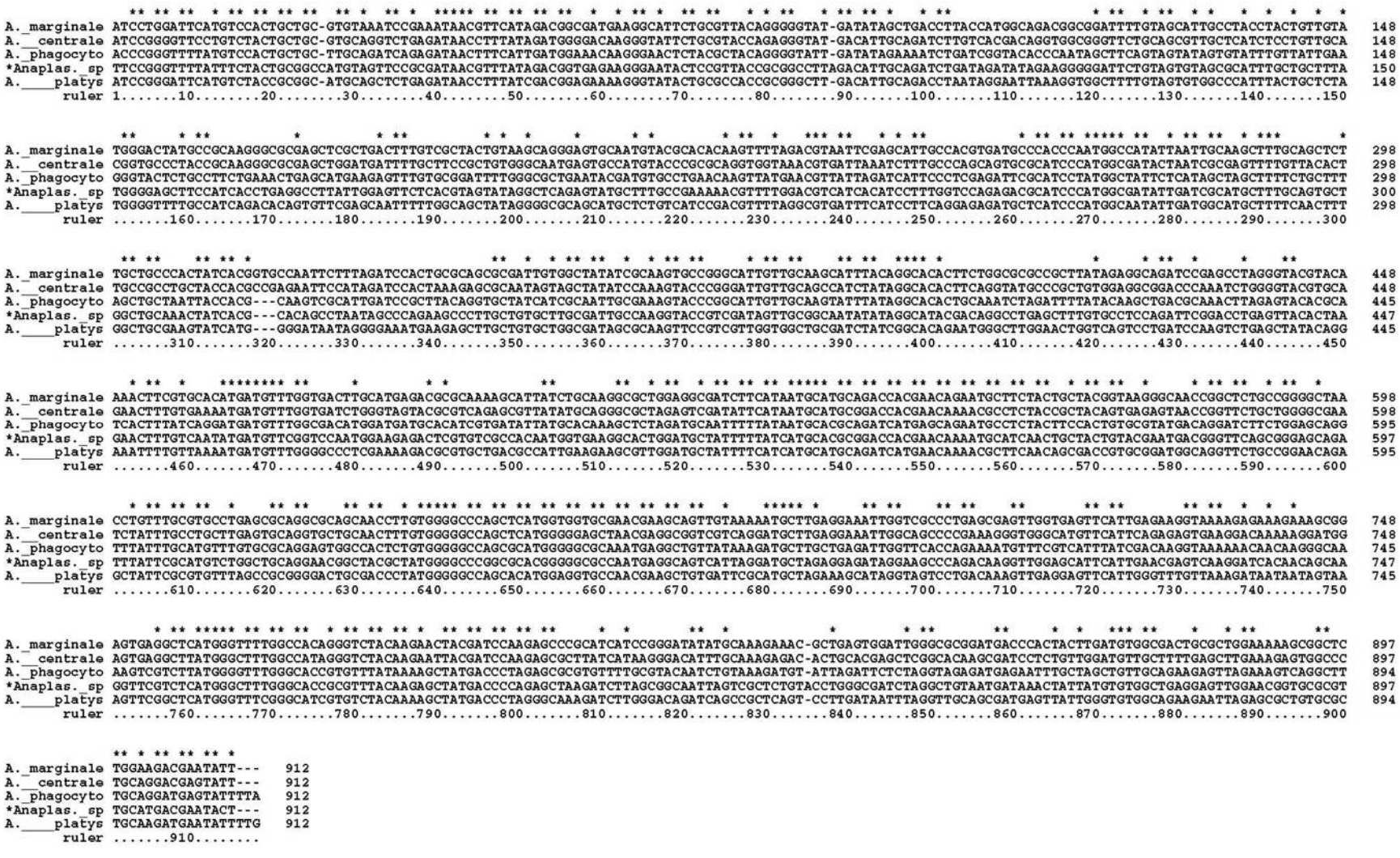

A._marginale
A._centrale
A._phagocyto
*Anaplas._sp
A._____platys
ruler

Table 3.5 Pilot transmission trials of the *Anaplasma* sp. with *Amblyomma americanum*

	Trial 1	Trial 2	
Donor deer ID	WTD76	WTD135	
DPI tick trial began	119 DPI	41 DPI	
Infection status of donor deer during tick feeding	16S RT-nPCR POSITIVE	16S RT-nPCR and CULTURE POSITIVE	
# nymphs acquisition fed on donor deer	110	900	
# live, fed nymphs recovered from donor deer	103	390	
# successfully molted adult ticks	55	222	
RT-nPCR of molted adults (# tested / positive)	10 / 0	20 / 0	
Recipient deer ID	WTD82	WTD130	WTD140
# adult ticks placed on recipient deer	45	90 F 12 M	88 F 12 M
# fed adult ticks recovered from recipient deer	21	10	17
Infection status of recipient deer:			
# times RT-nPCR tested	6	8	8
Test period; days post tick (DPT)	13 – 79 DPT	4 – 60 DPT	4 – 74 DPT
Results	NEGATIVE	NEGATIVE	NEGATIVE

CHAPTER 4

CONCLUDING REMARKS

This research was conducted to gain insight into the course of infection, pathology, and natural history of *Anaplasma* organisms harbored by white-tailed deer (WTD). With this broad goal in mind, a study was undertaken comprised of two objectives. The first objective was to experimentally assess the ability of WTD to serve as hosts of and possibly as sentinels for *A. phagocytophilum*. WTD have been identified as both a potential sentinel species for *A. phagocytophilum* (Belongia et al., 1997; Little et al., 1998; Magnarelli et al., 1999; Magnarelli et al., 2004; Walls et al., 1998) as well as a potential reservoir host of *A. phagocytophilum* (Arens et al., 2003; Bakken and Dumler, 2000; Barlough et al., 1995; Belongia et al., 1997; McQuiston et al., 2003); however, these potential roles have not been analyzed because data on susceptibility to and course of infection with *A. phagocytophilu*m in WTD have previously been unavailable. To accomplish this first objective, a human isolate of *A. phagocytophilum* was used to infect four WTD via needle inoculation. Two WTD served as negative controls. All 4 deer developed antibody titers ≥ 64, and two deer maintained detectable titers through 60 days. In three deer, circulating organisms were detectable by 16S reverse transcription nested polymerase chain reaction (RT-nPCR) for more than two weeks post-inoculation, and in one deer *A. phagocytophilum* was detectable in post-mortem bone marrow at 66 days post inoculation (DPI). These data confirm that WTD are susceptible to infection with a human isolate of *A. phagocytophilum*, and they suggest that WTD may maintain circulating organisms for a sufficient time period hypothetically to infect ticks. Nonetheless, even though deer have a high probability of natural infection with *A. phagocytophilum*, WTD are unlikely to be a significant source of *A. phagocytophilum* for immature ticks. This is because adults are the predominant life stage of *I. scapularis* found on deer and because adult *I. scapularis* do not transmit *A. phagocytophilum* transovarially. However, the susceptibility and immunologic response of

WTD to *A. phagocytophilum* should render them suitable as natural sentinels for this emerging zoonotic tick-borne organism.

The second objective of this study was to characterize a previously undescribed *Anaplasma* sp. first detected by 16S PCR in the blood of wild WTD in the southeastern U.S. (Dawson et al., 1996). Specific goals of this second objective were: 1) to visualize and characterize this *Anaplasma* sp. in deer tissues, 2) to develop a serologic assay for detection of this *Anaplasma* sp., 3) determine the kinetics of the antibody response of deer to infection with this *Anaplasma* sp. and assess the degree of antigenic cross-reactivity among selected *Anaplasma* and *Ehrlichia* spp. known to infect WTD, and 4) investigate the ability of *Amblyomma americanum*, a suspected tick vector, to transmit this *Anaplasma* sp. among WTD. To this end, six captive WTD were experimentally infected with either blood from infected deer or with a tick cell culture isolate of the *Anaplasma* sp. All six deer became persistently infected with this *Anaplasma* sp., as determined by 16S RT-nPCR of blood. Light microscopy of Giemsa-stained thin-blood smears revealed tiny, dark, spherical structures in platelets of acutely infected deer, and TEM of platelets of infected deer demonstrated cytoplasmic, membrane-bound *Anaplasma*-like organisms. Immunohistochemistry and in situ hybridization localized to *Anaplasma* sp. organisms in platelets of infected deer. Nested PCR was used to amplify 16S rDNA of this *Anaplasma* sp. from purified platelets of experimental deer.

Five of six deer developed antibodies reactive to the *Anaplasma* sp. antigen, as detected by indirect fluorescent antibody testing (IFA), and remained seroreactive for 10 or more weeks. Slight antigenic cross-reactivity was detected via IFA among this *Anaplasma* sp., *A. phagocytophilum*, and *A. marginale*. An RT-nPCR assay was developed and used to amplify a partial *gltA* gene sequence of this *Anaplasma* sp. and phylogenetic analysis demonstrated that the

gltA gene sequence was most similar to *gltA* sequences of *A. platys*, a result that is concordant with previous analyses of the *Anaplasma* sp. 16S and *GroESL* genes. Two attempts to transmit this *Anaplasma* sp. from donor deer to naïve deer via *A. americanum* were unsuccessful; however, further investigation of the vector capacity of *A. americanum* for this *Anaplasma* sp. is warranted, based on the small scale of these attempts. The ultrastructural, antigenic, and phylogenetic characteristics of this organism confirm that it is a distinct new species of *Anaplasma* which produces subclinical, persistent infection of platelets of WTD. The name *Anaplasma odocoilei* is proposed for this new species.

Although the research presented in this dissertation has provided new insight into infection of WTD with these two *Anaplasma* species, it also generates new questions. The validation of WTD as a suitable sentinel species for *A. phagocytophilum* may lead to a deeper understanding of the epidemiology of human granulocytic anaplasmosis in North America. Molecular evidence of WTD populations co-infected with *A. phagocytophilum* and this *Anaplasma* sp. has been reported (Little et al., 1998), and an area of future investigation might be evaluation of the dynamics of simultaneous and sequential co-infection of WTD with these organisms, to include assessment of potential interaction such as immunologic cross-protection, competitive infection exclusion, or enhanced pathology. Characterization and description of *Anaplasma odocoilei* as a new species should allow development of standardized diagnostic tools for detection of this platelet-infecting organism of WTD and such new diagnostic tools should aid in assessing whether it also is a zoonotic organism as are some other tick-borne rickettsiae of wild animals.

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