NATURAL HISTORY OF ZOONOTIC *EHRLICHIA* SPECIES IN THE UNITED STATES AND DISCOVERY OF A NOVEL EHRLICHIAL PATHOGEN

Natuurlijke historie van zoönotische *Ehrlichia* soorten in the Verenigde Staten en ontdekking van een nieuw *Ehrlichia* pathogeen

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Ehrlichia are pathogens of both domestic and wild animals, transmitted by ixodid (hard) ticks; an increasing number of species are known to be zoonotic. *Ehrlichia* are obligate intracellular bacteria and have a tropism for mammalian endothelium and leukocytes (Allsopp et al., 2005a; Camus et al., 1996; de Castro et al., 2004; Dumler et al., 2007; Okada et al., 2003). Five valid species have been described, summarized in Table 1, and the severity of clinical signs varies by the species and strain of pathogen as well as by host immune factors. With the exception of *E. canis* and *E. ruminantium*, both of which are significant veterinary pathogens (Cowdry, 1925b; Donatien and Lestoquard, 1935), most ehrlichiae have been discovered within the last two decades (Allsopp et al., 1996; Anderson et al., 1991; Anderson et al., 1992; Inokuma et al., 2004; Koutaro et al., 2005; Parola et al., 2000; Parola et al., 2001; Parola et al., 2003; Sarih et al., 2005; Wen et al., 1995); limited information is available about their life cycles, natural history, or, in most cases, pathogenicity for domestic animals and people.

Table 1. Host associations, tick vectors, and geographic distribution of *Ehrlichia*

	Domestic animal hosts	Zoonotic Pathogen	Natural Reservoirs	Tick Vectors	Geographic Distribution
Taxonomically valid species					
E. canis	Dogs	yes	Dogs	*R. sanguineus*	Worldwide
E. chaffeensis	Dogs	yes	White-tailed deer	*A. americanum*	United States
E. ewingii	Dogs, goats	yes	White-tailed deer	*A. americanum*	United States
E. muris	Dogs, mice	no	Wild rodents	*I. ovatus* *I. persulcatus*	Japan, Asiatic Russia
E. ruminantium	Cattle, sheep, goats	yes	Wild African ruminants (12 species)	*Amblyomma* (13 species)	Africa, Caribbean
Unnamed species with vertebrate models of infection					
HF strain *Ehrlichia*	Mice	no	Unknown	*I. ovatus*	Japan, Asiatic Russia
Panola Mountain *Ehrlichia*	Goats	yes	White-tailed deer	*A. americanum* *A. maculatum*	United States

Understanding of the natural transmission cycles of tick-borne diseases is an essential component of targeted control efforts. For instance, acaricide treatment of white-tailed deer and white-footed mice was demonstrated to reduce *Borrelia burgdorferi* infections in the northeast USA (Dolan et al., 2004; Piesman, 2006; Schulze et al., 1994). Identification of the natural reservoirs of ehrlichiae requires a laboratory system for assessing tick-host transmission. Surveys of wild animals can identify species which have ehrlichial DNA in their blood, but these studies do not address reservoir competence; vertebrate reservoirs are identified by experimentally infesting naïve animals with infected ticks and monitoring the animals for active infection (e.g., (Massung et al., 2006)). Similarly, transmission competence of ticks can be assessed by infesting a susceptible animal with ticks from a PCR-positive cohort and determining if the ticks transmitted the infection to the animal (e.g., (Levin et al., 2002)).

Pathogenicity data, both for humans and domestic animals, have been difficult to obtain due to poor clinician recognition and diagnostic capability. The diagnosis of ehrlichiosis traditionally relies on serology, but serologic cross-reactivity and the need for paired acute and convalescent sera hampers accurate and rapid diagnosis (Childs et al., 1999; Dumler et al., 2007; Knowles et al., 2003; Rikihisa et al., 1994; Unver et al., 1999). Molecular biology offers additional options for the rapid diagnosis of acute ehrlichiosis, if PCR assays are designed to be both specific and sensitive enough to detect the small number of ehrlichiae in the blood of infected vertebrates (Childs et al., 1999; Dawson et al., 1996; Dawson et al., 2001; Dumler et al., 2007; Goodman et al., 2003; Mahan et al., 1998; Prince et al., 2007).

1.2. Historical Background

Ehrlichia species of veterinary importance were first described in the early 20th century; the first bacteriological description of the causative agent of "heartwater" was published in 1925, with the description of *E. ruminantium* (Cowdry, 1925b). *Ehrlichia canis*, which causes tropical canine pancytopenia, was first described in domestic dogs from Algeria in 1935 (Donatien and Lestoquard, 1935). Both species were originally described as members of the genus *Rickettsia* but were ultimately classified into *Ehrlichia*, a different genus in the same order (Dumler et al., 2001). Both *E. ruminantium* and *E. canis* cause significant pathology in domestic animals, with frequent fatalities; this might have contributed to their relatively early discovery.

Ehrlichiae were newly recognized as causative agents of human disease in the last few decades. In the United States, human infection with an unknown monocytic ehrlichia was first described in 1986, resulting in the discovery of *E. chaffeensis* (Anderson et al., 1991; Dawson et al., 1991). This pathogen can also causes a monocytic ehrlichiosis in dogs but is less pathogenic than *E. canis* (Dawson et al., 1996; Dawson and Ewing, 1992). *Ehrlichia ewingii* was discovered shortly afterward, initially as a cause of canine granulocytic ehrlichiosis (Anderson et al., 1992) and later as a cause of human disease (Buller et al., 1999). Both *E. chaffeensis* and *E. ewingii* typically cause sporadic cases of mild to moderate illness; severe illness and mortality can occur, especially in immuno-compromised hosts, and most clinical reports of *E. chaffeensis* and *E. ewingii* in humans are based on severely ill patients (e.g., (Buller et al., 1999; Dawson et al., 2001; Paddock et al., 1997; Paddock et al., 2001)).

Increased awareness of ehrlichioses, combined with the development of molecular biological techniques to detect ehrlichiae, has resulted in the discovery of additional species and strains identified from wildlife and ticks. *Ehrlichia muris* and the "HF strain" of

Ehrlichia, both isolated from Japan, cause ehrlichiosis in mice (Inokuma et al., 2001; Shibata et al., 2000; Wen et al., 1995). The "Panola Mountain *Ehrlichia*" was described from an infected goat in the United States and causes ehrlichiosis in both goats and humans (Loftis et al., 2006; Reeves et al., 2008). A multitude of other, unnamed ehrlichiae have been identified from ticks in several countries (Inayoshi et al., 2004; Inokuma et al., 2004; Koutaro et al., 2005; Parola et al., 2001; Parola et al., 2003; Sarih et al., 2005; Wen et al., 2003); these agents were identified solely by molecular techniques and their transmissibility and pathogenicity for vertebrates is unknown.

Additionally, the advent of molecular tools has assisted in the identification of ehrlichial infections in novel hosts. Cases of human ehrlichiosis caused by a strain of *Ehrlichia canis*, confirmed using DNA sequencing, were reported from Venezuela (Perez et al., 1996). The first cases of human and canine ehrlichioses associated with *E. ruminantium* were recently reported from South Africa, more than seven decades after the bacterium was first described (Allsopp et al., 2005b; Allsopp and Allsopp, 2001). Additionally, several unnamed *Ehrlichia* have been detected in animals that appeared clinically to be suffering from *E. ruminantium* infections (Allsopp et al., 1996; Allsopp et al., 1997).

1.3. Taxonomy of *Ehrlichia*

Ehrlichia are small, coccoid to pleomorphic, Gram-negative alphaproteobacteria in the order Rickettsiales, family Anaplasmataceae (Dumler et al., 2001). Rickettsiales are obligately intracellular bacteria, and *Ehrlichia* are seen in membrane-bound cytoplasmic vacuoles, or "morulae", inside cells. Anaplasmataceae also includes the closely related genus *Anaplasma*, as well as more distantly related *Neoehrlichia* and *Wolbachia* (Dumler et al., 2001). *Ehrlichia* and *Anaplasma* are sister taxa, and these two genera show significant biologic similarities, including ixodid tick transmission and similar adaptations to

intracellular parasitism. A new genus within this family has been proposed, intermediate to *Ehrlichia* and *Anaplasma*, based upon the isolation and characterization of a novel species from Japan, "*Candidatus* Neoehrlichia mikurensis" (Kawahara et al., 2004). Figure 1 illustrates the relationship between species and genera within the family Anaplasmataceae.

Taxonomically valid species of *Ehrlichia* are: *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, and *E. ruminantium* (Table 1). The present-day members of the genus *Ehrlichia* were initially described in the genera *Rickettsia* or *Ehrlichia*; additionally, *E. ruminantium* was known as *Cowdria ruminantium*, the sole member of a monotypic genus, from 1947 through 2001 (Dumler et al., 2001). Several other species that were originally described as *Ehrlichia* (*Ehrlichia equi*, *E. phagocytophila*, *E. risticii*, and *E. sennetsu*) have been moved to the genera *Anaplasma* or *Neorickettsia* (Dumler et al., 2001). A phylogenetic reconstruction of

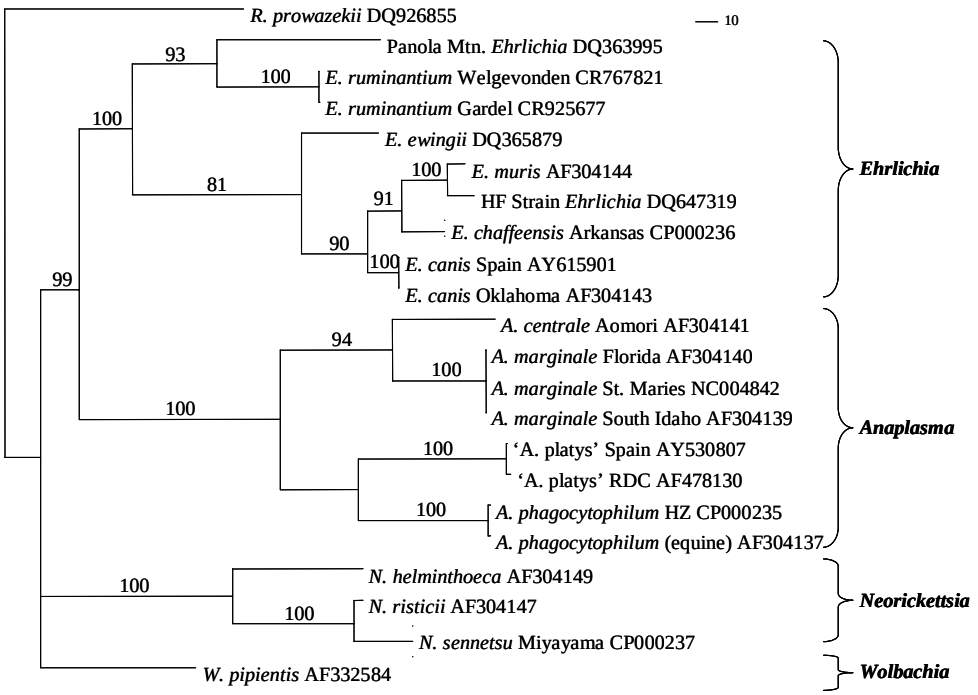

Figure 1. Amino acid sequences of the citrate synthase (*glt*A) gene were used to create a phylogenetic reconstruction of species within the family Anaplasmataceae. A neighbor-joining tree was produced using 100 bootstrap replicates; only nodes with bootstrap support >80 are shown. The scale bar shows the number of changes per 100 amino acid residues.

the recognized species within Anaplasmataceae, illustrating the close relationship of *Ehrlichia* and *Anaplasma*, is shown in Figure 1.

Within the genus *Ehrlichia*, invalid names are as numerous as valid names. Some taxa were described historically, prior to the initiation of the modern bacterial nomenclature code, and have no extant isolates (e.g., "*E. ovina*", "*E. ondiri*" (Davies, 1993; Neitz, 1968)). Other taxa have recently been identified using molecular biological techniques that also do not meet the criteria for valid publication of bacterial names (Lapage et al., 1992) (e.g., "*E. extremiorentalis*" (unpublished, #AY584851), "*E. shimanensis*" (Kawahara et al., 2006), and "*E. walkeri*"(Brouqui et al., 2003)). Some emerging species have been isolated in vertebrate animals and are known only by informal strain designations: the "HF strain" and "Panola Mountain *Ehrlichia*". The requirement for *in vitro* cultivation is a major deterrent to the valid naming of *Ehrlichia* spp., as ehrlichiae are fastidious and not easy to isolate. Of the validly named species, *E. ewingii* has not yet been cultivated; the type strain material, a frozen stabilate of infected dog blood, was submitted before the revision of the bacterial code (Anderson et al., 1992). Of the unnamed species, only "*E. walkeri*" has reportedly been cultivated and is therefore eligible for taxonomic status. Cultivatable species of *Ehrlichia* have been maintained in endothelial, myeloid, and tick cell lines (Allsopp et al., 2005a; Bell-Sakyi et al., 2007; Chen et al., 1995; Dawson et al., 1993; Zweygarth et al., 2002).

1.4. *Ehrlichia* spp. Natural History

Ehrlichiae are maintained in nature by a tick-host cycle, typically involving one ixodid tick species or genus and several mammalian host species. Wild animals are the typical reservoir for all of the ehrlichiae except *E. canis*, which is maintained primarily by transmission among dogs, but livestock can also be competent reservoirs. These bacteria are primarily transmitted horizontally, via infected host animals; immature ticks acquire

ehrlichiae while blood-feeding on a host and maintain the bacteria transstadially to become infected nymphal or adult ticks. Horizontal transmission of some ehrlichiae is also described by male ticks, infected as adults, that could sequentially feed on multiple hosts (Andrew and Norval, 1989; Bremer et al., 2005). Vertical (transovarial) transmission of ehrlichiae is either nonexistent or very rare (Bezuidenhout, 1987; Cowdry, 1925a; Groves et al., 1975; Long et al., 2003; Rikihisa, 1991). Infection rates within naturally occurring populations of ticks are frequently low, less than 5%, with 15-20% infection in exceptionally active foci (Faburay et al., 2007; Gueye et al., 1993; Mixson et al., 2006). However, data suggest that a single infected tick can be sufficient to produce clinical infection in an infested vertebrate. Similar to *Anaplasma* spp., at least some *Ehrlichia* spp. can be found in the salivary glands of infected ticks prior to attachment (Yunker et al., 1993). In these cases, the infection might be transmitted within a few hours of tick attachment, with the probability of transmission increasing throughout the first 24 hours of tick feeding, as has been described for *Anaplasma phagocytophilum* (Levin and Troughton, 2006).

1.4.1. *Ehrlichia ruminantium.* *Ehrlichia ruminantium* is enzootic in sub-Saharan Africa and occurs in livestock on several islands in the Caribbean (Allsopp et al., 2005a; Camus et al., 1996). *Ehrlichia ruminantium* is the causative agent of heartwater in domestic ruminants, an acute disease with high morbidity and up to 90% mortality in cattle, sheep, and goats (Allsopp et al., 2005a; Martinez et al., 2008). Significant annual funding is invested by the United States Department of Agriculture to prevent the pathogen from spreading from the Caribbean into the USA (Pegram and Eddy, 2002). The pathogen is transmitted by *Amblyomma* spp.; at present, thirteen species are known to be experimental or natural vectors of *E. ruminantium*, including the Gulf Coast tick (*A. maculatum*) found in the southeastern USA (Bezuidenhout, 1987; Camus et al., 1996; Mahan et al., 2000; Uilenberg, 1982). The

wildlife reservoirs of *E. ruminantium* include several wild African ruminants (Peter et al., 2002), but other species, such as tortoises, can be alternate hosts for infected ticks (Burridge et al., 2000). Domestic ruminants are also competent reservoirs for *E. ruminantium* and might contribute to the propagation of the pathogen in epizootic loci. Finally, domestic dogs are experimentally and naturally susceptible to infection but their reservoir competence has not been assessed (Allsopp and Allsopp, 2001; Kelly et al., 1994). The wide range of tick and vertebrate hosts for this bacterium might contribute to its broad geographic range and its ability to become established in new locations, such as occurred in the Caribbean (Barré et al., 1995).

Recently, *Ehrlichia ruminantium* was associated with cases of human illness in Africa, with PCR and sequence confirmation of the pathogen's presence (Allsopp et al., 2005b; Louw et al., 2005), raising the possibility of undiagnosed zoonotic infections in endemic regions.

1.4.2. *Ehrlichia canis.* *Ehrlichia canis* is a worldwide pathogen of domestic dogs, originally described in Algeria (Donatien and Lestoquard, 1935; Rikihisa, 1991). The tick vector is *Rhipicephalus sanguineus* (Bremer et al., 2005; Groves et al., 1975), and the majority of infections are seen in tropical and subtropical regions that are hospitable to this tick species. Dogs serve as both the reservoir and domestic animal host for this pathogen due to the strict host specificity of the tick vector. Some wild canids are susceptible to infection, including coyotes, suggesting that these animals might serve as secondary reservoirs (Ewing et al., 1964).

1.4.3. *Ehrlichia chaffeensis.* *Ehrlichia chaffeensis* is enzootic in the eastern half of the United States. The only proven tick vector is *Amblyomma americanum*, lone star ticks,

and the only confirmed wildlife reservoir is *Odocoileus virginianus* (white-tailed deer) (Childs and Paddock, 2003; Paddock and Childs, 2003). Using molecular techniques, *E. chaffeensis* has also been detected in *Dermacentor variabilis* and *Ixodes pacificus* ticks (Holden et al., 2003; Roland et al., 1998); the vector competence of these alternate tick species has not been evaluated. Domestic dogs infected with *E. chaffeensis* are competent reservoirs (Long et al., 2003). Wild canids can be infected with *E. chaffeensis*, including coyotes (*Canis latrans*) and red foxes (*Vulpes vulpes*), but grey foxes (*Urocyon cinereoargenteus*) were refractory to infection (Davidson et al., 1999; Kocan et al., 2000). The reservoir competence of wild canids species was not assessed. Raccoons (*Procyon lotor*) become transiently infected with *E. chaffeensis* from *in vitro* culture, but transmission to ticks was unsuccessful (Yabsley et al., 2008b). Although *A. americanum* feeds on a wide variety of other hosts, including ground-dwelling birds and many species of medium-sized mammals, the reservoir competence of these other vertebrates for *E. chaffeensis* has not been evaluated (Brillhart et al., 1994; Koch and Dunn, 1980; Kollars, Jr., 1993; Kollars, Jr. et al., 2000; Mock et al., 2001; Morlan, 1952; Tugwell and Lancaster, Jr., 1962; Wehinger et al., 1995; Zimmerman et al., 1988).

Ehrlichiae similar to *E. chaffeensis* were identified from ticks in Asia using PCR, but these sequences are not identical to *E. chaffeensis* and no further studies have been undertaken to conclusively determine if the amplicons are from *E. chaffeensis* or a closely related species (Kim et al., 2006; Wen et al., 2002; Wen et al., 2003).

1.4.4. *E. ewingii.* Similar to *E. chaffeensis*, *E. ewingii* is enzootic in the eastern half of the United States, the only proven tick vector is *Amblyomma americanum*, and the only confirmed wildlife reservoir is *Odocoileus virginianus* (white-tailed deer) (Childs and Paddock, 2003; Yabsley et al., 2002). DNA from *E. ewingii* has been detected in *D.*

variabilis and *Rhipicephalus sanguineus* ticks (Murphy et al., 1998), but the vector competence of these species has not been evaluated. Domestic dogs may be competent reservoirs for *E. ewingii*, with proven susceptibility to infection and the ability to transmit the pathogen to ticks (Murphy et al., 1998). Raccoons were resistant to experimental infection with *E. ewingii* (Yabsley et al., 2008b). No other potential vertebrate reservoirs have been assessed.

In one survey of dogs in Cameroon, amplicons from the *dsb* gene with 100% homology to *E. ewingii* were obtained, so it is possible that the geographic distribution of this pathogen extends beyond the United States (Ndip et al., 2005).

1.4.5 *Ehrlichia muris*. Originally isolated in Japan, *E. muris* has also been detected in animals and ticks in Russia (Alekseev et al., 2001; Eremeeva et al., 2006; Rar et al., 2005; Shpynov et al., 2006; Smetanova et al., 2007; Wen et al., 1995). *Ixodes ricinus* and *I. persulcatus* ticks are associated with *E. muris*, and wild rodents are the only recognized reservoirs at this time (Kawahara et al., 1999; Smetanova et al., 2007; Wen et al., 1995). Recently, DNA from *E. muris* was detected in the blood of a sika deer in Japan, suggesting the possibility of natural infection, but no experimental studies have been performed (Tamamoto et al., 2007).

1.4.6 Panola Mountain *Ehrlichia*. Similar to *E. chaffeensis* and *E. canis*, Panola Mountain *Ehrlichia* (PME) is enzootic in the eastern United States. *Amblyomma americanum* is a natural vector for the pathogen, but *A. maculatum* has also been shown to be a competent vector in the laboratory (Loftis et al., 2006; Loftis et al., 2008). White-tailed deer (*Odocoileus virginianus*) are natural reservoirs for PME (Yabsley et al., 2008a); no other

wild animals have been assessed for their susceptibility to infection. Domestic goats are also competent reservoirs for this emerging pathogen.

1.5. Comparative pathogenesis of *Ehrlichia* spp.

Ehrlichiae are obligately intracellular and are classically seen as clusters of organisms in cytoplasmic vacuoles ("morulae") in nucleated cells. *Ehrlichia* are highly adapted to an intracellular lifestyle. They are sensitive to mechanical stress and desiccation, and, even in enriched cell culture media, cell-free *E. chaffeensis* organisms remain infectious for fewer than 24 hours (Li and Winslow, 2003). They do not have classical bacterial cell walls, instead incorporating cholesterol scavenged from the host cell into a cell membrane; genome sequences from *E. chaffeensis* and *E. ruminantium* show a lack of genes required for the synthesis of lipopolysaccharide (LPS) and peptidoglycan (Collins et al., 2005; Lin and Rikihisa, 2003). Pathways for the *de novo* synthesis of amino acids are similarly reduced. However, genes involved with pathogenesis are consistently present: type IV secretion systems and multicopy gene families for major antigenic surface proteins, possibly used for immune evasion, are found in the genomes of *E. chaffeensis*, *E. canis*, and *E. ruminantium* (Collins et al., 2005; Ohashi et al., 2002). These multicopy genes are differentially expressed in tick and mammalian cells (Ganta et al., 2007; Postigo et al., 2008; Zhang et al., 2004).

Following a tick bite, ehrlichiae invade either the endothelium or leukocytes in the tick bite site. Systemic dissemination is thought to be hematogenous, although experimental data is lacking and monocyte-associated pathogens might disseminate via the reticuloendothelial system. Ehrlichiae commonly concentrate in the spleen, liver, lungs, kidneys, and bone marrow (Allsopp et al., 2005a; Dumler, 2005; Okada et al., 2003; Rikihisa, 1991).

Clinical infection with *E. ruminantium* produces several possible syndromes, from the acute, typically fatal, infection known as heartwater to mild febrile illness. *Ehrlichia ruminantium* has a tropism primarily for vascular endothelium, but morulae are also seen in circulating neutrophils (Camus et al., 1996). Acute infection with *E. ruminantium* is typical in cattle, sheep, and goats, with fatality rates up to 90% in susceptible animals. Clinical signs are caused primarily by increased permeability of vascular endothelium; hydropericardium, hydrothorax, pulmonary edema, and encephalitis are common (Allsopp et al., 2005a; Van de Pypekamp and Prozesky, 1987; Yunker, 1996). On the other end of the spectrum is a mild febrile response that is largely asymptomatic; this has been associated with natural wildlife reservoirs, very young animals, or with strains of *E. ruminantium* that have low pathogenicity (Allsopp et al., 2005a; Allsopp et al., 2007; Camus et al., 1996; Jongejan et al., 1984; Peter et al., 2002). Some animals that survive the acute disease, whether mild or severe, develop persistent, clinically silent infections (Bekker et al., 2002; Peter et al., 1998).

Ehrlichia canis primarily infects monocytes. Infections of dogs with *E. canis* often manifest as febrile illness with lymphadenopathy, anorexia, lethargy, depression, and thrombocytopenia. Clinical symptoms of acute infection are primarily caused by thrombocytopenia; infection with *E. canis* results in an increase in autoantibodies against host platelets and alters platelet aggregation (Harrus et al., 1999; Waner et al., 2001). In severe cases, symptoms include edema, ascites, hemorrhage including epistaxis, and inflammation of the spleen, liver, and kidneys (de Castro et al., 2004; Rikihisa, 1991). The prognosis for recovery, even with appropriate therapy, depends upon the severity of thrombocytopenia and clotting abnormalities prior to treatment (Shipov et al., 2008). Ocular infections and meningitis are also seen (Panciera et al., 2001), suggesting some compromise of the blood-brain barrier. The severity of infection varies according to breed of dog, with German Shepherds considered to be extremely susceptible (Rikihisa, 1991). Persistent infections,

resulting either in clinically silent carrier status or long-term chronic illness, can develop in dogs and last for years (Harrus et al., 1999).

Infections with *E. chaffeensis*, also a pathogen of monocytes, range from mild to fatal. Infections can be mild in immuno-competent individuals, characterized by fever, headache or myalgia, and leukogram abnormalities. Severe infections with *E. chaffeensis* are most commonly reported in humans co-infected with HIV or who have otherwise compromised immune systems, and the overall case fatality rate in humans is 3% (Paddock et al., 2001). Severe infections manifest as respiratory distress or pneumonia, hepatic failure, acute renal failure, coagulopathies, and severe neurological dysfunction (Dumler, 2005; Paddock et al., 2001; Rikihisa, 1999). In dogs infected with *E. chaffeensis*, febrile illness with thrombocytopenia, followed by chronic infection lasting for months, has been recorded (Breitschwerdt et al., 1998; Dawson and Ewing, 1992; Zhang et al., 2003).

Infections with *E. ewingii* are clinically similar to infections with *E. chaffeensis*, although *E. ewingii* has a primary tropism for neutrophils. Overall, virulence in humans appears to be lower with *E. ewingii*; fewer fatal cases are reported, and almost all reported cases of *E. ewingii* have been from immuno-compromised people (Buller et al., 1999; Paddock et al., 2001). Although some dogs develop asymptomatic infections with *E. ewingii*, severe infections with neurological deficits and polyarthritis are also seen (Anderson et al., 1992; Goodman et al., 2003). Persistent infection has been documented in infected domestic goats.

Overall, the pathology of infections with *Ehrlichia* is variable, ranging from mild to severe for almost all reported species, and the potential for persistent infections is conserved across the genus. The most severe infection is seen with *E. ruminantium*, which destroys vascular endothelium in multiple organs, with lesser infections caused by ehrlichiae with a primary tropism for leukocytes. Clinical symptoms of mild infections are similar across the

genus and include fever, lethargy, myalgia and headache in humans, inappetance or depression in animals, leukogram abnormalities, possible thrombocytopenia, elevated liver enzymes, and decreased alkaline phosphatase. Severe infections are more variable; they often include significant leukogram abnormalities, anemia, and thrombocytopenia, but overt clinical signs vary depending on which organ system is most affected. Overall, the severity of infection is determined by the species and strain of *Ehrlichia*, the species and strain of tick vector, the species and breed of host animals, and the immune status of the infected vertebrate.

1.6. Diagnosis of Ehrlichial Infections

Classically, diagnosis of infections with *Ehrlichia* spp. in humans and animals relies on serology, especially indirect immunofluorescent assays (IFA) (Allsopp et al., 2005a; Martinez et al., 2008; McQuiston et al., 1999; Rikihisa, 1991; Waner et al., 2001). In human practice, a significant rise in titer between acute and convalescent sera, defined as a 4-fold increase using IFA, is diagnostic for acute infection with *Ehrlichia* (Centers for Disease Control and Prevention, 2008). Commercial ELISA kits designed for detection of *E. canis* antigens are also available, including a tabletop rapid flow assay; these assays are less sensitive than IFA and are not quantitative, but they are used clinically to identify seropositive animals (Harrus et al., 2002). Because chronic infection of dogs with *E. canis* is common, all seropositive dogs are treated as though they are actively infected (Waner et al., 2001), with less need to discriminate acute infections.

Serologic techniques have several limitations, however. Convalescent serum is best collected 3-4 weeks after the acute infection; by this time, the infected vertebrate has either recovered, died, or established a chronic infection. This limits the usefulness of this diagnostic tool as a guide to treatment. Serologic cross-reactivity occurs between species of

Ehrlichia and can occur between *Ehrlichia* and *Anaplasma* spp. (Comer et al., 1999; Katz et al., 1996; Rikihisa et al., 1994; Waner et al., 2001), confounding the specificity of serologic diagnoses. Since not all *Ehrlichia* spp. have been cultivated, a limited number of antigens are available, and testing with heterologous species is common. However, serologic cross-reactivity is not seen in all individuals and between all species of *Ehrlichia*, and serologic testing using a heterologous species of *Ehrlichia* can fail to detect antibodies. Finally, there are reports of animals with chronic infections with *Ehrlichia* spp. that become seronegative over time.

Confirmation of infections with *Ehrlichia* spp. sometimes relies upon cultivation of the organism or direct visualization of morulae. Since *Ehrlichia* spp. are not easy to cultivate, passage of suspected materials into a naïve vertebrate host has also been recorded (Allsopp et al., 2005a); however, both cultivation and animal passage are expensive and time-consuming. Direct visualization of ehrlichiae is possible during acute infections with *E. canis*, *E. chaffeensis*, and *E. ewingii*, but leukocytes with morulae are infrequent and the absence of morulae has no diagnostic significance (Childs et al., 1999). In cases of severe, acute infections with *E. ruminantium*, postmortem diagnoses can often be made by visualizing ehrlichiae in the endothelium of brain capillaries (Allsopp et al., 2005a; Martinez et al., 2008).

DNA-based testing has become more common in recent years, and PCR assays for *Ehrlichia* spp. have been applied to ticks, animals, and human samples. Ticks maintain ehrlichiae transstadially, remaining infected for weeks to months, and PCR can detect DNA from pathogens throughout this time. Ehrlichemia occurs in vertebrates during the acute phase of infection and intermittently during chronic infections (Breitschwerdt et al., 1998; Davidson et al., 2001; Rikihisa, 1991). However, ehrlichioses associated with infrequent cell types (e.g., monocytes) can exist in the bloodstream at very low levels. Successful diagnosis

of *Ehrlichia* spp. using molecular techniques requires the use of sensitive and specific PCR assays that can selectively amplify the trace amounts of ehrlichial DNA that might be found in the vertebrate bloodstream.

1.7. Aims and Outline of this Thesis

Limited data are available on the life cycles, tick reservoirs, or wildlife hosts of emerging zoonotic ehrlichioses in the USA, including *E. chaffeensis* and *E. ewingii*. The goal of this thesis was to further define the natural history of *Ehrlichia* spp. in the USA, both by characterizing laboratory animal models for tick transmission and by examining ticks and animal samples collected from the wild.

The first three chapters deal specifically with *E. chaffeensis*. In order to assess the kinetics of *E. chaffeensis* in a laboratory model, molecular biologic tools for the detection and evaluation of ehrlichiae were developed. **Chapter 2** describes the development of a sensitive, specific quantitative PCR assay for the detection of *E. chaffeensis*; the assay was validated for use on tick samples as well as animal samples. In **Chapters 3 and 4**, this assay was applied to laboratory transmission studies. Small laboratory mammals were assessed for their susceptibility to infection with *E. chaffeensis* and their ability to transmit the infection to immature *A. americanum* ticks. Furthermore, four strains of immunocompromised mice were examined for their susceptibility to *E. chaffeensis*, the quantitative burden of ehrlichiae in the blood and tissues, and their ability to transmit the pathogen to ticks (**Chapter 3**). Guinea pigs and gerbils were also examined as potential laboratory animal models for *E. chaffeensis* (**Chapter 4**).

The next five chapters describe the discovery and characterization of a novel *Ehrlichia*. Domestic goats were initially evaluated as animal models for naturally occurring *E. chaffeensis*, but none of the goats infested with wild *A. americanum* developed infections with this pathogen. However, one of the goats became infected with a novel *Ehrlichia* that is closely related to *E. ruminantium*, as described in **Chapter 5**. Other goats became infected with *E. ewingii*, and in **Chapter 6**, the clinical features of tick-transmitted infections of goats by both the novel *Ehrlichia* and by *E. ewingii* are described. The reservoir competence of

white-tailed deer (*Odocoileus virginianus*) for the novel *Ehrlichia* is described in **Chapter 7**. The zoonotic potential of the novel *Ehrlichia* is documented by the first report of human infection with this agent following the bite of a nymphal *Amblyomma* tick (**Chapter 8**). The geographic range of the pathogen is described in **Chapter 9**, which details the development of a sensitive and specific nested PCR assay for the novel *Ehrlichia* and a survey of 3799 *A. americanum* from the USA, including 1835 ticks collected from people. Finally, sequence analysis of the MAP-1 gene revealed two distinct genetic clades. Taken as a whole, these chapters describe the discovery and initial characterization of a previously unrecognized *Ehrlichia* in the USA, including pathogenicity for domestic goats, the identification of a wild animal reservoir, illustration of zoonotic potential, and geographic distribution.

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Quantitative Real-Time PCR Assay for Detection of *Ehrlichia chaffeensis*

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A real-time PCR assay was developed for the detection of *Ehrlichia chaffeensis*. The assay is species specific and provides quantitative results in the range 10 to 10^{10} gene copies. The assay is not inhibited by the presence of tick, human, or mouse DNA and is compatible with high sample throughput. The assay was compared with previously described assays for *E. chaffeensis*.

Ehrlichia chaffeensis is the causative agent of human monocytic ehrlichiosis, a potentially fatal disease (11). The bacterium, first isolated in 1991 (4), is a tick-borne zoonotic pathogen classified within the order *Rickettsiales*, family *Anaplasmataceae* (6). The pathogen is maintained in a natural transmission cycle between the Lone Star tick (*Amblyomma americanum*) and mammalian hosts (1, 7, 9). Experimental studies of the acquisition and transmission of *E. chaffeensis* between mammals and ticks require a quantitative assay for the organism that is compatible with high sample throughput.

Existing PCR assays for *E. chaffeensis* include a direct PCR assay for the 16S rRNA gene (2) and a nested PCR assay that amplifies the variable-length PCR target (VLPT) (13). These PCR assays can detect the presence of pathogen DNA but do not provide quantitative data. Nested PCR is typically more sensitive than direct PCR but requires more handling of amplified PCR products, decreasing throughput capacity and increasing the risk of sample cross-contamination.

Real-time (TaqMan) PCR. The single-copy 16S rRNA gene of *E. chaffeensis* was selected for the development of the real-time TaqMan PCR assay (10). Primers that amplify an 81-bp region of the gene (bases 17 to 97, GenBank accession no. U86665) were selected. The assay was optimized with a Brilliant quantitative-PCR core reagent kit (Stratagene, La Jolla, Calif.), with a final reaction volume of 25 μl. The reaction mixture contained a 200 nM concentration of the forward primer ECH16S-17 (5′-GCGGCAAGCCTAACACATG-3′), an 800 nM concentration of the reverse primer ECH16S-97 (5′-CCCGTCTGCCACTAACAATTATT-3′), a 100 nM concentration of the probe ECH16S-38 (5′-6-carboxyfluorescein-AGTCGAACGGACAATTGCTTATAACCTTTTGGT-3′), and 3.0 μM magnesium chloride. Real-time PCRs and fluorescence detection were performed using an iCycler thermal cycler and iQ software (Bio-Rad Laboratories, Hercules, Calif.). The optimized thermal cycler program was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 57°C for 1 min.

Sensitivity and specificity. Quantitative results were based on a 10-fold dilution series of a plasmid encoding the 16S rRNA gene of *E. chaffeensis*. The specificity of the assay was determined by testing genomic DNA from three strains of *E. chaffeensis*, several closely related organisms, uninfected ticks, and uninfected mammalian blood and tissues. *E. chaffeensis* isolates (Arkansas, St. Vincent, and Jax) were obtained from clinical samples, as described previously (4, 12). All three strains of *E. chaffeensis* were detected by the TaqMan PCR, and agarose gel electrophoresis revealed a single band of the appropriate size. The TaqMan assay did not amplify genomic DNA from *Ehrlichia muris* (AS145 strain) (15), *Ehrlichia canis* (Oklahoma strain) (5), *Neorickettsia sennetsu* (formerly *Ehrlichia sennetsu*, Miyayama strain) (14), or *Rickettsia prowazekii* (Breinl strain). As revealed by agarose gel electrophoresis, the primers used for the TaqMan PCR assay amplified the 16S rRNA genes from *Ehrlichia ewingii* (human clinical sample) (3) and *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, USG3 strain) (16). However, neither *A. phagocytophilum* nor *E. ewingii* was detected during real-time amplification and fluorescence detection, indicating that the TaqMan probe is species specific for *E. chaffeensis* (data not shown).

Genomic DNA was extracted from uninfected ticks, blood, and tissue samples using the IsoQuick nucleic acid extraction kit (Orca Research, Inc., Bothell, Wash.). The TaqMan PCR assay did not amplify genomic DNA from mouse blood, spleen, or liver (BALB/c and C57BL/6 strains), human or guinea pig blood, or uninfected ticks (*Amblyomma americanum*, *Dermacentor variabilis*, or *Ixodes scapularis*).

The specificity of the TaqMan PCR assay was further tested using DNA extracted from human clinical samples submitted to the Centers for Disease Control and Prevention (Atlanta, Ga.). Three *E. chaffeensis*-positive samples and three *E. chaffeensis*-negative samples (one *A. phagocytophilum*-positive blood sample, one negative blood sample, and one negative lymph node sample) were tested. The TaqMan PCR was positive for all three samples containing *E. chaffeensis* DNA but was negative for two samples lacking *E. chaffeensis* DNA and for the sample containing *A. phagocytophilum* DNA.

Effect of background DNA. The effect of background DNA on the efficiency and sensitivity of the TaqMan PCR assay was determined. Genomic DNA was pooled from 10 adult female *Amblyomma americanum* ticks (35.3 μg of DNA/ml) and 10 uninfected BALB/C mice (19.9 μg of DNA/ml), and human

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TABLE 1. Effect of host DNA on the efficiency and sensitivity of the real-time TaqMan PCR assay for *E. chaffeensis*[a]

Diluent	% Reaction efficiency		Reaction sensitivity	
	Avg	95% CI	Avg C_T	95% CI
Water	89.3	85.5–93.1	23.34	22.94–23.74
Tick DNA	95.8	90.9–100.6	23.08	23.03–23.12
Mouse DNA	104.5	96.1–112.9	22.91	22.76–23.06
Human DNA	105.1	104.4–105.9	23.06	23.03–23.09

[a] Results are reported as the averages and 95% confidence intervals (CI) of results from three separate amplifications. C_T, threshold cycle.

genomic DNA (25 μg of DNA/ml) was obtained from Promega (Madison, Wis.). Reaction efficiencies were compared using a fivefold dilution series of genomic *E. chaffeensis* DNA in water, tick DNA, mouse DNA, or human DNA. As summarized in Table 1, the presence of background DNA did not decrease the efficiency of the TaqMan PCR. The effect of host DNA on sensitivity was determined by comparing the threshold cycles in which genomic *E. chaffeensis* DNA was detected in the presence of water, tick DNA, mouse DNA, or human DNA. *E. chaffeensis* DNA was tested at a dilution of approximately 620 bacteria per μl of blood, corresponding with a moderate degree of ehrlichemia (unpublished results). The sensitivity of the assay in the presence of background DNA was not significantly different from that in water (Table 1).

Comparison with other assays. The analytical sensitivity and specificity of the TaqMan PCR were compared with those of three previously described PCR assays for *E. chaffeensis*: a direct PCR assay for the 16S rRNA gene using primers HE1 and HE3 (2), a nested PCR assay for the VLPT of *E. chaffeensis* (13), and a real-time PCR assay for the 16S rRNA gene, based on the SYBR Green detection system (8). All PCR assays were performed in a 25-μl final volume. For the SYBR Green assay, real-time fluorescence detection was performed by use of an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, Calif.).

The sensitivities of all four PCR assays were compared by testing a fivefold dilution series of *E. chaffeensis* genomic DNA (Table 2). All assays were repeated to ensure reproducible results. The sensitivity of the TaqMan assay was significantly greater than that of the direct (HE1/HE3) assay and comparable to those of the real-time SYBR Green and nested VLPT assays.

TABLE 2. Relative sensitivities of four different PCR assays for *E. chaffeensis* DNA

Dilution[a]	Test result with:			
	TaqMan PCR	HE1/HE3 PCR	VLPT nested PCR	SYBR Green PCR
1/50	+	+	+	+
1/250	+	+	+	+
1/1,250	+	+	+	+
1/6,250	+	−	+	+
1/31,250	+	−	+	+
1/156,250	+	−	+	+
1/781,250	−	−	±[b]	−

[a] The 1/156,250 dilution corresponds to approximately 10 genomes of *E. chaffeensis.*
[b] The VLPT assay was positive in 7 of 10 replicates.

TABLE 3. Specificities of four different PCR assays for *E. chaffeensis* DNA

DNA sample	Test result with:			
	TaqMan PCR	HE1/HE3 PCR[a]	VLPT nested PCR[a]	SYBR Green PCR
E. chaffeensis	+	+	+	+
E. muris	−	ND[b]	−	+
E. canis	−	−	−	+
E. ewingii	−	−	−	+
A. phagocytophilum	−	−	−	+
N. sennetsu	−	−	ND	−
R. prowazekii	−	−	ND	−
Amblyomma americanum ticks	−	−	−	−
Mouse DNA	−	ND	ND	−
Human DNA	−	−	−	−

[a] Data derived from published results (2, 13).
[b] ND, not done.

The specificity of the TaqMan PCR was compared with those of the other three PCR assays for *E. chaffeensis* (Table 3). The HE1/HE3 direct and VLPT nested PCR assays have been evaluated by using genomic DNA from several species of *Ehrlichia* and *Anaplasma* and have been demonstrated to be species specific for *E. chaffeensis* (2, 13). As reported above, the TaqMan PCR is species specific for *E. chaffeensis*. However, the SYBR Green assay detected *E. canis*, *E. ewingii*, *E. muris*, and *A. phagocytophilum*, as well as *E. chaffeensis*.

Conclusions. In conclusion, the real-time PCR assay that we have developed for *E. chaffeensis* is very sensitive (10 gene copies), is species specific, is suitable for high-throughput applications, and is not inhibited by the presence of human, mouse, or tick DNA. The assay was found to be superior to three assays previously described for the detection of *E. chaffeensis* based on one or more criteria. It is more sensitive than the HE1/HE3 direct PCR assay, more specific than the SYBR Green real-time PCR assay, and has a higher throughput capacity and less sample handling than the VLPT nested PCR assay. Most importantly, the real-time TaqMan PCR assay provides quantitative data, allowing for simultaneous detection of the pathogen and determination of the infectious loads in ticks and mammals. This assay provides a powerful tool for examining the kinetics of infection with *E. chaffeensis* and the transmission of the pathogen between mammalian hosts and arthropod vectors.

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Research Paper

Evaluation of Immunocompetent and Immunocompromised Mice (*Mus musculus*) for Infection with *Ehrlichia chaffeensis* and Transmission to *Amblyomma americanum* Ticks

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ABSTRACT

Experiments on the natural history of *Ehrlichia chaffeensis*, the agent of human monocytic ehrlichiosis (HME), would be facilitated by the availability of a laboratory animal model for transmission to vector ticks. Five strains of mice were evaluated for their susceptibility to infection with *E. chaffeensis* and transmission competence: C57BL/6 mice, inducible nitric oxide synthase (iNOS) deficient mice, MHC I deficient (β2m −/−) mice, MHC II deficient mice (Abb −/−), and B and T cell deficient (Rag1 −/−) mice. Mice were inoculated with a low passage isolate of *E. chaffeensis*, and infection and morbidity were monitored for 57 days. Three xenodiagnostic infestations with *A. americanum* nymphs were performed 1, 8, and 15 days following inoculation. C57BL/6 mice cleared the organism in less than 17 days, with no indication of morbidity, and mounted a rapid, strong antibody response. Transmission to feeding *A. americanum* nymphs was seen in 1/30 nymphs fed on C57BL/6 mice immediately after inoculation. In MHC I and iNOS deficient mice, pathogen DNA was detected up to 17 or 24 days, respectively, after inoculation. Persistent infection for the duration of the experiment (57 days) was observed in MHC II deficient mice. However, *E. chaffeensis* was not detected in ticks fed on iNOS, MHC I, or MHC II deficient mice. Susceptibility to infection was greatest in Rag1 knockout mice, with significant morbidity and mortality within 24 days after inoculation. *E. chaffeensis* DNA was detected in up to 55% of replete nymphs that fed on Rag1 mice. However, *E. chaffeensis* was not detected in molted adult ticks from the same cohorts. Key Words: Mice—*Mus musculus*—Ticks—*Amblyomma americanum*—*Ehrlichia chaffeensis*. Vector-Borne Zoonotic Dis. 4, 323–333.

INTRODUCTION

Ehrlichia chaffeensis, the causative agent of human monocytic ehrlichiosis (HME), is a zoonotic tick-borne disease transmitted by the vector tick, *Amblyomma americanum* ("lone star" tick). The natural history of this pathogen is poorly understood. The white-tailed deer (*Odocoileus virginianus*) is the only wild animal that has been shown to be reservoir competent for *E. chaffeensis* (Ewing et al. 1995, Lockhart et al. 1997). However, *A. americanum* larvae and nymphs also feed on a variety of small- and medium-sized mammals, including squirrels, cottontail rabbits, raccoons, opossums, and skunks (Cooney and Burgdorfer 1974, Koeh and Dunn 1980, Kollars et al. 2000, Lockhart et al. 1997, Sonenshine and Levy 1971, Zimmerman et al. 1988), and dogs and coyotes are naturally exposed to *E. chaffeensis* (Breitschwerdt et al. 1998, Kocan et al. 2000, Murphy et al. 1998). Establishment of a laboratory animal

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model for infection with *E. chaffeensis* and transmission to feeding ticks would facilitate studies of the natural history of this agent, including the reservoir competence of other wild animals.

Previous investigators have reported that immunocompromised laboratory mice (*Mus musculus*) are susceptible to infection with *E. chaffeensis*. Mice with the severe combined immunodeficiency (SCID) mutation were highly susceptible to experimental infection with cultured *E. chaffeensis*, and significant morbidity and mortality were associated with infection (Li et al. 2002, Li et al. 2001, Winslow et al. 1998, Winslow et al. 2000). In other experiments, T-cell receptor β chain and β/δ chain deficient mice developed low level persistent infection with *E. chaffeensis* ($\geq$24 days), but no morbidity was noted (Winslow et al. 2000). Similarly, mice lacking the MHC II molecule developed persistent infection with the pathogen ($\geq$92 days), with no morbidity or mortality (Ganta et al. 2002). In contrast, immunocompetent mice (C57BL/6, C3H, or BALB/c) were resistant to *E. chaffeensis* and eliminated experimental infection within 14–17 days (Lockhart and Davidson 1999, Telford and Dawson 1996, Winslow et al. 1998). However, these experiments did not assess the competence of these animals to transmit the pathogen to feeding vectors.

We evaluated five strains of mice for their susceptibility to infection and transmission competence for *E. chaffeensis*. Three mutations have previously been studied with regards to *E. chaffeensis*: "wild-type" C57BL/6 mice, MHC II deficient mice, and B- and T-cell deficient mice. We extend previously published data to assess the competence of these mice for transmission of *E. chaffeensis* to *A. americanum* nymphs and to document the infectious burden of the pathogen in tissues. We also hypothesized that two previously untested strains of mice would be susceptible to infection with *E. chaffeensis*: MHC I deficient mice and inducible nitric oxide synthase (iNOS) deficient mice. The $CD8^+$ "cytotoxic" T-cell response, which is dependent on MHC I expression, has been shown to be a critical component of immunity to other rickettsiaceae, including the closely related bacterium *Ehrlichia muris* (Feng et al. 1997, Feng and Walker 2004, Walker et al. 2001, Walker et al. 2000). Nitric oxide production by iNOS is one mechanism by which macrophages exert antimicrobial activity. Increased nitric oxide production has been associated with *E. chafeensis* infection of both immunocompetent and MHC II deficient mice (Ganta et al. 2002), and iNOS deficient laboratory mice display delayed clearance of the closely related pathogen, *Anaplasma* (formerly *Ehrlichia*) *phagocytophilum* (Banerjee et al. 2000).

MATERIALS AND METHODS

Mice

Mice were obtained from commercial sources and maintained at the Centers for Disease Control and Prevention, Atlanta, GA, in accordance with approved Institutional Animal Care and Use Committee protocols. C57BL/6 mice, MHC I deficient mice (β2m $-/-$), iNOS knockout mice (iNOS $-/-$), and B and T cell deficient mice (Rag1 $-/-$) were obtained from Jackson Laboratory (Bar Harbor, ME). MHC II deficient mice (Abb $-/-$) were obtained from Taconic (Germantown, NY). All mutant mouse strains represent targeted gene knockouts on a C57BL/6 genetic background. Twenty-eight to 35 mice from each strain were inoculated with *E. chaffeensis*, and an additional 12 mice from each strain were used as uninfected controls.

Inoculum

Mice were infected by intraperitoneal injection with 100 μL of a frozen stabilate of infected mouse livers, containing approximately 1.4×10^6 *E. chaffeensis* in each dose. The stabilate was produced by inoculating 12 Rag1 knockout mice with 7.5×10^5 disrupted DH82 cells infected with a low passage strain of *E. chaffeensis* (St. Vincent strain, pass 10) (Paddock et al. 1997) and harvesting the livers 7 days after infection. Livers were homogenized in a small volume of sterile phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA) and stored in 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) at -80°C. Aliquots of the inoculum were tested by quantitative PCR to determine the infectious dose.

Ticks

Uninfected *A. americanum* nymphs were obtained from a laboratory colony at the Centers for Disease Control and Prevention, Atlanta, GA. Ticks were housed at 24°C, 90% relative humidity, with a day/night photoperiod of 16:8 h, both before and after molting. Cohorts of four infected mice per strain were infested with 25–30 *A. americanum* nymphs 1, 8, or 15 days after infection. Four uninfected mice per strain were also infested with ticks and served as a control for tick feeding success. For each infestation, ticks were counted and contained in plastic capsules attached to mice. Tick feeding capsules were constructed from the barrel of a 6-mL syringe, as previously described (Burkot et al. 2001) and attached over the shaved back of each mouse using Kamar Adhesive (Kamar, Inc., Steamboat Springs, CO). Infested animals were checked daily and ticks were collected and counted for the duration of infestation (7–10 days).

Monitoring of infection

All mice were weighed weekly during the experiment as an indicator of morbidity. Whole blood was obtained from uninfested mice once every 7–20 days. Mice were divided into two groups, and bleeding dates for the groups were staggered; one group of mice was bled 0, 7, 14, 21, 28, and 57 days post-infection, and the other group was bled 0, 10, 17, 24, 31, and 45 days post-infection. Blood (100 μL/sample) was collected into serum separator tubes, for indirect immunofluorescence antibody testing (IFA), and/or into EDTA tubes, for PCR, and stored at −20°C until testing. Cohorts of four to six infected mice and two uninfected mice were humanely euthanized 10, 17, 24, 45, and 57 days post-infection. Spleens were collected by sterile technique and weighed. Samples of both spleen and liver tissue (10–15 mg each) were reserved for PCR. The remainder of the spleen was processed for *in vitro* culture.

Culture

For diagnostic culture, spleens were homogenized in a small volume of sterile PBS. Red blood cells were removed using Red Blood Cell Lysing Buffer (Sigma-Aldrich), and nucleated cells were resuspended in sterile PBS. Splenocytes (1×10^5 cells/well) were co-cultured with uninfected DH82 cells in each well of a 24-well plate. Cultures were maintained at 37°C, 5% CO_2, in Minimal Essential Medium (MEM) containing Earle's salts (Mediatech, Inc., Herndon, VA), 10% heat-inactivated, tetracycline-free fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), and 10 mM HEPES buffer (Invitrogen). The medium was changed every 3–4 days. Starting ten days after establishment, cultures were monitored for DH82 cell lysis. In addition, the supernatant was tested weekly for the presence of *E. chaffeensis* DNA for 8 weeks.

PCR

DNA from tissue samples and ticks was extracted using the IsoQuick Nucleic Acid Extraction Kit (Orca Research, Inc., Bothell, WA). Prior to DNA extraction, tissue samples were digested using 1 mg/mL Proteinase K (Sigma-Aldrich) at 55°C for 1 h, and ticks were individually frozen in liquid nitrogen and crushed using sterile Teflon pestles. All DNA samples were resuspended in 50 μL of sterile, DNAse/RNAse-free water. Samples were tested in duplicate using a quantitative real-time PCR assay with a sensitivity of 10 *E. chaffeensis* genomes/2.5 μL of DNA (Loftis et al. 2003), and positive samples were verified by repetition. The double-stranded DNA content of blood and tissue samples was determined using the PicoGreen dsDNA Quantitation kit (Molecular Probes, Eugene, OR), according to the manufacturer's directions. Final quantitative PCR results are reported as the number of *E. chaffeensis*/μg total DNA.

Serology

Antibody titers against *E. chaffeensis* were determined by IFA, using the methods outlined previously (Comer et al. 1999). Sera were screened at a dilution of 1/16, and detection was achieved with goat anti-mouse $IgG_{(\gamma)}$ conjugated to fluorescein isothiocyanate (KPL, Inc., Gaithersburg, MD). Positive sera were titered out, using a twofold dilution series.

Serologic data are reported as the reciprocal of the last dilution showing positive fluorescence.

Statistical analysis

The total body weight of infected mice was compared to that of strain- and age-matched control mice using analysis of variance (ANOVA). For each euthanasia group, the one-tailed *T* test was used to compare spleen weights of infected mice to those of control mice. Chi-square analysis was used to compare the percent of PCR positive samples between types of tissues and between groups of mice. Quantitative PCR data (number of *E. chaffeensis*/μg spleen DNA) was compared between infected mice of different strains using a two-tailed *T* test for populations with unequal variance. Serologic data was compared using ANOVA. Tick feeding and molting success for each xenodiagnostic infestation was compared to an infestation of uninfected mice of the same strain using a chi-square test.

RESULTS

C57BL/6 mice

No morbidity was seen in immunocompetent mice that were inoculated with *E. chaffeensis*, and the total body weight of inoculated mice was similar to control mice throughout the experiment (Fig. 1A). Significant splenomegaly ($p < 0.05$) was observed 10 days after infection but was not seen in mice euthanized on later dates (Fig. 2A). *E. chaffeensis* DNA was detected in the blood of nine of 22 (41%) mice on day 7 post-inoculation (P.I.) and in two of 17 (12%) mice on day 10 P.I. Liver PCR was significantly more sensitive than blood PCR, and six of 13 (43%, $p = 0.035$) mice were positive on day 10 P.I. Spleen PCR was the most sensitive test for the detection of *E. chaffeensis*, and 13/13 (100%, $p < 0.01$) spleens from C57BL/6 mice were positive on day 10 P.I. (Table 1). This is similar to the other four strains of mice, in which detection of the organism was most consistent in the spleen; for this reason, quantitative PCR data for spleens was used to compare the infectious burden between strains of mice. Ten days after inoculation, spleens from C57BL/6 mice had an average of 3958 *E. chaffeensis* genomes/μg total DNA (95% C.I. 1398–5784). However, *in vitro* culture recovered live *E. chaffeensis* organisms from only one of these PCR-positive spleens. Neither blood nor tissues were PCR positive for *E. chaffeensis* at any time point after day 10 P.I. These data are consistent with transient infection and complete clearance of the pathogen between 10 and 17 days P.I.

C57BL/6 mice mounted a rapid IgG class antibody response to *E. chaffeensis*, in which 100% of the mice seroconverted by day 10 P.I. These mice developed a geometric mean titer (GMT) of 2702 by day 14 P.I, corresponding with clearance of the organism. Titers remained high for the duration of the 57-day experiment, with the GMT ranging from 1448 to 2896.

Three cohorts of infected mice were infested with *A. americanum* nymphs on days 1, 8, and 15 P.I., and ticks fed to repletion over the following 4–9 days. Engorged nymphs and molted adults were tested for the presence of *E. chaffeensis* using a real-time PCR assay with a lower limit of detection of 200 organisms per tick. One engorged nymph out of 30 (3.3%) was positive for *E. chaffeensis* DNA in the first xenodiagnostic infestation (Table 2). No other nymphs and no molted adults from any of the three infestations tested positive for the pathogen.

iNOS knockout mice

No morbidity was seen in iNOS $-/-$ mice inoculated with *E. chaffeensis* (Fig. 1B). Splenomegaly was greatest on day 10 P.I. ($p = 0.009$) and remained significant through day 17 P.I. ($p = 0.024$) (Fig. 2B). Similar to C57BL/6 mice, *E. chaffeensis* DNA was detected in the blood of iNOS knockout mice at 7 days (10 of 18 mice) and 10 days (one of 10 mice) after inoculation, but not at later dates (Table 1). Liver PCR was more sensitive than blood PCR, and the pathogen was detected in five of six liver samples (83.3%) 10 days P.I. Similar to C57BL/6 mice, *E. chaffeensis* DNA was detected in the spleens of five of six mice on day 10 P.I., with an average of 882 organisms/μg DNA (95% C.I. 238–1527). Live organism was recovered from three of these spleens using *in vitro*

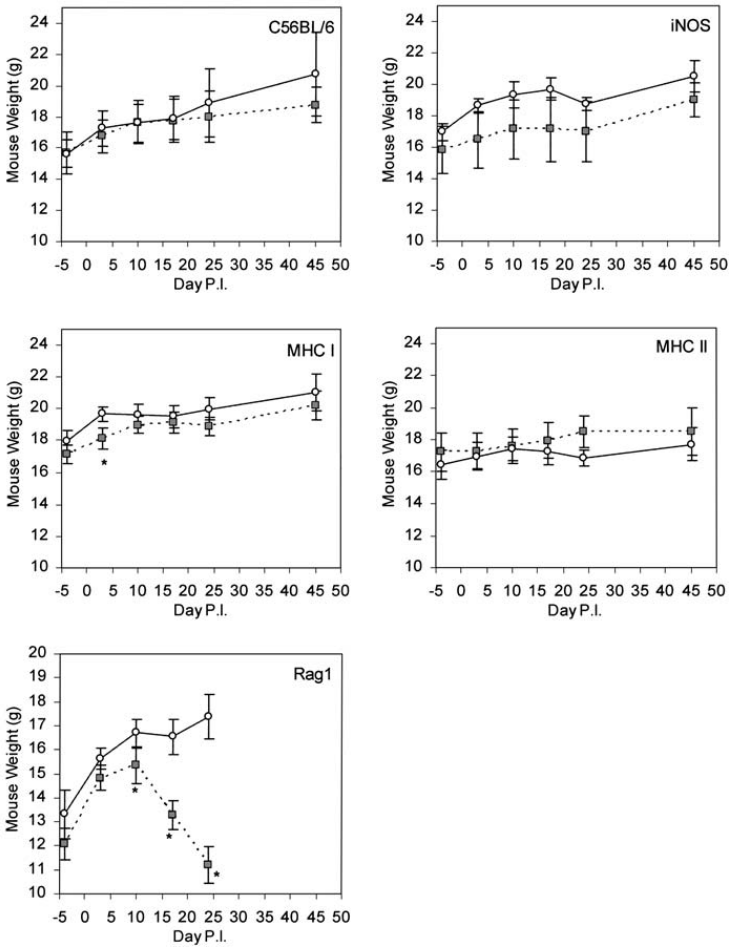

FIG. 1. Total body weight of *E. chaffeensis*–infected (---■---) and control (—○—) mice. Statistically significant ($p < 0.05$) difference in body weight is indicated (*).

culture with DH82 cells. Unlike C57BL/6 mice, in which no pathogen was detected after day 10 P.I., pathogen DNA was also detected in three of six (50.0%, $p = 0.045$) spleens collected from iNOS −/− mice on day 24 P.I. However, live *E. chaffeensis* was not recovered from these spleens by *in vitro* culture. Poor recovery *in vitro* may reflect poor viability of the pathogen and/or the low quantity of the cultured organism. The serologic response of iNOS −/− mice was similar to C57BL/6 mice, with 100% seroconversion by day 10 P.I. and statistically similar titers throughout the 57-day experiment.

When uninfected *A. americanum* nymphs were fed on three sequential cohorts of iNOS −/− mice, as for C57BL/6 mice, no *E. chaffeensis* DNA was detected in engorged nymphs or molted adults fed on iNOS −/− mice (Table 2).

MHC I deficient mice

No morbidity was seen in MHC I deficient (β2m −/−) mice inoculated with *E. chaffeensis*

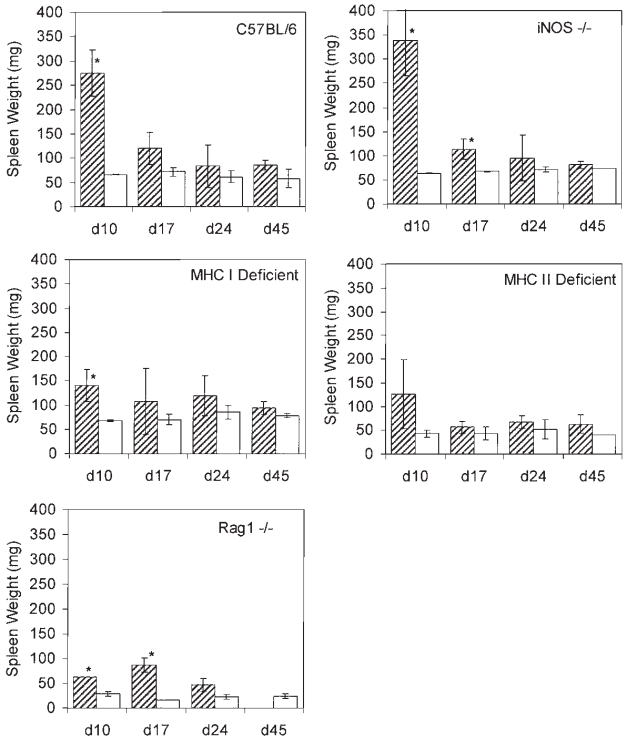

FIG. 2. Average weight of spleens harvested from *E. chaffeensis*–infected (▨) and control (□) mice, by day post-inoculation. Error bars reflect 95% confidence intervals. Statistically significant ($p < 0.05$) splenomegaly is indicated (*).

(Fig. 1C). Mild, transient splenomegaly was seen ten days P.I. and was resolved by day 17 P.I. (Fig. 2C). *E. chaffeensis* DNA was detected in blood samples collected from one of 18 (5.6%) mice on day 7 P.I. and three of 12 (25.0%) mice on day 14 P.I. (Table 1). Similar to C57BL/6 mice, *E. chaffeensis* was detected in three of six (50.0%) liver samples on day 10 P.I., but not at any later time points. Six of six (100%) spleens tested on day 10 P.I. were also PCR positive for the pathogen, and the number of ehrlichiae was comparable to C57BL/6 mice (2031 organisms/μg DNA, 95% C.I. 1041–3022). In addition, three of six (50.0%) spleens from MHC I deficient mice tested on day 17 P.I. were PCR positive for the pathogen, whereas none of the six C57BL/6 mice were PCR positive at this time point ($p = 0.045$). However, *in vitro* culture failed to recover live *E. chaffeensis* from any spleens collected from MHC I deficient mice at any time point.

MHC I deficient mice also mounted a delayed IgG antibody response against *E. chaffeensis*. Mice seroconverted by day 17 P.I., 10 days later than C57BL/6 mice, and antibody titers of MHC I deficient were significantly lower ($p < 0.05$) than C57BL/6 mice until day 24 P.I. These data suggest that MHC I deficient mice are more susceptible to *E. chaffeensis* infection than immunocompetent mice, with delayed clearance of the organism and delayed antibody response.

When uninfected *A. americanum* nymphs were fed on MHC I deficient mice, as for C57BL/6 mice, no *E. chaffeensis* DNA was detected in either engorged nymphs or molted adults fed on MHC I deficient mice (Table 2).

MHC II deficient mice

No morbidity was seen in MHC II deficient (Abb −/−) mice inoculated with *E. chaffeensis*

TABLE 1. NUMBER OF PCR-POSITIVE BLOOD, SPLEEN, AND LIVER SAMPLES FROM *E. CHAFFEENSIS*–INFECTED MICE

Day P.I.	C57BL/6			iNOS			MHC I			MHC II			Rag1		
	Blood	*Liver*	*Spleen*	*Blood*	*Liver*	*Spleen*	*Blood*	*Liver*	*Spleen*	*Blood*	*Liver*	*Spleen*	*Blood*	*Liver*	*Spleen*
7	9/22			10/18			1/18			12/16			15/18		
10	2/17	6/13	13/13	1/10	5/6	5/6	0/10	3/6	6/6	7/10	6/6	6/6	13/15	10/11	11/11
14	0/11			0/12			3/12			4/10			2/12		
17	0/13	0/9	0/9	0/10	0/6	0/6	0/10	0/6	3/6	0/10	0/6	4/6	3/10	7/9	9/9
21	0/2			0/6			0/6			2/4			0/4		
24	0/10	0/6	0/6	0/10	0/6	3/6	0/10	0/6	0/6	1/10	1/6	3/6	0/8	6/7	7/7
28	0/2			0/6			0/6			0/4					
31	0/4			0/4			0/4			1/4					
45	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/3	0/4	4/4			
57	0/2	0/2	0/2	0/5	0/5	0/5	0/6	0/6	0/6	1/4	2/4	3/4			

TABLE 2. NUMBER OF PCR-POSITIVE TICKS FED AS NYMPHS ON *E. CHAFFEENSIS*–INFECTED MICE

	C57BL/6	*iNOS −/−*	*β2m −/−*	*Abb −/−*	*Rag1 −/−*
Xeno 1					
Nymphs	1/30 (3.3%)	0/21 (0%)	0/20 (0%)	0/20 (0%)	3/40 (7.5%)
Adults	0/72 (0%)	0/47 (0%)	0/32 (0%)	0/37 (0%)	0/80 (0%)
Xeno 2					
Nymphs	0/28 (0%)	0/9 (0%)	0/20 (0%)	0/15 (0%)	2/14 (14.3%)
Adults	0/68 (0%)	0/29 (0%)	0/45 (0%)	0/33 (0%)	0/42 (0%)
Xeno 3					
Nymphs	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	11/20 (55.0%)
Adults	0/65 (0%)	0/54 (0%)	0/58 (0%)	0/78 (0%)	0/52 (0%)

(Fig. 1D), and significant splenomegaly was not detected at any time following inoculation (Fig. 2D). *E. chaffeensis* DNA was intermittently detected in the blood of MHC II deficient mice throughout the 57-day experiment (Table 1); this is significantly different from the results seen with C57BL/6 mice, in which *E. chaffeensis* could not be detected in the blood after day 10 P.I. The pathogen was detected in the livers of all six of six (100%) mice on day 10 P.I., one of six (16.7%) mice on day 24 P.L, and two of four (50.0%) mice on day 57 P.I. *E. chaffeensis* DNA was consistently detected in 50–100% of the spleen samples collected from MHC II deficient mice at all time points, and spleen PCR was the most reliable test for detection of the pathogen. The initial infectious burden in the spleen was significantly higher than in C57BL/6 mice, with 31617 organisms/μg DNA on day 10 P.I. (95% C.I. 15212–48023). Ehrlichial DNA sharply decreased by day 17 P.I., with an average of 130 organisms/μg spleen DNA (95% C.I. 37–224, range 0–299), then increased again through day 45 P.I., to an average of 1596 organisms/μg DNA (95% C.I. 0–3246, range 382–4084). The pathogen persisted in the spleen at low levels ($<$1000/μg DNA) throughout the remainder of the experiment. Live *E. chaffeensis* was successfully cultured from all six (100%) mouse spleens harvested 10 days P.I., but not from spleens harvested on later dates. Serologic testing confirmed that MHC II deficient mice were incapable of developing an IgG antibody response to *E. chaffeensis*.

When uninfected *A. americanum* nymphs were fed on MHC II deficient mice, *E. chaffeensis* DNA was not detected in either engorged nymphs or molted adults fed on MHC II deficient mice during any of the three xenodiagnostic infestations (Table 2).

Rag1 knockout mice

Significant morbidity and mortality were seen in Rag1 knockout mice inoculated with *E. chaffeensis*. Infected mice began to lose weight after 10 days P.I. and displayed statistically significant weight loss, compared to uninfected mice (Fig. 1E). Infected mice became moribund after day 14 P.I., and 11/16 mice (68.8%) died between days 14 and 23 P.I. The remaining mice were euthanized on day 24 P.I. Statistically significant splenomegaly was noted on days 10 and 17 P.I., but not on day 24 P.I. (Fig. 2E).

E. chaffeensis was detected in 15 of 18 (83.3%) and 13 of 15 (86.7%) blood samples collected on days 7 and 10 P.I., respectively, and in a lower proportion of blood samples collected on days 14 and 17 P.I. (Table 1). The pathogen was also detected in the majority of liver samples collected from Rag1 $-/-$ mice on days 10, 17, and 24 P.I. The proportion of PCR-positive liver samples on days 17 and 24 P.I. was significantly ($p < 0.05$) higher than that seen with any of the other strains of mice tested. However, the detection of the pathogen was most consistent in the spleen; *E. chaffeensis* was detected in all spleens from all inoculated Rag1 $-/-$mice at all three time points. The number of ehrlichiae in the spleen was very high throughout the experiment, reaching an average of 753,528 organisms/μg DNA (95% C.I. 246,158–1,260,898) by day 10 P.I. The infectious burden remained in the same range through day 24 P.I., when all remaining Rag1 mice were euthanized. Live *E.*

chaffeensis organisms were successfully cultured from the spleens of all infected Rag1 −/− mice at all three time points. Serology was not performed on Rag1 −/− mice.

Three separate cohorts of Rag1 knockout mice were infested with xenodiagnostic ticks 1 ($n = 8$ mice), 8 ($n = 4$), and 15 ($n = 4$) days after inoculation with *E. chaffeensis*. *E. chaffeensis* DNA was detected in engorged nymphs from all three infestations (Table 2). From the first infestation, three of 40 (7.5%) nymphs tested positive, with an average of 901 DNA copies per infected tick. Two of 14 (14.3%) engorged nymphs tested positive from the second infestation, with an average of 1956 organisms per positive tick. The proportion of infected ticks increased significantly in the third infestation, with 11 of 20 (55.0%, $p < 0.01$) ticks testing positive and an average of 3504 organisms per infected tick. Ticks from these cohorts were allowed to molt to adults and were tested for the presence of the pathogen. There was no significant difference in molting success between *A. americanum* nymphs fed on infected and uninfected Rag1 knockout mice (data not shown). All 174 adult ticks that fed as nymphs on infected Rag1 −/− mice were negative for *E. chaffeensis* DNA by real-time PCR.

DISCUSSION

We evaluated the susceptibility of five strains of mice to infection with *E. chaffeensis* and used a series of xenodiagnostic infestations to assess their competence to transmit the infection to feeding *A. americanum* nymphs. The most sensitive test for detection of the pathogen was PCR of the spleen, followed by liver PCR, spleen culture, and, finally, blood PCR. Quantitative data added the ability to compare infectious burdens in the spleen and liver, as well as between strains of mice. In general, the burden of *E. chaffeensis* in the spleen (organisms/μg DNA) was 10-fold higher than in the liver and 100-fold higher than in the blood (data not shown). With the exception of Rag1 −/− mice, *in vitro* culture of *E. chaffeensis* from infected mouse splenocytes had poor success. In all cases, culture was a reliable method of detection only when the infectious burden exceeded approximately 10,000 ehrlichiae/μg spleen DNA (data not shown), suggesting that *in vitro* recovery of E. *chaffeensis* has a very low sensitivity for detection of the organism.

Consistent with previously published results, wild-type C57BL/6 mice were not susceptible to infection with *E. chaffeensis*, and no organism or DNA could be detected after 10 days post-inoculation. Although most iNOS deficient mice in the experiment rapidly cleared *E. chaffeensis*, similar to C57BL/6 mice, three of six iNOS deficient mice had detectable DNA in the spleen 24 days after inoculation. Similarly, three of six MHC I deficient mice had detectable *E. chaffeensis* DNA in the spleen 17 days P.I. This suggests that mice deficient in iNOS or MHC I are slightly more susceptible to infection with *E. chaffeensis*, with slightly delayed clearance of the organism relative to immunocompetent mice. Furthermore, MHC I deficient mice developed a significantly delayed antibody response to the pathogen, approximately 10 days slower than seen in C57BL/6 mice. Mice deficient in MHC II were initially permissive to *E. chaffeensis* infection, and the number of ehrlichiae in the spleen 10 days following inoculation was at least 10-fold that of immunocompetent mice. MHC II deficient mice controlled the level of *E. chaffeensis* infection by day 17 P.I. but did not successfully eliminate the organism; low-level, persistent infection was revealed by intermittent detection of the pathogen in blood and spleen samples through day 57 P.I. As expected, B- and T-cell deficient (Rag1 −/−) mice developed overwhelming infection with *E. chaffeensis*, and mortality was significant by day 24 P.I. Quantitative PCR revealed that titers of ehrlichiae in the spleens of these mice was more than 200 times higher than that seen in C57BL/6 mice.

The increased susceptibility of MHC II deficient mice is consistent with previously published results (Ganta et al. 2002) and supports the critical role of $CD4^+$ cells in immunity against *E. chaffeensis*. However, the poor susceptibility of MHC I deficient mice to infection with *E. chaffeensis* suggests that activation of $CD8^+$ cytotoxic T cells is not a significant effector mechanism for immunity against *E. chaffeensis*. Although iNOS is a significant component of immunity to other rickettsiaceae

(Banerjee et al. 2000, Feng et al. 1994, Walker et al. 1997), lack of iNOS did not significantly alter the course of infection by *E. chaffeensis*. The correlation between seroconversion and pathogen clearance, seen in C57BL/6, iNOS −/−, and MHC I deficient mice, provides indirect support for the hypothesis (Li et al. 2002, Li et al. 2001, Winslow et al. 2000) that antibody-mediated clearance of *E. chaffeensis is* a critical component of immunity against this organism.

The competence of mice for the transmission of *E. chaffeensis* to feeding *A. americanum* nymphs was generally poor. One PCR-positive nymph was recovered from the first infestation of C57BL/6 mice, and no PCR-positive nymphs or adults were obtained from any infestations of iNOS, MHC I, or MHC II deficient mice, although infection of these mice was well-documented for the time frame in which ticks fed. These data suggest that infection of an animal with *E. chaffeensis* does not necessarily result in successful host-to-tick transmission of the pathogen; other factors, including the burden of infection, properties of the bacterial strain used, and macrophage trafficking, may play a role in transmission competence. Tick transmission was seen in Rag1 −/− mice, in which an increasing proportion of engorged nymphs were positive for *E. chaffeensis* DNA over the time course of the study. During the third xenodiagnostic infestation, which corresponded with peak morbidity and mortality of infected Rag1 −/− mice, 55% of the engorged nymphs tested were PCR positive for *E. chaffeensis. In vitro* recovery of live *E. chaffeensis* from all infected Rag1 mice suggests that nymphs feeding on those mice acquired live pathogen. However, the pathogen was not transmitted transstadially and could not be detected in any molted adults from these cohorts. Transstadial transmission of *E. chaffeensis* has been demonstrated in *A. americanum* nymphs and larvae fed on infected white-tailed deer, a competent reservoir (Ewing et al. 1995), suggesting that the lack of transstadial transmission in nymphs fed on infected mice is a significant finding.

In summary, Rag1 deficient mice were the most appropriate laboratory hosts identified for infection with *E. chaffeensis* and transmission to feeding *A. americanum* ticks. The mice were susceptible to the pathogen, rapidly developing a high level of infection that persisted for up to 24 days. *E. chaffeensis* was consistently cultured *in vitro* from the spleens of infected Rag1 −/− mice. These mice made the pathogen available to feeding *A. americanum* nymphs, and up to 55% of the nymphs fed on Rag1 −/− mice during the third week post-inoculation acquired the pathogen. We attribute the apparent lack of transstadial transmission to the properties of the strain and passage of *E. chaffeensis* used in this experiment, rather than to properties inherent in Rag1 −/− mice. It is possible that the isolate of *E. chaffeensis* used in this experiment could have lost infectivity for *A. americanum* ticks during *in vitro* culture. This phenomenon has been described for the closely related bacterium, *Ehrlichia canis*, which lost infectivity for its vector tick, *Rhipicephalus sanguineus*, after numerous passages *in vitro* (Mathew et al. 1996). Although a low-passage strain of *E. chaffeensis* (10 passages *in vitro*) was selected for this experiment, no rigorous studies have been performed to determine how rapidly *Ehrlichia* species maintained *in vitro* lose the capacity for tick infection. This hypothesis must be confirmed in future experiments involving tick-derived isolates of *E. chaffeensis* that have not been passaged *in vitro*.

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Research Paper

Lack of Susceptibility of Guinea Pigs and Gerbils to Experimental Infection with *Ehrlichia chaffeensis*

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ABSTRACT

Guinea pigs and Mongolian gerbils were experimentally infected with *Ehrlichia chaffeensis* (St. Vincent strain, 10 passages *in vitro*). The infection was monitored by serial blood sampling for PCR and by xenodiagnosis with *Amblyomma americanum* larvae. Exposure to the pathogen was confirmed using serology. Neither guinea pigs nor gerbils were susceptible to infection with *E. chaffeensis*, and ticks fed upon these animals did not become infected with the pathogen. Key Words: Guinea pigs—*Cavia porcellus*—Gerbils—*Meriones unguiculatus*—Ticks—*Amblyomma americanum*—*Ehrlichia chaffeensis*. Vector-Borne Zoonotic Dis. 4, 319–322.

INTRODUCTION

Ehrlichia chaffeensis, the causative agent of human monocytic ehrlichiosis, is a tick-borne zoonotic pathogen in the order *Rickettsiales*, family *Anaplasmataceae* (Dumler et al. 2001). The pathogen is maintained in a natural transmission cycle between wild animals and the tick vector, *Amblyomma americanum*. At present, the white-tailed deer (*Odocoileus virginianus*) is the only known wild animal reservoir for *E. chaffeensis* (Ewing et al. 1995, Lockhart et al. 1997). All three life stages of *A. americanum* feed on white-tailed deer, but larvae and nymphs also feed on a variety of small- and medium-sized mammals, including squirrels, cottontail rabbits, raccoons, opossums, and skunks (Cooney and Burgdorfer 1974, Kollars et al. 2000, Lockhart et al. 1997, Zimmerman et al. 1988). The reservoir competence of small- and medium-sized mammals for *E. chaffeensis* has not been evaluated. The availability of a small laboratory animal model for *E. chaffeensis* infection and transmission to ticks would facilitate experiments in the natural transmission cycle of this pathogen.

We evaluated guinea pigs (*Cavia porcellus*) and Mongolian gerbils (*Meriones unguiculatus*) for their suitability as laboratory animal models for *E. chaffeensis* infection and transmission to feeding ticks. Guinea pigs are susceptible to several species of *Rickettsia* and to *Coxiella burnetii* (Heggers et al. 1975, Sammons et al. 1977). Mongolian gerbils are susceptible to the tick-borne pathogens *Anaplasma phagocytophilum* (M.L. Levin, unpublished results), *Borrelia burgdorferi*, and *Babesia microti* (Gray et al. 2002, Stanek et al. 1986). Animals were infected by inoculation with the lowest available passage strain of *E. chaffeensis*, and their response to infection was monitored by PCR, xenodiagnostic feeding of *A. americanum* larvae, and serology.

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MATERIALS AND METHODS

The St. Vincent strain of *E. chaffeensis* was isolated from human blood, as described previously, and maintained in DH82 cells *in vitro* (Paddock et al. 1997). The isolate was propagated for a total of 10 passages following isolation. Infection of DH82 cell culture was monitored by Diff-Quik staining (Dade Behring Inc., Newark, DE), and cells were harvested when the infection rate exceeded 85%. The inoculum was prepared by suspending the cells in sterile PBS (Invitrogen, Carlsbad, CA) at 4°C and disrupting the cells by needle passage immediately prior to inoculation. Inocula produced using this protocol are infectious for susceptible strains of mice (unpublished results).

Female guinea pigs (HsdPoc:DH, 4–6 weeks old) and Mongolian gerbils (12 weeks old) were obtained from Harlan (Indianapolis, IN) and maintained in accordance with Institutional Animal Care and Use Committee protocols. Four guinea pigs and three gerbils per group were inoculated via intraperitoneal injection with 1×10^4, 1×10^5, or 1×10^6 disrupted, *E. chaffeensis*–infected DH82 cells. Two guinea pigs and one gerbil were also maintained as uninfected control animals. All animals were observed daily for normal appetite and activity level.

Blood samples (100 μL each) were collected from gerbils and guinea pigs 3, 7, 10, and 14 days after inoculation. DNA was extracted using the IsoQuick Nucleic Acid Extraction Kit (Orca Research, Inc., Bothell, WA) and rehydrated in 50 μL of RNAse/DNAse free water. Blood samples were tested for the presence of *E. chaffeensis* DNA by a quantitative real-time PCR assay with a sensitivity of 10 gene copies/2.5 μL DNA sample (Loftis et al. 2003), which corresponds to approximately 2 bacteria/μL of whole blood, and by a nested PCR assay (VLPT) with a slightly higher sensitivity (Sumner et al. 1999).

Serum was collected from each animal 45 days after inoculation with *E. chaffeensis*, and an indirect immunofluorescence assay (IFA) was used to confirm the presence of antibodies against *E. chaffeensis*. For the IFA, sera were diluted 1/16, 1/32, 1/64, and 1/128 and applied to glass slides coated with acetone-fixed, *E. chaffeensis*–infected DH82 cells. Detection was achieved using secondary antibodies conjugated to fluorescein isothiocyanate: goat anti-guinea pig $IgG_{(H+L)}$ (KPL, Inc., Gaithersburg, MD) was used at 1:150, and rabbit anti-gerbil $IgG_{(H+L)}$, (Immunology Consultants Laboratory, Inc., Newberg, OR) was used at 1:300. Sera from gerbils exhibited a significant reactivity against DH82 cells and were adsorbed using uninfected DH82 cells prior to IFA testing. Adsorption was not necessary with sera from guinea pigs. Titers are reported as the inverse of the last dilution at which antibodies were detected.

For xenodiagnosis, animals were infested with uninfected *A. americanum* larvae from a colony maintained at the Centers for Disease Control and Prevention (Atlanta, GA). Larvae were placed on two guinea pigs per group, three days after inoculation, and were contained within 1-inch tubular stockinette attached over the back with Kamar Adhesive (Kamar, Inc., Steamboat Springs, CO). Larvae were placed on all 10 gerbils, 4 days after inoculation, and were contained within plastic capsules made from 10-mL syringe barrels (Burkot et al. 2001), attached with the same adhesive. Engorged larvae collected from infested animals were maintained at 21°C and 80% relative humidity until molting. DNA was extracted from 50 molted nymphs (10 pools of five nymphs each) from each animal and was tested for *E. chaffeensis* by real-time PCR.

RESULTS AND DISCUSSION

Four guinea pigs each were inoculated with 1×10^4, 1×10^5, or 1×10^6 disrupted, *E. chaffeensis*-infected DH82 cells (Table 1). According to quantitative PCR, each infected DH82 cell included approximately 75 *E. chaffeensis* genomes. Forty-five days after infection, antibodies against *E. chaffeensis* were detected in two of four guinea pigs inoculated with 1×10^5 infected DH82 cells, with titers of at least 128, and in two of four guinea pigs inoculated with 1×10^6 infected DH82 cells (titers >128). However, none of the four guinea pigs inoculated

TABLE 1. EXPERIMENTAL INFECTION OF GUINEA PIGS AND GERBILS WITH *E. CHAFFEENSIS*[a]

Inoculum: no. of infected DH82 cells	*No. of animals*	*Blood PCR: day post-inoculation*				*No. of PCR (+) ticks*	*Anti–*E. chaffeensis *antibodies*[b]
		3	*7*	*10*	*14*		
Guinea pigs							
1×10^4	4	0/4	0/4	0/4	0/4	0/100	0/4
1×10^5	4	0/4	0/4	0/4	0/4	0/100	2/4
1×10^6	4	0/4	0/4	0/4	0/4	0/100	2/4
Mongolian gerbils							
1×10^4	3	0/3	0/3	0/3	0/3	0/150	3/3
1×10^5	3	0/3	0/3	0/3	0/3	0/150	3/3
1×10^6	3	0/3	0/3	0/3	0/3	0/150	3/3

[a]Number of positive samples/number tested.
[b]Antibodies detected at IFA titers greater than or equal to 128.

with 1×10^4 infected DH82 cells seroconverted. Blood samples collected from each animal 3, 7, 10, and 14 days after inoculation were all negative for *E. chaffeensis* DNA. Xenodiagnostic *A. americanum* larvae placed on guinea pigs fed to repletion and detached 7–9 days after inoculation. This corresponds with the expected time period of peak ehrlichemia, as seen in mice (unpublished results). Molting success was >90% for all ticks that fed on inoculated guinea pigs. A total of 100 molted nymphs were tested from each group of inoculated guinea pigs; all ticks were negative for *E. chaffeensis* DNA.

Three gerbils each were inoculated with 1×10^4, 1×10^5, or 1×10^6 disrupted, *E. chaffeensis*–infected DH82 cells (Table 1), in which each cell contained approximately 250 *E. chaffeensis*. All inoculated gerbils seroconverted by 45 days post-inoculation (titers >128); however, none of the gerbil blood samples were PCR positive for *E. chaffeensis* at any time point tested. Xenodiagnostic *A. americanum* larvae placed on gerbils fed to repletion and detached 6–9 days after inoculation. Molting success for engorged larvae was >80% for all gerbils, and there was no significant difference in the molting success between ticks fed on inoculated and control gerbils. Nymphs fed as larvae on inoculated gerbils (150 nymphs/group) tested negative for *E. chaffeensis* DNA.

Thus, the susceptibility of two laboratory animal species—guinea pigs and gerbils—to infection by *E. chaffeensis* was evaluated. Animals were inoculated with 1×10^4 to 1×10^6 DH82 cells infected with the lowest available passage of *E. chaffeensis* (St. Vincent strain, 10 passages *in vitro*). On average, each infected DH82 cell contained 75 (guinea pigs) or 250 (gerbils) *E. chaffeensis* organisms, underscoring the difficulty of standardizing inocula based on DH82 cell numbers. These dosages of *E. chaffeensis* are infectious to susceptible strains of laboratory mice and consistently produce *E. chaffeensis* bacteremia by day 7 post-inoculation with 100% seroconversion within three weeks following exposure (unpublished data). Antibodies against *E. chaffeensis* were elicited by exposure to the pathogen in all inoculated gerbils. Seroconversion was less consistent in guinea pigs and may reflect poor antigenicity of the inoculum in this species. However, *E. chaffeensis* DNA could not be detected in blood samples or xenodiagnostic ticks from any inoculated guinea pigs or gerbils. These results indicate that guinea pigs and gerbils are not susceptible to infection by *E. chaffeensis* and, therefore, are not appropriate laboratory animals for the generation of infected ticks.

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Infection of a goat with a tick-transmitted *Ehrlichia* from Georgia, U.S.A., that is closely related to *Ehrlichia ruminantium*

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ABSTRACT: We detected a novel tick-transmitted *Ehrlichia* in a goat following exposure to lone star ticks (*Amblyomma americanum*) from a park in the metropolitan area of Atlanta, GA, U.S.A. Nineteen days after infestation with field-collected adult ticks, the goat developed a fever of two days duration, which coincided with mild clinical pathologic changes and the presence of DNA from a novel *Ehrlichia* in peripheral blood. The goat transmitted ehrlichiae to uninfected nymphal *A. americanum* that fed upon the goat, and the ticks maintained the pathogen transstadially. Five months after exposure, immunosuppression of the goat resulted in transient ehrlichemia with transmission of ehrlichiae to feeding ticks. Sequencing and phylogenetic reconstructions of the 16S rRNA, *glt*A, *map*1, *map*2, and ribonuclease III genes suggest the agent might be a divergent strain of *Ehrlichia ruminantium*, the agent of heartwater, or a new, closely related species. Convalescent serum from the goat reacted with the MAP-1 protein of *E. ruminantium* and with whole-cell *Ehrlichia chaffeensis* antigen. DNA from the novel *Ehrlichia* was detected in 5/302 field-collected adult *A. americanum* from the park. Our data suggest that *A. americanum* is a natural vector and reservoir of this *Ehrlichia* and that domestic goats can be reservoirs The geographic range of the agent and its pathogenicity to humans and livestock needs to be evaluated. ***Journal of Vector Ecology* 31 (2): 213-223. 2006.**

Keyword Index: *Ehrlichia*, tick-borne diseases, emerging infectious diseases, Ixodidae, ruminants, animal disease models, polymerase chain reaction.

INTRODUCTION

Ehrlichia spp. cause disease in humans, dogs, horses, cattle, sheep, goats, and wildlife (Childs and Paddock 2003, Peter et al. 2002, Rikihisa 1991). In the United States, *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* are enzootic, circulating between ticks and animals. *Ehrlichia canis*, transmitted by brown dog ticks (*Rhipicephalus sanguineus*), causes disease in dogs and possibly humans (Rikihisa 1991, Perez et al. 1996). *Ehrlichia chaffeensis* and *E. ewingii* are transmitted by lone star ticks (*Amblyomma americanum*) and infect humans, dogs, goats, and white-tailed deer (*Odocoileus virginianus*) (Anderson et al. 1992a, Buller et al. 1999, Childs and Paddock 2003, Yabsley et al. 2002, M.L. Levin, unpublished data). *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) infects ruminants and causes heartwater, which can be fatal in up to 80% of infected cattle and 100% of infected sheep and goats (Bram et al. 2002, Camus et al. 1996, Jongejan et al. 1984). Thirteen *Amblyomma* spp. transmit *E. ruminantium*, which is enzootic in the Caribbean and sub-Saharan Africa (Bezuidenhout 1987, Camus et al. 1996). The pathogen was introduced to the Caribbean from Africa in the 1800s and spread throughout the region in the 1970s-1980s, possibly as a result of livestock movement or cattle egret (*Bubulcus ibis*) migration (Barre et al. 1995). An eradication program to eliminate the Caribbean vector, *Amblyomma variegatum*, was initiated in 1995 and has been only partially successful (Pegram and Eddy 2002). Recent evidence suggests that *E. ruminantium* might also infect people (Allsopp et al. 2005b).

Heartwater has not been reported in the United States, but the disease could be introduced by the importation of tick-infested animals from endemic areas (Bram et al. 2002). In one instance, *E. ruminantium* was detected in Zambian ticks from a shipment of tortoises to Florida in 1999. The ticks were exterminated, and there were no reported cases of heartwater in animals (Burridge et al. 2000). In the latter half of the twentieth century, eight *Amblyomma* spp. that can transmit *E. ruminantium* were collected in the United States from imported animals and animal skins (Keirans and Durden 2001).

If *E. ruminantium* or a similar agent were introduced into the United States, the pathogen might establish an enzootic cycle, similar to that of *E. chaffeensis* or *E. ewingii*. White-tailed deer have been experimentally infected with *E. ruminantium* (Peter et al. 2002). Ticks indigenous to the United States, including *Amblyomma cajennense* and *Amblyomma maculatum*, are experimental vectors of *E. ruminantium* (Mahan et al. 2000, Uilenberg 1982, 1983), and the vector competence of *A. maculatum* is similar to

that of African ticks. Mahan et al. (2000) reported that *A. americanum* acquired *E. ruminantium* by feeding on infected sheep and maintained the pathogen transstadially but failed to demonstrate transmission to naive animals.

We report here the discovery of a tick-borne *Ehrlichia* from the United States that is genetically and antigenically similar to *E. ruminantium*.

MATERIALS AND METHODS

Ticks and infestations

Questing adult *A. americanum* were collected in February-May, 2005 by flagging at Panola Mountain State Park, Georgia, U.S.A. Tick feeding chambers were attached to a goat as previously described for rabbits (Loftis et al. 2004). Xenodiagnostic infestations were performed with uninfected nymphs from laboratory colonies. *Amblyomma americanum* and *A. cajennense* were obtained from a tick colony at CDC, and *A. maculatum* were donated from a colony maintained by P.D. Teel, College Station, TX. The first and second xenodiagnostic infestations were performed with approximately 100 *A. americanum*, and the third infestation (following immunosuppression) was performed with 100 nymphs each of *A. americanum*, *A. cajennense*, and *A. maculatum*. Five to ten engorged nymphs from each species and infestation were extracted for PCR analysis; remaining ticks were allowed to molt into adults prior to testing.

Goat maintenance, sample collection, and immunosuppression

A female Nigerian cross-breed goat, 12 months old, was obtained from a farm in southern Georgia and maintained in accordance with a protocol approved by the Institutional Animal Care and Use Committee, CDC. Upon arrival, the goat was deloused with a non-toxic botanical soap (Bug Arrest, Heartland Products, Valley City, ND). No internal parasites were detected with a fecal float. The goat was seronegative for antibodies against *E. chaffeensis* using an indirect fluorescent antibody (IFA) assay. The goat was housed in an air-conditioned indoor facility for 11 months prior to the beginning of the study and was not exposed to ticks during this time. Rectal temperatures were obtained from the goat using a digital thermometer; fever was confirmed using an additional reading from a second thermometer. The goat was evaluated daily for changes in activity level, appetite, fecal consistency, or presence of oculonasal discharages.

Blood was collected by jugular venipuncture on 35 occasions prior to the experiment, three times weekly for the first month of the experiment, and three times weekly during and after immunosuppression. Blood was drawn into serum separator and EDTA anticoagulant tubes. An aliquot of each serum was stored at -20°C for serologic testing and two 100 µL aliquots of each whole blood sample were stored at -20°C for PCR. Remaining serum and whole blood were submitted to Antech Diagnostics (Atlanta, GA) for biochemical profile and complete blood count with differential (CBC). Baseline values for serum chemistry and CBC parameters were calculated from the average +/- 2 standard deviations for the 20 blood samples drawn in the six months prior to this study, with the following exception: alkaline phosphatase (ALP) activity increased steadily throughout the months prior to this study, so baseline values were based on the six samples from the 30 days prior to the study.

Skin biopsies were collected on days 20 and 73 using local lidocaine analgesia and 2 mm dermal biopsy punches (Miltex, Inc., York, PA). Spleen, liver, and bone marrow biopsies were obtained percutaneously, under general sedation (11 mg/kg ketamine with 0.22 mg/kg xylazine, I.M.), on day 73. Spleen and liver biopsies were collected using ultrasound guidance and an automatic 18 gauge core biopsy system (Microvasive, Boston, MA), and bone marrow was collected from the iliac crest using a 16 gauge, 1 5/16 inch Rosenthal needle. The goat was euthanized on day 186, and postmortem samples of the bone marrow, brain, heart, kidney, liver, lung, lymph node, and spleen were collected.

Immunosuppression was attempted on days 136-140 and 178-182 post-exposure. The goat was injected with sterile, alcohol-free dexamethasone (4 mg/kg IM) once daily for five consecutive days (Koptopoulos et al. 1992).

Serology

Sera were diluted 1/32 and tested with an IFA assay (Nicholson et al. 1997), using slides coated with acetone-fixed, *E. chaffeensis*-infected DH82 cells. Detection was achieved using rabbit anti-goat $IgG_{(H+L)}$-FITC (KPL, Inc., Gaithersburg, MD). Serial two-fold dilutions of positive sera were used to determine the endpoint titer. Selected sera were also evaluated using a MAP-1B indirect ELISA based upon a recombinant peptide from the MAP1 protein of *E. ruminantium*; antibodies were detected using rabbit anti-goat IgG-HRP (KPL, Inc.), and the cutoff for positivity was calculated as previously described (Van Vliet et al. 1995). Western blots were performed on pre-exposure and convalescent sera using whole-cell *E. ruminantium*, as previously described (Mahan et al. 1993).

DNA extraction and polymerase chain reaction (PCR)

DNA extraction, PCR setup, and detection of amplicons were performed in separate, dedicated areas. DNA extractions, PCR, and sequencing were performed in a laboratory that had never cultured *E. ruminantium* or handled DNA from *E. ruminantium*. DNA was extracted using an IsoQuick Nucleic Acid Extraction kit (Orca Research, Inc., Bothell, WA). Prior to extraction, tissue samples were digested with 1 mg/ml Proteinase K (Sigma, St. Louis, MO) for 2 h at 55 °C. Ticks were pulverized in liquid nitrogen using sterile pestles (Kontes, Vineland, NJ). PCR reactions were performed using a Taq Master Mix kit (Qiagen, Valencia, CA) with 1.0 µM each of the forward and reverse primers. Thermocycler conditions were 95 °C for 3 min, 40 cycles of 95 °C for 30 sec/ XX °C for 30 sec/ 72 °C for 1 min, and an extension of 5 min at 72 °C, where XX was the annealing temperature for each assay. Primers and annealing temperatures for *Ehrlichia* spp. PCR assays are shown in Table 1. Positive controls were included for the 16S rRNA and *glt*A assays, consisting of DNA from a tick infected with *E. ewingii*; a positive control

template was not available for *E. ruminantium*-specific PCR assays. Amplicons were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light.

DNA-DNA hybridization

DNA-DNA hybridization was performed in a heartwater research laboratory (S.M. Mahan). Products of pCS20 PCR assays were obtained from selected ticks and from a positive control (*E. ruminantium* Crystal Springs), using primers AB128/AB129, HH1f/HH2r, and pCS20-IntF/pCS20-IntR. Amplicons were hybridized with a chemiluminescent DNA probe derived from the pCS20 plasmid, as previously described (Mahan et al. 1992).

Gene sequencing and phylogenetic analysis

Amplicons were prepared with a QIAquick PCR Purification Kit (Qiagen) or a Wizard SV gel cleanup system (Promega, Madison, WI) and sequenced using PCR primers and BigDye Terminator v.3.1 (Applied Biosystems, Foster City, CA). Excess dye was removed using DyeEx 2.0 columns (Qiagen). Fragments were assembled and primer sequences removed using GCG SeqMerge (Accelrys, San Diego, CA). Sequences were submitted to GenBank, as follows: 16S rDNA (DQ324367); *map*1 (DQ324368); *map*1-1 (DQ324369); *map*2 (DQ324370); pCS20 (DQ324371); and *glt*A (DQ363995).

The 16S rRNA sequence was analyzed as nucleic acids, and the *map*1, *map*2, ribonuclease III (pCS20), and *glt*A protein-coding genes were translated into amino acids. Similar sequences were identified using BLAST 2.0 (NCBI, Bethesda, MD), sequences were aligned using GCG SeqLab (Accelrys) and checked by hand, and phylogenetic analysis was performed using PAUP 4.0 Beta 10 (Sinauer Associates, Inc., Sunderland, MA). The most parsimonious trees were constructed using heuristic bootstrap analysis (100 replicates); starting trees were obtained by stepwise addition with tree-bisection-reconnection as the branch-swapping algorithm.

RESULTS

During the 11 months prior to the experiment, the goat was clinically healthy. DNA samples from 35 time points were PCR negative for *Ehrlichia* spp. using a hemi-nested PCR assay for the 16S rDNA. The animal did not have detectable antibodies against *Ehrlichia* spp. using *E. chaffeensis* IFA, a MAP-1B subunit ELISA, or a western blot with whole-cell *E. ruminantium*.

On day zero, the goat was infested with 50 adult *A. americanum* (25 males and 25 females) from Panola Mountain State Park, Georgia, U.S.A. Ticks attached and fed for 10-13 days. Several ticks died during feeding, and 15 male and 5 female engorged ticks were recovered. During the infestation (days 9-11), the goat developed transient, mild pyrexia (Figure 1). The goat became febrile 19-20 days after infestation with adult ticks, with clear nasal discharge and a peak temperature of 41.3 °C (Figure 1) but remained alert, active, and had a normal appetite. Uninfected nymphal *A. americanum* were placed on the goat on days 14 and 21 and engorged over three to five days; these two xenodiagnostic infestations corresponded with the immediate pre-febrile and post-febrile periods.

Clinical pathologic changes were observed during the infestation with adult ticks and during, and immediately after, the febrile reaction. Monocytosis, slightly increased neutrophil counts, and slightly decreased alkaline phosphatase activity (ALP), relative to baseline values (Figure 2), were noted during the infestation. During and after the febrile reaction, we observed decreased serum albumin, decreased ALP, slightly decreased red blood cells (RBC), transient monocytosis, and transient neutropenia with increased lymphocytes (Figure 2). The nadir of ALP activity (107 U/L, day 21) was significantly below baseline values for this animal but was within the reference range (93-387 U/L) for the commercial hematology laboratory. Aspartate aminotransferase activity (AST) was below the reference range for the commercial laboratory at every time point for the 17 months the goat was in our care, but significant increases above baseline were noted during tick feeding and during the febrile reaction. Platelets could not be counted, due to clumping, but did not appear to be decreased when blood smears were visually examined.

Forty-one post-exposure sera, collected on days 3-182, were tested for antibodies cross-reactive with *E. chaffeensis* using IFA. A significant rise in titer was detected on day 26 (titer 1/64), with a peak titer of 1/256 on day 38. Serum from day 38 was weakly positive using a recombinant subunit ELISA based on the immunodominant MAP-1 protein of *E. ruminantium*. Western blot analysis confirmed the presence of antibodies against the MAP-1 protein of *E. ruminantium* in the convalescent goat serum (Figure 3). Antibody titers decreased below the limit of detection by day 112 and remained at this level until the end of the experiment.

Using sensitive, species-specific nested PCR assays (Table 1), DNA from *E. ewingii* or *E. chaffeensis* was not detected in goat blood, tissue samples, or xenodiagnostic *A. americanum*. Using a generic 16S rDNA PCR assay (#1, Table 1), followed by sequencing, DNA from a novel *Ehrlichia* was detected in goat blood from days 19 and 21, coinciding with the febrile reaction, and in one of two skin biopsies from tick feeding sites from day 20. When the 16S rDNA assay was hemi-nested (16S #1, Table 1), the novel agent was also detected in blood from day 34. Five of 10 engorged nymphs, 2/20 molted adults from the first xenodiagnostic infestation, and 3/13 molted adults from the second xenodiagnostic infestation contained DNA from the novel *Ehrlichia*. DNA from *Ehrlichia* spp. was not detected from skin, liver, spleen, or bone marrow samples collected on day 73. All 16S rDNA PCR amplicons obtained from the goat and xenodiagnostic ticks were sequenced, and the sequences were identical to each other and >99% similar to *E. ruminantium* (394/395 bp). No evidence of mixed infection with either *E. chaffeensis* or *E. ewingii* was obtained.

To evaluate the possibility that these samples contained *E. ruminantium*, the ten positive tick DNAs were tested using assays described for *E. ruminantium*: a *map*1 assay (Peixoto et al. 2005), a nested assay for the "MAP1-like protein" (*map*1-1), and two pCS20 assays (Peter et al. 1995, Van

Table 1. PCR primers used for amplification of genes from ticks and goat blood samples. Reactions were performed using the published optimal annealing temperature for each primer pair; annealing temperatures for previously unpublished assays are noted.

Assay Target	Name of Primer	Sequence	Reference
Ehrlichia spp. 16S rDNA #1 [B]	EC12a	TATAGGTACCGTCATTATCTTCCCTATT	R.F. Massung
primary round	HE3	GATCCTGGCTCAGAACGAACG	Anderson et al. 1992b
Ehrlichia spp. 16S rDNA #1 [B]	ECH-SYBR-F	AACACATGCAAGTCGAACGG	Li et al. 2001
hemi-nested round	HE3	(as above)	
Ehrlichia spp. 16S rDNA #2	EHR16SD	GGTACCYACAGAAGAAGTCC	Parola et al. 2000
	EHR16SR	TAGCACTCATCGTTTACAGC	Parola et al. 2000
Ehrlichia spp. 16S rDNA #3 [C]	3EH	AATAGGGAAGATAATGACGGTACCTATA	reverse comp. of HE3
	RP2	ACGGCTACCTTGTTACGACTT	Weisburg et al. 1991
Ehrlichia spp. gltA [A,C]	EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	A.D. Loftis
primary round	EHRCS-1226R	CCAGTATATAAYTGACGWGGACG	A.D. Loftis
Ehrlichia spp. gltA [A]	EHRCS-131F	(as above)	
hemi-nested round 5′	EHRCS-879R	TIGCKCCACCATGAGCTG	A.D. Loftis
Ehrlichia spp. gltA [A]	EHRCS-754F	ATGCTGATCATGARCAAAATG	A.D. Loftis
hemi-nested round 3′	EHRCS-1226R	(as above)	
Ehrlichia ewingii – P28 [B]	Eew-28F	CAACTGTATCACATTTCGGTAACTTTTC	A.D. Loftis
primary round	Eew-28R	TGACACTAAATCAGCACCAACAC	A.D. Loftis
E. ewingii – P28	EEM2F	GGAGCTAAAATAGAAGATAATC	Gusa et al. 2001
nested round	EEM1R	GTGCCAAAAGGTAATACAT	Gusa et al. 2001
Ehrlichia chaffeensis – VLPT	FB5A	GTGACATCTTAGTTTAATAGAAC	Sumner et al. 1999
primary round	FB3A	AAGACTGAAACGTTATAGAG	Sumner et al. 1999
E. chaffeensis – VLPT	FB5C	GTTGATCATGTACCTGTGTG	Sumner et al. 1999
nested round	FB3	GCCTAATTCAGATAAACTAAC	Sumner et al. 1999
Ehrlichia ruminantium – pCS20	AB128	ACTAGTAGAAATTGCACAATCTAT	Peter et al. 1995
	AB129	TGATAACTTGGTGCGGGAAATCCTT	Peter et al. 1995
E. ruminantium – pCS20	HH1F	CCCTATGATACAGAAGGTAACCTCGC	Van Heerden et al. 2004
	HH2R	GATAAGGAGATAACGTTTGTTTGG	Van Heerden et al. 2004
E. ruminantium – pCS20 nested	PCS20-intF	GGAGAAAGRAGTTGTGGTGGAG	A.D. Loftis
	PCS20-intR	ACAGAATATGCTGTATAATGGYACTGAAG	A.D. Loftis
Ehrlichia – Ribonuclease III 5' [A]	EOM-274F	GGTASAACYATTTCTTACTATGA	A.D. Loftis
	PCS20-intR	ACAGAATATGCTGTATAATGGYACTGAAG	A.D. Loftis
E. ruminantium – *map*1 gene	(forward	ATTTTTACCTGGTGTGTCCTTTTCTGA	Peixoto et al. 2005
	(reverse	CCTTCCTCCAATTTCTATACC	Peixoto et al. 2005
E. ruminantium – *map*1 USA [B]	Pmap-38F	GAAGATAGTAGTACGAGAGCCAACG	A.D. Loftis
primary round	Pmap-581R	CTTGGTAAGATAACTTGGGATTTG	A.D. Loftis
E. ruminantium – *map*1 USA [B]	Pmap-2F	GACACCAAGGCAGTATACGG	A.D. Loftis
nested round	Pmap-2R	CTAAGTCAGTACCAATACCTGCAC	A.D. Loftis
E. ruminantium – *map*1-1 [B]	map1.orf2.73F	GCAGAACCTGTAAGTTCAAATA	J. Robinson
primary round	map1.orf2.782R	CAAGAGTTACTGAAGCGGAAG	J. Robinson
E. ruminantium – *map*1-1 [B]	map1.orf2.134F	GTGCAAAATACAACCCAAGCAT	J. Robinson
nested round	map1.orf2.679R	TCCGCCAATAAATGCAGAAAT	J. Robinson
E. ruminantium – *map*2	AB249	AAACTCTAATTTTATACA	Bowie et al. 1999
	AB251	AAAATAAGACTAAAAGAAAC	Bowie et al. 1999

[A]Annealing temperature 50 °C.
[B]Annealing temperature 55 °C.
[C]The thermocycler program for this primer pair used 2 min extensions.

Table 2. Percent DNA similarity of 16S rRNA (1409 bp), *glt*A (1046 bp), pCS20 (785 bp), *map*1 (730 bp), *map*1-1 (523 bp), and *map*2 (627 bp) gene sequences between strains of *E. ruminantium* and between the *Ehrlichia* sp. from Panola Mountain State Park, Georgia, and *Ehrlichia* sequences available on GenBank.

			16S rRNA	gltA	pCS20 [a]	MAP1	MAP1-1	MAP2
Similarity between strains of *E. ruminantium*			99.5-100%	99.4-100% [b]	85.6-100%	79.2-99.7%	97.5-100%	98.1-100%
Panola Mountain *Ehrlichia* sp.	vs.	*E. ruminantium*	99.4-99.6%	85.5-85.6%	83.9-91.2%	76.5-78.8%	88.5-89.7%	84.0-84.6%
	vs.	*E. chaffeensis*	97.6%	78.3-78.4%	<60%	65.4%	<60%	74.0%
	vs.	*E. canis*	97.1-97.7%	78.3%	<60%	<60%	<60%	76.0%
	vs.	*E. ewingii*	96.6%	NA	NA	<60%	NA	NA

NA: Homologous sequence not available.
[a]Sequence from a portion of the pCS20 target was obtained using degenerate PCR primers and includes the full-length ribonuclease III gene (687 bp) and 102 bp of the overlapping *cta*G gene.
[b]Only two sequences available (Welgevonden and Gardel strains).

Heerden et al. 2004). The *map*1 and *map*1-1 assays produced amplicons from 1/10 and 3/10 of the ticks, respectively. No amplicons were seen following PCR amplification with published assays for the pCS20 target of *E. ruminantium* (Table 1, AB128/AB129 and HH1f/HH2r), which includes the ribonuclease III and cytochrome C oxidase assembly protein (*cta*G) genes. Hybridization of these PCR products with a DNA probe, a sensitive method for detecting low level amplification (Mahan et al. 1992), was also negative. Degenerate nested primers pCS20-IntF and pCS20-IntR (Table 1) were designed to amplify a 482 bp fragment of the pCS20 target. Using these primers, amplicons were obtained from 1/10 ticks (Figure 4) and, after hybridization with a DNA probe from *E. ruminantium*, a faint signal was seen after 15 min of exposure (readily visible after 60 min). Amplicons from *E. ruminantium* Crystal Springs hybridized well and were readily visible after 15 min.

Phylogenetic reconstruction, to determine the relationship of the novel *Ehrlichia* to *E. ruminantium*, was attempted. Products obtained by amplification of *map*1, *map*1-1, and pCS20 were sequenced, and xenodiagnostic ticks were selected for amplification and sequencing of the 16S rRNA gene (1409 bp, Table 1, #2 and #3), the citrate synthase gene (*glt*A, 1046 bp) gene, the entire ribonuclease III gene (687 bp), and the *map*2 gene (627 bp), a putative electron transport protein. The 16S rRNA gene was >99% similar to sequences from *E. ruminantium* (Table 2). Parsimony analysis of this gene supported the association of the Panola Mountain *Ehrlichia* with *E. ruminantium* in 100/100 replicates (Figure 5), and the new agent was the sister taxa to *E. ruminantium* in 90/100 replicates. Similar results were obtained for *map*2 and *glt*A, in which the agent was a sister taxa to *E. ruminantium* in 92/100 replicates (Table 2; Figures 5 and 6). The sequence of *map*1, a gene that varies significantly between strains of *E. ruminantium* (Table 2), was within the clade formed by *E. ruminantium* strains in 100/100 replicates (Figure 6). The ribonuclease III sequence formed a clade with the Kumm2 strain of *E. ruminantium* in 96/100 replicates (Figure 6); Kumm2 produces fatal infection in mice but has low pathogenicity for ruminants (Zweygarth et al. 2002, Allsopp et al. 2005a). According to pairwise similarities, the *map*1-1 sequence was more similar to sequences from *E. ruminantium* than to homologous gene sequences from other ehrlichiae (Table 2); phylogenetic reconstruction was not attempted due to the limited database of sequences.

A PCR assay for the Panola Mountain *Ehrlichia* was designed based on the *map*1 gene sequence (Pmap primers, nested PCR, Table 1). This assay was used to confirm samples that were positive using the 16S rDNA assay (#1) and to test *A. americanum* adults from Panola Mountain. Five of 302 field-collected ticks (1.6%, three females and two males) contained DNA from the new ehrlichial agent. Three of these ticks yielded amplicons using the nested PCR primers pCS20-IntF/pCS20-IntR. Hybridization of the three pCS20 amplicons with an *E. ruminantium* probe produced a weak signal, similar to that seen from a xenodiagnostic tick (Figure 4).

The goat was immunosuppressed 19 weeks post-

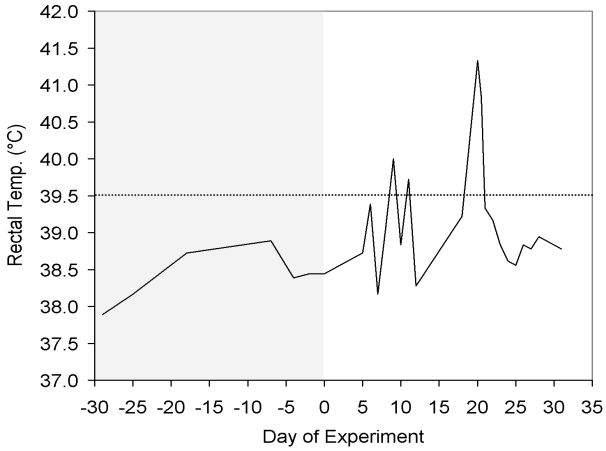

Figure 1. Rectal temperature of the goat before and after infestation with field-collected ticks (day 0). Pyrexia was defined as a temperature greater than 39.5° C.

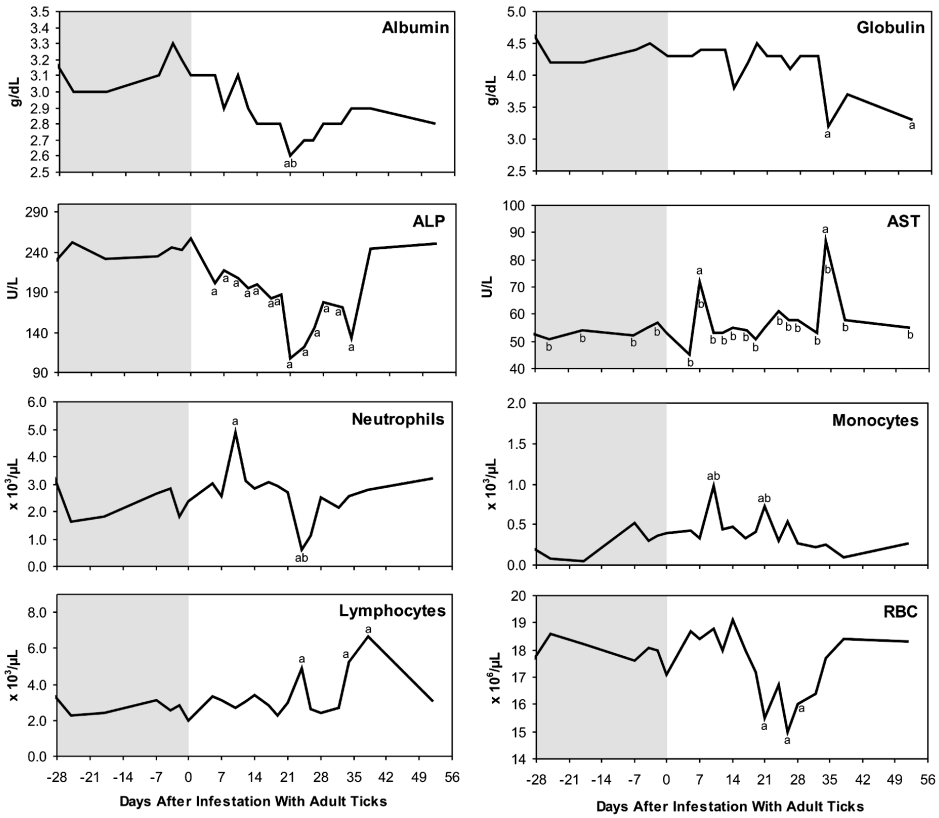

Figure 2. Selected biochemical and hematological parameters from a goat infected with the *Ehrlichia* sp. from Panola Mountain. ALT = aspartate aminotransferase, ALP = alkaline phosphatase, RBC = red blood cells. Values that were significantly different from the baseline for this individual animal (average ± 2 SD) are indicated (a), as are values outside the reference range for the commercial hematology laboratory (b).

exposure (days 136-140). DNA from the Panola Mountain *Ehrlichia* was detected in blood samples from days 140-145. Ehrlichemia corresponded with a significant decrease in ALP; ALP activity was less than 100 U/L on days 143-150 and was below the reference range for the laboratory on days 147-150. Leukogram changes (neutrophilia, lymphopenia, and monocytosis) were compatible with the administration of dexamethasone. The goat remained normothermic and seronegative for *Ehrlichia* spp. Uninfected nymphal *A. americanum*, *A. cajennense*, and *A. maculatum* fed upon the goat during this time; 1/10 engorged *A. americanum*, 1/10 engorged *A. cajennense*, and 0/5 engorged *A. maculatum* contained DNA from the Panola Mountain *Ehrlichia*.

Immunosuppression was attempted on days 178-182. The goat did not become ehrlichemic prior to euthanasia on day 186. No gross abnormalities were noted during necropsy. Ehrlichiae were not detected in the bone marrow, brain, heart, kidney, liver, lung, mesenteric lymph nodes, or spleen, using either the Pmap or 16S rRNA nested PCR assays.

DISCUSSION

We report the discovery of a novel *Ehrlichia* in *A. americanum* from Panola Mountain State Park, within the metropolitan area of Atlanta, GA, U.S.A. This genotype is distinct from *E. chaffeensis*, *E. ewingii*, *Anaplasma phagocytophilum*, and the "white-tailed deer agent" from the southeastern U.S.A., which is similar to *Anaplasma platys* (Arens et al. 2003). Preliminary genetic, antigenic, and biologic characterizations of the novel agent suggest it is either a new species of *Ehrlichia* or a divergent strain of *E. ruminantium*. Further phenotypic and genotypic analysis, including consideration of the criteria used to define species within *Ehrlichia*, will be required to conclusively resolve the taxonomic status of this agent.

Phylogenetic reconstructions of the 16S rRNA, *glt*A, and *map*2 genes suggest the Panola Mountain *Ehrlichia* is distinct from, but closely related to, previously described isolates of *E. ruminantium*. Reconstructions of the *map*1 and ribonuclease III (pCS20 target) genes from the agent suggest this genotype is phylogenetically indistinguishable from one or more strains of *E. ruminantium*. However, published assays for the pCS20 target, including PCR primers that are internationally recognized as the standard for detection of *E. ruminantium* (AB128/AB129) (Camus et al. 2004), did not detect the new agent, and hybridization of amplicons obtained from Panola Mountain ticks, using degenerate pCS20 primers, with the *E. ruminantium* Crystal Springs pCS20 DNA probe produced a weak signal.

Seroconversion of the goat was confirmed using three assays: a whole cell IFA that can detect antibodies against *E. canis*, *E. chaffeensis*, *E. ewingii*, and *E. ruminantium* (Buller et al. 1999, Jongejan et al. 1993); an ELISA, based on the MAP-1B peptide from *E. ruminantium*, that cross-reacts with *E. chaffeensis* and *E. canis* (Van Vliet et al. 1995); and a western blot with whole-cell *E. ruminantium* that cross-reacts with related ehrlichiae (Mahan et al. 1993). These data confirm the goat was infected with an ehrlichial agent, but

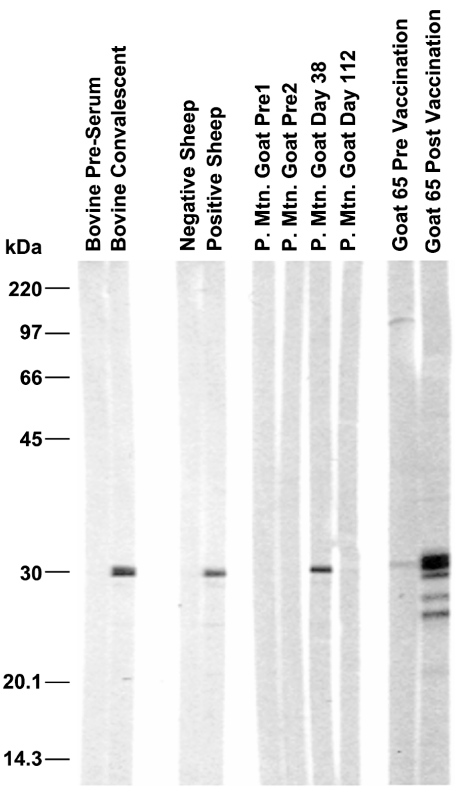

Figure 3. Western blot analysis, using whole-cell *E. ruminantium* antigen, of two pre-exposure and two convalescent sera from the goat infected with the Panola Mountain *Ehrlichia*. Negative and positive sera from a cow and pre-exposure and convalescent sera from a sheep and a goat vaccinated with an inactivated *E. ruminantium* vaccine are shown for comparison. The immunodominant MAP-1 antigen is approximately 32 kDa in size.

cross-reactivity of serologic assays precludes species-level diagnosis.

The Panola Mountain *Ehrlichia* caused transient febrile illness in a goat which was similar to "heartwater fever," the mildest manifestation of *E. ruminantium* (Jongejan et al. 1984, Camus et al. 1996). Clinical pathologic changes were mild but were compatible with ehrlichial infection. Infection with *E. ruminantium* causes leukopenia, neutropenia, thrombocytopenia, and anemia (Camus et al. 1996, Allsopp et al. 2005a). Increased AST, leukopenia, anemia, and decreased albumin and ALP are recorded in goats infected with *E. ewingii* (M.L. Levin unpublished data). Goats infected with *A. phagocytophilum* (formerly *Ehrlichia phagocytophila*) display a characteristic decrease in ALP with anemia, thrombocytopenia, and neutrophilia followed by neutropenia

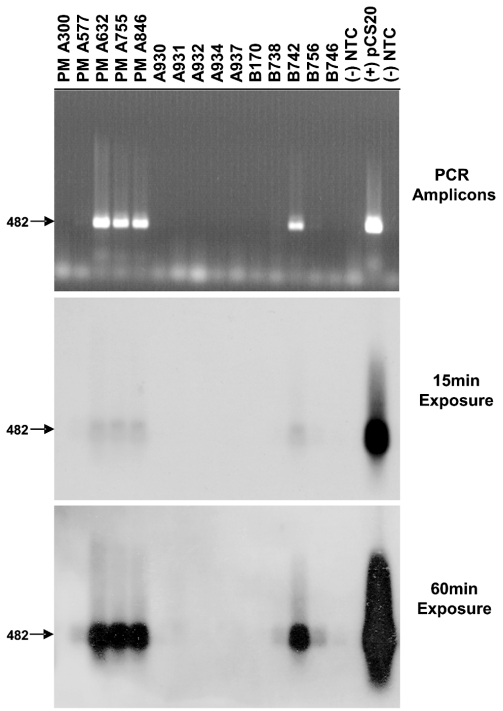

Figure 4. PCR amplification (primers PCS20-intF/PCS20-intR) and DNA-DNA hybridization of the pCS20 target from the Panola Mountain *Ehrlichia*, compared to *E. ruminantium* Crystal Springs (pCS20 recombinant plasmid). Xenodiagnostic ticks (engorged nymphs, A930—A937, and molted adults, B170—B746) and wild *A. americanum* (PM-A300—PM-A846) that were positive using 16S rDNA PCR are shown.

(Gokce and Woldehiwet 1999a, 1999b). The agent also established a chronic infection in the otherwise healthy animal and was reactivated by immunosuppression approximately five months after the acute infection. The capacity for chronic infection is common among *Ehrlichia* spp. (Anderson et al. 1992a, Childs and Paddock 2003, Camus et al. 1996, Rikihisa 1991). In a clinical setting, recrudescence of ehrlichemia and tick transmission might occur in response to stress, malnutrition, or other circumstances that compromise immune function.

Our data suggest that *A. americanum* is a natural vector for this agent: adult *A. americanum* infected the goat, nymphs acquired the pathogen by feeding on the goat, and 15.2% of the nymphs that fed during the acute infection maintained the pathogen transstadially. Nymphal *A. cajennense* that fed upon the goat during reactivation of the chronic infection also acquired pathogen DNA, but we did not assess transstadial maintenance and transmission capability of this species. Transmission of the agent by *A. americanum* was unexpected, because previous investigators failed to demonstrate transmission of *E. ruminantium* from *A. americanum* to sheep (Mahan et al. 2000, Uilenberg 1982). However, inconsistencies in published data regarding *A. cajennense* underscore the difficulty of assessing species that might be inefficient vectors, and the transmission dynamics of *E. ruminantium* can vary according to the strain of pathogen and colony of ticks (Camus et al. 1996, Uilenberg 1982, Mahan et al. 1995). Transmission of the pathogen to a small proportion of *A. americanum* is biologically relevant, because a single animal, such as a white-tailed deer, can be infested by thousands of nymphs and larvae at once (Patrick and Hair 1977).

The origin and distribution of the Panola Mountain *Ehrlichia* has yet to be established. The Panola Mountain *Ehrlichia* could have been in North America for years. Previous surveys of ticks from the United States (e.g., Schulze et al. 2005, Varela et al. 2004) utilized species-specific PCR assays for *E. chaffeensis* and *E. ewingii*, which would not detect DNA from *E. ruminantium* or the Panola Mountain agent. Alternatively, a previously undescribed strain of *E. ruminantium*, similar to Kumm2, could have been recently introduced into the United States. The geographic distribution of the novel *Ehrlichia* must be established to evaluate this possibility. The possible introduction of *E. ruminantium* into the United States is an issue of major veterinary concern, especially in cities, such as Atlanta, which are ports of entry for imported animals.

Although the Panola Mountain *Ehrlichia* is genetically similar to *E. ruminantium*, the capacity of this agent to produce the severe disease classically known as "heartwater" is unknown. Because the pathogenicity of *E. ruminantium* isolates varies between breeds and species of animals (Jongejan et al. 1984, Camus et al. 1996), we cannot extrapolate the clinical characteristics of the novel *Ehrlichia* in this goat to other goats, sheep, cattle, or white-tailed deer. There is also evidence that this agent might cause human disease (W.K. Reeves, unpublished data). The pathogenicity of the agent should be evaluated by *in vitro* isolation, infection of additional animals, diagnosis using brain smears from febrile animals, and transmission of the agent by competent vectors of *E. ruminantium*. This information is necessary to determine whether control programs are needed to prevent the dissemination of the Panola Mountain *Ehrlichia* and whether diagnostic testing of clinical specimens for this agent is warranted.

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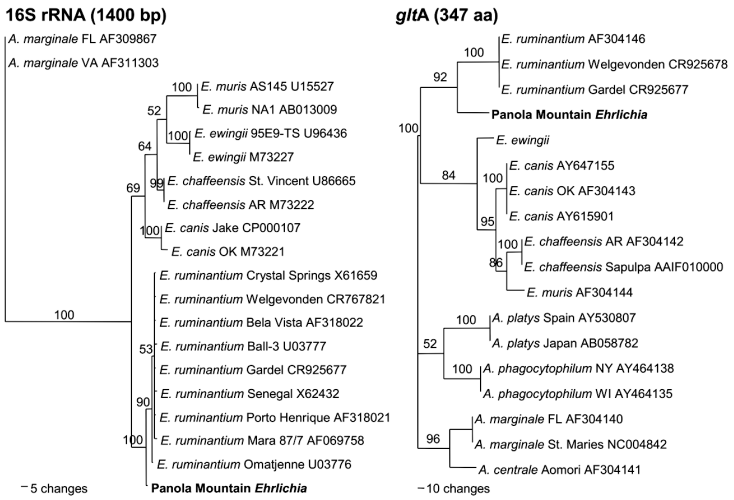

Figure 5. Majority-rule consensus trees comparing the16S rRNA and *glt*A gene sequences obtained from the *Ehrlichia* sp. detected in a goat and in ticks from Panola Mountain State Park, Georgia, with published sequences from known *Ehrlichia* and *Anaplasma* spp. Trees were constructed, with distances (number of changes per 100 residues), using parsimony analysis. Bootstrap values (100 replicates) are noted on individual branches.

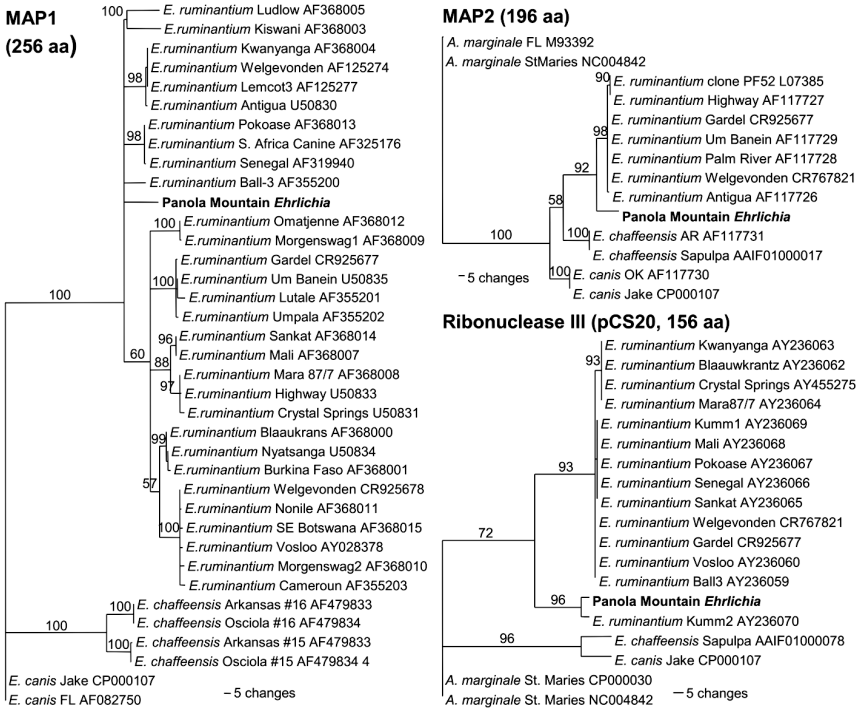

Figure 6. Majority-rule consensus trees comparing the MAP1 (256 amino acids), MAP2 (196 amino acids), and ribonuclease III (pCS20, 119 amino acids) sequences obtained from the Panola Mountain *Ehrlichia* with published sequences from *E. ruminantium* strains. Trees were constructed as described for Figure 5.

for design of the EC12a primer and help with phylogenetic analyses. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the United States Department of Health and Human Services or the Centers for Disease Control and Prevention.

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Short communication

Two USA *Ehrlichia* spp. cause febrile illness in goats☆

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Abstract

Ehrlichia spp. are not currently recognized as a cause of illness in goats in the USA, but three *Ehrlichia* are enzootic in lone star ticks (*Amblyomma americanum*) in the eastern USA, and related bacteria in other countries cause illness in goats. We exposed naïve goats to *Ehrlichia*-infected *Amblyomma* and demonstrated that infection and clinical illness can be caused by two USA species, *E. ewingii* and the recently discovered Panola Mountain *Ehrlichia* sp. Clinical features in all five goats are described; ehrlichioses were associated with pyrexia, serous nasal discharge, inappetance, lethargy, decreased alkaline phosphatase, and, in most cases, neutropenia. Goats remained chronically infected for several months following exposure to ehrlichiae and transmitted the pathogens to uninfected ticks. In the eastern USA, undifferentiated febrile illness in goats might be caused by previously unrecognized ehrlichial infections, and pastures housing-infected goats could become infested with a large number of infected ticks.
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Keywords: Ruminants; Ehrlichiosis; Zoonoses; Animal diseases; Tick-borne diseases; Ixodidae; Disease reservoirs

1. Introduction

Ehrlichia are obligately intracellular bacteria transmitted by hard ticks. In the United States, *Ehrlichia* spp. are recognized as pathogens of dogs and people (Anderson et al., 1991, 1992; Murphy et al., 1998; Buller et al., 1999). Infections with *Ehrlichia* are not recognized in goats in the USA, but in Africa and the Caribbean, *Ehrlichia ruminantium* causes disease in ruminants, varying from mild febrile illness to fatal heartwater disease (Camus et al., 1996; Allsopp et al., 2007). Additionally, the closely related species *Anaplasma phagocytophilum*, previously known as *Ehrlichia phagocytophila*, causes illness in ruminants in Europe (Rikihisa, 1991; Gokce and Woldehiwet, 1999).

Of the three *Ehrlichia* species known to occur in the USA, only *E. chaffeensis* has been studied with respect to goats, with data suggesting that goats are largely resistant to infection (Dugan et al., 2000, 2004). No work has been performed with *E. ewingii* in ruminants.

☆ The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the United States Department of Health and Human Services or the Centers for Disease Control and Prevention.

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Recently, Loftis et al. (2006) reported the discovery of a novel *Ehrlichia* from Panola Mountain State Park, GA, which caused illness in a goat, but clinical parameters of the infection were not reported. All three species of *Ehrlichia* are transmitted by lone star ticks (*Amblyomma americanum*), which are common throughout the eastern USA (Fig. 1) (Long et al., 2004; Mixson et al., 2006).

We evaluated the susceptibility of naïve goats to natural infection with *Ehrlichia* spp. from the USA, using lone star ticks from a wild population infected with *E. chaffeensis*, *E. ewingii*, and the Panola Mountain *Ehrlichia* (PME). Of the four goats exposed to wild ticks, three were infected with *E. ewingii* and one with PME; Gulf Coast ticks (*A. maculatum*) that fed on the goat with PME transmitted the infection to an additional naïve goat. We describe here the clinical features of infection in these five goats.

2. Materials and methods

All work was performed under an approved animal care and use protocol. A 1-year-old female Nigerian cross-breed (goat 4), a 2-year-old female Nubian cross-breed (goat 20) and three 1-year-old, castrated male Nubian cross-breed goats (goats 5, 209, and 210) were included in this study. Prior to the study, serum and PCR samples were collected from all goats and used to confirm that the goats had not been exposed to ehrlichiae. Baseline ranges of rectal temperatures, blood chemistry, and blood cell counts were established for goats 5, 20, 209, and 210; published reference values were used for goat 4. All goats were housed in an indoor laboratory animal facility.

Goats 4, 20, 209, and 210 were each infested with 50 adult lone star ticks (*A. americanum*) from northwest Georgia. At the time of this study, the prevalence of infection in adult ticks was as follows: 1.6% with PME, 5.2% with *E. ewingii*, and 4.9% with *E. chaffeensis*. Goat 5 was infested with 22 adult Gulf Coast ticks (*A. maculatum*) produced by feeding uninfected nymphs on goat 20. Rectal temperatures, appetite, and attitude were assessed daily for the first month following tick exposure. Blood and serum samples were collected three times weekly and submitted to a commercial laboratory for CBC and serum biochemical profile (Antech, Atlanta, GA). DNA was extracted from whole blood and ticks and tested for *E. ewingii*, *E. chaffeensis*, PME, and *Rickettsia* spp. using PCR followed by sequencing, as previously described (Loftis et al., 2006). Antibody response against *Ehrlichia* spp. was assayed using a

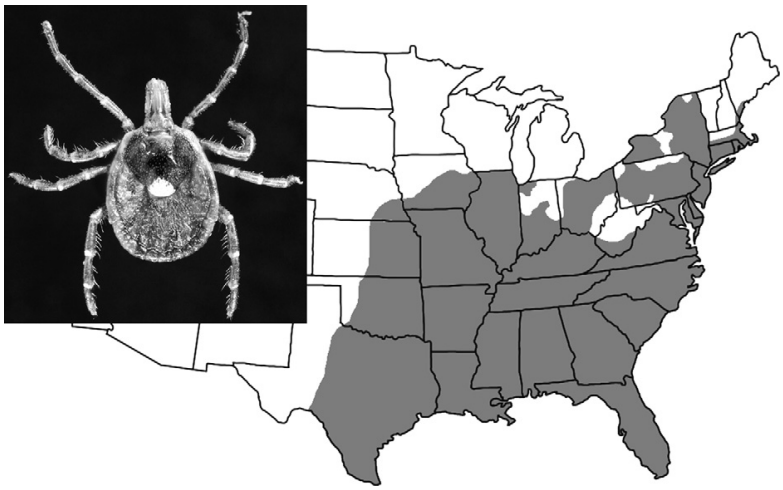

Fig. 1. Photograph of an adult female lone star tick, showing the distinctive white ‘‘star’’ on the back of the female, and approximate distribution map for this tick species.

clinically diagnostic indirect fluorescent antibody (IFA) assay with whole-cell *E. chaffeensis* (Comer et al., 1999). Goats 5 and 20 were also assessed using a recombinant subunit ELISA based on the MAP1 protein of *E. ruminantium* (Van Vliet et al., 1995). Four to 6 months after infection, goats 5, 20, 209, and 210 were immunosuppressed using dexamethasone (4 mg/kg IM for five consecutive days), according to published protocols (Koptopoulos et al., 1992).

3. Results

3.1. Infection with E. ewingii

Goats 4, 209, and 210 became infected with *E. ewingii* after exposure to ticks; representative sequences were deposited in GenBank # (DQ365885, DQ365881). Goats exhibited mild pyrexia, lethargy, inappetance, and serous nasal discharge; goat 4 also exhibited lameness and coughing. Rectal temperatures were above the baseline established for each goat but below typical reference ranges (Table 1). Decreased alkaline phosphatase (ALP) activity was seen in all three goats, beginning 3 days after tick exposure and lasting for 2–4 months; the lowest activity was seen 4 weeks after exposure (Table 1). Neutropenia was a consistent finding during the acute infection. During days 19–21, toxic neutrophils and rare ehrlichial morulae in mononuclear cells were seen in blood smears from goats 209 and 210. DNA from *E. ewingii* was detected in blood for 4–5 weeks during the acute infection, and uninfected nymphal ticks fed upon the goats became infected with *E. ewingii*.

All three goats developed chronic infections with *E. ewingii*; pathogen DNA was detected intermittently throughout the 5 months following exposure (Table 1). Detection of ehrlichiae was temporally associated with any combination of: elevated rectal temperatures, serous nasal discharge with or without sneezing, neutropenia, and decreased ALP.

E. ewingii has not been cultivated and antigen is not available for serologic assays. Using an IFA for a related species, *E. chaffeensis*, cross-reactive antibodies were first detected 17–21 days after tick exposure, coinciding with acute illness. Antibody titers peaked 4–7 weeks after tick exposure, and bimodal peaks were seen in goats 4 and 209 (Table 1). Titers for goats 4 and 209 fluctuated between 1/32 and 1/512 for the remainder of the study. Titers for goat 210 remained above 1/512 until 16 weeks post-exposure before falling to undetectable levels.

Six months after exposure to ticks, goats 209 and 210 were immunosuppressed. ALP decreased slightly in both goats (Table 1) and DNA from *E. ewingii* was detected in the blood of goat 209. No change in antibody titers was observed during the 2 weeks following immunosuppression.

3.2. Infection with the Panola Mountain Ehrlichia sp.

Goats 5 and 20 were infected with PME (GenBank # DQ324367) after exposure to ticks. Three weeks

Table 1
Summary of clinical findings for five goats infected with USA *Ehrlichia* spp.

Goat ID	Infected with	PCR positive on days	Peak rectal temperature	Acute ALP Nadir[a]	Acute neutropenia[b]	Peak IFA titer *E. chaffeensis*	ALP Nadir post-immunosuppression
4	*E. ewingii*	12–40, 63–70, 101–103, 136, 140, 150–154, 189, 199–203	102.6 °F	88 U/L (d25)	0.824 (d26)	1/4096 (d24–28), 1/1024 (d77–84)[c]	Not done
209	*E. ewingii*	19–56, 91–98, and 140–150, 189–192	103.4 °F	70 U/L (d28)	0.158 (d25)	1/1024 (d28–31), 1/1024 (d82–88)[c]	123 U/L
210	*E. ewingii*	14–49, 66–68, and 88–103	104.3 °F	48 U/L (d26)	0.240 (d28)	1/2048 (d52–54)	175 U/L
5	PME	73[d]	104.1 °F	201 U/L (d14)	None	1/128 (d42)	105 U/L
20	PME	19–21, 34, and 140–145[e]	106.2 °F	107 U/L (d21)	0.610 (d24)	1/256 (d38)	80 U/L

[a] Reference range for ALP (alkaline phosphatase) activity 93–387 U/L.
[b] Reference range for neutrophil counts 1.2–7.2 × 10^3 μL^{-1}.
[c] Two distinct antibody peaks were detected; both peaks are reported.
[d] PCR data are not available from days 0 to 38, due to technical problems with DNA extraction.
[e] Samples were not collected from days 52 to 129.

after tick exposure, both goats exhibited serous nasal discharge and pyrexia but were otherwise active and had normal appetite. Goat 20 developed significant pyrexia for 2 days; temperatures for goat 5 were lower but intermittent pyrexia was seen over 7 days (Table 1). Clinical pathologic changes were limited to decreases in ALP (Table 1) and leukogram alterations in goat 20, in which neutropenia, toxic neutrophils (1–2% of WBCs), and rare morulae in mononuclear cells (<0.5% of WBCs) were observed. Uninfected nymphal ticks that fed upon the goats during the acute infection became infected with PME.

PCR testing for PME was performed on whole blood samples. Goat 20 was ehrlichemic during the febrile period and up to 145 days after exposure (Table 1) Acute whole blood specimens were not available from goat 5 due to technical problems, but ehrlichemia was detected on day 73.

Using IFA with *E. chaffeensis* as the antigen, both goats developed antibodies 1–2 weeks after fever. Antibody titers were low (Table 1) and decreased to undetectable levels within a few weeks. Using an ELISA based on the MAP1 protein of *E. ruminantium*, the closest taxonomic relative to PME, both goats seroconverted, and peak ELISA antibody titers for goat 5 were slightly higher than titers for goat 20.

Following immunosuppression with dexamethasone, 15 weeks (goat 5) and 21 weeks (goat 20) after tick exposure, decreased ALP was observed (Table 1). Goat 20 became ehrlichemic and transmitted PME to uninfected Gulf Coast ticks. These nymphal ticks molted into adults and subsequently infected goat 5. Both goats remained seronegative during and after immunosuppression.

4. Discussion and conclusion

The goats described here were infected with two tick-transmitted *Ehrlichia* species enzootic in the USA. Each goat was infected after exposure to 22–50 adult ticks from cohorts in which <5% of the ticks were infected with ehrlichiae, suggesting that exposure to 1–2 infected ticks may produce clinical infection. None of the goats developed infections with *E. chaffeensis*. This finding is consistent with other work that has failed to produce experimental *E. chaffeensis* infection of goats (Dugan et al., 2004). Co-infection with both ehrlichiae was not observed, although the four goats infested with field-collected ticks were probably exposed to both *E. ewingii* and PME. The lack of co-infection might be a function of our small sample size or the differing susceptibility of individual animals.

Clinical symptoms were first observed 3 weeks after exposure and primarily consisted of pyrexia, serous nasal discharge, decreased ALP activity, and neutropenia. Toxic neutrophils and morulae were seen, but careful examination of 200–300 WBCs was required to detect these rare abnormalities. Decreased ALP activity was a reliable finding and has been reported in goats in Europe infected with the closely related pathogen *A. phagocytophilum* (Gokce and Woldehiwet, 1999); data on ALP in goats with *E. ruminantium* could not be located. All of the goats in this study developed chronic infections, with ehrlichiae detected intermittently for several months after exposure.

The clinical diagnosis of acute ehrlichiosis can be assisted by the use of diagnostic serologic assays, such as IFA using *E. chaffeensis* antigen, or by the detection of DNA using PCR assays. Neither *E. ewingii* nor PME have been cultivated, in spite of attempts to isolate both agents *in vitro*, and the lack of homologous antigen makes it difficult to interpret serologic results. Antibodies against PME appeared to be less cross-reactive with *E. chaffeensis* than antibodies against *E. ewingii*; titers from PME-infected animals were lower, seen later in infection, and were only detected for a short time. PME is closely related to *E. ruminantium*, and sera from goats infected with PME also reacted with a recombinant ELISA based on *E. ruminantium*. Overall, detection of ehrlichial DNA was more reliable than serology. These findings have direct implications for the diagnosis of these emerging ehrlichioses; *E. chaffeensis* IFA is a widely available clinical diagnostic assay, but the PCR assays used in this study are primarily research tools.

Both *E. ewingii* and PME are found in lone star ticks throughout the range of this vector (Murphy et al., 1998; Long et al., 2004; Mixson et al., 2006; Loftis et al., in press), and veterinarians should consider the possibility of ehrlichiosis in goats in the eastern USA with fever of unknown origin and compatible hematological changes. Our study consisted of young, healthy, animals in an otherwise

pathogen-free environment, and clinical ehrlichiosis could be more severe in pregnant animals or animals simultaneously infected with other pathogens, as described for people (Paddock et al., 2001). Acute ehrlichiosis should occur during the peak season for the tick, May through October (Patrick and Hair, 1977; Kollars Jr. et al., 2000). PME was also transmitted by Gulf Coast ticks, which are found in the southeastern USA; this is the first report of transmission by this tick, and further study is needed to determine its significance.

Individuals working in pastures housing-infected goats should be aware that these pastures might contain infected ticks, since infected goats transmit ehrlichiae to ticks for weeks to months after exposure. Both *E. ewingii* and PME have been associated with illness in people, and the most common symptoms include headache, acute myalgia, fever, and malaise (Buller et al., 1999; Paddock et al., 2001; Reeves et al., in press).

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NATURAL AND EXPERIMENTAL INFECTION OF WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) FROM THE UNITED STATES WITH AN *EHRLICHIA* SP. CLOSELY RELATED TO *EHRLICHIA RUMINANTIUM*

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ABSTRACT: An *Ehrlichia* sp. (Panola Mountain [PM] *Ehrlichia* sp.) closely related to *Ehrlichia ruminantium* was recently detected in a domestic goat experimentally infested with lone star ticks (LSTs, *Amblyomma americanum*) collected from Georgia, USA. The infected goat exhibited pyrexia and mild clinical pathologic abnormalities consistent with ehrlichiosis. At least two other *Ehrlichia* species (*Ehrlichia chaffeensis* and *Ehrlichia ewingii*) are maintained in nature by a cycle involving LSTs as the primary vector and white-tailed deer (*Odocoileus virginanus*) as a known or suspected reservoir. To investigate the possibility that white-tailed deer are potential hosts of the PM *Ehrlichia* sp., whole blood samples collected from 87 wild deer from 2000 to 2002 were screened with a species-specific nested PCR assay targeting the citrate synthase gene. In addition, two laboratory-raised white-tailed deer fawns were each infested with 120 wild-caught LST adults from Missouri, USA, and blood samples were periodically collected and tested for the PM *Ehrlichia* sp. Of 87 deer tested from 20 locations in the southeastern United States, three (3%) deer from Arkansas, North Carolina, and Virginia were positive for the PM *Ehrlichia* sp. Wild-caught ticks transmitted the PM *Ehrlichia* sp. to one of two deer fawns, and colony-reared nymphal LSTs acquired the organism from the deer, maintained it transstadially as they molted to adults, and transmitted the PM *Ehrlichia* sp. to two naïve fawns. These findings indicate that white-tailed deer are naturally and experimentally susceptible to infection with an *Ehrlichia* sp. closely related to *E. ruminantium* and are able to serve as a source of infection to LSTs.

Key words: *Amblyomma*, cervid, *Cowdria*, *Ehrlichia chaffeensis*, *Ehrlichia ruminantium*, heartwater, lone star tick.

INTRODUCTION

Ehrlichia spp. are a group of tick-transmitted, intracellular, Gram-negative bacteria that cause disease in a wide range of hosts, including humans, domestic dogs, ruminants, equids, and felids (Rikihisa, 1991). *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis, is maintained in a cycle involving white-tailed deer (WTD; *Odocoileus virginianus*) as a primary reservoir and the lone star tick (LST; *Amblyomma americanum*) as a primary vector (Ewing et al., 1995; Lockhart et al., 1997a, b; Yabsley et al., 2003). Field evidence and experimental infection trials also suggest that WTD are important hosts for at least two other LST-vectored organisms, *Ehrlichia ewingii* and *Borrelia. lonestari*, and natural infections of WTD with all three organisms have been reported in much of the range of the LST (Lockhart et al., 1997a; Yabsley et al., 2002; Arens et al., 2003; Moore et al., 2003; Moyer et al., 2006).

Ehrlichia ruminantium, previously *Cowdria ruminantium*, the causative agent of heartwater (cowdriosis) in ruminants, is widely distributed in sub-Saharan Africa and is established on some islands in the Caribbean (Deem, 1998). Numerous species of *Amblyomma* ticks can transmit *E. ruminantium*, but *Amblyomma variegatum* and *Amblyomma hebraeum* are the two primary vectors in Africa. There is great concern that, were *E. ruminantium* introduced into the United States, the organism could readily establish in wildlife reservoirs and native ticks. White-tailed deer are experimentally

TABLE 1. Polymerase chain reaction (PCR) results for PM *Ehrlichia* sp. in 87 white-tailed deer (*Odocoileus virginianus*) collected from 20 locations in the southeastern United States.

Location[a]	Coordinates	County, state	PM *Ehrlichia* sp. no. positive/no. tested (%)
Pea Ridge NMP	36°26′58″N, 94°06′48″W	Benton, AR	1/4 (25)
Roanoke River NWR	35°59′37″N, 76°56′33″W	Bertie, NC	0/8
Unknown	37°26′15″N, 78°34′15″W	Buckingham, VA	0/1
Lakens Island	34°53′14″N, 76°35′39″W	Carteret, NC	0/7
Unknown	36°19′30″N, 79°22′45″W	Caswell, NC	0/1
Okefenokee NWR	30°44′35″N, 82°34′37″W	Charlton/Ware, GA	0/10
Unknown	37°26′38″N, 78°22′22″W	Cumberland, VA	1/1 (100)
Cape Hatteras NS	35°15′10″N, 75°35′03″W	Dare, NC	0/3
Unknown	36°11′27″N, 78°41′59″W	Granville, NC	0/1
Scatter Creek WMA	36°11′58″N, 90°32′36″W	Greene, AR	0/5
Mattamukseet NWR	35°27′24″N, 76°11′40″W	Hyde, NC	1/4 (25)
Piedmont NWR	33°10′55″N, 83°38′31″W	Jones, GA	0/5
West Kentucky WMA	37°08′00″N, 88°49′00″W	McCracken, KY	0/5
Elk City WMA	37°12′21″N, 95°47′59″W	Montgomery, KS	0/7
White Oak Plantation	30°43′33″N, 81°42′31″W	Nassau, FL	0/3
B.F. Grant Memorial Forest	33°22′34″N, 83°28′42″W	Putnam, GA	0/2
Big Hammock WMA	31°55′36″N, 81°56′10″W	Tattnall, GA	0/6
New Hill area	35°38′25″N, 78°56′52″W	Wake, NC	0/2
St. Marks NWR	30°09′01″N, 84°08′56″W	Wakulla, FL	0/10
Panther Swamp NWR	32°46′15″N, 90°32′56″W	Yazoo, MS	0/2
Total			3/87 (3.5%)

[a] NWR = National Wildlife Refuge; NMP = National Military Park; WMA = Wildlife Management Area; CC = Conservation Center; NS = National Seashore.

susceptible to infection with *E. ruminantium* (Dardiri et al., 1987), and the Gulf Coast, USA, tick, *Amblyomma maculatum*, has been experimentally shown to be a competent vector (Mahan et al., 2000). In addition, *E. ruminantium* has recently been recognized as a zoonotic disease in South Africa (Allsopp et al., 2005).

Recently an *Ehrlichia* sp. (Panola Mountain [PM] *Ehrlichia* sp.) closely related to *E. ruminantium* was detected in LSTs from Panola Mountain State Park in Georgia, USA (Loftis et al., 2006). The organism was detected in a domestic goat that was experimentally infested with wild-caught LSTs. This goat developed pyrexia and clinical pathologic changes consistent with ehrlichiosis (monocytosis, neutropenia with increased lymphocytes, decreased alkaline phosphatase activity). The DNA of the PM *Ehrlichia* sp. was detected in blood samples collected on days post–tick exposure (DPTE) 19, 21, and 34. Antibodies cross-reactive with *E. chaffeensis* were detected in serum samples, with a maximum titer of 256 on DPTE 38. Laboratory-raised LST nymphs acquired the PM *Ehrlichia* sp. from the goat and transstadially maintained the infection (Loftis et al., 2006). Recently the PM *Ehrlichia* sp. was detected via polymerase chain reaction (PCR) in a blood sample from a human patient from Atlanta, Georgia, with a history of a LST bite, fever, and muscle pain that resolved after treatment with doxycycline (Reeves et al., unpubl. data).

Because the PM *Ehrlichia* sp. is suspected to be transmitted by LSTs, and because WTD are susceptible to infection with three other LST-vectored organisms (Lockhart et al., 1997a, b; Yabsley et al., 2002; Moore et al., 2003; Yabsley et al., 2003), we hypothesized that WTD might be natural hosts for the PM *Ehrlichia* sp. In this study, we conducted a PCR-based survey of WTD blood samples collected

from WTD populations with known exposure to LST-vectored organisms. In addition, we infected WTD with the PM *Ehrlichia* sp. by transmission-feeding wild-caught LSTs from Missouri, USA, on two laboratory-raised WTD fawns, and we evaluated their ability to infect colony-reared nymphal LSTs.

MATERIALS AND METHODS

From 2000 to 2002, whole blood samples from 87 deer from 20 sites (Table 1) in the southeastern United States with known exposure to *A. americanum*–transmitted organisms were collected in Vacutainer EDTA tubes and frozen at −20 C until PCR testing. For PCR, DNA was extracted from 200 µl of whole blood using the GFX Genomic Blood Purification kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) according to the manufacturer's instructions. To detect the PM *Ehrlichia* sp., a nested PCR protocol for the citrate synthase gene (~433 bp) (Loftis et al., 2008) was conducted using the external primers Ehr3CS-185F (5′-GCCACCGCAGATAGTTAGGGA) and Ehr3CS-777R (5′-TTCGTGCTCGTGGATCATAGTTTT) in a 25 µl reaction that contained 11 µl molecular biology-grade water, 2.5 µl 25 mM $MgCl_2$, 5 µl 5X colorless buffer (Promega, Madison, Wisconsin, USA), 0.25 µl 20 mM dNTPs (Promega), 0.5 µl of each primer (50 µM), 0.25 µl GoTaq® Flexi polymerase (Promega), and 5 µl of sample DNA. For the nested PCR, 1 µl of primary product was used as template in a 25 µl reaction containing the same PCR components except primers Ehr3CS-214F (5′-TGTCATTTCCACAGCATTCTCATC) and Ehr3CS-619R (5′-TGAGCTGGTCCCCACAAAGTT) (Loftis et al., 2008). Cycling conditions in both the primary and secondary reactions were 40 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec. This protocol has a sensitivity of 10 gene copies as determined by PCR with a cloned plasmid containing the *gltA* gene from the *Ehrlichia* sp. (Loftis et al., 2008).

Assays of DNA from *E. chaffeensis*, *E. ewingii*, *Ehrlichia canis*, *Ehrlichia* sp. of raccoons, *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Anaplasma* sp. of WTD with this PCR protocol were uniformly negative. DNA from a naturally infected *A. americanum* was used as a positive control. Stringent protocols and controls were utilized in all PCR assays to prevent and detect contamination. DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate dedicated laboratory areas. A negative water control was included in each set of DNA extractions, and one water control was included in each set of primary and secondary PCR reactions.

Two 6-mo-old, laboratory-reared white-tailed deer fawns (ID nos. 12 and 18) were housed in a tick-proof facility for the duration of these experiments. Before experimental exposure to *A. americanum*, both fawns were negative for antibodies reactive to *E. chaffeensis* and PCR-negative for *E. chaffeensis*, *E. ewingii*, the PM *Ehrlichia* sp., *A. phagocytophilum*, the *Anaplasma* sp. of WTD, and *Borrelia* spp. Adult *A. americanum* wild-caught in Missouri ($n=120$ per fawn) were placed in tick chambers secured in a mid-lateral position on each deer fawn. Whole blood samples were collected on DPTE 3, 7, 14, 24, 27, 29, 31, 34, 42, 49, and 56 and tested for DNA of the PM *Ehrlichia* sp. by PCR as described above. At DPTE 27, laboratory-raised *A. americanum* nymphs from Oklahoma State University ($n=200$ per deer) were fed on fawns 12 and 18. Replete nymphs from both deer fawns were collected, pooled, allowed to molt, and a subset of resulting adults ($n=20$) was individually dissected, all internal organs were removed and digested overnight in SDS/proteinase K, and nucleic acid was isolated by standard phenol/chloroform extraction followed by ethanol precipitation (Sambrook et al., 1989). Water controls were included during each extraction process. Resultant pellets were dissolved in 100 µl of molecular biology-grade water, and 5 µl were used in nested PCR for the PM *Ehrlichia* sp. as previously described. At necropsy, samples of skin, inguinal lymph node, mesenteric lymph node, spleen, kidney, bone marrow, lung, liver, and ear were collected from both deer and processed for routine histopathology and PCR testing for the PM *Ehrlichia* sp. as described above. For DNA extraction, tissues (~5 mg) were digested with proteinase K and extracted using the GFX extraction kit.

Remaining molted adult ticks that were acquisition fed as nymphs ($n=120$) were divided randomly into two pools of 60 ticks each and allowed to feed on two naïve deer fawns (ID numbers 8 and 32). Blood samples were collected from each deer every 3–4 days for 56 days post-transmission feeding and evaluated for evidence of infection. At necropsy, the same organs, plus the brain, were collected and processed as described for fawns 12 and 18.

RESULTS

The PM *Ehrlichia* sp. was detected in three of 87 (3%) deer from the southeastern United States by nested PCR (Table 1). A single 1-yr-old deer was coinfected with the PM *Ehrlichia* sp. and *E. ewingii*, and the two remaining PM *Ehrlichia*-positive deer (both 1.5-yr-old) were coinfected with *E. chaffeensis*. Sequence analysis of *gltA* amplicons from two deer were 100% identical to the PM *Ehrlichia* sp. detected in an experimentally infected goat (GenBank DQ363995) and from *A. americanum* from numerous southeastern states (Loftis et al., 2008). One *gltA* amplicon from a wild deer had a single polymorphic base (nucleotide 289 AR as numbered by DQ363995).

One of the two deer fawns (no. 18) experimentally infested with wild-caught adult *A. americanum* became PCR positive for the PM *Ehrlichia* sp. on DPTE 24 and was positive until DPTE 42. The *gltA* sequence of the PM *Ehrlichia* sp. from deer fawn 18 was 100% identical to GenBank no. DQ363995. The second fawn (no. 12) was PCR negative for the PM *Ehrlichia* sp. on all sampling dates. Eight of 20 (40%) adult LSTs that had been fed as nymphs on the two deer fawns were PCR positive for the PM *Ehrlichia* sp. Fawns 8 and 32, who each received 60 adult LSTs acquisition fed as nymphs, both became PCR positive for the PM *Ehrlichia* sp. by DPI 24 and 27, respectively, and one fawn remained PCR positive until DPI 52 (Table 2). At necropsy, samples of both of the lymph nodes that were examined, lung, and bone marrow were PCR positive for the PM *Ehrlichia* sp. (Table 2). No histopathologic lesions were noted in any deer fawns.

DISCUSSION

Our data provide the first evidence that deer are naturally infected with and experimentally susceptible to infection

TABLE 2. Polymerase chain reaction results for PM *Ehrlichia* sp. from blood samples and tissues collected at necropsy from four white-tailed deer fawns (*O. virginianus*) exposed to *Amblyomma americanum* adults.

	Days post–tick exposure (DPTE)																Tissues positive at necropsy[a]
	0	2 or 3	7	10	14	17	21	24	27	29	31	34 or 35	38	41 or 42	45	49	52	56	
Deer exposed to wild-caught ticks																			
Deer 12	—	—	—	nd[b]	—	nd	nd	—	—	—	—	—	nd	—	nd	—	nd	—	None
Deer 18	—	—	—	nd	—	nd	nd	+	+	+	+	+	nd	+	nd	—	nd	—	None
Deer exposed to experimental ticks																			
Deer 8	—	—	—	—	—	—	—	—	+	nd	nd	+	+	—	—	—	—	—	MLN, BM, LU
Deer 32	—	—	—	—	—	—	—	+	+	nd	+	+	+	+	+	+	+	—	ILN, MLN, BM

[a] ILN = inguinal; MLN = mesenteric lymph node; BM = bone marrow; LU = lung.

[b] nd = not done.

with the PM *Ehrlichia* sp., which is closely related to *E. ruminantium.* We also demonstrated that LSTs, the suspected vector of PM *Ehrlichia* sp., can transmit this agent to naïve deer fawns, and that LSTs can acquire infection from infected deer as nymphs and maintain that infection transstadially when they molt to adults; similar results were previously shown with LSTs fed on an infected goat (Loftis et al., 2006). This study further expands the known geographic range of the PM *Ehrlichia* sp. beyond Georgia to include parts of four additional US states (Arkansas, Missouri, North Carolina, and Virginia) and confirms the vector capacity of LSTs.

Because of the association of the PM *Ehrlichia* sp. and LSTs, we tested only deer populations with known exposure to LSTs and evidence of infection with other LST-vectored organisms. Coinfections of humans and domestic animals with multiple pathogens transmitted by the same vector are being increasingly recognized (Sexton et al., 1998; Kordick et al., 1999; Loebermann et al., 2006), as are coinfected individual ticks (Schulze et al., 2005; Mixson et al., 2006). These reports of coinfection resulted from infestations with single or limited numbers of ticks that are coinfected; because WTD are frequently infested with hundreds to thousands of LSTs, the likelihood of coinfection in wild WTD is increased (reported as high as 25%) (Yabsley et al., 2002, 2003; Arens et al., 2003; Moore et al., 2003). In the current study, all three wild deer that were positive for the PM *Ehrlichia* sp. also were infected with either *E. chaffeensis* or *E. ewingii.* Coinfection of individual WTD with *E. chaffeensis/E. ewingii*, *E. chaffeensis/B. lonestari*, and *E. ewingii/B. lonestari* has been detected in previous studies (Yabsley et al., 2002; Arens et al., 2003). Infestation of deer fawns with as few as 300 wild-caught *A. americanum* resulted in coinfection with *E. chaffeensis*, *E. ewingii*, and *B. lonestari* (Varela-Stokes, 2007). The majority of WTD that are PCR positive for *E. chaffeensis*, *E. ewingii*, and *B. lonestari* are ≤1.5 yr old (Lockhart et al., 1997b; Yabsley et al., 2002; Moore et al., 2003; Yabsley et al., 2003); a similar association with age was observed with the PM *Ehrlichia* sp.

WTD are highly susceptible to experimental infection with *E. ruminantium* and display significant morbidity and mortality (Dardiri et al., 1987). However, the PM *Ehrlichia* sp. did not cause mortality in the three experimentally infected deer. No health data were available for the three hunter-killed wild deer. Although one of our experimentally infected fawns did develop pyrexia, mild anemia, and depression concomitant with development of PM *Ehrlichia* sp. infection, simultaneous coinfection with a *Theileria* sp. and *E. chaffeensis* precluded accurate interpretation of serology or hemograms (Little et al., unpubl. data). Future work will be aimed at the in vitro isolation of the PM *Ehrlichia* sp. so that experimental infection and transmission trials can be conducted in monospecifically infected hosts.

Data from this study and others demonstrate that WTD are exposed to at least five ehrlichial species (*E. chaffeensis*, *E. ewingii*, PM *Ehrlichia* sp., *Anaplasma phagocytophilum*, and *Anaplasma* sp. of WTD) (Dawson et al., 1996; Belongia et al., 1997; Lockhart et al., 1997a; Yabsley et al., 2002, 2003; Arens et al., 2003; Moore et al., 2003). Four of these species are known to be zoonotic, and WTD have been shown to be competent reservoirs of *E. chaffeensis* and PM *Ehrlichia* sp. Because of the reported low level of serologic cross-reactivity between *E. chaffeensis* and the PM *Ehrlichia* sp. in goats (Loftis et al., 2006), it is possible that low-titer *E. chaffeensis* seroreactors in prior studies of WTD actually represent infection with the PM *Ehrlichia* sp, *E. chaffeensis*, *E. ewingii*, or mixed infections. The presence of multiple, serologically cross-reactive ehrlichiae also raises the possibility that prior infection of WTD

with *E. chaffeensis* or other ehrlichiae could confound serologic surveys for *E. ruminantium*, should it be introduced into the United States. Future studies should utilize an array of diagnostic assays for epidemiologic studies, and experimental infections should investigate coinfection dynamics.

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Case report

The first report of human illness associated with the Panola Mountain *Ehrlichia* species: a case report

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Abstract

Introduction: Two species of *Ehrlichia* are known to cause human illness. Several other species have been discovered in ticks and animals, and recent reports suggest that some of these *Ehrlichia* species might be human pathogens. We report here the first association of a recently discovered pathogen, the Panola Mountain *Ehrlichia* species, with a case of human illness.

Case presentation: A 31-year-old man from Atlanta, Georgia (GA) in the United States of America (USA) presented with a persistent sore neck of 3 weeks duration following a tick bite. DNA from the Panola Mountain *Ehrlichia* species, which was recently discovered in a goat in Georgia, was detected in an acute blood sample. Serologic testing was inconclusive. Polymerase chain reaction tests for other tick-borne diseases found in this region were negative. The patient rapidly improved in response to doxycycline therapy.

Conclusion: Detection of *Ehrlichia* DNA in an acute blood sample meets the Centers for Disease Control and Prevention laboratory confirmation criteria for ehrlichiosis, and response to doxycycline provides supporting clinical evidence. The Panola Mountain *Ehrlichia* species, an emerging pathogen transmitted by ticks in the eastern USA, should be considered as a possible cause of tick-borne illness in this region.

Introduction

Ehrlichia species are tick-transmitted intracellular bacteria closely related to *Anaplasma, Neorickettsia* and *Rickettsia*. *Ehrlichia chaffeensis* was first described as a human pathogen in 1986 and *E. ewingii* in 1996 [1], and recent evidence suggests that other species of *Ehrlichia* might also cause illness [2,3]. A new species of *Ehrlichia*, the Panola Mountain *Ehrlichia* species, was recently discovered in the USA [4]. Clinical signs of ehrlichial infection are often non-specific, and the most common signs are fever, headache, myalgia and malaise [1]. Laboratory diagnosis of ehrlichiosis depends on the detection of ehrlichiae in samples collected during the acute illness or on demonstration of a significant rise (four-fold or greater) in antibody titer between the acute and convalescent phases of the illness [1,5]. Serology is limited because acute serology alone is insufficient to diagnose infection, paired serology does not provide a diagnosis until 3 to 4 weeks

Table 1: Complete blood count results for blood drawn on 26 September 2005

Hematocrit	46.7%
Red blood cell count	5.32×10^7/μl
Hemoglobin	15.9 g/dl
Mean corpuscular volume	87.8 fl
Mean corpuscular hemoglobin	29.9 pg
Mean corpuscular hemoglobin concentration	34.1 g/dl
Red blood cell distribution width	12.7%
Platelet count	272,000/μl
White blood cell count	7500/μl
Absolute neutrophils (%)	4868 cells/μl (64.9%)
Absolute lymphocytes (%)	2243 cells/μl (29.9%)
Absolute monocytes (%)	233 cells/μl (3.1%)
Absolute eosinophils (%)	143 cells/μl (1.9%)
Absolute basophils (%)	15 cells/μl (0.2%)

after empirical treatment has been initiated, and only *E. chaffeensis* is available for serologic testing of humans [1]. Diagnosis of ehrlichiosis during the acute infection increasingly relies on polymerase chain reaction (PCR) [1,6]. According to the official case definition used by the Centers for Disease Control (CDC) and the USA National Notifiable Diseases System, a positive PCR reaction, with confirmation of the amplicon identity, is sufficient for laboratory confirmation of a case of human ehrlichiosis [5].

Case presentation

On 23 September 2005, a 31-year-old Caucasian man from Atlanta, Georgia, USA presented with a complaint of neck soreness for 3 weeks. The patient reported hiking at Panola Mountain State Park in Georgia on 31 August 2005 and later removing a partially engorged nymphal tick from his upper arm on 3 September. The patient stored the tick in an empty vial at room temperature, and the tick was later identified as *Amblyomma americanum*. On 8 September, the patient began suffering from a persistent sore neck, characterized by musculoskeletal pain upon turning his head and insomnia due to pain. The pain was refractory to anti-inflammatory medications, including acetaminophen, aspirin and ibuprofen. Physical examination on 23 September was unremarkable. Pyrexia was not observed and no erythema or edema was noted at the site of the tick bite; however, the patient had taken 500 mg aspirin prior to examination. The patient was treated for a presumptive tick-borne illness with 100 mg of oral doxycycline twice daily for 10 days. The patient reported that neck soreness was improved by 48 to 60 hours after doxycycline therapy was initiated.

Laboratory testing

Blood was drawn from the patient on 23 September for PCR testing for tick-borne diseases, on 26 September for a complete blood count (CBC) and acute serology, and on 15 October for convalescent serology. Whole blood from 23 September and sera from 26 September and 15 October were submitted to the CDC for tick-borne disease testing. The CBC was performed by Quest Diagnostics (Nichols Institute, Chantilly, VA), and CBC results (Table 1) were within the normal reference range for this laboratory.

For PCR testing, DNA was extracted from 100 μl of clotted blood and from the dead tick, using an IsoQuick Nucleic Acid Extraction Kit (ORCA Research Inc., Bothell, WA). We detected DNA from *Ehrlichia* using a genus-specific, hemi-nested PCR with the outer primers EC12A and HE3 [4], followed by a hemi-nested reaction using the 'Forward' primer [7] and HE3. DNA from *Rickettsia* species was detected using primer-1 and primer-2 [8]. We assessed the quality of the tick DNA using primers T1B and T2A [9]. Positive and negative controls were used for all assays and consisted of genomic DNA from *Rickettsia rickettsii*, *Ehrlichia ewingii* or distilled water. All PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced in duplicate using PCR primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequences were determined using an ABI 3100 (Applied Biosystems). Primer sequences were removed and sequences assembled with Seqmerge (Accelrys, San Diego, CA).

Using the hemi-nested PCR, an amplicon from the 16S rRNA gene of an *Ehrlichia* species was obtained from the acute blood sample. The amplicon was sequenced, and the 361-bp sequence (GenBank accession number DQ217573) was 100% identical to the sequence reported from the Panola Mountain *Ehrlichia* species (*Ehrlichia* species P-Mtn, GenBank accession number DQ324367). The amplicon was not identical to sequences from any other species represented in GenBank. No DNA from *Rickettsia* was detected in the patient's blood. The tick was poorly preserved by the patient, and DNA could not be amplified from it.

For acute and convalescent serology, sera from 26 September and 15 October were tested using indirect immunofluorescence assays (IFA), performed as previously described [10], to detect antibodies against *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Coxiella burnetii*, *Francisella tularensis*, *Rickettsia africae*, *R. akari*, '*R. amblyommii*', *R. conorii*, *R. parkeri*, *R. prowazekii*, *R. rickettsii* and *R. typhi*, and antibodies cross-reactive with *E. chaffeensis*. We could not test the patient's serum against the Panola Mountain *Ehrlichia* species because this emerging agent has not yet been cultured. Antibody was detected using isotype-specific goat antihuman immunoglobulin G (IgG) and human immunoglobulin M (IgM), labeled with fluorescein isothiocyanate (FITC) (KPL, Gaithersburg, Maryland). Prior to testing for IgM, sera were depleted of IgG

by use of a recombinant Protein G kit (Rapi-Sep-M, Pan-Bio, Columbia, MD). Acute and convalescent samples were tested side-by-side, and positive, negative and diluent controls were assayed with the test samples and gave expected results. Serology did not support recent infection with any of the agents tested. With *E. chaffeensis* antigen, the patient's serum reacted with a small proportion of the organisms on the slide, as compared with positive control sera, and was considered cross-reactive in both acute and convalescent samples. Titers are expressed as the reciprocal of the last dilution exhibiting specific fluorescence and were as follows: IgM 32 (26 September), and IgG 16 (26 September) and IgG 32 (15 October). Convalescent IgM data were not available.

Conclusion

We report that an emerging pathogen, the Panola Mountain *Ehrlichia* species, was detected in blood from a human patient following the bite of a nymphal *Amblyomma* that was probably acquired at Panola Mountain State Park in Georgia in the United States of America. The Panola Mountain *Ehrlichia* species was originally described from a goat fed upon by *A. americanum* collected at this park [4], but this is the first report associating the agent with human illness.

The Panola Mountain *Ehrlichia* species is genetically closely related to *E. ruminantium* and more distantly related to *E. chaffeensis* [4]. The patient exhibited myalgia for 3 weeks prior to presentation, had ehrlichemia, which was confirmed by DNA sequencing at presentation, and rapidly recovered after treatment with doxycycline. Although PCR and serological testing for other tick-borne agents was negative, suggesting that ehrlichiosis was the cause of illness, we cannot conclusively rule out the possibility that the patient's symptoms were caused by an unknown factor. Serological confirmation of infection with the Panola Mountain *Ehrlichia* species could not be obtained, with only a two-fold rise in IgG titer between the two serum samples. This might be due to the initiation of antibiotic therapy prior to optimal immune response or due to the lack of an appropriate antigen; antibodies against ehrlichial agents are often, but not always, cross-reactive with other species of *Ehrlichia* [3]. In this case, PCR testing of whole blood was of significantly greater diagnostic value than serological testing.

Abbreviations

CBC: complete blood count; CDC: Centers for Disease Control; FITC: fluorescein isothiocyanate; IFA: immunofluorescence assays; IgG: immunoglobulin G; IgM: immunoglobulin M; PCR: polymerase chain reaction.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WKR identified the tick, isolated DNA from the blood sample, performed sequencing reactions, analyzed data, and was a major contributor in writing the manuscript. ADL tested the DNA from the blood sample, recorded patient information, and was a major contributor in writing the manuscript. WLN performed serological tests and reviewed drafts of this manuscript. AGC was the attending physician for the patient and contributed to drafts, collecting clinical specimens, and patient treatment and observations. All authors read and approved the final manuscript.

Consent

Written informed consent was obtained from the patient for publication of this case report. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

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Research article

Geographic distribution and genetic diversity of the *Ehrlichia* sp. from Panola Mountain in *Amblyomma americanum*

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Abstract

Background: A novel *Ehrlichia*, closely related to *Ehrlichia ruminantium*, was recently discovered from Panola Mountain State Park, GA, USA. We conducted a study to determine if this agent was recently introduced into the United States.

Methods: We developed a sensitive PCR assay based on the conserved *glt*A (citrate synthase) gene and tested DNA samples extracted from 1964 field-collected and 1835 human-biting *Amblyomma americanum* from 23 eastern states of the USA.

Results: The novel agent was detected in 36 ticks collected from 10 states between 1998 and 2006. Infected ticks were collected both from vegetation (n = 14, 0.7%) and from humans (n = 22, 1.2%). Fragments of the conserved *glt*A gene and the variable *map*1 gene were sequenced from positive samples. Two distinct clades, with 10.5% nucleic acid divergence over the 730 bp *map*1 sequence, were identified.

Conclusion: These data suggest that the Panola Mountain *Ehrlichia* was not recently introduced to the United States; this agent has an extensive distribution throughout the range of its tick vector, has been present in some locations for several years, and displays genetic variability. Furthermore, people in several states were exposed to this agent through the bite of infected ticks, underscoring the potential public health risk of this emerging ehrlichiosis.

Background

A novel *Ehrlichia* transmitted by *Amblyomma americanum* (lone star ticks) was recently discovered in Panola Mountain State Park, Georgia, USA. The "Panola Mountain *Ehrlichia*" (PME), which is closely related to *E. ruminantium*, caused transient febrile illness, followed by chronic latent infection, in a goat [1]. This agent was also associated, using PCR and sequencing, with a case of human illness following a bite from a nymphal *Amblyomma* acquired at Panola Mountain State Park [2]. White-tailed deer (*Odocoileus virginanus*) are a probable vertebrate reservoir for PME in the United States; deer are susceptible to infection, are naturally exposed to the agent, and are competent reservoirs for tick transmission of this agent [3].

Ehrlichia ruminantium is endemic in southern Africa and the Caribbean, and its pathogenicity in cattle, sheep, and goats varies from mild febrile illness to fatal heartwater [4-7]. Several species of *Amblyomma* vector *E. ruminantium* in Africa and *A. variegatum* is the vector in the Caribbean [4]. This exotic disease has not been detected in the United States, but it could be introduced into the country by the importation of animals and tick vectors from endemic areas [8,9]. White-tailed deer are also susceptible to infection with *E. ruminantium* [10], and could provide a sylvatic reservoir for *E. ruminantium* in the United States. Thus, the *Ehrlichia* discovered at Panola Mountain State Park could be a divergent strain of *E. ruminantium* that was recently introduced to this country. This park is located within the Atlanta, Georgia, metropolitan area and is less than twenty miles from the Hartsfield International Airport, a port of entry for imported animals and animal products.

To evaluate the geographic distribution and public health risk posed by this emerging agent, we developed a sensitive nested PCR assay based on the *glt*A (citrate synthase) gene, which is conserved within species of *Ehrlichia* [11], and screened DNA extracts from *A. americanum* collected from vegetation and submitted through four different human-biting tick surveillance programs. All PCR amplicons were confirmed by sequencing the *glt*A fragment, and the genetic diversity of this novel agent was evaluated by amplification and sequencing of the variable Major Antigenic Protein 1 gene (*map*1) [12].

Methods

Collection of ticks

Ticks were collected in the years 1998–2006. Questing ticks were collected by flagging vegetation or by using carbon dioxide baited traps, as described by Fleetwood et al. [13]. Ticks removed from humans were collected through surveillance programs established by the Georgia Division of Public Health, Ohio Department of Health, University of North Texas Health Science Center, and U.S. Army Center for Health Promotion and Preventive Medicine. Ticks were voluntarily submitted by individuals who reported the location in which they believed the tick was acquired. All ticks were identified to species and life stage, and gender of adult ticks was recorded. DNA was extracted from individual questing adult ticks as previously described [1,14]. For human-biting tick collections, DNA was extracted from individual nymphal and adult ticks, or from pools of ticks collected from a single person, using previously described methods [15].

PCR detection of PME

A nested PCR assay for the Panola Mountain *Ehrlichia* was designed based on the published *glt*A sequence (GenBank:DQ363995). Nested PCR primers were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) and GCG Seqlab (Accelrys, San Diego, CA), and evaluated for specificity using a panel of DNA from *A. americanum* known to be PCR-positive for the agent and negative DNA samples extracted from colony-reared ticks. Sensitivity was determined using a cloned plasmid containing the *glt*A gene from the *Ehrlichia* sp. Reaction conditions were optimized to ensure a sensitivity of at least 10 gene copies. The outer PCR reaction included 5.0 μL of Taq Master Mix (Qiagen, Valencia, CA), 500 nM each of primers Ehr3CS-185F (5'-GCC ACC GCA GAT AGT TAG GGA) and Ehr3CS-777R (5'-TTC GTG CTC GTG GAT CAT AGT TTT), and 1.0–2.0 μL of DNA in a 10 μL final reaction volume. The volume of DNA used in each reaction was based upon the elution volume of DNA; 1.0 μL was used for DNA samples in a final volume of 50–100 μL, and 2.0 μL were used for samples extracted in a volume of 200 μL. The thermocycler program was as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, with a final extension at 72°C for 5 min. The inner PCR reaction included 10.0 μL of Taq Master Mix, 500 nM each of primers Ehr3CS-214F (5'-TGT CAT TTC CAC AGC ATT CTC ATC) and Ehr3CS-619R (5'-TGA GCT GGT CCC CAC AAA GTT), and 1.5 μL of the primary reaction product in a 20 μL final reaction volume. The thermocycler program was similar to the primary reaction, but with a 60°C annealing temperature rather than 55°C.

All PCR reactions were prepared in a dedicated hood equipped with an ultraviolet light source. Positive (10 copy) and negative controls were included on every plate; positive control material was handled only after all unknown samples were loaded. Ticks that were identified as positive using the *glt*A primer set were confirmed by repeating the assay and by sequencing. Positive ticks were tested using a PCR assay for *map*1, as previously described [1,16], using the outer primer pair map1-forward/map1-reverse and, if needed, paired heminested reactions with

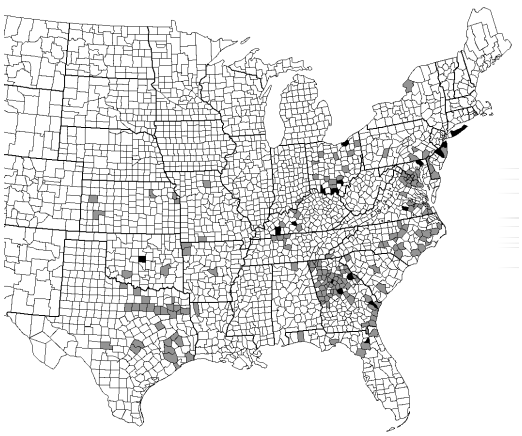

Figure 1
Geographic distribution of the Panola Mountain Ehrlichia sp. Summary of collection sites for *Amblyomma americanum*, by county. Counties from which ticks were tested and PME was not detected are shaded gray (n = 185); counties from which at least one tick contained DNA from PME are shaded black (n = 18). In addition to the counties listed in Table 3, Rockdale County, Georgia, which contains Panola Mountain State Park, is shaded black.

Pmap-2F/map1-reverse and map1-forward/Pmap-2R or nested reactions with Pmap-2F/Pmap-2R.

Sequence analysis

All PCR amplicons obtained using the *glt*A assay and *map*1 assay were sequenced, with 2- to 4- fold coverage, using PCR primers. When mixed sequences were obtained, PCR amplicons were cloned using TOPO TA Cloning kits with the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) and sequencing was repeated using several clones. Fragments were assembled using GCG Seqmerge (Accelrys), translated into the respective predicted amino acid sequences, and aligned. All sequences were submitted to GenBank: *glt*A, EU272374–EU272410 and *map*1, EU272339–EU272373. Related sequences were identified using BLAST (NCBI, Bethesda, MD). Phylogenetic reconstructions based on the amino acid sequences of the *map*1 amplicons were performed using PAUP 4.0 Beta 10 (Sinauer Asosciates, Inc., Sunderland, MA). The most parsimonious trees were constructed using heuristic bootstrap analysis (1000 replicates); starting trees were obtained by stepwise addition with tree-bisection-reconnection as the branch-swapping algorithm.

Table 1: Summary of test results for ticks collected from vegetation. *Amblyomma americanum* adults were collected from vegetation and tested for the presence of the Panola Mountain *Ehrlichia* sp.

Collection State	# Ticks (M/F)	# POS (M/F)	% POS
Florida	151 (57/94)	1 (1/0)	0.66%
Georgia	705 (292/413)	6 (3/3)	0.85%
Kentucky	6 (1/5)	1 (0/1)	16.7%
New Jersey	120 (68/52)	2 (2/0)	1.67%
New York	475 (246/229)	4 (4/0)	0.84%
North Carolina	383 (152/231)	0	0%
South Carolina	80 (37/43)	0	0%
Texas	44 (14/30)	0	0%
TOTAL	1964 (867/1097)	14 (10/4)	0.71%

Results are summarized here by state of collection and gender (M = male, F = female).

Results and Discussion

Amblyomma americanum were collected from 203 counties in 23 states, representing much of the geographic range of this tick (Figure 1).

Questing ticks were collected from vegetation at 32 sites in 8 states. Of 1964 questing adult ticks, including 867 adult males and 1097 adult females, 14 ticks (10 males, 1.15%, and 4 females, 0.36%) were positive for PME (Table 1). The overall prevalence of infection in questing ticks was 0.71%, and the prevalence of infection in sites with infected ticks ranged from 1.32–2.88%.

Human-biting ticks were obtained, via surveillance programs, from 21 states. Of 1835 *A. americanum* obtained from surveys of human-biting ticks, 22 DNA samples from 5 states were positive (Table 2). Six of these positive samples were pools of 2–9 ticks, with the remainder representing individual ticks. Given the low prevalence of infection, we assumed that only one tick in a positive pool was likely to be positive; the infection rate among human-biting ticks was therefore 22/1835, or 1.20%. Eleven of the positive samples were from nymphal ticks (1.12%), three from males (0.71%), five from females (1.17%), and five from pools containing more than one life stage.

Overall, 35 positive ticks were obtained from 18 counties in ten states: Florida, Georgia, Kentucky, Maryland, Missouri, New Jersey, New York, Ohio, Oklahoma, and Virginia (Figure 1). Four of these ticks were co-infected with *E. chaffeensis* and/or *E. ewingii* (Table 3). None of the positive ticks were co-infected with "*Borrelia lonestari*". All of the positive ticks were collected between the months of April and September. Positive ticks were identified from collections made in 1998 through 2006; on Fire Island, in Suffolk Co., New York, individual positive ticks were collected from vegetation in both 1998 and

Table 2: Summary of test results for human-biting ticks. *Amblyomma americanum* nymphs and adults were collected from human-biting tick surveillance programs and tested for the presence of the Panola Mountain *Ehrlichia* sp. Results are summarized here by the state of reported tick acquisition and life stage or gender (N = nymph, M = male, F = female).

Collection State	# Ticks (N/M/F/Not Recorded)	# POS (N/M/F/unknown)[a]	% POS
Alabama	8 (2/2/4/0)	0	
Arkansas	8 (1/2/4/1)	0	
District of Columbia	7 (1/3/3/0)	0	
Delaware	12 (8/2/2/0)	0	
Florida	7 (3/2/1/1)	0	
Georgia	343 (157/87/98/1)	0	
Indiana	1 (1/0/0/0)	0	
Kansas	67 (32/12/22/1)	0	
Kentucky/Tennessee	199 (114/40/45/0)	1 (0/0/1/0)	0.50%
Louisiana	1 (1/0/0/0)	0	
Maryland	266 (178/51/37/2)	4 (3/1/0/0)	1.50%
Missouri	10 (3/3/4/0)	1 (0/0/1/0)	10.0%
North Carolina	93 (45/25/23/0)	0	
Nebraska	1 (1/0/0/0)	0	
New Jersey	265 (163/57/45/3)	7 (3/1/1/2[a])	2.64%
New York	1 (1/0/0/0)	0	
Ohio	22 (11/7/4/0)	6 (3/1/2/0)	27.3%
Oklahoma	21 (14/3/3/1)	1 (0/0/0/1[a])	4.76%
Pennsylvania	4 (1/1/2/0)	0	
Rhode Island	1 (0/0/1/0)	0	
South Carolina	33 (17/7/9/0)	0	
Texas	81 (23/32/26/0)	0	
Virginia	368 (199/80/89/0)	1 (1/0/0/0)	0.27%
Unknown/Unrecorded	16 (7/5/4/0)	1 (1/0/0/0)	6.25%
TOTAL	1835 (983/421/426/10)	22 (11/3/5/3)	1.20%

a: When a pool of ticks (2–9 ticks each) was positive, the minimum infection rate (1 tick) is reported. The life stage of the infected tick could not be determined for 3 pools that included more than one life stage.
b: Kentucky and Tennessee ticks were combined, since most of the ticks in this collection centered on the border between these two states.

2003, suggesting that the agent was maintained in this area over several years. Similarly, positive human-biting ticks in Burlington Co., New Jersey were collected in 2001 and 2006, and ticks collected from Scioto Co., Ohio in 2000 and 2001 were positive.

The nested *glt*A amplicon was sequenced from all positive ticks. All positive amplicons had sequences consistent with PME; *E. chaffeensis* or *E. ewingii* were not amplified. The *glt*A sequence was highly conserved and was identical to the reported PME sequence (GenBank: DQ363995) in 35/36 ticks; one sequence included a single nucleotide polymorphism (EU272407).

A portion of *map*1 was successfully amplified and sequenced from 31 of the 36 positive tick DNA samples; the outer PCR amplicon, when available, produced 730 bp of sequence, and the inner amplicon produced 384 bp. Two *map*1 genogroups were identified and were seen both in questing ticks and in human-biting ticks (Table 3): one group was 100% homologous to the sequence reported from PME (GenBank:DQ324368), and the other group was 89.5% similar to PME, with two polymorphic loci identified within this group of sequences. There were five base pair mismatches between the sequences in the latter genogroup and the outer pair of PCR primers previously developed for the detection of the *map*1 gene from this agent (Pmap-38F/Pmap-581R, [1]). The two different PME clades were not geographically isolated; both clades were detected in ticks from Jones County, Georgia and Monmouth County, New Jersey. Additionally, one pool of ticks from Ocean County, New Jersey, contained both genotypes. The amplicons produced from this mixed template pool were cloned and sequenced. Sequence analysis indicated representatives of both genotypes were present, as well as several sequences that appear to be combinations of these two genotypes and may indicate a crossover event in *map*1 (GenBank: EU272356–EU272360).

Phylogenetic reconstructions based on the *map*1 amino acid sequences were attempted (Figure 2) using all of the DNA sequences that were 730 bp in length. The sequences from the ticks formed two distinct clades, with 100% bootstrap support for each clade (1000 psuedoreplicates), and they formed a cluster within the larger taxonomic group of *E. ruminantium* strains in 82% of the replicates.

Table 3: Collection details and map1 sequence homologies of the positive ticks. Collection details for the 36 *Amblyomma americanum* harboring DNA from the Panola Mountain *Ehrlichia* sp. (PME) are shown, including the source of the tick, date of collection, coinfectionn status, and homology of the *map*1 DNA amplicons to reference sequences for PME.

Location Tick Was Collected or Acquired		Collected From	Life Stage	Date Collected	Coinfection with Other *Ehrlichia*	*map*1 GenBank Sequence ID	map1 Homology[a]
FL	Bradford Co.	vegetation	M	6/25/2003		EU272339	730/730
GA	Jones Co.	vegetation	F	6/9/2003		EU272340	730/730
	Jones Co.	vegetation	M	6/9/2003		EU272341	654/730
	Jones Co.	vegetation	M	6/9/2003		EU272342	654/730
	Wilkes Co.	vegetation	M	6/11/2004		EU272343	730/730
	Wilkes Co.	vegetation	F	6/11/2004		EU272344	730/730
	Bryan Co.	vegetation	F	6/18/2004		EU272345	730/730
KY	Edmonson Co.	vegetation	F	6/30/2002		EU272346	654/730
	Christian Co.	22 yo female	2 FF	6/13/2006		EU272347	654/730
MD	Harford Co.	20 yo male	N	7/18/2001		EU272348	384/384[b]
	Harford Co.	52 yo male	N	5/31/2006		EU272349	730/730
	Harford Co.	25 yo male	M	6/7/2006		EU272350	730/730
	Harford Co.	50 yo male	9 NN	6/28/2006	*E. ewingii*	no amplification	
MO	Not recorded	human[c]	F	6/23/2000		no amplification	
NJ	Burlington Co.	35 yo male	N	6/29/2001		EU272351	384/384[b]
	Burlington Co.	34 yo male	F	6/29/2001		EU272352	277/277[b]
	Monmouth Co.	vegetation	F	4/22/2003	*E. ewingii E. chaffeensis*	EU272353	654/730
	Monmouth Co.	vegetation	F	4/22/2003		EU272354	730/730
	Ocean Co.	41 yo male	M+N	5/22/2006	*E. chaffeensis*	EU272355	654/730
	Ocean Co.	46 yo male	F+N	6/27/2006		EU272356–EU272360[d]	730/730, 654/730[d]
	Burlington Co.	41 yo male	M	7/20/2006		EU272361	730/730
	Burlington Co.	31 yo male	N	7/20/2006		EU272362	730/730
	Burlington Co.	46 yo male	2 NN	8/1/2006		EU272363	730/730
NY	Suffolk Co.	vegetation	M	7/3/1998	*E. ewingii E. chaffeensis*	EU272364	730/730
	Suffolk Co.	vegetation	M	6/4/2003		EU272365	730/730
	Suffolk Co.	vegetation	M	6/4/2003		EU272366	730/730
	Suffolk Co.	vegetation	M	7/9/2003		EU272367	730/730
OH	Scioto Co.	human[c]	F	5/8/2000		EU272368	377/377[b]
	Clermont Co.	human[c]	M	5/30/2000		EU272369	384/384[b]
	Cuyahoga Co.	human[c]	F	6/7/2000		EU272370	375/375[b]
	Vinton Co.	human[c]	N	6/23/2000		no amplification	
	Hocking Co.	human[c]	N	9/13/2000		EU272371	376/377[b]
	Scioto Co.	human[c]	N	6/27/2001		EU272372	313/313[b]
OK	Oklahoma Co.	human[c]	F+7NN	7/25/2006		EU272373	730/730
VA	Nottoway Co.	25 yo male	N	6/27/2006		no amplification	
State not recorded		human[c]	N	5/23/2000		no amplification	

a: Percent homology to the reference sequence for PME, GenBank: DQ324368
b: Only the internal, nested fragment was available for sequencing.
c: Age and gender not recorded.
d: The *map*1 PCR amplicon was a mixture of more than one sequence, and the product was cloned prior to sequencing.

The PME/*E. ruminantium* clade was a sister taxa to *E. chaffeensis* and *E. canis* in 100% of the replicates. Bootstrap support for separation of PME and *E. ruminantium* into separate taxa was weak (< 500/1000).

Conclusion

We detected the novel Panola Mountain *Ehrlichia* sp. (PME), recently discovered in *A. americanum* from north central Georgia, USA, in 36 ticks from 18 counties in ten states. DNA from PME was detected in ticks from the traditional southeastern range of *A. americanum* as well as from recently established populations in the northeastern USA. The overall prevalence of infection with PME, approximately 1–3%, was similar to that previously reported from Panola Mountain [1]. Similar prevalences of *E. ewingii* and *E. ruminantium* are seen in populations of *Amblyomma* infected with these agents [14,17-19].

Earlier assays for PME were based on the gene encoding the Major Antigenic Protein 1 (*map*1) [1]; this gene can be

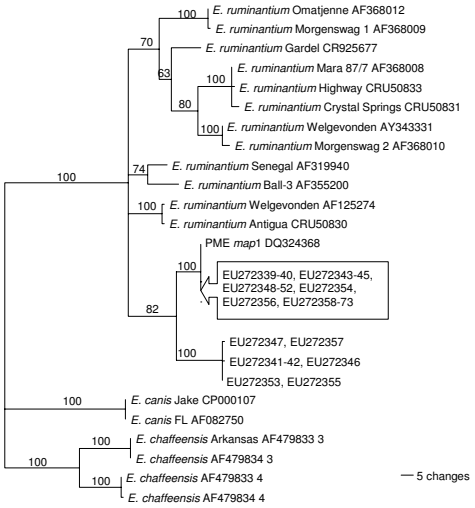

Figure 2
Genetic diversity of the Panola Mountain Ehrlichia sp. Phylogenetic reconstruction of the *map*1 predicted amino acid sequences from 31 *Amblyomma americanum* harboring DNA from the Panola Mountain *Ehrlichia* sp. (PME). Numbers indicate the bootstrap support for each node, as a percentage of 1000 replicates, and the scale represents the number of changes per 100 residues.

highly variable [12], and polymorphisms in the primer annealing sites could prevent the detection of some genotypes of PME. We therefore developed a sensitive and specific nested PCR assay based on the conserved *glt*A gene. As expected, *glt*A amplicons obtained from PME-infected ticks were 99.5–100% identical to each other and to the sequence previously reported from this agent. Significant mismatches were seen between the previously reported *map*1 primers and the *map*1 sequences from PME-infected ticks. Additionally, *map*1 could not be amplified from all of the *glt*A-positive ticks, presumably due to primer site mismatches, confirming that detection of PME using *map*1 assays is limited by the variability of this gene.

This gene is, however, a valuable tool to assess genetic variability in PME across the geographic range of *A. americanum*. Using phylogenetic reconstruction of *map*1 sequences, we identified two clades, one of which includes the genotype previously reported from Panola Mountain. Both PME clades were closely associated with each other and were more closely related to *E. ruminantium* than to any other species of *Ehrlichia*, similar to previous reports. Sequences that suggest the possibility of crossover between these two genotypes were also obtained from a few clones from a single DNA sample; collection and analysis of other specimens, to determine if this is an isolated finding, are ongoing.

The extensive geographic distribution of PME and the presence of genetic variability within the species suggest that this agent was not recently introduced to the United States. A recent point source introduction should have resulted in a limited geographic distribution of a single genotype or possibly of closely related genotypes with minimal divergence. Although two distinct genotypes of PME were detected, less genetic diversity was seen than is reported to occur between African strains of *E. ruminantium*, suggesting that the *map*1 gene of PME might evolve more slowly than that of its African counterparts, that the diversity of susceptible *Amblyomma* and vertebrate species in Africa might have contributed to the diversity of *E. ruminantium* on that continent, or that the introduction of *E. ruminantium*-like bacteria to North America might have been more recent than the introduction into Africa.

Finally, the lack of reports of heartwater, or heartwater-like disease, in ruminants, wild or domestic, from areas in which PME was detected provides indirect support that this agent has low pathogenicity for these animals. However, this emerging ehrlichiosis has been associated with a human case of illness following the bite of a nymphal *A. americanum* from Panola Mountain State Park. *Amblyomma americanum* is an abundant and aggressive human-biting tick throughout its geographic range [20]. Our detection of PME in 22 human-biting ticks underscores the potential public health risk of this emerging tick-transmitted disease. The broad geographic range and patchy distribution of ticks harboring PME suggests that human cases could occur throughout the eastern United States but be sporadic in nature and therefore difficult to diagnose. Sensitive and specific PCR assays, performed on whole blood samples collected during the acute febrile period, might assist in the rapid clinical diagnosis of undifferentiated ehrlichial infections in people with a history of tick bite by *A. americanum*.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AL conceived of the study, carried out portions of the molecular genetic studies, participated in the sequence alignment and analysis, performed statistical analysis, and drafted the manuscript. TM, ES, and KB participated in the design of the study, collected tick samples, carried out portions of the molecular genetic studies, and helped to draft the manuscript. PW participated in the design of the study, collected tick samples, carried out portions of the molecular genetic studies, participated in the sequence

analysis, and helped to draft the manuscript. MY and RF carried out portions of the molecular genetic studies, participated in the sequence alignment, and helped to draft the manuscript. LG, PF, and DK participated in the design of the study, collected tick samples, and helped to draft the manuscript. All authors read and approved the final manuscript.

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CHAPTER 10

GENERAL DISCUSSION

10.1 Introduction

The natural history of *Ehrlichia* species in the USA is poorly understood, with only one recognized vertebrate host and one recognized invertebrate vector for both *E. chaffeensis* and *E. ewingii* (Dumler et al., 2007; Paddock and Yabsley, 2007). The difficulty of working with these agents in a laboratory setting has limited further studies of their natural transmission cycles. Some immunocompromised mice are susceptible to *E. chaffeensis*, but immunocompetent mice are largely resistant to infection; prior to the work reported in this thesis, no tick transmission experiments had been performed (Ganta et al., 2002; Telford and Dawson, 1996; Winslow et al., 1998). Dogs are susceptible to infection with *E. ewingii* (Anderson et al., 1992; Murphy et al., 1998), but laboratory dogs are expensive to maintain and animal welfare regulations in the USA make it difficult to perform infection trials in dogs. Goats were reported to naturally harbor *E. chaffeensis*, but the subsequent experimental inoculation trial of goats was unsuccessful (Dugan et al., 2000; Dugan et al., 2004). This thesis reports a series of experiments designed to develop a laboratory model for natural transmission of ehrlichiae between mammals and ticks. During the course of these experiments, a novel *Ehrlichia* was discovered in the USA; a second series of studies was performed to evaluate the pathogenicity, zoonotic potential, and natural history of this emerging pathogen.

10.2 Laboratory transmission of *E. chaffeensis*

Studies of the natural transmission cycles of *E. chaffeensis* require a working system for producing infected ticks and a competent animal reservoir with which to evaluate cohorts of potentially infectious ticks (e.g., (Levin et al., 2002; Massung et al., 2006)). To this end, efforts were made to develop a laboratory model for tick-host transmission of *E. chaffeensis*. There were no extant tools to assess infection kinetics in animals; Chapter 2 describes the

development of a sensitive, quantitative real-time PCR assay for the measurement of *E. chaffeensis* in animal and tick DNA samples. The assay is species specific, reliably detects as few as 10 genomes per reaction, and yields quantitative data in the range of $10\text{-}10^{10}$ gene copies. This assay provided a standardized method with which to measure the amount of *E. chaffeensis* in experimentally infected animals and also to detect transmission to ticks.

A low-passage strain of *E. chaffeensis* from *in vitro* culture was used to experimentally infect laboratory mice, and the mice were monitored using PCR on blood and tissues, tick acquisition, and serology. Previous workers had shown that immunocompetent mice are resistant to infection but that MHCII deletion mutants and SCID mice were susceptible (Ganta et al., 2002; Winslow et al., 1998), so the study emphasized the susceptibility of immunocompromised mice. A low-passage isolate of *E. chaffeensis* was used because previous work had demonstrated the loss of vector transmission of *E. canis* after numerous *in vitro* passages in mammalian cells (Mathew et al., 1996). Similar to previous reports, immunocompetent wild-type mice were not susceptible to infection, MHCII deletion mutants were susceptible to infection with delayed clearance of ehrlichiae, and mice with no adaptive immunity (Rag1 deletion mutants) developed fatal infections with *E. chaffeensis* (Chapter 3). We extended the previous work by showing that MHCI and iNOS deletion mutants were more susceptible to infection than wild-type mice. Additionally, the use of quantitative PCR revealed that the burden of *E. chaffeensis* was ten-fold higher in the spleen than in the liver and 100-fold higher in the spleen than in circulating blood. We also demonstrated *E. chaffeensis* DNA in nymphal *A. americanum* fed upon infected wild-type mice and Rag1 deletion mutants but not in ticks fed upon other mouse strains; however, none of the ticks maintained the pathogen transstadially. Similar work was done with laboratory gerbils and guinea pigs, neither of which were susceptible to infection with *E. chaffeensis* and neither of which was a competent reservoir for tick transmission (Chapter 4). After these

experiments to develop a small laboratory animal model for tick transmission were unsuccessful, a larger animal with putative susceptibility to *E. chaffeensis* was assessed: domestic goats (Dugan et al., 2000; Dugan et al., 2004). However, although several goats were infested with ticks from an infected cohort, none of the goats developed infections with *E. chaffeensis*, suggesting that goats are not naturally susceptible to tick-transmitted *E. chaffeensis*.

10.3 Characterization of other USA ehrlichiae

Field-collected *A. americanum* from Panola Mountain State Park, Georgia, USA, were used to infest naïve laboratory goats. The ticks were tested and the cohort was found to harbor both *E. chaffeensis* and *E. ewingii*. Three of four goats became infected with *E. ewingii*, and the fourth goat became infected with an unknown pathogen that caused a transient high fever. Further analysis revealed infection with a novel *Ehrlichia*, genetically and antigenically similar to *E. ruminantium* (Chapter 5). The novel agent, referred to as "Panola Mountain *Ehrlichia*" or PME, was confirmed to be present within the cohort of ticks from Panola Mountain. Following the acute febrile reaction, PME caused chronic asymptomatic infection of the goat, lasting for several months, during which the goat was reservoir competent. The pathogen is genetically and antigenically similar to *E. ruminantium* but is distinct from known African and Caribbean strains. The exact taxonomic placement of PME, either as a new species of *Ehrlichia* or as a new strain of *E. ruminantium*, could not be determined due to the close relationships of species within this genus, the lack of a consensus within the field as to the exact definition of a species, and the inability to isolate the agent and perform whole-genome comparisons with *E. ruminantium*.

Additional goats were infected with PME by tick passage, both using *A. americanum* and *A. maculatum*, and the clinical presentation of selected goats infected with *E. ewingii* and

with PME is summarized in a clinical veterinary case report (Chapter 6). Ehrlichiosis and related anaplasmoses are known to cause disease in goats in Europe and Africa, but ehrlichiosis is not a recognized clinical entity in goats in the USA (Bekker et al., 2001; Gokce and Woldehiwet, 1999; Pennisi, 1999; Yunker, 1996). All of the goats infected with *E. ewingii* or PME developed transient acute febrile illness and neutropenia, followed by chronic infections, with intermittent ehrlichemia detected for several months. Acutely and chronically infected goats were competent reservoirs for ehrlichiae, providing laboratory animal models for *E. ewingii* and PME transmission between ticks and animals. From a public health standpoint, goats naturally infected with either species of *Ehrlichia* could transmit the pathogen to ticks in the field, providing a peridomestic source for human exposures to infected ticks.

The discovery of a novel ehrlichiosis vectored by *A. americanum* raised further questions as to the natural history of this pathogen. Studies using naïve white-tailed deer that were infested with field-collected *A. americanum* revealed that these mammals are susceptible to PME and are natural reservoirs for tick transmission (Chapter 7). Three of 87 samples from wild deer were also PCR positive for PME, confirming natural infection of this wildlife reservoir. Three different ehrlichiae in the USA are now known to be vectored by *A. americanum* and to have white-tailed deer as a natural reservoir: *E. chaffeensis* (Ewing et al., 1995; Varela-Stokes, 2007), *E. ewingii* (Varela-Stokes, 2007; Yabsley et al., 2002), and PME (Chapter 7). Given the serological cross-reactivity that is common within the genus *Ehrlichia*, the presence of three co-circulating ehrlichial pathogens will confound any efforts at serological diagnosis within white-tailed deer populations in the USA. Accurate species-specific diagnosis will have to rely on molecular diagnostic methods until specific serologic assays are available.

The zoonotic potential of this agent was discovered (Chapter 8) after a person who visited Panola Mountain State Park reported a severe neck ache following attachment of a nymphal *A. americanum*. Blood samples were tested using PCR and serology, and DNA from PME was detected in acute blood from the patient; clinical response to doxycycline was rapid and complete. Serology using *E. chaffeensis* as the antigen was inconclusive, with low titers reported overall. No other tick-borne pathogens could be identified using either PCR or serology. This finding underscores the similarity of PME to closely related ehrlichiae; *E. chaffeensis* and *E. ewingii* are zoonotic (Dumler et al., 2007), and recent evidence supports the zoonotic potential of African strains of *E. ruminantium* (Allsopp et al., 2005). As with animals, the presence of three closely related co-circulating pathogens within a single population of ticks confounds the ability of clinicians to accurately identify tick-transmitted illnesses in people.

The discovery of a novel pathogen that infects both animals and humans led to further questions as to the origin and distribution of PME in the United States. The risk of undiagnosed human and animal infections is higher if the pathogen has a broad distribution or is present in a large proportion of ticks. To this end, a large-scale survey of 3799 *A. americanum* from the eastern United States, collected both from vegetation and from people, was performed. The pathogen was found in 36 ticks collected throughout the entire geographic range of the tick vector and from people as well as vegetation (Chapter 9). This finding, combined with the presence of genetic diversity within PME in the United States, rules out the possibility of recent introduction into the United States. Overall, though, the prevalence of infection was low, with fewer than 3% of the ticks in any infected cohort harboring DNA from PME. The discovery of the pathogen in several locations, but at low levels, confirms that people and animals in several states are exposed to PME but that clinical cases will be sporadic.

10.4 Conclusion

The work presented in this thesis, collectively, adds to the body of work on the natural history of *Ehrlichia* spp. in the USA. Although attempts to develop a laboratory model for *E. chaffeensis* tick transmission were unsuccessful, the discovery of a novel *Ehrlichia* that infects domestic ruminants and people is highly significant. The ultimate origin of the Panola Mountain *Ehrlichia* (PME) in the USA is unknown, but its broad geographic distribution and the presence of two genetic clades of the pathogen within the country suggests that PME is not new to this continent. An unknown number of undiagnosed febrile illnesses in people and in goats, both historically and recently, may have been caused by this emerging ehrlichiosis.

10.5 Future directions

Attempts to define a laboratory model for *E. chaffeensis* tick transmission were unsuccessful, but the need for a laboratory model remains. Small rodents do not appear to be very susceptible to infection; medium-sized mammals that naturally harbor *A. americanum*, such as opossums, raccoons, and cottontail rabbits (Kollars, Jr., 1993; Sonenshine and Levy, 1971; Zimmerman et al., 1988), would be good candidates for future attempts to establish a laboratory model. For other pathogens, vector ticks have been infected in the laboratory using microinjection techniques and membrane feeding; membrane feeding, using mouse skins, was attempted and was unsuccessful (A. Loftis, unpublished), and microinjection was not attempted due to the laborious nature of the process and small number of ticks that can be generated. Recently, a new system for membrane feeding ixodid ticks has been developed; this system should be assessed for use with *A. americanum* and ehrlichiae.

Clinical diagnosis of ehrlichiosis in both humans and animals has traditionally relied on serology, but the discovery of diverse ehrlichiae, some of which cannot be cultivated for

use as serologic antigens (e.g., *E. ewingii* and PME) and all of which are serologically cross-reactive to various degrees, confounds the diagnosis of ehrlichiosis in modern medicine. Several attempts were made to isolate *E. ewingii* and PME in myeloid and endothelial vertebrate cell lines as well as in an *Ixodes scapularis* derived cell line (ISE6) but all attempts were unsuccessful; other tick cell lines, including lines derived from *Amblyomma* spp., are available (Bell-Sakyi et al., 2007) and should be evaluated. Amplification in a highly susceptible or immunocompromised vertebrate host might increase the probability of successful isolation; re-isolation of *E. chaffeensis* from experimentally infected mice was only successful in Rag1 deletion mutants, when more than 1 x 10^5 ehrlichiae per microgram of DNA were present (Chapter 3). However, passage of a blood stabilate from the initial goat infected with PME to immunodeficient mice did not produce patent infection of the mice (A. Loftis, unpublished), suggesting that more work is needed to identify a highly susceptible vertebrate. Once isolates are ultimately established, genome sequencing would be invaluable for studies of tick-pathogen and tick-host-pathogen interactions (Jongejan et al., 2007) and would also help to resolve phylogenetic relationships within the genus.

Given the difficulty in primary isolation of ehrlichiae, as well as the presence of serological cross-reactivity between isolates, more emphasis should be placed upon the development of species specific serologic assays based upon defined recombinant antigens. Attempts to develop such assays using the immunodominant proteins of *E. ruminantium* have been hampered by the presence of antigenic epitopes that cross-react between species of *Ehrlichia* (e.g., (Bowie et al., 1999; Van Vliet et al., 1995)), but assays based on less dominant antigens show promise in their ability to discriminate between infections with *E. canis* and *E. chaffeensis* (Knowles et al., 2003). Recombinant antigens have been used to develop commercial ELISA assays for *E. canis*, with good specificity (Harrus et al., 2002). Further work using antigens with secondary immunogenicity is warranted, and molecular

biologic techniques can be used to produce recombinant antigens for *E. ewingii* and PME without the need for prior isolation of the organisms.

However, all serology is limited by the delayed nature of the diagnosis; convalescent phase sera are required for diagnosis, 3-4 weeks after the acute presentation of illness (e.g., (Centers for Disease Control and Prevention, 2008)). Decisions about patient treatment must be made long before a specific diagnosis is available. Acute diagnosis using PCR has been shown to be an effective solution (Prince et al., 2007). To this end, a "tick pathogen array" of DNA-targeted assays should be developed for use on acute blood and tissue samples from humans or animals. Specific assays have been developed for *E. chaffeensis*, *E. ewingii*, and PME, as well as for *Rickettsia* and *Borrelia* spp., but these assays are not all compatible with multiplexed diagnosis, and laboratories that offer PCR testing typically run only the specific assays requested by the primary care physician. Efforts should be made to develop a unified diagnostic system for rapid multiplexed analysis of patient samples for diagnosis of tick-borne illness during the acute phase of infection. Furthermore, the system should have the capacity to expand as new, emerging tick-borne agents are discovered. Line blotting technology, multiplexed bead assays, and microarrays are all compatible with these criteria. Multiplexed assays using line blots have already been published for some tick-borne pathogens, and similar assays might be adapted for samples from the United States (Bekker et al., 2002; Matjila et al., 2008; Nijhof et al., 2007).

Finally, the veterinary and economic significance of the novel PME agent needs to be better understood. Prospective studies of people and goats in PME endemic areas who have febrile illnesses of unknown origin and exposure to ticks should be performed to determine the clinical economic significance of this pathogen. Of more significant veterinary importance, the USA has never reported any cases of heartwater disease, in spite of the close geographic proximity to infected loci in the Caribbean (Barré et al., 1987). This finding

might be due to the exceptional efforts put forth by the United States government to prevent the introduction of heartwater, but this seems improbable: the disease has been present in the Caribbean since the late 1800s, federal efforts to eliminate *Amblyomma variegatum* from the Caribbean only date to 1995, and importation of infected ticks on exotic reptiles from Africa does occur (Barré et al., 1987; Barré et al., 1995; Burridge et al., 2000; Faburay et al., 2007). Alternately, one could speculate that the presence of PME in the southeastern USA, an agent that is highly similar to pathogenic *E. ruminantium*, might confer partial or complete immunity to the white-tailed deer and domestic livestock that are physically in closest proximity to the Caribbean islands.

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SUMMARY

Ehrlichia are obligate intracellular pathogens, transmitted by ixodid ticks, of both animals and humans. Three species are recognized as zoonotic agents in the USA (*E. chaffeensis*, *E. ewingii*, and "Panola Mountain *Ehrlichia*" (PME)), but the discovery of new species proceeds more rapidly than the development of laboratory models to study these agents. This thesis examined two aspects of ehrlichioses in the USA: the first part describes a series of experiments designed to develop a laboratory animal model for tick transmission studies with *E. chaffeensis*, and the second part describes a model for *E. ewingii* and the discovery and characterization of PME.

A quantitative real-time PCR assay was developed in order to follow and measure the kinetics of infection with *E. chaffeensis* (Chapter 2). The assay is species specific, sensitive to as few as 10 genomes per reaction, and was validated with both tick and vertebrate animal samples. Using this assay to detect infection, laboratory mice, gerbils, and guinea pigs were experimentally infected with a low passage strain of *E. chaffeensis* from *in vitro* culture and then followed using PCR, serology, and acquisition feeding by the tick vector (*Amblyomma americanum*). Five strains of mice were evaluated, including the wild-type strain (C57BL/6) and four immunocompromised strains (MHCI -/-, MHCII -/-, iNOS -/-, and Rag1 -/-) (Chapter 3). Wild-type mice developed transient infections and transmitted ehrlichiae to some ticks, but the ticks did not maintain the pathogen transstadially. MHCI, MHCII, and iNOS deletion mutants were more susceptible to infection, to varying degrees, but tick transmission was not observed. The Rag1 deletion mutants developed fulminant, fatal infections; as with wild-type mice, some ticks acquired ehrlichiae but failed to maintain them transstadially. Although these studies provided more data on the mechanisms of immunity to *E. chaffeensis*, revealing that MHCII is more critical to clearance of infection than either

MHCI or iNOS, and we documented that the concentration of ehrlichiae in the spleen is approximately 100-fold that in blood, a laboratory reservoir was not identified. Laboratory guinea pigs and gerbils were resistant to infection, and there was no evidence of transmission to ticks (Chapter 4).

In a further attempt to develop a model for *E. chaffeensis*, laboratory goats were infested with field-collected ticks. Goats became infected with *E. ewingii* and with a novel ehrlichiosis, designated PME, but not with *E. chaffeensis*. PME is genetically and antigenically similar to *E. ruminantium* but is not identical, and phylogenetic reconstructions of five separate genes confirmed the close relationship between these taxa (Chapter 5). The goats infected with *E. ewingii* and PME displayed acute febrile syndromes, followed by months of chronic infection; Chapter 6 details the clinicopathological findings and is the first description of illness in goats caused by ehrlichioses in the USA. Laboratory transmission between goats was demonstrated using both *A. americanum* (Chapter 5) and *A. maculatum* (Chapter 6), two species of USA ticks.

The discovery of a novel ehrlichiosis in the USA led to further questions as to its wildlife reservoir, zoonotic potential, and geographic distribution. White-tailed deer (*Odocoileus virginianus*), the reservoir for both *E. chaffeensis* and *E. ewingii*, are naturally infected with PME by field-collected ticks and are also reservoirs for PME (Chapter 7). Deer are naturally parasitized by all three life stages of *A. americanum* and could therefore acquire the pathogen from a mature tick and transmit it to immature life stages, perpetuating PME in a natural cycle. The zoonotic potential of this agent was discovered after a person who acquired a tick from the Panola Mountain field site developed persistent neck pain; molecular testing on acute blood samples detected DNA from PME, and clinical signs rapidly responded to doxycycline (Chapter 8). Serological testing using *E. chaffeensis* as a heterologous antigen was inconclusive, with low antibody titers. Combined, these studies

underscored the potential that PME is established in the USA and its medical and veterinary significance but did not address the origin of PME or its actual geographic distribution. Therefore, a large-scale survey of ticks collected from the entire geographic range of *A. americanum* was undertaken, as reported in Chapter 9. A total of 3799 ticks were tested for PME, and ticks containing DNA from the pathogen were detected from throughout the vector's range. Infected ticks were collected both from the vegetation and, notably, from people, confirming that people are naturally exposed to this pathogen. The conserved *glt*A gene and the variable *map*1 gene were sequenced from the positive ticks, revealing complete identity of *glt*A and two distinct clades of *map*1. These data support the hypothesis that PME is not newly introduced to the USA and has undergone some genetic differentiation.

The work described in this thesis adds significantly to the understanding of zoonotic ehrlichioses from the USA. Although studies to validate a laboratory animal model for transmission of *E. chaffeensis* to ticks were not successful, a goat model for *E. ewingii* was established, and a new ehrlichiosis was discovered that infects goats and people. The further description of natural vectors and reservoirs for PME, combined with the description of its geographic range and *map*1 diversity, provides a model for the natural transmission cycle of this emerging zoonosis. Prior to this work, ehrlichioses endemic to the USA were not recognized as agents of disease in goats, and PME was still undiscovered; it is unknown how many clinical infections with these zoonotic agents may have gone undiagnosed and untreated.

SAMENVATTING

Ehrlichia soorten zijn obligaat intracellulaire bacteriën, die door teken worden overgedragen op mens en dier. In de Verenigde Staten zijn tot dusver drie zoönotische *Ehrlichia* soorten beschreven: *Ehrlichia chaffeensis, E.ewingii* en Panola Mountain *Ehrlichia* (PME). Hierbij is gebleken dat de ontdekking van nieuwe *Ehrlichia* soorten sneller verloopt dan de ontwikkeling van laboratorium modellen om deze agentia te bestuderen. In dit proefschrift zijn twee aspecten van ehrlichiose beschreven: het eerste deel betreft een serie experimenten met als doel te komen tot een diermodel voor transmissie studies voor *Ehrlichia chaffeensis*, in het tweede deel is een model voor *Ehrlichia ewingii* beschreven, alsmede de ontdekking en karakterisering van een nieuwe zoönose veroorzaakt door Panola Mountain *Ehrlichia.*

In hoofdstuk 2 werd een kwantitatieve real-time PCR test ontwikkeld om de infectie dynamiek van *E. chaffeensis* in kaart te kunnen brengen. De test bleek soortspecifiek te zijn, gevoelig tot minimaal 10 kopieën per reactie, en werd gevalideerd met monsters afkomstig van teken en van verschillende diersoorten. De test werd vervolgens toegepast voor detectie van *E. chaffeensis* infecties in verschillende soorten laboratoriumdieren, die werden geïnfecteerd met een lage *in vitro* passage van *E. chaffeensis* en waarop vervolgens *Amblyomma americanum* teken werden gevoed. Er werden vijf muizenstammen geëvalueerd: het wild-type C57BL/6 en vier immuun-gecompromitteerde stammen (MHCI -/-, iNOS -/-, en Rag1 -/-). (Hoofdstuk 3). In de wild-type muizen ontwikkelde zich een milde infectie die weliswaar werd opgenomen door een beperkt aantal teken, maar die waren niet in staat om de infectie transstadieel over te dragen. MHCI, MHCII en iNOS mutante muizenstammen bleken wel gevoeliger voor de infectie, maar niet infectieus voor teken. De Rag1 mutante muizen ontwikkel-

den een fatale infectie, waarbij enkele teken wel in staat waren om de infectie op te nemen, maar ook niet om over te dragen. Verder bleek dat de MHCII muizen beter in staat waren om de infectie te elimineren dan de MHCI of iNOS muizen, waarbij de concentratie van ehrlichiae in the milt ongeveer 100 maal hoger was dan in het bloed. Echter in géén van de muizenstammen bleek *E. chaffeensis* zich te kunnen handhaven, waardoor ze ongeschikt waren als laboratoriummodel. Tenslotte bleken cavia's en woestijnratten eveneens ongeschikt als laboratoriummodel voor *E. chaffeensis* (Hoofdstuk 4).

Vervolgens werd getracht om een model voor *E. chaffeensis* te ontwikkelen in geiten, waarop teken afkomstig uit de vegetatie werden gevoed. De geiten werden echter positief voor *E. ewingii* en bovendien voor een nieuwe *Ehrlichia* soort, Panola Mountain *Ehrlichia* (PME), maar niet voor *E. chaffeensis*. PME bleek genetisch nauw verwant met *Ehrlichia ruminantium* (de veroorzaker van heartwater bij herkauwers in Afrika), op basis van een fylogenetische analyse van 5 verschillende genen (Hoofdstuk 5). De geiten die werden geïnfecteerd met *E. ewingii* en PME ontwikkelden een ziektebeeld dat gepaard ging met koorts in de acute fase, gevolgd door een chronische fase die maanden lang in beslag nam. Het ziektebeeld, dat verder is beschreven in hoofdstuk 6, betreft de eerste beschrijving van ehrlichiose in geiten in the V.S. Bovendien bleek het mogelijk om PME over te dragen tussen geiten met twee Amerikaanse tekensoorten, *Amblyomma americanum* (Hoofdstuk 5) en *Amblyomma maculatum* (Hoofdstuk 6).

De ontdekking van een nieuwe ehrlichiose in de V.S. wierp vervolgens nieuwe vragen op met betrekking tot een reservoir in wild, mogelijke infectie voor de mens, alsmede de geografische verspreiding. Het witstaarthert (*Odocoileus virginianus*), reservoir voor zowel *E. chaffeensis* als *E. ewingii*, bleek ook als reservoir voor PME

te fungeren na besmetting via teken (Hoofdstuk 7). Aangezien alle stadia van *A. americanum* op herten kunnen voeden, is het mogelijk dat de infectie tijdens het voeden van de volwassen teken op het hert worden overgedragen en vervolgens weer wordt opgenomen door de onvolwassen teken, zodat een natuurlijke cyclus van PME in stand kan worden gehouden. Het zoönotische aspect van PME werd ontdekt bij een persoon die, na een tekenbeet in het Panola Mountain gebied, last kreeg van een persisterende nekpijn. PME werd aangetoond door middel van PCR in het bloed van deze patient, die overigens goed reageerde op een behandeling met doxycyclinen (Hoofdstuk 8). Serologisch onderzoek op basis van heteroloog antigeen afkomstig van *E. chaffeensis* kon niet leiden tot een eenduidig resultaat.

Nadat het medisch en veterinair belang van PME was vastgesteld, werd verder onderzoek gericht op de vraag waar Panola Mountain *Ehrlichia* vandaan is gekomen en waar het momenteel voorkomt in de V.S. Hiervoor werd een grootschalig teken survey opgezet binnen het geografische verspreidingsgebied van *A. americanum* (Hoofdstuk 9). In totaal werden 3799 teken getest voor PME, waarbij geïnfecteerde teken werden aangetroffen over het gehele verspreidingsgebied van *A. americanum*. PME werd in teken gevonden uit de vegetatie, maar ook in teken die verwijderd waren van mensen, hetgeen bevestigt dat mensen aan PME zijn blootgesteld. Uit de DNA sequentie analyse van het geconserveerde *glt*A gen en het variabele *map*1 gen uit geïnfecteerde teken bleek dat het *glt*A in alle teken identiek was, terwijl er twee aparte clusters konden worden onderscheiden op basis van het *map*1 gen. Deze gegevens ondersteunen de hypothese dat PME niet recent in the V.S. is geïntroduceerd, maar dat het pathogeen wel enige genetische differentiatie heeft ondergaan.

Het onderzoek zoals beschreven in dit proefschrift heeft bijgedragen aan het begrip omtrent zoönotische ehrlichioses in de V.S. Alhoewel het niet is gelukt om een

laboratorium model op te zetten voor *E. chaffeensis*, kon er wel een model voor *E. ewingii* in geiten worden opgezet en werd bovendien een nieuwe *Ehrlichia* soort ontdekt die pathogeen is voor de mens en de geit. De beschrijving van de natuurlijke vectoren en reservoir gastheren voor PME, alsmede de geografische distributie en genetische diversiteit, heeft geresulteerd in een model waarin de transmissie cyclus van deze nieuwe emerging zoönose verder kan worden bestudeerd.

Curriculum Vitae

Amanda Dawn Loftis was born in Pocatello, Idaho, USA on 9th August 1977. She graduated with a BS in microbiology in 1998 and with a Doctor of Veterinary Medicine from Washington State University, USA, in 2000. She participated in research during this time, with an emphasis on ruminant immunology, and was awarded the John Gorham Veterinary Research Award upon graduation. Between 2000 and 2001, she worked as a clinical veterinarian; a series of cases of tick-borne disease prompted her to return to research, with an emphasis in tick-borne zoonoses. During 2001-2003, she completed an Emerging Infectious Diseases post-doctoral fellowship at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, USA, and began studies in tick-borne ehrlichioses and anaplasmoses. In 2003, she was awarded the American Society for Rickettsiology Travel Award for the presentation, "Infection of immunocompromised mice with *Ehrlichia chaffeensis* and transmission to feeding *Amblyomma americanum* ticks." After this fellowship, she continued to work at the CDC as a Senior Service Fellow in the Rickettsial Zoonoses Branch from 2003-2007. She worked on serological and molecular diagnostic assays for tick-borne rickettsial diseases and continued her work on animal models for *Ehrlichia*, discovering the Panola Mountain *Ehrlichia* agent in 2005. Since 2008, she has been employed at Idaho State University, USA.

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