EHRLICHIA SPP. OF DOGS:

RISK OF INFECTION,

PERSISTENCE OF RICKETTSEMIA,

AND IMPACT ON REINFECTION

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Abstract:

The research presented in this dissertation was conducted to further understanding of *Ehrlichia* spp. infections in dogs in the U.S. and Haiti. In chapter 3, to determine the risk of infection posed to dogs exposed to ticks in a natural setting, dogs were walked through tick habitat in northcentral Oklahoma and monitored clinically, serologically, and molecularly for four months for evidence of tick-borne infection. All 10 dogs were shown by serologic and molecular methods to be infected with *E. ewingii*, and 7 were co-infected with *E. chaffeensis*; however, no dog exhibited clinical signs of ehrlichiosis. Four of these *Ehrlichia* spp. infected dogs were monitored for two years (Chapter 4) following tick-exposure to evaluate long-term *Ehrlichia* spp. infection. In this study, three of four dogs were infected with *E. ewingii* for at least 460 days while infection with *E. chaffeensis* was only detectable through day 55. These data demonstrate that dogs may serve as a reservoir host for maintaining *E. ewingii*. The third study (Chapter 5) was a pilot trial designed to evaluate the impact that previous infection with an *Ehrlichia* spp. has on reinfection through tick feeding and intravenous sub-inoculation. The *Ehrlichia* spp. infected dogs from Chapter 4 were exposed to ticks by walks and monitored for four months for evidence of reinfection with an *Ehrlichia* spp. as previously described in Chapter 3. No reinfections by tick feeding were detected; however, three dogs sub-inoculated with *E. ewingii* and monitored for 46 additional days demonstrated molecular evidence of reinfection with *E. ewingii*. Lastly, Chapter 6 determined the prevalence of *Ehrlichia* spp. infections in 210 dogs from Haiti. Ticks infesting dogs were collected for identification and blood samples were evaluated by serologic and molecular methods for evidence of *Ehrlichia* spp. infections. *Rhipicephalus sanguineus* was the only tick collected from dogs. *Ehrlichia canis* was the only *Ehrlichia* spp. identified in these dogs; antibodies were present in 69 dogs and DNA was detected in 15 of those dogs. In summary, the risk of infection with an *Ehrlichia* spp. is high and infections may persist for years and alter future *Ehrlichia* spp. infections.

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CHAPTER I

INTRODUCTION

Canine and human tick-borne ehrlichioses are increasingly common infections seen in the United States and throughout the world (McQuiston et al., 1999; Parola and Raoult, 2001; Ndip et al., 2005; Rani et al., 2011; Wu et al., 2013). *Ehrlichia chaffeensis* is the most common, potentially fatal tick-borne infection of people in the U.S., while *E. ewingii* is the most common *Ehrlichia* spp. detected in dogs (Little et al., 2010; Beall et al., 2012; CDC, 2014). Although difficult to track precisely, tick populations and the *Ehrlichia* spp. they transmit appear to be on the rise as well (Ogden et al., 2006; Gilbert, 2010; Nicholson et al., 2010; Dahlgren et al., 2011; Adams et al., 2013; CDC, 2013; CDC, 2014). Based on existing survey data, dogs and people in the central U.S., particularly in Oklahoma, Arkansas, and Missouri, have an increased prevalence of *Ehrlichia* spp. infections compared to surrounding states, however many of these surveys lack a thorough patient history regarding travel as well as lifestyle of the person or pet which are important to consider when evaluating the risk for tick-borne infections in animals and people (Little et al., 2010; Dahlgren et al., 2011; Beall et al., 2012).

Diagnosis of infection with *Ehrlichia* spp. can be a challenge (Ismail et al., 2010; Little, 2010; Harrus and Waner, 2011; Allison and Little, 2013). Detection of antibodies

is the most widely used diagnostic approach, although detectable antibodies may remain in circulation for years, even after treatment for *Ehrlichia* spp. infection, leading to clinical confusion regarding resolution of infection (Baneth et al., 1996; Bartsch and Greene, 1996; da Costa et al., 2005; Cardenas et al., 2007; Little et al., 2010). Molecular methods such as PCR are also utilized to detect DNA present during an active infection, but false negative PCR results may occur if inhib itors are present in the sample or if the DNA is not properly handled and stored (Beutler et al., 1990; Al-Soud and Radstrom, 2001; Schrader et al., 2012). Isolation in cell culture and morulae visualization during examination of a cytologic preparation are also often employed in an effort to diagnose infection with *Ehrlichia* spp., but these diagnostic methods have their limitations as well, especially if the level of rickettsemia in the sample is low (Nyindo et al., 1971; Dawson et al., 1991; Wen et al., 1995; Popov et al., 1998; Chapman et al., 2006; Dumler et al., 2007; Reeves et al., 2008). Incorporating the use of multiple diagnostic modalities in an infected dog or person should enhance accurate detection of infection with *Ehrlichia* spp.

Although relatively common infections, there is much to be learned about the *Ehrlichia* spp. that infect dogs and people, especially regarding longevity of infection. Asymptomatic dogs have been shown to harbor *E. chaffeensis* and *E. ewingii* for months after experimental infection, and DNA of *E. ewingii* has been detected in naturally infected asymptomatic dogs (Breitschwerdt et al., 1998a; Liddell et al., 2003; Zhang et al., 2003; Yabsley et al., 2011). To date, no publications describe the persistence of infection with *E. chaffeensis* or *E. ewingii* in dogs when infection was acquired through natural tick exposure and feeding. Evaluation of dogs as a competent reservoir host for

Ehrlichia spp. would provide important information regarding how infected dogs should be managed to help prevent transmission of *Ehrlichia* spp. to naïve ticks.

Prevention of infection to dogs and people with *Ehrlichia* spp. also warrants additional exploration. Currently there is no vaccine available to prevent infection with any of the canine or human *Ehrlichia* spp. Acaricide use and avoidance of ticks are the mainstays of preventing infection (Stafford, 2007; Cisak et al., 2012; Domingos et al., 2013; CDC, 2014). Experimental studies with *Ehrlichia* spp. have shown that animals previously infected have fewer clinical signs or decreased bacterial loads when challenged subsequently with the same *Ehrlichia* spp. compared to cohort animals without previous infection (Buhles et al., 1974; Breitschwerdt et al., 1998b; Yabsley et al., 2011). Determination of the potential protection afforded subsequent to natural infection with different *Ehrlichia* spp. could provide insight into future vaccination prospects.

To address these deficits in our understanding of canine ehrlichiosis, a series of experiments were undertaken:

1) Determine the risk of infection to dogs posed by natural exposure to lone star ticks (*Amblyomma americanum*) and identify the *Ehrlichia* species involved.

Infection with *Ehrlichia* spp. is common in dogs from the southeastern U.S., an area where *Amblyomma americanum* ticks are also commonly identified infesting dogs (Bowman et al., 2009; Beall et al., 2012; CDC, 2013; Barrett et al., 2014; Little et al., 2014). Our study design mimicked how dogs become infested with ticks in a natural setting in order to determine the risk of infection with *Ehrlichia* spp. Dogs were walked

weekly for seven weeks to provide exposure to ticks in nature. During and after the tick-exposure period, dogs were examined for presence of ticks and monitored clinically as well as by hematologic, serologic, and molecular methods to detect *Ehrlichia* spp. infections. *Amblyomma americanum* ticks were present on 10/10 dogs after every tick-exposure opportunity and were the predominant tick (90.5%) seen infesting dogs (Barrett et al., 2014). Over the four-month study period, dogs developed antibodies reactive to *E. chaffeensis* (IFA: 10/10; VLPT: 7/10), *E. ewingii* (p28: 9/10), or both *Ehrlichia* spp. (VLPT + p28: 6/10). Detection of DNA of *E. chaffeensis* and *E. ewingii* were detected in 7/10 and 10/10 dogs, respectively, yet no dog developed clinical signs indicative of a tick-borne infection. These data show that dogs have a high-risk—100% of dogs in this study— for acquiring an *Ehrlichia* spp. infection, especially in locations where *A. americanum* ticks are common.

2) Characterize the persistence of infection with *Ehrlichia chaffeensis* and *E. ewingii* in dogs after natural tick exposure.

Ehrlichia chaffeensis and *E. ewingii* infections have been detected in both experimentally and naturally infected dogs in the absence of clinical signs, and there is evidence to show that dogs can maintain active infection for months with either of these *Ehrlichia* spp. following experimental inoculation (Breitschwerdt et al., 1998a; Liddell et al., 2003; Zhang et al., 2003; Little et al., 2010; Yabsley et al., 2011). To evaluate the persistence of infection, dogs (n=4) from Study 1 were maintained in a tick-free environment for two years and monitored by serology and PCR for evidence of *E. chaffeensis* or *E. ewingii* infection. Dogs produced antibodies reactive to *E. chaffeensis*

(IFA: 4/4; VLPT: 2/4) and *E. ewingii* (p28: 4/4), with antibodies persisting in 3/4 dogs for the entire two-year study period. Detection of DNA was also observed in 4/4 dogs: *E. chaffeensis* in 2/4 and *E. ewingii* in 4/4. *Ehrlichia chaffeensis* DNA was not detected in any dog after day 55 while *E. ewingii* DNA was detected in 3/4 dogs through day 460 with one dog remaining *E. ewingii* PCR-positive at the end of the two-year study. Our results provide evidence of long-term infection with *E. ewingii* in asymptomatic dogs with *E. ewingii* following natural tick-exposure.

3) Conduct a pilot trial to evaluate the effect of previous natural infection with *Ehrlichia chaffeensis* or *E. ewingii* on challenge through tick feeding and intravenous inoculation.

To date, there is no vaccine available for the prevention of *Ehrlichia* spp. infection. Studies have demonstrated that animals previously infected with an *Ehrlichia* spp. show less severe clinical signs and shorter durations of rickettsemia when later challenged with the same *Ehrlichia* spp. (Breitschwerdt et al., 1998b; Yabsley et al., 2011). More recent work involving attempted peptide vaccinations in mice and dogs have shown that even though prevention of reinfection is not provided, disease is less severe and rickettsemia is more short-lived in the vaccinated animals (Croquet-Valdes et al., 2011; Thomas et al., 2011; Rudoler et al., 2012).

To determine if animals previously infected with *E. chaffeensis* or *E. ewingii* through natural tick feeding could be reinfected, the four dogs from Study 2 were re-exposed to tick habitat weekly for seven weeks and monitored clinically, hematologically, serologically, and molecularly for four months for evidence of

reinfection with an *Ehrlichia* spp. None of the dogs developed clinical signs, hematologic abnormalities, or increases in antibody titer for four months following tick-exposure. Additionally, detection of *E. chaffeensis* or *E. ewingii* DNA was absent in 3/4 dogs; the only PCR-positive dog was *E. ewingii* PCR-positive prior to tick-exposure and remained positive throughout the four month period. The three dogs with no PCR evidence of *Ehrlichia* spp. infection following re-exposure to ticks were intravenously sub-inoculated with *E. ewingii* and monitored by the same methods for an additional 46 days. Clinical signs and hematologic abnormalities were absent in all dogs (3/3), however, a three-fold or more increase in antibody titer (*E. chaffeensis* IFA) was detected in 2/3 dogs and DNA of *E. ewingii* was detected in 3/3 dogs.

Data from this study indicate that dogs may be protected from reinfection with *Ehrlichia* spp. if exposed by the same route of infection, tick feeding. Challenge by a different route of infection, intravenous sub-inoculation, did result in reinfection; however, durations of rickettsemia were brief and clinical signs were absent which is consistent with previously reported results (Yabsley et al., 2011).

4) Determine the prevalence and identity of *Ehrlichia* spp. in dogs from Haiti, an area where lone star ticks are not present.

Infection with *Ehrlichia canis* and other vector-borne pathogens has been identified in dogs from a number of Caribbean islands where *Rhipicephalus sanguineus* is the predominant tick, yet information regarding these infections in dogs from Haiti is lacking (Bool and Sutmoller; 1957; Huxsoll et al., 1970; Georges et al., 2008; L'Hostis et al., 1998; Yabsley et al., 2008; Asgarali et al., 2012; Kelly et al., 2013; Loftis et al., 2013;

Qurollo et al., 2014). To determine the species of ticks infesting dogs and the prevalence of vector-borne pathogens in dogs from Haiti, ticks and whole blood samples were collected from 210 dogs throughout Haiti. Ticks were detected on 28/210 dogs; all collected ticks were morphologically identified as *Rhipicephalus sanguineus* sensu lato. Whole blood revealed that approximately one-third (69/210) of dogs had antibodies reactivdee to *E. canis* or *E. ewingii* as detected by a commercially-available antibody assay. Furthermore, 15 of the antibody-positive dogs also had circulating DNA of *E. canis*. Many of the *E. canis*-positive dogs (39/210) had evidence of a co-infection with one or more vector-borne pathogens. *Ehrlichia chaffeensis* and *E. ewingii* were not detected in any dog from Haiti. Our results are the first to document the prevalence of *R. sanguineus* infestations and *E. canis* infections in dogs from Haiti.

SUMMARY

These studies provide information regarding the high-risk of infection with an *Ehrlichia* spp. in dogs in Oklahoma following natural tick-exposure, the ability of *E. ewingii* to persist long-term in dogs following a naturally acquired infection through tick feeding, the potential for a previous infection with an *Ehrlichia* spp. to protect against reinfection by the same *Ehrlichia* spp. through the same route of infection, and the prevalence of infestation with *R. sanguineus* and infection with *E. canis* and other vector-borne pathogens in dogs from Haiti. Taken together, these data provide for a greater understanding of *Ehrlichia* spp. infections in dogs. First, the risk of infection to dogs in the absence of acaricide use is high. Furthermore, the diagnosis of *Ehrlichia* spp. infections in naturally infected, asymptomatic dogs provides insight into how long dogs

may remain infected with *E. chaffeensis* and *E. ewingii* following natural infection. Additionally, our data indicate that a previous *Ehrlichia* spp. infection in a dog may protect that dog against reinfection with the same *Ehrlichia* spp. through the same route of infection, thus providing helpful data to be used in future vaccine development. Lastly, our documentation of *E. canis* infections in dogs from Haiti along with reports of *E. canis* infections from other Caribbean islands suggests that animal and human health would benefit from the establishment and use of vector-control programs.

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CHAPTER II

REVIEW OF LITERATURE

EHRLICHIA SPP. OF PEOPLE AND DOGS

Members of the genus *Ehrlichia* are alpha-proteobacteria within the order Rickettsiales and the family Anaplasmataceae. *Ehrlichia* spp. are gram-negative staining intracellular coccobacilli that exist in either reticulate or dense-core cell forms (Rikihisa, 1991; Dumler et al., 2001). Reticulate cells divide by binary fission. The dense-core cell is thought to be a dormant stage; however division has been observed, although to a lesser extent than what occurs with reticulate cells (Popov et al., 1995; Rar and Golovljova, 2011). The genome of *Ehrlichia* spp. ranges between 1.18 – 1.32 Mb and is contained on one circular chromosome (Dunning Hotopp et al., 2006; Mavromatis et al., 2006). The genome of *Ehrlichia* spp., like many intracellular pathogens, is reduced compared to extracellular bacteria, and the majority of the retained genes typically code for vitamin, co-factor, nucleotide, and protein biosynthesis (Andersson and Kurland, 1998; Dunning Hotopp et al., 2006; Rar and Golovljova, 2011). In the vertebrate host, the organisms replicate within intracytoplasmic parasitophorous vacuoles and are visualized as inclusions termed morulae. Replication site preference differs between the *Ehrlichia* spp., but occurs within leukocytes, including neutrophils, monocytes, and macrophages,

or within endothelial cells (Cowdry, 1925; Rikihisa, 1991; Popov et al., 1995; Dumler et al., 2001). Although direct transmission can occur through infected blood products, tick vectors are the primary means of transmission to vertebrates (Ewing, 1969; Groves et al., 1975; Ristic and Huxsoll, 1984; Regan et al., 2013). *Ehrlichia* spp. are maintained transstadially and intrastadially in the tick host; there is no evidence of transovarial transmission as is seen with some other tick-borne disease agents (Groves et al., 1975; Anziani et al., 1990; Ewing et al., 1995; Johnson et al., 1998; Long et al., 2003; Bremer et al., 2005). However, infected vertebrate reservoirs are believed to maintain the organisms in the environment.

Ehrlichia canis

Many *Ehrlichia* spp. are capable of infecting dogs which may lead to the development of clinical disease. *Ehrlichia canis*, the agent responsible for canine monocytic ehrlichiosis, also known as tropical canine pancytopenia, was described in 1935 as the first *Ehrlichia* spp. in a dog and was later detected in dogs from the United States as early as 1962 (Donatien and Lestoquard, 1935; Ewing and Buckner, 1965b). Clinical signs associated with *E. canis* infection in dogs include anorexia, lethargy, fever, epistaxis, and hemorrhages visible in the mucous membranes; severely affected dogs may die from this infection (Wilkins et al., 1967; Huxsoll et al., 1969, 1970a,b; Walker et al., 1970; Huxsoll et al., 1972). *Ehrlichia canis* is transmitted between dogs by *Rhipicephalus sanguineus* ticks, but *Dermacentor variabilis* has also been shown to be a competent vector (Groves et al., 1975; Johnson et al., 1998). Rickettsial agents, including *Ehrlichia* sp., are transmitted more quickly, within 4 – 24 hours, when compared to other tick-borne

disease agents (Nicholson et al., 2010). Recent work described transmission of *E. canis* to dogs in as little as 3 hours after initiation of tick feeding (Fourie et al., 2013).

Reservoir hosts for *E. canis* include domestic and wild canids (Ewing et al., 1964; Amyx and Huxsoll, 1973). Dogs surviving acute infection can circulate the agent for periods exceeding five years and serve as a source of infection to ticks (Ewing and Buckner, 1965a; Groves et al., 1975; Harrus et al., 1998). *Ehrlichia canis* is considered a low-risk zoonosis, but people have been shown to have antibody titers to *E. canis*, and there are case reports of clinical illness in humans from Venezuela that have 16S rRNA sequences matching sequences of *E. canis* obtained from local canines (Perez et al., 2006; Vieira et al., 2013).

Ehrlichia chaffeensis

Perhaps the most important and pathogenic *Ehrlichia* spp. of humans to date is *E. chaffeensis*, the causative agent of human monocytic ehrlichiosis, which was first documented in people in 1986 (Maeda et al., 1987; Anderson et al., 1991; Paddock and Childs, 2003; Dahlgren et al., 2011). Not long after its description in people, *E. chaffeensis* was experimentally transmitted to dogs as well as detected in naturally infected dogs and wild canids (Dawson and Ewing, 1992; Dawson et al., 1996; Kocan et al., 2000; Zhang et al., 2003). Clinical abnormalities in dogs are milder than those associated with *E. canis*; signs range from inapparent illness to mild fever or thrombocytopenia (Dawson and Ewing, 1992; Zhang et al., 2003). A variety of other vertebrate species in the U.S., including cattle, goats, raccoons, lemurs, red fox, and white-tailed deer, have been reported to be naturally or experimentally susceptible to infection with *E. chaffeensis* with clinical illness ranging from imperceptible to death in

certain experimental studies (Davidson et al., 1999; Dugan et al., 2000; Williams et al., 2002; de los Santos et al., 2007; de los Santos et al., 2008; Yabsley et al., 2008c). Humans often present with flu-like symptoms including fever, myalgia, headache, and joint pain; nausea and a rash are sometimes present as well (Anderson et al., 1991; Paddock and Childs, 2003).

The primary tick vector for *E. chaffeensis* is *Amblyomma americanum*, which has been experimentally shown to transmit *E. chaffeensis* to deer but not to dogs (Ewing et al., 1995; Long et al., 2003; Varela-Stokes, 2007). All life stages of this tick are found primarily on white-tailed deer but can be found on dogs, people, and other hosts in the southeastern U.S. (Bishopp and Trembley, 1945; Koch, 1982; Felz et al., 1996; Merten and Durden, 2000; Paddock and Yabsley, 2007). The geographical range of this tick is expanding to include areas along the east coast and into the upper Midwest (Keirans and Lacombe, 1998; Walker et al., 1998; Ijdo et al., 2000; Merten and Durden, 2000). Other potential vectors of *E. chaffeensis* include *D. variabilis* and *R. sanguineus*, as *E. chaffeensis* DNA has been detected in each of these tick species (Steiert and Gilfoy, 2002; Ndip et al., 2007; Fritzen et al., 2011, Stoffel et al., 2014). White-tailed deer serve as the primary reservoir host for *E. chaffeensis* due to the vast number of PCR positive naturally infected animals identified in the southeastern U.S., their ability to transmit infection to naïve tick vectors, their lack of clinical illness and hematologic abnormalities, and their ability to remain rickettsemic and sequester bacteria in their lymph nodes and bone marrow long-term (Ewing et al., 1995; Little et al., 1997; Davidson et al., 2001; Arens et al., 2003; Yabsley et al., 2003a,b; Yabsley, 2010). Dogs may also serve as a natural host given their mild reaction to infection and the potential to

circulate *E. chaffeensis* for 2 – 4 months post infection (Dawson et al., 1996; Zhang et al., 2003).

Ehrlichia ewingii

Ehrlichia ewingii also infects dogs and people; however, neutrophils are the target vertebrate host cell. First thought to be a granulocytic form of *E. canis*, *E. ewingii* was later recognized in 1992 as a new agent of canine ehrlichiosis (Ewing et al., 1971; Anderson et al., 1992a). Disease in dogs varies from clinically absent to an acute febrile illness with lethargy and anorexia; lameness, joint effusion, and polyarthritis also can be seen during infection with *E. ewingii* (Cowell et al., 1988; Anderson et al., 1992a; Goodman et al., 2003, Yabsley et al., 2011). Dogs may also, albeit rarely, exhibit central nervous system signs and hemorrhage (Goldman et al., 1998; Goodman et al., 2003). People can also be infected with *E. ewingii*; clinical illness presents similar to that caused by *E. chaffeensis* with fever and flu-like symptoms (Buller et al., 1999; Salinas et al., 2010). The primary tick vector for *E. ewingii* is also *A. americanum* (Anziani et al, 1990). Although DNA of *E. ewingii* has been detected in *D. variabilis* and *R. sanguineus*, experimental transmission by these ticks has not been confirmed (Steiert and Gilfoy, 2002; Ndip et al., 2007; Lee et al., 2014). White-tailed deer are the proposed reservoir host, but dogs may play a role as well (Murphy et al., 1998; Steiert and Gilfoy, 2002; Yabsley et al., 2002; Liddell et al., 2003; Ndip et al., 2007).

Panola Mountain *Ehrlichia* species

In 2006, a new *Ehrlichia* species was described in *A. americanum* ticks collected from Panola Mountain State Park, Georgia. Feeding of these ticks on a goat resulted in fever and mild clinical illness, however, the genetic sequence isolated from the infected

goat was genetically distinct from other *Ehrlichia* spp. found in the U.S. The sequence most closely resembled that of *E. ruminantium,* the causative agent of heartwater disease in ruminants outside of the U.S. (Loftis et al., 2006). To date, infection with the Panola Mountain *Ehrlichia* species (PME) has been reported in people, dogs, goats, and white-tailed deer (Loftis et al., 2006; Loftis et al., 2008b; Reeves et al., 2008; Yabsley et al., 2008a; Qurollo et al., 2013). *Amblyomma americanum* is the primary vector of PME which has been found in *A. americanum* ticks collected from 10 different states, including states in the northeastern U.S. where this tick has more recently been reported (Loftis et al., 2006; Loftis et al., 2008a; Schulze et al., 2011). *Amblyomma maculatum,* a competent vector for the related *E. ruminantium,* was shown experimentally to be capable of transmitting PME to a naïve goat (Mahan et al., 2000; Loftis et al., 2008b). White-tailed deer are a likely wildlife reservoir host for the Panola Mountain *Ehrlichia* species (Yabsley et al., 2008a).

Ehrlichia muris and *E. muris*-like Agent

The most recent *Ehrlichia* spp. to be described in dogs from the U.S. is *E. muris* (Hegarty et al., 2012). This agent was first described in 1995 in a mouse from Japan followed by a report in mice and humans from Russia (Wen et al., 1995; Nefedova et al., 2008). A review of data collected from veterinary clinics in the U.S. from 2001 – 2007 revealed that a higher than expected number of animals in Wisconsin and Minnesota had antibodies to an *Ehrlichia* spp. (Bowman et al., 2009). An earlier survey in Minnesota (2004 – 2005) had documented 11 of 731 naturally exposed pet dogs had *Ehrlichia canis* antibodies as detected by the SNAP® 4Dx® (Beall et al., 2008). Because these states are north of the historic geographic range of *A. americanum*, and *R. sanguineus* ticks are less

common in colder climates, infection with a novel *Ehrlichia* spp. was suspected (Bishopp and Trembley, 1945; Merten and Durden, 2000; Childs and Paddock, 2003). Molecular techniques detected *E. muris* DNA in a dog from Minnesota shortly after *E. muris*-like DNA was detected in people from the same region (Pritt et al., 2011; Hegarty et al., 2012).

The *E. muris* infected dog exhibited a stiff gait with painful elbow joints, a fever, and decreased activity. Doxycycline therapy was instituted for three weeks, but the dog's clinical signs recurred three months later. Additional laboratory diagnostics revealed mild thrombocytopenia and amplification of a sequence identical to that in a mouse from Japan (Hegarty et al., 2012). In people, disease due to the *E. muris*-like agent mimics that of the other *Ehrlichia* spp. infections, including fever, lethargy, headache, and thrombocytopenia (Pritt et al., 2011).

Ixodes scapularis is the proposed vector of *E. muris* and the *E. muris*-like agent in the U.S. (Pritt et al., 2011; Hegarty et al., 2012). *Ixodes scapularis* ticks are prevalent in the upper Midwest while other known vectors of *Ehrlichia* spp. are scarce or absent (Schrock, 1982; Pritt et al., 2011). Evaluation of 760 *I. scapularis* collected from Wisconsin from 1992 – 1997 revealed that DNA of *E. muris* was present in 1% of ticks (Telford et al., 2011). *Ehrlichia muris*-like agent has also been detected in *I. scapularis*; co-infections of EML with the known *I. scapularis* transmitted pathogens *Borrelia burgdorferi*, *Babesia microti*, and *Anaplasma phagocytophilum* were detected in ticks collected from military personnel from Minnesota and Wisconsin (Stromdahl et al., 2014). Additionally, the dog in the above case-report had antibodies to *Anaplasma phagocytophilum*, a rickettsial pathogen known to be vectored by *I. scapularis*, indicating

exposure of this dog to *I. scapularis* ticks (Pusterla et al., 2002; Hegarty et al., 2012). Reservoir hosts for *E. muris* and EML remain unknown. Recent work has shown DNA of EML in white-footed mice collected from Minnesota and Wisconsin; EML DNA was absent in white-tailed deer tested from Minnesota (Castillo et al., 2015).

DIAGNOSTIC METHODS

Blood Smear Evaluation

Definitive diagnosis of ehrlichiosis in people or animals can be difficult, and often a variety of diagnostic assays are used (Little, 2010; Allison and Little, 2013). A positive diagnosis can be achieved by direct microscopic visualization of morulae within the cytoplasm of an infected host cell on stained cytologic preparations, but the sensitivity is poor, especially if the patient is no longer in the acute stage of infection (Mylonakis et al., 2004b; Chapman et al., 2006; Nicholson et al., 2010; Allison and Little, 2013). Sensitivity of direct microscopic visualization is influenced somewhat by the cell type infected and the sample type examined. For monocytic morulae (*E. canis, E. chaffeensis,* and *E. muris*), examination of peripheral blood smears is considered less sensitive than examination of buffy coat smears or lymph node aspirates (Mylonakis et al., 2003). Direct visualization of granulocytic morulae (*E. ewingii*) is often more readily achieved due, in part, to the relative number of neutrophils in circulation (Goodman et al., 2003; Allison and Little, 2013). Cytologic evaluation is also not specific for certain *Ehrlichia* spp., as the monocytic species (*E. canis, E. chaffeensis,* and *E. muris*) morphologically resemble one another (Wen et al., 1995; Popov et al., 1998); *Ehrlichia ewingii* is also morphologically indistinguishable from the related *A. phagocytophilum*, which also

forms morulae within the cytoplasm of granulocytes (Chapman et al., 2006). One benefit of cytologic evaluation is that it allows potential visualization of other blood related pathogens, as co-infections with vector-borne disease agents do occur in nature (Ewing and Buckner, 1965b; Breitschwerdt et al., 1998a; Kordick et al., 1999; Mylonakis et al., 2004a; Gal et al., 2007; Yabsley et al., 2008b; Al Izzi et al., 2013).

Cell Culture

Another means of diagnosing infection with *Ehrlichia* spp. is to attempt to culture the organism *in vitro*, however, isolation of *Ehrlichia* spp. in cell culture is limited by the level of rickettsemia in the test sample as well as availability of viable cell lines in which the different *Ehrlichia* spp. can multiply (Nyindo et al., 1971; Dawson et al., 1991; Wen et al., 1995; Dumler et al., 2007; Reeves et al., 2008). This diagnostic approach has only been successful for the monocytic species, *E. canis, E. chaffeensis*, and *E. muris* (Nyindo et al., 1971; Dawson et al., 1991; Wen et al., 1995). There is one report in the literature of PCR amplification of *E. ewingii* from cell culture, however all attempts to isolate *E. ewingii* or Panola Mountain *Ehrlichia* sp. in continuous culture have failed thus far (Dumler et al., 2007; Reeves et al., 2008; Breitschwerdt et al., 2014).

Serology

Serologic approaches are often utilized to identify antibodies reactive to *Ehrlichia* spp. Different methods have been developed, ranging in specificity from the cross-reactive indirect fluorescence antibody (IFA) test to species-specific enzyme-linked immunosorbent assays (ELISA) (Ristic et al., 1972; Ohashi et al., 1998b; Sumner et al., 1999; Cardenas et al., 2007; Daniluk et al., 2007; O'Connor et al., 2010). Some serologic diagnostic methods have been made available in patient-side assay format, while others

require the sample to be sent to a reference or a research laboratory for diagnosis (O'Connor et al., 2002; O'Connor et al., 2006; Abaxis Veterinary Reference Laboratories, 2015; Antech Diagnostics, 2015; IDEXX Laboratories, 2015). Cross-reactivity between different *Ehrlichia* spp. is a major drawback when evaluating some serologic test results, as the antibodies developed to one species of *Ehrlichia* may cross-react and bind to the antigen of a different *Ehrlichia* spp. in non-specific assays (Ohashi et al., 1998a; Waner et al., 2001). A serologic test may also fail to detect antibodies in the acute stage of infection before detectable levels are produced (Prince et al., 2007). A detectable antibody (IgG) response first develops by two to four weeks post-infection, but it has been shown that antibodies (IgG) can be detected in dog sera by *E. canis* IFA in as little as two days post-inoculation of *E. canis* infected macrophages (Iqbal et al., 1994; Cardenas et al., 2007). Another disadvantage of antibody-based tests is that they do not differentiate between current and previous infection, as antibodies are readily detected in clinically healthy persons and dogs (Baneth et al., 1996; da Costa et al., 2005; Little et al., 2010). Persistently seropositive animals have been reported in the literature. Dogs were followed post-treatment for *Ehrlichia* spp. infection and 27 of 39 animals with an initial titer of 1:2,560 or greater remained seropositive for up to a year post-treatment (Bartsch and Greene, 1996). Additionally, 12 dogs were monitored post-treatment, and all dogs had a detectable *Ehrlichia* spp. titer at the last serologic evaluation 6 – 12 months following treatment (Breitschwerdt et al., 1998a). Chronic infection despite antibiotic therapy and reinfection are potential reasons for the persistent elevated titers as six of the 12 dogs were PCR or tissue culture positive for *Ehrlichia* spp. following treatment (Breitschwerdt et al., 1998a; McClure et al., 2010).

Polymerase Chain Reaction

Molecular methods are also available for diagnosing ehrlichial infection. Polymerase chain reaction (PCR) assays, including nested and real-time, are sensitive and specific methods used to amplify circulating rickettsial DNA. Targets for *Ehrlichia* spp. PCR include ribosomal DNA (16S rDNA), disulfide bond formation protein gene (dsb), heat shock protein (GroESL), citrate synthase (gltA), and a variable length PCR target (VLPT) (Anderson et al., 1992b; Sumner et al., 1999; Felek et al., 2001; Inokuma et al., 2001; Loftis et al., 2003; Bell and Patel, 2005; Doyle et al., 2005). When utilizing proper positive and negative controls and in the absence of contamination, interpretation of a positive PCR is straightforward if specific primers were used; sequence confirmation can be performed on the amplicons to confirm accurate diagnosis (Anderson et al., 1992b; Iqbal et al., 1994; Allison and Little, 2013). Identification of an infection prior to antibody formation and differentiation between previous or current infection in a seropositive host are two advantages of DNA detection (Iqbal et al., 1994; Harrus and Waner, 2011). Another advantage is the capability of detecting multiple tick-borne pathogens through the utilization of multiplex PCR assays (Sirigireddy and Ganta, 2005; Kledmanee et al., 2009; Peleg et al., 2010; Rufino et al., 2013; Killmaster et al., 2014). Conversely, interpretation of a negative PCR result is more difficult and may indicate that there wasn't sufficient amplifiable DNA present in the sample type collected, that inhibitors were present in the clinical sample rendering the assay falsely negative, or that the animal is truly negative (Beutler et al., 1990; Al-Soud and Radstrom, 2001; Schrader et al., 2012).

TREATMENT

Antibiotics

The treatment of choice for ehrlichial infections is doxycycline at 10 mg/kg orally once a day for 28 days (Neer et al., 2002). Other antibiotic options for the treatment of ehrlichiosis include rifampin, imidocarb, and other tetracyclines (Price and Dolan, 1980; Matthewman et al., 1994; Sainz et al., 2000; Schaefer et al., 2008). Improvement in clinical signs is usually seen within the first week after treatment has been initiated (Neer et al., 2002).

Treatment Does Not Guarantee Clearance of Infection

Without treatment, dogs can maintain infection long-term with *Ehrlichia canis* and potentially other *Ehrlichia* spp. and serve as a potential source of infection; however, appropriate treatment with antibiotics does not ensure clearance of infection (Harrus et al., 1998; Zhang et al., 2003; Yabsley et al., 2011). *Ehrlichia canis* has been isolated from blood and tissues of dogs that were experimentally infected by intravenous inoculation or tick feeding and subsequently treated with doxycycline (Iqbal and Rikihisa, 1994; Schaefer et al., 2007). DNA of *E. canis* has also been detected in naturally infected dogs after treatment with doxycycline (Wen et al., 1997). Furthermore, nymphal and adult ticks fed on *E. canis* infected dogs post-treatment became infected, and molted adults were able to transmit *E. canis* to naïve dogs (Schaefer et al., 2007; McClure et al., 2010). Elimination of *E. canis* infection was seen, however, in dogs experimentally infected by intravenous inoculation after a 14 day regimen of doxycycline (Breitschwerdt et al., 1998b). Route of infection, duration of antibiotic therapy, and stage of infection when treatment is implemented could all factor in to these conflicting data.

PREVALENCE OF *EHRLICHIA* SPP. INFECTIONS

The prevalence of *Ehrlichia* spp. infection in dogs, people, and ticks has been established in different states and regions of the U.S. and in some Caribbean islands. Prevalence is often determined in a host by using one or more of the previously discussed diagnostic methods.

Prevalence in Dogs

Two large surveys testing more than 7 million dogs throughout the U.S. for the presence of antibody to *Ehrlichia* spp. were conducted from 2001 – 2007 and 2010 – 2012 using a combination of patient-side ELISAs (SNAP® 3Dx®, 4Dx®, and 4Dx® Plus; IDEXX Laboratories, Inc., Westbrook, Maine) designed to detect antibodies to *E. canis, E. chaffeensis*, and *E. ewingii* (Bowman et al., 2009; Little et al., 2014). Dogs in the Southeast had the highest prevalence of *Ehrlichia* spp. antibodies (1.3% [3,865/290,636] – 3.2% [65,191/2,057,984]); regional *Ehrlichia* spp. antibody prevalence for the Northeast, Midwest, and West were 0.3% (777/271,070) – 0.9% (24,011/2,806,112), 0.4% (1,354/373,090) – 1.0% (17,337/1,720,168), and 0.6% (299/47,540) – 1.3% (5,134/410,419), respectively (Table 1). The state with the highest seroprevalence of *Ehrlichia* spp. antibodies from both surveys was Arkansas (Table 1). Two additional nationwide surveys testing over 15,000 dogs were conducted utilizing a species-specific peptide assay (SNAP® M-A, IDEXX Laboratories, Inc., Westbrook, Maine) for the detection of antibodies to *E. canis, E. chaffeensis*, and *E. ewingii* (Beall et al., 2012; Qurollo et al., 2014). Antibodies in dogs to *E. ewingii* were most common in both surveys (3.8% [251/6,582] – 5.1% [#439/8,662]) (Table 1).

Presence of *Ehrlichia* spp. infections in dogs has also been explored in local or regional studies (Table 1); states with additional local data on *Ehrlichia* spp. antibody presence in dogs include Arizona, Arkansas, Connecticut, Louisiana, Missouri, New York, North Carolina, Oklahoma, Tennessee, and Virginia (Stephenson & Ristic, 1978; Hoskins et al., 1988; Rodgers et al., 1989; Stockham et al., 1992; Magnarelli & Anderson, 1993; Dawson et al., 1996; Murphy et al., 1998; Kordick et al., 1999; Suksawat et al., 2000; Liddell et al., 2003; Seaman et al., 2004; Diniz et al., 2010; Little et al., 2010). A number of studies have also evaluated the prevalence of canine infection with *E. canis* from different Caribbean islands where *R. sanguineus* is common; *Ehrlichia* spp. antibodies have been detected in 24.1% (41/170) – 47.6% (89/187) of dogs (Table 2) (Georges et al., 2008; Hoff et al., 2008; Yabsley et al., 2008b; Asgarali et al., 2012; Kelly et al., 2013; Loftis et al., 2013; Qurollo et al., 2014).

In addition to seroprevalence studies in dogs, several researchers have evaluated dogs by PCR assays from Arizona, Arkansas, Missouri, North Carolina, Ohio, Oklahoma, Tennessee, Virginia for the presence of circulating rickettsemia indicating active infection with *Ehrlichia* spp. (Table 3)(Dawson et al., 1996; Wen et al., 1997; Murphy et al., 1998; Kordick et al., 1999; Liddell et al., 2003; Seaman et al., 2004; Diniz et al., 2010; Little et al., 2010). Infection with *E. canis* was most common in Arizona (36.5% [85/233]), whereas *E. ewingii* (12.2% [59/482]) and *E. chaffeensis* (5.4% [26/482]) were more commonly detected in dogs tested from the other states (Table 3). Dogs from three Caribbean islands, Grenada, St. Kitts, and Trinidad, have also been tested for presence of *Ehrlichia* spp. DNA (Georges et al., 2008; Yabsley et al., 2008b; Kelly et al., 2013; Loftis et al., 2013). Prevalence of *E. canis* infection was highest in dogs from Grenada

(24.7% [18/73]); overall prevalence of *E. canis* infection was 17.0% (163/958) in dogs from all three islands (Table 2).

Prevalence in People

Since 1999, ehrlichiosis has been a reportable disease in the U.S. with data compiled annually; infection with *E. chaffeensis* accounts for the majority of the human cases, although infection with *E. ewingii* does occur (CDC, 2013). People in Oklahoma, Missouri, and Arkansas had the highest incidence rates (12.0, 11.4, and 10.3 cases per million persons per year, respectively) of *E. chaffeensis* reported from 2000 – 2007 in the U.S. (Dahlgren et al., 2011). In addition, recent reports document additional *Ehrlichia* spp. that infect people in the U.S., namely, Panola Mountain *Ehrlichia* sp. and an *E. muris*-like agent (Reeves et al., 2008; Pritt et al., 2011).

Prevalence in Ticks

Ticks have also been surveyed for the presence of different ehrlichial agents that cause disease in animals and people (Table 4). Surveys report *E. chaffeensis* in *A. americanum* from Arkansas, Connecticut, Georgia, Indiana, Iowa, Kentucky, Maryland, Missouri, New Jersey, New York, North Carolina, Oklahoma, Rhode Island, and Tennessee (Anderson et al., 1993; Lockhart et al., 1997; Burket et al., 1998; Roland et al., 1998; Steiner et al., 1999; Ijdo et al., 2000; Irving et al., 2000; Whitlock et al., 2000; Steiert and Gilfoy, 2002; Mixson et al., 2004; Schulze et al., 2005; Mixson et al., 2006; Varela et al., 2004; Cohen et al., 2010; Fritzen et al., 2011; Blanton et al., 2014; Killmaster et al., 2014; Lee et al., 2014; Harmon et al., 2015). In *E. chaffeensis* endemic regions, prevalence of infection in adult *A. americanum* is typically between 5% and 15% (Childs and Paddock, 2003; Paddock and Yabsley, 2007). *Dermacentor variabilis* is also

suspected of transmitting *E. chaffeensis*; amplifiable DNA was present in 0% to 14.6% of unfed ticks collected from Arkansas, Kentucky, Missouri, North Carolina, and Tennessee (Roland et al., 1998; Steiert and Gilfoy, 2002; Cohen et al., 2010; Blanton et al., 2014, Lee et al., 2014). *Ehrlichia chaffeensis* DNA was not detected in any *Ixodes scapularis* tested from Connecticut, North Carolina, Rhode Island, and Tennessee (Ijdo et al., 2000; Cohen et al., 2010; Lee et al., 2014).

Ehrlichia ewingii has been reported in *A. americanum* ticks from Florida, Georgia, Kentucky, Missouri, New Jersey, New York, North Carolina, Tennessee, and Texas (Table 4) (Wolf et al., 2000; Steiert and Gilfoy, 2002; Long et al., 2004; Varela et al., 2004; Schulze et al., 2005; Mixson et al., 2006; Cohen et al., 2010; Fritzen et al., 2011; Killmaster et al., 2014; Lee et al., 2014; Harmon et al., 2015). Additionally, DNA of *E. ewingii* has been detected in *D. variabilis* from Missouri and North Carolina (Steiert and Gilfoy, 2002; Lee et al., 2014). Evidence of *E. ewingii* was not present in *I. scapularis* from NC (Wolf et al., 2000; Lee et al., 2014).

Fewer studies have been conducted evaluating ticks for the presence of Panola Mountain *Ehrlichia* sp., *E. muris*, or *E. muris*-like agent (Table 4). To date, DNA of Panola Mountain *Ehrlichia* sp. has been detected in *A. americanum* collected from vegetation or human hosts from Florida, Georgia, Kentucky, Maryland, Missouri, New Jersey, New York, North Carolina, Ohio, Oklahoma, and Tennessee (Loftis et al., 2008a; Fitak et al., 2014; Killmaster et al., 2014; Lee et al., 2014; Harmon et al., 2015). I*xodes scapularis* from three states, Minnesota, Wisconsin, and Pennsylvania, have been tested for presence of *E. muris* or *E. muris*-like agent with positive ticks detected only in Minnesota and Wisconsin (Telford et al., 2011; Stromdahl et al., 2014).

Co-infections with *Ehrlichia* spp.

Co-infection with multiple tick-borne disease agents have been induced in experimental settings in dogs but are also seen in naturally infected people and dogs in the U.S. and in many other parts of the world (Klag et al., 1991; Breitschwerdt et al., 1998a; Kordick et al., 1999; Suksawat et al., 2001; Mylonakis et al., 2004a; Beall et al., 2008; Yabsley et al., 2008b; Nieto and Foley, 2009; Gaunt et al., 2010; Little et al., 2010; Beall et al., 2012; Al Izzi et al., 2013; Kelly et al., 2013; Loftis et al., 2013). Tick-borne agents frequently seen together in a co-infected person or dog are often transmitted by the same tick. For example, *E. chaffeensis* and *E. ewingii* are both vectored by *A. americanum* and are commonly seen in co-infected dogs (Anziani et al., 1990; Ewing et al., 1995; Breitschwerdt et al., 1998a; Little et al., 2010; Beall et al., 2012). Additionally, *R. sanguineus*, the most common vector for *E. canis* also transmits *Hepatozoon canis* and *Babesia canis vogeli*; dogs world-wide have been diagnosed with co-infections of these agents (Yabsley et al., 2008b; Kelly et al., 2013; Loftis et al., 2013; Rojas et al., 2014; Baneth et al., 2015). Evaluation of infectious agents in *A. americanum* from nine states concluded that 4.3% of the ticks had more than one infectious agent present (Mixson et al., 2004). Furthermore, *E. muris* along with *A. phagocytophilum, Borrelia burgdorferi,* or *Babesia microti* DNA was identified in ticks collected from Minnesota and Wisconsin (Telford et al., 2011; Stromdahl et al., 2014).

PREVENTION OF *EHRLICHIA* SPP. INFECTION

Vaccination

Prevention of *Ehrlichia* spp. infection is difficult. Vaccines for preventing ehrlichial infection in people or dogs have not yet been developed although some promising research has been reported (Maender and Tyring, 2004; Little, 2010; McBride and Walker, 2010; Rudoler et al., 2012; Thirumalapura et al., 2013). Many factors contribute to the difficulty in developing a vaccine against *Ehrlichia* spp. (McBride and Walker, 2010). First, each species of *Ehrlichia* displays genetic diversity among strains of the organism. The amino acid sequences of the 28-kDa outer membrane protein (p28) of different strains of *E. chaffeensis* vary by greater than 10% from one another, and immunization with the p28 protein of one strain may not provide protection against infection with different strains of *E. chaffeensis* (Yu et al., 1999). However, in mice challenged with *E. muris*, those vaccinated with certain p28 or Hsp60 peptides had lower bacterial loads after inoculation than unvaccinated mice, suggesting vaccination in this scenario may have provided partial protection (Crocquet-Valdes et al., 2011; Thomas et al., 2011).

Another obstacle is the number of different zoonotic *Ehrlichia* spp. present in the U.S. that a vaccine would be expected to prevent. *Ehrlichia chaffeensis, E. ewingii*, and Panola Mountain *Ehrlichia* sp. have been shown to infect both dogs and people in the U.S. (Ewing et al., 1971; Maeda et al., 1987; Dawson et al., 1996; Buller et al., 1999; Reeves et al., 2008; Qurollo et al., 2013); *E. muris* and the closely related *E. muris*-like agent have been isolated from dogs and people, respectively, in the upper Midwestern states, while *E. canis* is seen in dogs worldwide, but reports of infection in people remain

isolated to South America (Ristic and Huxsoll, 1984; Perez et al., 2006; Pritt et al., 2011; Hegarty et al., 2012).

Development of an appropriate, yet feasible, animal model has also been a challenge facing vaccine development because the majority of *Ehrlichia* spp. that infect humans and dogs don't sustainably infect mice (McBride and Walker, 2010). Recently, a model has been developed utilizing the *E. muris*-like agent in mice for use in vector-transmission and immunity studies (Saito and Walker, 2015; Saito et al., 2015). An added challenge to vaccine development is the lack of an *in vitro* model for cultivation of the granulocytic *Ehrlichia* spp. (Dumler et al., 2007; Reeves et al., 2008).

Lastly, the nature of the pathogen itself poses a unique impediment to vaccine development as it has evolved to survive within the host cells and evade the immune mechanisms targeted towards clearance of intracellular bacteria (Mavromatis et al., 2006; Rikihisa, 2006; Nandi et al., 2009; Wakeel et al., 2010).

Acquired Immunity

Even though vaccine development has not yet yielded a viable means of protection, it appears that acquired immunity may help clear an existing infection or lessen clinical signs with future infections. Experimental infection, treatment, and subsequent reinfection with two strains of *E. canis* showed decreased disease severity in the dogs reinfected with a homologous strain as compared to dogs given a heterologous strain post-treatment (Breitschwerdt et al., 1998b). These results could be due to potential virulence differences between the two strains of *E. canis* or to the primed immune system which may offer some protection regarding severity of a future infection. That same study showed that in the absence of antibiotics, four control animals were able to

eliminate or reduce infection to the point that it could no longer be detected by tissue culture isolation, PCR, or inoculation of blood into a naïve dog (Breitschwerdt et al., 1998b). In dogs, previous infection with *E. ewingii* does not prevent experimental reinfection by blood sub-inoculation, but previously infected dogs became PCR negative for *E. ewingii* earlier than dogs without previous infection (Yabsley et al., 2011). These studies further support the potential for acquired immunity to lessen clinical signs or assist in clearance of infection.

Limit Tick Exposure

The mainstay of preventing tick-borne infections, including human and canine ehrlichioses, is to limit contact with the tick. By limiting exposure to environments with ticks and thus reducing the number of ticks encountered, the chance of being fed upon and acquiring an infection decreases (Cisak et al., 2013; CDC, 2014). If absolute avoidance of tick habitat is not feasible, tick-control products and tick repellents should be used on pets and people, respectively (Nentwig, 2003; Stafford, 2007; Cisak et al., 2013; CDC, 2014). Adult and juvenile stages of ticks are active at different times throughout the year in the U.S. spending the majority of their developmental time off-host in an outdoor environment. *Amblyomma americanum* are often active spring through fall; adult activity peaks May – June, nymphs are active throughout summer, and the larval activity peaks in August (Goddard, 2007; Goddard and Varela-Stokes, 2009). Activity of the adults and nymphs of *D. variabilis* peaks May – July, while larval activity peaks both in September and again during the winter (Sonenshine, 1972; Kollars et al., 2000). *Ixodes scapularis* activity differs in different regions: in the northeastern U. S., the activity of nymphs and larvae peaks from May – July, while adult activity peaks in

November and February (Platt et al., 1992; Kollars et al., 1999). *Rhipicephalus sanguineus* is unusual in that all life stages can be seen year round and this tick can survive indoors and develop under low relative humidity (35%) (Dantas-Torres, 2010).

Cumulatively, tick activity occurs during all months of the year, and transmission of *Ehrlichia* spp. can occur within hours of tick attachment thus highlighting the need for year-round implementation of an approved tick-control product on dogs (Stafford, 2007; Little, 2010; CAPC, 2014). Tick protection recommendations for people include wearing protective, light-colored clothing such as long pants taped to boots and a tucked-in long sleeve shirt (Stafford, 2007; Vazquez et al., 2008; CAPC, 2014; CDC, 2014). Light-colored clothing allows better visualization of ticks and limits skin exposure, thereby limiting attachment sites. Lastly, routine examination for ticks is recommended, especially after spending time in a potential tick environment (Stafford, 2007; CAPC, 2014; CDC, 2014).

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Table 1: Reported seroprevalence of *Ehrlichia* spp. in dogs from the U.S.

State	*Ehrlichia* spp. (p30/30-1[a,b,c] ± p28[d])	*E. canis* (p16)[c]	*E. chaffeensis* (VLPT)[c]	*E. ewingii* (p28)[c]	*Ehrlichia* spp. (IFA)[e,f]	Citation
Alabama	0.3% (64/18,998)[a,b]	--	--	--	--	Bowman et al., 2009
	1.6% (856/53,339)[d]	--	--	--	--	Little et al., 2014*
	--	0.3% (1/337)	1.5% (5/337)	3.6% (12/337)	--	Beall et al., 2012
	5.0% (2/40)[c]	7.5% (3/40)	0.0% (0/40)	2.5% (1/40)	--	Qurollo et al., 2014
Alaska	--	0.0% (0/2)	0.0% (0/2)	0.0% (0/2)	--	Beall et al., 2012
Arizona	43.8% (102/233)[b]	--	--	--	--	Diniz et al., 2010
	3.2% (32/992)[a,b]	--	--	--	--	Bowman et al., 2009
	2.4% (1,349/55,865)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/1)	0.0% (0/1)	0.0% (0/1)	--	Beall et al., 2012
	0.0% (0/15)[c]	0.0% (0/15)	0.0% (0/15)	6.7% (1/15)	--	Qurollo et al., 2014
	--	--	--	--	11.8% (40/339)[e]	Stephenson & Ristic, 1978
Arkansas	3.9% (324/8,391)[a,b]	--	--	--	--	Bowman et al., 2009
	9.4% (4,029/42,774)[d]	--	--	--	--	Little et al., 2014*
	--	3.6% (3/84)	21.4% (18/84)	36.9% (31/84)	--	Beall et al., 2012
	5.6% (2/36)[c]	0.0% (0/36)	2.8% (1/36)	5.6% (2/36)	--	Qurollo et al., 2014
	17.9% (7/39)[b]	0.0% (0/39)	59.0% (23/39)	25.6% (10/39)	--	Little et al., 2010
California	0.8% (225/29,454%)[a,b]	--	--	--	--	Bowman et al., 2009
	0.8% (2,258/270,190)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	--	Beall et al., 2012
	0.0% (0/121)[c]	0.0% (0/121)	0.0% (0/121)	0.0% (0/121)	--	Qurollo et al., 2014
Colorado	0.2% (19/11,557)[a,b]	--	--	--	--	Bowman et al., 2009
	1.1% (217/19,467)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	--	Beall et al., 2012
	3.3% (8/246)[c]	3.7% (9/246)	0.0% (0/246)	0.6% (1/246)	--	Qurollo et al., 2014

Connect-	0.2% (21/10,209)[a,b]	--	--	--	--	Bowman et al., 2009
icut	0.8% (1,434/183,776)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/97)	0.0% (0/97)	0.0% (0/97)	--	Beall et al., 2012
	6.3% (3/48)[c]	4.2% (2/48)	4.2% (2/48)	2.1% (1/48)	--	Qurollo et al., 2014
	--	--	--	--	20.8% (5/24)[e]	Magnarelli & Anderson, 1993
Delaware	1.0% (48/4,595)[a,b]	--	--	--	--	Bowman et al., 2009
	2.3% (1,114/49,131)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/27)	0.0% (0/27)	0.0% (0/27)	--	Beall et al., 2012
	0.0% (0/4)[c]	0.0% (0/4)	0.0% (0/4)	0.0% (0/4)	--	Qurollo et al., 2014
Florida	0.8% (425/54,982)[a,b]	--	--	--	--	Bowman et al., 2009
	1.2% (4,644/403,622)[d]	--	--	--	--	Little et al., 2014*
	--	0.5% (4/733)	1.2% (9/733)	2.6% (19/733)	--	Beall et al., 2012
	4.2% (21/501)[c]	2.6% (13/501)	2.2% (11/501)	3.0% (15/501)	--	Qurollo et al., 2014
Georgia	1.9% (444/23,333)[a,b]	--	--	--	--	Bowman et al., 2009
	2.6% (3,290/124,637)[d]	--	--	--	--	Little et al., 2014*
	--	0.2% (1/662)	3.5% (23/662)	5.4% (36/662)	--	Beall et al., 2012
	3.7% (6/162)[c]	1.2% (2/162)	3.7% (6/162)	7.4% (12/162)	--	Qurollo et al., 2014
Hawaii	7.0% (166/2,359)	--	--	--	--	Little et al., 2014
	--	0.0% (0/2)	0.0% (0/2)	0.0% (0/2)	--	Beall et al., 2012
Idaho	0.0% (0/369)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (1/167)[d]	--	--	--	--	Little et al., 2014*
Illinois	0.4% (135/31,976)[a,b]	--	--	--	--	Bowman et al., 2009
	0.8% (2,155/277,174)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/489)	1.8% (9/489)	1.6% (8/489)	--	Beall et al., 2012
	1.3% (5/383)[c]	0.3% (1/383)	0.5% (2/383)	1.0% (4/383)	--	Qurollo et al., 2014
Indiana	0.3% (54/20,515)[a,b]	--	--	--	--	Bowman et al., 2009
	1.3% (1,480/112,477)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/553)	0.5% (3/553)	0.7% (4/553)	--	Beall et al., 2012
	2.2% (2/93)[c]	0.0% (0/93)	1.1% (1/93)	1.1% (1/93)	--	Qurollo et al., 2014

Iowa	0.4% (61/17,390)[a,b]	--	--	--	--	Bowman et al., 2009
	0.7% (751/111,518)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/14)	0.0% (0/14)	0.0% (0/14)	--	Beall et al., 2012
	1.3% (1/78)[c]	0.0% (0/78)	0.0% (0/78)	3.8% (3/78)	--	Qurollo et al., 2014
Kansas	2.2% (119/5,473)[a,b]	--	--	--	--	Bowman et al., 2009
	2.3% (1,228/52,429)[d]	--	--	--	--	Little et al., 2014*
	--	0.9% (4/457)	1.1% (5/457)	6.8% (31/457)	--	Beall et al., 2012
	1.9% (1/53)[c]	1.9% (1/53)	1.9% (1/53)	3.8% (2/53)	--	Qurollo et al., 2014
Kentucky	0.8% (152/18,935)[a,b]	--	--	--	--	Bowman et al., 2009
	4.3% (2.420/56,027)	--	--	--	--	Little et al., 2014*
	--	6.3% (1/16)	0.0% (0/16)	18.8% (3/16)	--	Beall et al., 2012
	7.2% (5/69)[c]	0.0% (0/69)	2.9% (2/69)	4.3% (3/69)	--	Qurollo et al., 2014
Louisiana	0.2% (18/11,197)[a,b]	--	--	--	--	Bowman et al., 2009
	1.1% (140/12,406)[d]	--	--	--	--	Little et al., 2014*
	--	1.5% (4/274)	0.0% (0/274)	0.7% (2/274)	--	Beall et al., 2012
	3.7% (1/27)[c]	3.7% (1/27)	3.7% (1/27)	3.7% (1/27)	--	Qurollo et al., 2014
	--	--	--	--	22.1% (19/86)[e]	Hoskins et al., 1988
Maine	0.1% (39/28,230)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (1,214/221,555)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/13)	0.0% (0/13)	0.0% (0/13)	--	Beall et al., 2012
	0.0% (0/4)[c]	0.0% (0/4)	0.0% (0/4)	0.0% (0/4)	--	Qurollo et al., 2014
Maryland	0.7% (165/22,945)[a,b]	--	--	--	--	Bowman et al., 2009
	1.9% (5,107/273,382)[d]	--	--	--	--	Little et al., 2014*
	--	0.8% (2/254)	3.5% (9/254)	3.5% (9/254)	--	Beall et al., 2012
	6.4% (20/313)[c]	1.0% (3/313)	8.0% (25/313)	3.8% (12/313)	--	Qurollo et al., 2014
Massachu-setts	0.3% (107/33,915)[a,b]	--	--	--	--	Bowman et al., 2009
	0.8% (3,315/406,476)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/241)	0.8% (2/241)	0.8% (2/241)	--	Beall et al., 2012
	0.0% (0/35)[c]	0.0% (0/35)	0.0% (0/35)	0.0% (0/35)	--	Qurollo et al., 2014

Michigan	0.1% (34/67,625)[a,b]	--	--	--	--	Bowman et al., 2009
	0.3% (781/236,798)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/9)	0.0% (0/9)	0.0% (0/9)	--	Beall et al., 2012
	5.0% (1/20)[c]	0.0% (0/20)	0.0% (0/20)	0.0% (0/20)	--	Qurollo et al., 2014
Minnesota	0.3% (202/76,610)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (1,426/234,558)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/7)	0.0% (0/7)	0.0% (0/7)	--	Beall et al., 2012
	0.0% (0/8)[c]	12.5% (1/8)	0.0% (0/8)	0.0% (0/8)	--	Qurollo et al., 2014
Mississippi	3.1% (68/2,198)[a,b]	--	--	--	--	Bowman et al., 2009
	4.6% (308/6,637)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/151)	1.3% (2/151)	6.0% (9/151)	--	Beall et al., 2012
	6.3% (1/16)[c]	0.0% (0/16)	0.0% (0/16)	0.0% (0/16)	--	Qurollo et al., 2014
Missouri	--	--	--	--	30.6% (26/85)[f]	Liddell et al., 2003
	1.9% (462/24,095)[a,b]	--	--	--	--	Bowman et al., 2009
	5.4% (5,888/108,573)[d]	--	--	--	--	Little et al., 2014*
	--	0.8% (5/663)	12.8% (85/663)	22.8% (151/663)	--	Beall et al., 2012
	11.1% (4/36)[c]	2.8% (1/36)	8.3% (3/36)	5.6% (2/36)	--	Qurollo et al., 2014
	--	--	--	--	62.7% (37/59)[e]	Stockham et al., 1992
Montana	0.0% (0/36)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/1)	0.0% (0/1)	0.0% (0/1)	--	Beall et al., 2012
Nebraska	0.3% (13/4,282)[a,b]	--	--	--	--	Bowman et al., 2009
	1.6% (70/4,485)[d]	1.6% (1/62)	0.0% (0/62)	0.0% (0/62)	--	Little et al., 2014*
	--	33.3% (1/3)	0.0% (0/3)	0.0% (0/3)	--	Beall et al., 2012
	33.3% (1/3)[c]				--	Qurollo et al., 2014
Nevada	0.5% (59/12,278)[d]	--	--	--	--	Little et al., 2014*
	0.0% (0/5)[c]	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	--	Qurollo et al., 2014
New Hampshire	0.2% (36/18,122)[a,b]	--	--	--	--	Bowman et al., 2009
	0.7% (949/129,829)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/28)	0.0% (0/28)	0.0% (0/28)	--	Beall et al., 2012
	0.0% (0/19)[c]	0.0% (0/19)	0.0% (0/19)	0.0% (0/19)	--	Qurollo et al., 2014

New Jersey	0.4% (89/20,575)[a,b]	--	--	--	--	Bowman et al., 2009
	1.2% (3,638/295,047)[d]	--	--	--	--	Little et al., 2014*
	--	0.4% (1/257)	1.9% (5/257)	2.7% (7/257)	--	Beall et al., 2012
	0.0% (0/12)c	0.0% (0/12)	0.0% (0/12)	0.0% (0/12)	--	Qurollo et al., 2014
New Mexico	1.0% (21/2,060)[a,b]	--	--	--	--	Bowman et al., 2009
	3.2% (858/26,706)[d]	--	--	--	--	Little et al., 2014*
	1.6% (1/61)[c]	1.6% (1/61)	1.6% (1/61)	0.0% (0/61)	--	Qurollo et al., 2014
New York	0.2% (179/81,305)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (3,176/536,968)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/188)	0.5% (1/188)	1.6% (3/188)	--	Beall et al., 2012
	2.0% (4/205)[c]	0.0% (0/205)	1.0% (2/205)	4.9% (10/205)	--	Qurollo et al., 2014
	--	--	--	--	5.6% (2/36)[e]	Magnarelli & Anderson, 1993
North Carolina	--	--	--	--	92.6% (25/27)[e]	Kordick et al., 1999
	2.1% (431/20,783)[a,b]	--	--	--	--	Bowman et al., 2009
	4.6% (11,431/249,132)[d]	--	--	--	--	Little et al., 2014*
	--	0.7% (3/403)	5.7% (23/403)	5.2% (21/403)	--	Beall et al., 2012
	11.8% (120/1,014)[c]	1.9% (19/1,014)	9.5% (96/1,014)	10.3% (104/1,014)	--	Qurollo et al., 2014
		--	--	--	2.4% (44/1,845)[e]	Suksawat et al., 2000
North Dakota	0.0% (1/4,558)[a,b]	--	--	--	--	Bowman et al., 2009
	0.3% (55/16,560)[d]	--	--	--	--	Little et al., 2014*
Ohio	0.2% (79/61,138)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (1,727/278,437)[d]	--	--	--	--	Little et al., 2014*
	--	0.9% (4/428)	0.0% (0/428)	0.2% (1/428)	--	Beall et al., 2012
	1.2% (5/430)[c]	0.2% (1/430)	0.2% (1/430)	0.5% (2/430)	--	Qurollo et al., 2014

Oklahoma	--	--	--	--	10.8% (7/65)[e]	Murphy et al., 1998
	--	--	--	--	9.3% (6/65)[f]	
	3.8% (439/11,549)[a,b]	--	--	--	--	Bowman et al., 2009
	5.4% (3,847/70,751)[d]	--	--	--	--	Little et al., 2014*
	--	1.8% (9/514)	1.0% (5/514)	7.2% (37/514)	--	Beall et al., 2012
	9.5% (4/42)[c]	2.4% (1/42)	0.0% (0/42)	14.3% (6/42)	--	Qurollo et al., 2014
	9.6% (10/104)[b]	1.9% (2/104)	39.4% (41/104)	14.4% (15/104)	--	Little et al., 2010
	--	--	--	--	53.3% (138/259)[e]	Rodgers et al., 1989
Oregon	0.1% (2/2,798)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (111/17,879)[d]	--	--	--	--	Little et al., 2014*
	0.0% (0/35)[c]	0.0% (0/35)	0.0% (0/35)	0.0% (0/35)	--	Qurollo et al., 2014
Pennsyl-	0.2% (80/40,948)[a,b]	--	--	--	--	Bowman et al., 2009
vania	0.6% (3,364/579,608)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/96)	0.0% (0/96)	1.0% (1/96)	--	Beall et al., 2012
	1.5% (3/203)[c]	0.5% (1/203)	0.5% (1/203)	3.4% (7/203)	--	Qurollo et al., 2014
Rhode	0.1% (6/6,508)[a,b]	--	--	--	--	Bowman et al., 2009
Island	0.3% (206/63,796)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/24)	0.0% (0/24)	0.0% (0/24)	--	Beall et al., 2012
	--	--	--	--	2.9% (8/277)[e]	Hinrichsen et al., 2001
South	0.8% (95/11,562)[a,b]	--	--	--	--	Bowman et al., 2009
Carolina	1.4% (1,151/82,677)[d]	--	--	--	--	Little et al., 2014*
	--	8.8% (3/34)	8.8% (3/34)	5.9% (2/34)	--	Beall et al., 2012
	7.5% (7/93)[c]	0.0% (0/93)	7.5% (7/93)	8.6% (8/93)	--	Qurollo et al., 2014
South	0.0% (0/358)[a,b]	--	--	--	--	Bowman et al., 2009
Dakota	0.6% (25/4,497)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/2)	0.0% (0/2)	0.0% (0/2)	--	Beall et al., 2012
Tennessee	2.3% (428/18,891)[a,b]	--	--	--	--	Bowman et al., 2009
	3.0% (3,307/111,312)[d]	--	--	--	--	Little et al., 2014*
	--	2.8% (5/181)	2.8% (5/181)	7.7% (14/181)	--	Beall et al., 2012
	8.9% (4/45)[c]	2.2% (1/45)	0.0% (0/45)	2.2% (1/45)	--	Qurollo et al., 2014
	1.1% (1/90)[a]	--	--	--	11.1% (10/90)[e]	Seaman et al., 2004

Texas	0.8% (441/58,088)[a,b]	--	--	--	--	Bowman et al., 2009
	1.8% (7,659/432,799)[d]	--	--	--	--	Little et al., 2014*
	--	2.0% (18/893)	0.1% (1/893)	0.6% (5/893)	--	Beall et al., 2012
	3.7% (36/966)[c]	3.1% (30/966)	0.5% (5/966)	0.3% (3/966)	--	Qurollo et al., 2014
Utah	0.0% (0/93)[a,b]	--	--	--	--	Bowman et al., 2009
	0.5% (4/783)[d]	--	--	--	--	Little et al., 2014*
	50.0% (1/2)[c]	50.0% (1/2)	0.0% (0/2)	0.0% (0/2)	--	Qurollo et al., 2014
Vermont	0.2% (7/3,718)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (381/59,515)[d]	--	--	--	--	Little et al., 2014*
	0.0% (0/6)[c]	0.0% (0/6)	0.0% (0/6)	0.0% (0/6)	--	Qurollo et al., 2014
Virginia	--	--	--	--	38.4% (28/73)[e,f]	Dawson et al., 1996
	1.8% (532/28,787)[a,b]	--	--	--	--	Bowman et al., 2009
	6.2% (21,770/350,437)[d]	--	--	--	--	Little et al., 2014*
	--	0.5% (2/385)	6.5% (25/385)	8.1% (31/385)	--	Beall et al., 2012
	5.8% (38/656)[c]	0.9% (6/656)	4.9% (32/656)	6.9% (45/656)	--	Qurollo et al., 2014
Washing-ton	0.0% (0/33)[a,b]	--	--	--	--	Bowman et al., 2009
	2.5% (109/4,330)[d]	--	--	--	--	Little et al., 2014*
	0.0% (0/12)[c]	0.0% (0/12)	0.0% (0/12)	0.0% (0/12)	--	Qurollo et al., 2014
West Virginia	0.1% (4/2,942)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (339/61,434)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/30)	3.3% (1/30)	0.0% (0/30)	--	Beall et al., 2012
	0.0% (0/2)[c]	0.0% (0/2)	0.0% (0/2)	0.0% (0/2)	--	Qurollo et al., 2014
Wisconsin	0.3% (194/59,070)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (1,751/282,662)[d]	--	--	--	--	Little et al., 2014*
	--	0.0% (0/9)	0.0% (0/9)	0.0% (0/9)	--	Beall et al., 2012
	0.0% (0/58)[c]	0.0% (0/58)	0.0% (0/58)	0.0% (0/58)	--	Qurollo et al., 2014

Wyoming	0.0% (0/184)[a,b]	--	--	--	--	Bowman et al., 2009
	0.6% (2/359)[d]	--	--	--	--	Little et al., 2014*
	0.0% (0/1)[c]	100.0% (1/1)	0.0% (0/1)	0.0% (0/1)	--	Qurollo et al., 2014

[a]SNAP® 3Dx® ELISA (IDEXX Laboratories Inc., Westbrook, Maine)

[b]SNAP® 4Dx® ELISA (IDEXX Laboratories Inc., Westbrook, Maine)

[c]SNAP® M-A ELISA (IDEXX Laboratories Inc., Westbrook, Maine)

[d]SNAP® 4Dx® Plus ELISA (IDEXX Laboratories Inc., Westbrook, Maine)

[e]*E. canis* IFA

[f]*E. chaffeensis* IFA

*unpublished data

Table 2: Reported serologic and molecular prevalence of *Ehrlichia* spp. in dogs from the Caribbean

Island	**Serology**					**Molecular**	**Citation**
	Ehrlichia spp. (p30/30-1)[a,b,c]	*E. canis* (p16)[c]	*E. chaffeensis* (VLPT)[c]	*E. ewingii* (p28)[c]	*E. canis* (IFA)	*E. canis* (PCR)	
Grenada	42.9% (76/177)[a,b]	--	--	--	--	24.7% (18/73)	Yabsley et al., 2008b
St. Kitts	24.1% (41/170)[a,b]	--	--	--	--	18.5% (69/372)	Kelly et al., 2013
	31.0% (48/155)[a,b]	--	--	--	--	16.4% (27/165)	Loftis et al., 2013
Trinidad	--	--	--	--	44.6% (41/92)	--	Asgarali et al., 2012
	--	--	--	--	--	14.1% (49/348)	Georges et al., 2008
Turks & Caicos	47.6% (89/187)[a]	--	--	--	--	--	Hoff et al., 2008
Caribbean*	31.0% (9/29)[c]	27.6% (8/29)	0.0% (0/29)	0.0% (0/29)	--	--	Qurollo et al., 2014

[a]SNAP® 3Dx® ELISA (IDEXX Laboratories Inc., Westbrook, Maine)

[b]SNAP® 4Dx® ELISA (IDEXX Laboratories Inc., Westbrook, Maine)

[c]SNAP® M-A ELISA (IDEXX Laboratories Inc., Westbrook, Maine)

*Island designation not provided

Table 3: Reported molecular prevalence of *Ehrlichia* spp. in dogs from the U.S. as detected by 16S rRNA PCR

State	***Ehrlichia* spp.**	***E. canis***	***E. chaffeensis***	***E. ewingii***	**Citation**
Arizona	--	36.5% (85/233)	0.0% (0/233)	0.0% (0/233)	Diniz et al., 2010[1,2]
Arkansas	--	0.0% (0/39)	2.6% (1/39)	23.1% (9/39)	Little et al., 2010[1]
Missouri	--	0.0% (0/88)	1.1% (1/88)	22.7% (20/88)	Liddell et al., 2003[1]
North Carolina	--	55.6% (15/27)	33.3% (9/27)	29.6% (8/27)	Kordick et al., 1999[1]
Ohio	--	16.7% (5/30)	--	--	Wen et al., 1997[1]
	15.4% (10/65)	3.1% (2/65)	6.2% (4/65)	6.2% (4/65)	Murphy et al., 1998[1,2]
Oklahoma	--	0.0% (0/49)	4.1% (2/49)	4.1% (2/49)	Little et al., 2010[1]
	--	1.2% (3/255)	0.4% (1/255)	3.9% (10/255)	Little et al., 2010[2]
Tennessee	--	0.0% (0/90)	--	--	Seaman et al., 2004[2]
Virginia	50.0% (19/38)	0.0% (0/19)	42.1% (8/19)	31.6% (6/19)	Dawson et al., 1996[1,2]

[1]samples collected from pet dogs

[2]samples collected from feral dogs or dogs in an animal shelter

Table 4: Reported molecular prevalence of *Ehrlichia* spp. in ticks from the U.S.

State	*E. chaffeensis*[a,b,c,d,e,f]	*E. ewingii*[a,b,g]	Panola mountain *Ehrlichia* sp.[c]	*E. muris* or *E. muris*-like[a,b,c]	Citation
Alabama	--	--	*Aa*: 0.0% (0/8)†‡[c]	--	Loftis et al., 2008a[2]
Arkansas	*Aa*: 1.2% (1/26*)†[d,e]	*Aa*: --	*Aa*: --	--	Blanton et al., 2014[1]
	0.0% (0/17*)‡[d,e]	--	--	--	
	--	--	0.0% (0/8)†‡[c]	--	Loftis et al., 2008a[2]
	Dv: 0.0% (0/9*)†[d,e]	*Dv*: --	*Dv*: --	--	Blanton et al., 2014[1]
Connecticut	*Aa*: 7.5% (8/106)†[a]	--	--	--	Ijdo et al., 2000[2]
	Is: 0.0% (0/50)†[a]	--	--	--	
Delaware	--	--	*Aa*: 0.0% (0/12)†‡[c]	--	Loftis et al., 2008a[2]
Florida	*Aa*: 0.0% (0/151)†[e]	*Aa*: 2.0% (3/151)†[g]	*Aa*: --	--	Mixson et al., 2006[1]
	--	--	0.7% (1/515)†[c]	--	Loftis et al., 2008a[1]
	--	--	0.0% (0/7)†‡[c]	--	Loftis et al., 2008a[2]
Georgia	*Aa*: 5.2% (13/250)†[a]	*Aa*: --	*Aa*: --	--	Whitlock et al., 2000[1]
	0.0% (0/38*)‡[a]	--	--	--	
	12.0% (6/50)†[a]	--	--	--	Lockhart et al., 1997[1]
	3.5% (14/79*)†[a]	--	--	--	
	1.7% (12/704)†[e]	3.3% (23/704)†[g]	--	--	Mixson et al., 2006[1]
	1.0% (4/398)†[a]	4.8% (19/398)†[a]	--	--	Varela et al., 2004[1]
	2.0% (8/398)†[e]	--	--	--	
	--	--	0.9% (6/705)†[c]	--	Loftis et al., 2008a[1]
	--	--	0.0% (0/343)†‡[c]	--	Loftis et al., 2008a[2]
	1.9% (23/1,183)†[a]	2.1% (25/1,183)†[a]	1.4% (16/1,183)†[c]	--	Killmaster et al., 2014[1]
	0.7% (20/598)‡[a]	0.8% (24/598)‡[a]	0.6% (18/598)‡[c]	--	
Indiana	*Aa*: 3.8% (10/55*)†[a]	--	*Aa*: --	--	Irving et al., 2000[1]
	0.0% (0/32*)‡[a]	--	--	--	
	--	--	0.0% (0/1)‡[c]	--	Loftis et al., 2008a[2]
	1.6% (15/184*)◊[a]	--	--	--	Steiner et al., 1999[1]
	4.9% (21/88*)†[a]	--	--	--	Burket et al., 1998[1]
	0.0% (0/19*)‡[a]	--	--	--	

Iowa	*Aa*: 5.3% (1/19)†[e]	*Aa*: 0.0% (0/19)†[g]	--	--	Mixson et al., 2006[1]
Kansas	*Aa*: 0.0% (0/3*)†[a]	--	*Aa*: --	--	Anderson et al., 1993[1]
	0.0% (0/5*)‡[a]	--	--	--	
	--	--	0.0% (0/67)†‡[c]	--	Loftis et al., 2008a[2]
Kentucky	*Aa*: 5.6% (6/108)†[e]	*Aa*: 3.7% (4/108)†[a]	*Aa*: --	--	Fritzen et al., 2011[2,3]
	3.6% (1/7*)†[a]	--	--	--	Anderson et al., 1993[1]
	0.0% (0/5*)‡[a]	--	--	--	
	0.0% (0/5*)†[a]	--	--	--	Anderson et al., 1993[2]
	--	--	16.7% (1/6)†[c]	--	Loftis et al., 2008a[1]
	Dv: 4.5% (8/179)†[e]	*Dv*: 0.0% (0/179)†[a]	*Dv*: --	--	Fritzen et al., 2011[2,3]
Louisiana	--	--	*Aa*: 0.0% (0/1)‡[c]	--	Loftis et al., 2008a[2]
Maryland	*Aa*: 3.5% (5/34*)†[a,e,f]	--	*Aa*: --	--	Stromdahl et al., 2000[1]
	0.8% (21/81*)‡[a,e,f]	--	--	--	
	--	--	1.5% (4/266)‡[c]	--	Loftis et al., 2008a[2]
Minnesota	--	--	--	*Is*: 7.5% (7/93)†[b]	Stromdahl et al., 2014[2]
	--	--	--	1.9% (2/103)‡[b]	
Missouri	*Aa*: 9.8% (57/579)†[a]	*Aa*: 5.4% (31/579)†[a]	*Aa*: --	--	Steiert & Gilfoy, 2002[1]
	1.7% (8/115*)‡[a]	0.6% (3/115*)‡[a]	--	--	
	28.8% (17/59)†[a]	--	--	--	Roland et al., 1998[1,2,3]
	1.2% (1/9*)†[a]	--	--	--	Anderson et al., 1993[1]
	0.0% (0/5*)‡[a]	--	--	--	
	--	--	10.0% (1/10)†‡[c]	--	Loftis et al., 2008a[2]
	Dv: 6.7% (8/120)†[a]	*Dv*: 3.3% (4/120)†[a]	*Dv*: --	--	Steiert & Gilfoy, 2002[1]
	14.6% (6/41)†[a]	--	--	--	Roland et al., 1998[1,2,3]
Nebraska	*Aa*: 0.0% (0/4)†[a]	--	*Aa*: --	--	Anderson et al., 1993[1]
	--	--	0.0% (0/1)‡[c]	--	Loftis et al., 2008a[2]
New Jersey	*Aa*: 12.4% (15/121)†[e]	*Aa*: 8.3% (10/121)†[g]	*Aa*: --	--	Schulze et al., 2005[1]
	--	--	1.7% (2/120)†[c]	--	Loftis et al., 2008a[1]
	--	--	2.6% (7/265)†‡[c]	--	Loftis et al., 2008a[2]

New York	*Aa*: 12.5% (59/473)†[e]	*Aa*: --	*Aa*: --	--	Mixson et al., 2004[1]
	1.4% (8/113*)‡[e]	--	--	--	
	12.8% (61/475)†[e]	1.3% (6/475)†[g]	--	--	Mixson et al., 2006[1]
	--	--	0.8% (4/475)†[c]	--	Loftis et al., 2008a[1]
	--	--	0.0% (0/1)‡[c]	--	Loftis et al., 2008a[2]
North Carolina	*Aa*: 1.2% (5/47*)†[a]	*Aa*: --	*Aa*: --	--	Anderson et al., 1993[1]
	0.0% (0/27*)‡[a]	--	--	--	
	1.0% (4/391)†[e]	5.9% (23/391)†[g]	--	--	Mixson et al., 2006[1]
	--	0.6% (3/462)†[a]	--	--	Wolf et al., 2000[1]
	--	0.4% (5/106*)‡[a]	--	--	
	--	--	0.0% (0/383)†[c]	--	Loftis et al., 2008a[1]
	--	--	0.0% (0/93)†‡[c]	--	Loftis et al., 2008a[2]
	1.8% (13/734)#[e]	7.1% (52/734)#[a]	0.4% (3/734)#[c]	--	Lee et al., 2014[2]
	Dv: 0.0% (0/37)†[a]	*Dv*: 8.1% (3/37)†[a]	*Dv*: --	--	
	--	0.0% (0/1,349)◊[a]	--	--	Wolf et al., 2000[1]
	Is: --	*Is*: 0.0% (0/51)◊[a]	*Is*: --	--	
	0.0% (0/8)†[a]	0.0% (0/8)†[a]	--	--	Lee et al., 2014[2]
	Am: 12.5% (1/8)†[a]	*Am*: 0.0% (0/8)†[a]	*Am*: --	--	
Ohio	*Aa*: 0.0% (0/327)◊[c]	--	*Aa*: 0.6% (2/327)◊[c]	--	Fitak et al., 2014[1,2,,3]
	--	--	27.3% (6/22)†‡[c]	--	Loftis et al., 2008a[2]
Oklahoma	*Aa*: 3.3% (2/60)†[e]	*Aa*: 0.0% (0/60)†[g]	*Aa*: --	--	Mixson et al., 2006[1]
	--	--	4.8% (1/21)†‡[c]	--	Loftis et al., 2008a[2]
Pennsylvania	--	--	*Aa*: 0.0% (0/4)†‡[c]	--	Loftis et al., 2008a[2]
	--	--	--	*Is*: 0.0% (0/252)†‡[b]	Stromdahl et al., 2014[2]
Rhode Island	*Aa*: 11.5% (6/52)†[a]	*Aa*: --	*Aa*: --	--	Ijdo et al., 2000[1]
	2.6% (1/38)†[e]	0.0% (0/38)†[g]	--	--	Mixson et al., 2006[1]
	--	--	0.0% (0/1)†[c]	--	Loftis et al., 2008a[2]
	Is: 0.0% (0/63)†[a]	*Is*: --	*Is*: --	--	Ijdo et al., 2000[1]
South Carolina	*Aa*: 0.0% (0/79)†[e]	*Aa*: 0.0% (0/79)†[g]	*Aa*: --	--	Mixson et al., 2006[1]
	--	--	0.0% (0/80)†[c]	--	Loftis et al., 2008a[1]
	--	--	0.0% (0/33)†‡[c]	--	Loftis et al., 2008a[2]

Tennessee	*Aa*: 2.0% (8/396)†‡[b]	*Aa*: 4.3% (17/396)†‡[b]	*Aa*: 2.0% (8/396)†‡[c]	--	Harmon et al., 2015[1]
	2.6% (4/153)†[a]	0.9% (1/114)†[a]	--	--	Cohen et al., 2010[1,2,3]
	2.6% (4/156)‡[a]	0.8% (1/124)‡[a]	--	--	
	Dv: 0.0% (0/277)†‡[a]	*Dv*: 0.0% (0/86)†‡[a]	--	--	
	Is: 0.0% (0/4)◊[a]	--	--	--	
	Am: 0.0% (0/2)◊[a]	--	--	--	
Texas	*Aa*: 0.0% (0/66)†[a]	*Aa*: 7.6% (5/66)†[a]	*Aa*: --	--	Long et al., 2004[1]
	--	--	0.0% (0/44)†[c]	--	Loftis et al., 2008a[1]
	--	--	0.0% (0/81)†‡[c]	--	Loftis et al., 2008a[2]
Virginia	--	--	*Aa*: 0.3% (1/368)‡[c]	--	Loftis et al., 2008a[2]
Wisconsin	--	--	--	*Is*: 2.1% (2/96)†[b]	Stromdahl et al., 2014[2]
	--	--	--	0.0% (0/269)‡[b]	
	--	--	--	0.9% (7/127*)†[a,b,c]	Telford et al., 2011[1]

Tick abbreviations: *Aa* (*Amblyomma americanum*), *Dv* (*Dermacentor variabilis*), *Is* (*Ixodes scapularis*), *Am* (*Amblyomma maculatum*)

Tick life stages: †Adult ticks, ‡Nymphal ticks, # all life stages, ◊ life stage not specified

PCR targets: [a]16S rRNA, [b]groESL, [c]Citrate Synthase/gltA, [d]dsb, [e]VLPT, [f]120 kDa, [g]p28

*Pools (prevalence for pooled ticks is based on minimum infection rate [MIR] assuming only one infected tick per positive pool)

[1]ticks collected from vegetation

[2]ticks collected from humans

[3]ticks collected from animal hosts

CHAPTER III

DEVELOPMENT OF ANTIBODIES TO AND PCR DETECTION OF *EHRLICHIA* SPP. IN DOGS FOLLOWING NATURAL TICK EXPOSURE[1]

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[1]Starkey, L.A., Barrett, A.W., Chandrashekar, R., Stillman, B.A., Tyrrell, P., Thatcher, B., Beall, M.J., Gruntmeir, J.M., Meinkoth, J.H., Little, S.E., 2014. *Veterinary Microbiology* 173(3-4):379-384. Reprinted here with permission of publisher.

ABSTRACT

Dogs exposed to ticks in the southern US may become infected with multiple species of *Ehrlichia*. To better define infection risk, blood samples collected from 10 dogs infested with ticks via a natural infestation model were evaluated by blood smear examination, PCR, patient-side ELISAs (SNAP® 4Dx® and SNAP® 4Dx® Plus), IFA, and peptide based ELISA for evidence of infection with *Ehrlichia canis, E. chaffeensis,* and/or *E. ewingii*. Although morulae were rarely identified in blood smears, every dog (10/10) became infected with *Ehrlichia* spp. as evidenced by nested PCR detection of *E. chaffeensis* (7/10) and *E. ewingii* DNA (10/10); real-time PCR detection of *E. chaffeensis* (0/10) and *E. ewingii* (9/10); seroconversion on two different patient-side ELISAs (4/10 or 10/10); seroconversion on IFA to *E. canis* (10/10, maximum inverse titer = 128 – 4,096, GMT_{MAX}=548.7) and *E. chaffeensis* (10/10, maximum inverse titer = 1,024 – 32,768, GMT_{MAX}= 4,096); and seroconversion on peptide specific ELISA to *E. chaffeensis* VLPT (7/10) and *E. ewingii* p28 (9/10). Rickettsemia with *E. chaffeensis* and *E. ewingii*, as determined by nested PCR, persisted in dogs for an average of 3.2 or 30.5 days, respectively. *Ehrlichia canis* was not detected in any dog by any method, and no dogs developed signs of clinical disease. Our data suggest that in areas where ticks are common, dogs are at high risk of infection with *Ehrlichia* spp., particularly *E. ewingii* and *E. chaffeensis*, and can serve as a sentinel for monitoring for the presence of these zoonotic pathogens.

Key words: dog, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, ehrlichiosis, ticks

INTRODUCTION

Dogs are known to be susceptible to infection with several different *Ehrlichia* spp. (Little, 2010). *Ehrlichia canis,* the causative agent of canine monocytic ehrlichiosis, is considered the most pathogenic; in some cases, fatalities result (Little, 2010). *Ehrlichia ewingii* has the capacity to set up long-term infections in dogs and may induce polyarthritis (Little, 2010). Other *Ehrlichia* spp., including *E. chaffeensis, E. muris,* and Panola Mountain *Ehrlichia* (PME), have also been reported from dogs (Little et al., 2010; Hegarty et al, 2012; Qurollo et al., 2013). A number of *Ehrlichia* spp. also have been reported to cause disease in humans, although *E. chaffeensis* is considered to be the most common and clinically severe (Nicholson et al., 2010).

Amblyomma americanum is responsible for transmission of *E. chaffeensis*, *E. ewingii,* and PME (Anziani et al., 1990; Ewing et al., 1995; Yabsley et al., 2008) in the southeastern US while *Ixodes scapularis* is the proposed vector for *E. muris*-like agents (Pritt et al., 2011; Hegarty et al., 2012). Infection with *E. canis* is most often seen only in dogs because the primary vector, *Rhipicephalus sanguineus,* prefers to feed on canine hosts (Dantas-Torres 2010). However, *Dermacentor variabilis* has also been shown to be capable of transmitting *E. canis* (Johnson et al., 1998).

Dogs in the southeastern United States have the highest seroprevalence for *Ehrlichia* spp. (Bowman et al., 2009; Beall et al., 2012). Infections are particularly common in areas where *A. americanum* populations are intense. All of the tick vectors mentioned are present in the southeastern US and may infest dogs in this region (Centers for Disease Control and Prevention, 2013). To determine the risk of ehrlichial infection to

dogs, we exposed dogs to tick habitat via weekly walks and evaluated them using clinical, serological, and molecular diagnostic techniques.

MATERIALS AND METHODS

Study Participants and Pre-screening

Oklahoma State University's Institutional Animal Care and Use Committee reviewed and approved all animal protocols prior to the initiation of this study. Class A Beagle dogs (n=10), five months of age, were tested for evidence of current or previous infection with *E. canis, E. chaffeensis,* and *E. ewingii* by patient-side ELISA, indirect fluorescence antibody (IFA), species-specific peptide analysis, and nested and real-time polymerase chain reaction as described below prior to inclusion in the study. In addition, dogs were screened for infection with *Anaplasma* spp. and *Borrelia burgdorferi* (SNAP® 4Dx®), as well as *Rickettsia* spp. (IFA) as previously described (Barrett et al., 2014).

Tick Exposure and Clinical Monitoring for Disease

To ensure exposure to ticks, dogs were walked once per week for seven weeks in May and June, 2011, at a field station in Payne County, Oklahoma, as previously described (Barrett et al., 2014). Ticks acquired were allowed to feed to repletion. After the last walk, ticks were allowed to feed for one additional week and then all of the ticks were removed from each dog. Dogs were monitored daily over the entire exposure period in addition to two months following final tick exposure for clinical signs of infection, including rectal temperature, activity level, myalgia, and ocular discharge.

Sample Collection

Prior to tick exposure and twice weekly throughout the 121 day study, whole blood and serum were collected via jugular venipuncture as previously described (Barrett et al., 2014). Blood smears were made weekly. Serum and whole blood were stored at –20°C until serology or PCR were performed.

Serology

Antibodies to *Ehrlichia* spp. were detected using IFA tests as previously described (Ristic et al., 1972). Commercially available slides were used to test sera for antibodies reactive to *E. chaffeensis* and *E. canis* (Fuller Laboratories, Fullerton, California) with FITC-labeled goat-anti-dog IgG (KPL, Gaithersburg, Maryland) used to detect bound antibody. Every sample was screened at a 1/128 dilution; serial, two-fold dilutions of positive samples were evaluated until fluorescence was no longer observed, and the highest dilution at which specific fluorescence was observed reported as the maximum titer.

Species-specific peptide analysis was used to detect antibodies specific for *Anaplasma* spp. (eenz1), *A. phagocytophilum* (p44 aph), *A. platys* (p44 apl), *Borrelia burgdorferi* (C6), *Ehrlichia* spp. (p30/p30-1), *E. canis* (p16), *E. chaffeensis* (VLPT), and *E. ewingii* (p28) using a research SNAP prototype (Qurollo et al., 2014). Positive peptide values were quantified using a densigraph to determine the SNAP spot intensity (Chandrashekar et al., 2010). Commercially available patient-side enzyme linked immunosorbent assays (SNAP® 4Dx® and SNAP® 4Dx Plus®, IDEXX® Laboratories, Westbrook, Maine) were also employed for detection of antibodies to *Ehrlichia* spp.,

Anaplasma spp., and *B. burgdorferi*. Serum was tested according to manufacturer's instructions.

Nested PCR and Sequencing

DNA was extracted from 200 µL of anticoagulated whole blood with the Illustra™ blood genomic Prep Mini Spin Kit (GE Healthcare UK Limited, Buckinghamshire, UK) according to manufacturer's instructions from each dog on each collection date, and nucleic acid eluted into a final volume of 200 µL. Separate, dedicated laboratory areas were used for DNA extraction, primary amplification, secondary amplification, and product analyses, and negative (water) controls were included in each extraction and amplification. Species-specific 16S rDNA fragments were amplified by nested PCR using primers ECC/ECB followed by ECA/HE3 (*E. canis*), HE1/HE3 (*E. chaffeensis*), and EE72/HE3 (*E. ewingii*) as previously described (Little et al., 2010). Starting at study day 51 (d51) and working both toward d0 and d121, samples were tested until all 10 dogs were negative on two consecutive sample days (1 week). Standard agarose gel electrophoresis was used to confirm presence of amplicons, and representative amplicons purified and concentrated according to manufacturer's instructions using a commercially available kit (Wizard PCR Preps, Promega Corporation, Madison, Wisconsin) were then submitted for sequencing at the Molecular Core Facility at Oklahoma State University (Stillwater, Oklahoma). Resultant sequences were compared to those available in the National Center for Biotechnology Information database, including *E. chaffeensis* (NR_074500) and *E. ewingii* (NR_044747).

Real-time PCR

To externally validate the nested PCR results, real-time *Ehrlichia* spp. PCR was performed at a separate facility using a different approach. Template DNA was extracted from 200 µL canine whole blood using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, Indiana) according the manufacturer's instructions. A final volume of 200 µL of eluted DNA was obtained for each sample. All sample DNA was stored at –20°C until testing.

Real-time PCR hybridization probe assays detecting the disulfide oxidoreductase gene of *E. ewingii* (DQ902688) and *E. chaffeensis* (AF403711) were used for the testing of the sample DNA. The real-time PCR assays were performed with the LightCycler 480 instrument (Roche Applied Science, Indianapolis, Indiana). PCR was carried out in a total reaction volume of 20 µl containing LightCycler 480 Genotyping Master mix (Roche Applied Science), species specific primers and probes and 5 µl of template DNA (Ndip et al., 2007). Cycling parameters for the real-time PCR consisted of a denaturation cycle of 95°C for 10 minutes, followed by a 55 cycle amplification profile (95°C for 20 seconds, 60°C for 30 seconds with a single data acquisition, 72°C for 20 seconds), a melting curve profile (95°C for 1 minute, 45°C for 1 minute and 80°C continuous with a ramp rate of 0.14°C per second and 4 data acquisitions per °C) and a cool cycle of 40°C for 30 seconds. In each run, 10^5 and 10^2 copies of recombinant plasmids containing an insert of the species specific target were tested as positive controls. PCR grade water (Roche Applied Science, Indianapolis, Indiana) was tested as the negative control. Analytical sensitivity was determined to be 10 gene copies using the assay specific plasmids.

Complete Blood Counts and Blood Smears

Whole blood was submitted for complete blood count to the clinical pathology laboratory service, Oklahoma State University, from each dog on days 16, 44, 58, 72, and 86. Thin blood smears were air dried, fixed in methanol, stained using Wright's-Giemsa, and then examined microscopically for morulae within leukocytes by a boarded clinical pathologist (JHM).

RESULTS

All dogs used in this study were seronegative for antibodies reactive to *Anaplasma* spp., *Borrelia burgdorferi*, and *Ehrlichia* spp. on all methods used, as well as PCR negative for *Ehrlichia* spp., prior to natural tick exposure. As previously reported, dogs were infested with ticks on every exposure date for a total infestation of 57 – 108 ticks per dog (Barrett et al., 2014). The majority of ticks present were *A. americanum*, however low numbers of *D. variabilis* and *A. maculatum* were also seen. Clinical signs of illness were never observed in any dog (Barrett et al., 2014).

All 10 dogs developed antibody titers on IFA to both *E. canis* and *E. chaffeensis* (Table 1). Maximum inverse titers ranged from 128 – 4,096 for *E. canis* and 1,024 – 32,768 for *E. chaffeensis*. The geometric mean of the maximum inverse titers (GMT_{MAX}) for *E. canis* and *E. chaffeensis* were 548.7 and 4,096, respectively (Figure 1).

Species-specific peptide analysis using the microtiter well based assays revealed antibodies to *E. chaffeensis* (VLPT) in 7/10 dogs, and *E. ewingii* (p28) in 9/10 dogs. Positive peptide values ranged from 0.03 – 0.45 for *E. chaffeensis* and 0.04 – 0.85 for *E. ewingii* (Figure 1). Antibodies to *E. canis* (p16), *B. burgdorferi* (C6), or *Anaplasma* spp.

(eenz1, p44 aph, p44 apl) were not detected (Table 1). On d121, 4/10 dogs had antibodies reactive to *Ehrlichia* spp. (p30/30-1) using the SNAP® 4Dx® patient-side ELISA, and 7/10 had antibodies reactive to *Ehrlichia* spp. (p30/30-1 and p28) using the SNAP® 4Dx® Plus (IDEXX Laboratories Inc., Westbrook, Maine). The three dogs which were negative on d121 on SNAP® 4Dx® Plus were positive on d61 or d93; in total, 10/10 dogs developed antibodies reactive to *Ehrlichia* spp. on SNAP® 4Dx® Plus during the study.

On nested PCR, 7/10 dogs tested positive on at least one study date for the presence of *E. chaffeensis* (Table 2a), 10/10 for *E. ewingii* (Table 2b), and 0/10 for *E. canis* (data not shown). Sequences from representative amplicons aligned with 100% identity to those for *E. chaffeensis* (NR_074500) or *E. ewingii* (NR_044747). Rickettsemia with *E. chaffeensis* and *E. ewingii* was detected intermittently in blood via nested PCR for an average of 3.2 and 30.5 total days, respectively. Real-time PCR detected *E. chaffeensis* DNA in 0/10 and *E. ewingii* DNA in 9/10 dogs throughout the study (Tables 2a and 2b).

As previously reported (Barrett et al., 2014), no significant changes were observed on any CBC. A single morula was found in a neutrophil from one dog on d61 and in two neutrophils from a second dog on d72.

DISCUSSION

Diagnosis of tick-borne infections is increasingly common in the United States, both in animals and people (Nicholson et al., 2010). Our data reveal that the risk of infection is high even with a relatively limited time period of tick exposure. As evidenced

by PCR and serologic data, all dogs in this study became infected with *Ehrlichia* sp(p)., although infection with *E. ewingii* was more common than infection with *E. chaffeensis*. Some dogs remained PCR positive intermittently throughout the study, with 4/10 dogs still PCR positive for *E. ewingii* on d121, over two months after the final exposure to ticks, indicating persistent infection had been established. Previous work has shown that dogs can become persistently infected with *E. ewingii* following intravenous inoculation (Yabsley et al., 2011). The finding of persistent *E. ewingii* rickettsemia following natural tick exposure in the present study supports dogs as the proposed reservoir host for *E. ewingii* (Yabsley et al., 2011), although further study is needed to confirm that interpretation.

Results of the various diagnostic assays used in these dogs largely agreed with one another, although non-specific antibodies were detected on IFA before the specific peptide-based assays (Figure 1), and peptide-based assays may detect antibodies against *Ehrlichia* spp. that have not yet been described (Little, 2010). In addition, occasional discordant PCR results were observed which could be due to the different targets used, the relative sensitivity of the different assays, lower amounts of target present, or loss of detectable nucleic acid during storage of samples (Allison and Little, 2013). When considered together, the data from the present study support using multiple diagnostic modalities to identify infection, particularly early in infection when disease is most likely to develop (Little, 2010). However, identifying the most reliable testing modality for detecting early infection requires further exploration.

Even though all dogs were shown to be infected with one or more *Ehrlichia* spp., none of the dogs exhibited clinical signs of illness, nor were there any blood work

abnormalities indicative of infection with an *Ehrlichia* spp. This observation mirrors what is commonly seen by practicing veterinarians: antibodies to one or more *Ehrlichia* spp. are commonly detected in dogs in which clinical disease is absent or inapparent (Little et al., 2010). Compared to *E. canis*, infection with *E. chaffeensis* is thought to be less pathogenic in dogs (Little, 2010), and disease from *E. ewingii* varies, with only a portion of infected dogs developing clinical illness (Anziani et al., 1990). Interestingly, the dogs in the present study were also infected with *Rickettsia* spp., albeit of unknown pathogenicity (Barrett et al., 2014). Despite this co-infection, disease was not evident. Previous work has shown co-infection with *E. canis* and *A. platys* does result in more severe clinical disease in dogs than either agent alone (Gaunt et al., 2010). Co-infection with multiple tick-borne disease agents has been reported in naturally infected dogs, although the influence of co-infection on disease severity is not always clear (Kordick et al., 1999; Little et al., 2010).

Results from the present study show that dogs are a sensitive indicator for the presence of *Ehrlichia* infections in ticks; all ten dogs naturally exposed to ticks became infected with at least one *Ehrlichia* species in a single transmission season (Table 1). Canine seroprevalence studies have shown that antibodies to *Ehrlichia* spp. are most common in dogs from southeastern United States, with some areas identified where more than 10% of dogs test positive (Murphy et al., 1998; Bowman et al., 2009). In hyperendemic areas of Arkansas and Missouri, prevalence may be even higher; as many as 36.9% of dogs had antibodies to *E. chaffeensis* and 21.4% to *E. ewingii* (Beall et al., 2012). Our data suggest that wide scale studies using canine serology may provide valuable insights into risk for human ehrlichiosis, similar to the success seen with this

approach in understanding the risk of other tick-borne disease agents, such as *Borrelia burgdorferi* (Duncan et al., 2005).

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CONFLICT OF INTEREST

In the past five years, SL and JM have received honoraria and/or research support from IDEXX Laboratories, Inc., a company that manufactures some of the assays used in this research. Salary support for LS is provided through funding from Bayer Animal Health, a manufacturer of tick control products for pets. In addition, RC, BS, PT, BT, and MB are employees of IDEXX Laboratories, Inc. The other authors have no conflicts of interest to report.

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Table 1. Antibodies detected to *Ehrlichia canis*, *E. chaffeensis*, *E. ewingii*, *Ehrlichia* spp., *Anaplasma platys*, *A. phagocytophilum*, and *Borrelia burgdorferi* in 10 dogs naturally infested with ticks.

Organism	**1° Tick Vector**	**Analyte**	**Months after initial tick infestation[a]**				
			0	**1**	**2**	**3**	**4**
E. canis	*R. sanguineus*	IFA	0/10	1/10	8/10	8/10	8/10
E. chaffeensis	*A. americanum*	IFA	0/10	5/10	9/10	9/10	8/10
Ehrlichia spp.	Various	p30/p30-1	0/10	0/10	6/10	6/10	7/10
E. canis	*R. sanguineus*	p16	0/10	0/10	0/10	0/10	0/10
E. chaffeensis	*A. americanum*	VLPT	0/10	1/10	5/10	6/10	2/10
E. ewingii	*A. americanum*	p28	0/10	0/10	6/10	8/10	8/10
B. burgdorferi	*I. scapularis*	C6	0/10	0/10	0/10	0/10	0/10
Anaplasma spp.	Various	eenz1	0/10	0/10	0/10	0/10	0/10
A. phagocytophilum	*I. scapularis*	p44 aph	0/10	0/10	0/10	0/10	0/10
A. platys	*R. sanguineus*	p44 apl	0/10	0/10	0/10	0/10	0/10

[a]Serum was tested by IFA on study days 30, 61, 93, and 121 and by ELISA using specific peptides on days 33, 65, 89, and 117.

Table 2a. Detection by nested (and real-time) PCR of *Ehrlichia chaffeensis* in 10 dogs naturally infested with ticks.

Study Day	Dog number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
13	–	–	–	–	–	–	–	–	–	–
16	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
19	–	–	–	–	–	–	–	–	–	–
23	–	+	–	–	–	–	–	–	–	–
26	–	–	–	–	–	–	–	–	–	–
30	–	–	–	–	–	–	+	–	–	–
33	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	+ (–)	– (–)	– (–)	– (–)
37	–	–	–	–	–	–	–	–	+	–
40	–	+	–	–	–	–	+	+	–	–
44	–	+	–	–	–	–	–	–	–	–
47	–	+	–	–	–	–	+	–	–	+
51	– (–)	+ (–)	– (–)	– (–)	– (–)	– (–)	+ (–)	– (–)	– (–)	– (–)
55	–	+	–	–	–	–	–	–	–	–
59	–	–	–	–	–	–	–	–	–	–
61	–	–	–	–	+	–	–	–	–	–
65	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	+ (–)	– (–)	– (–)
67	–	–	–	+	–	–	–	+	–	–
72	–	–	–	–	–	–	–	–	–	–
75	–	–	–	–	–	–	–	–	–	–
Total[a]	0	16	0	1	1	0	8	3	1	2

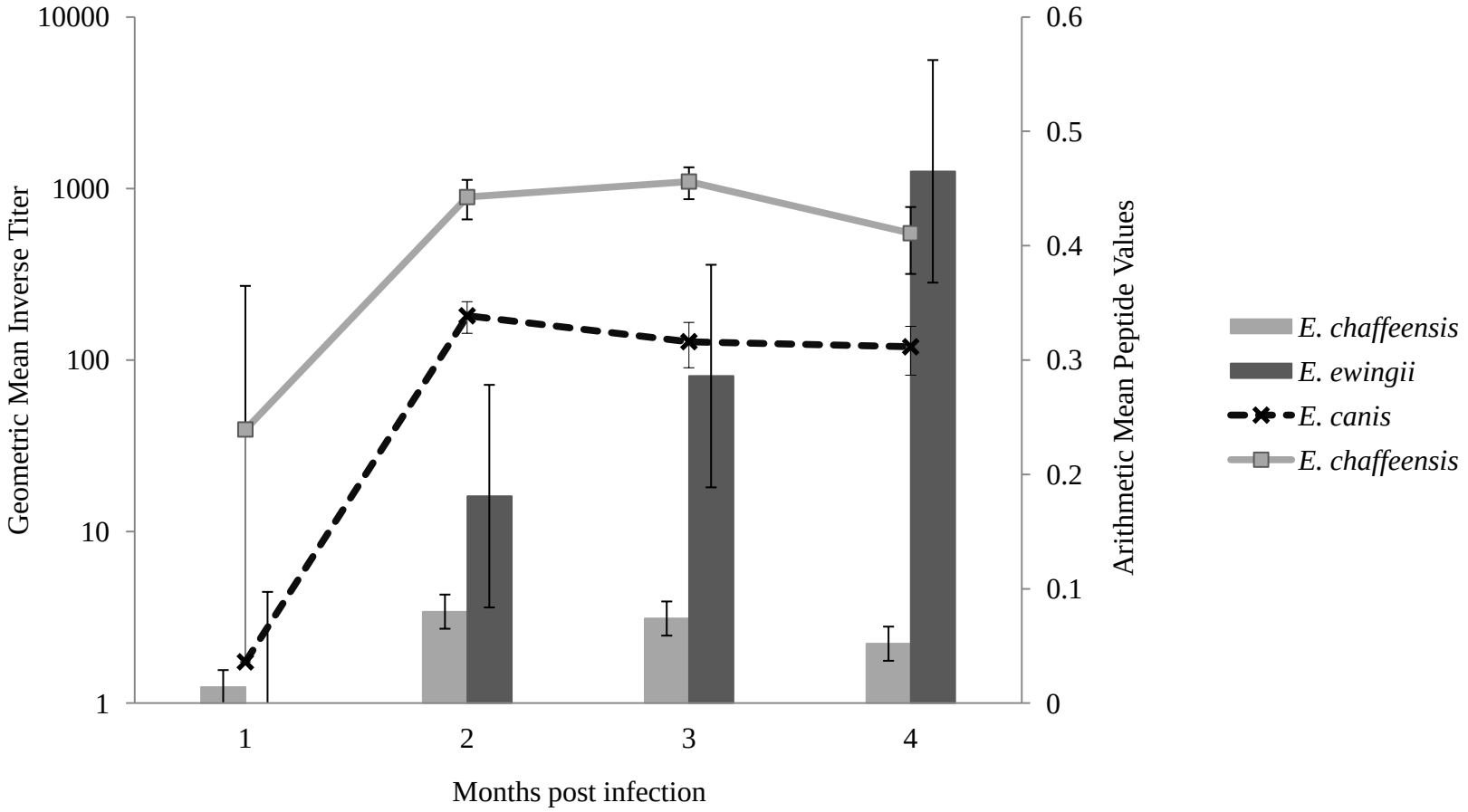
[a]Total number of consecutive days, inclusive, for which *E. chaffeensis* was detected by PCR.

Table 2b. Detection by nested (and real-time) PCR of *Ehrlichia ewingii* in 10 dogs naturally infested with ticks.

Study Day	Dog number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
30	–	–	–	–	–	–	–	–	–	–
33	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
37	–	–	–	–	–	–	–	+	–	–
40	–	–	–	–	–	–	–	+	–	–
44	–	–	–	–	–	–	–	+	–	–
47	–	–	+	–	–	–	+	+	–	–
51	– (–)	+ (+)	+ (+)	– (–)	– (–)	– (–)	+ (–)	+ (+)	+ (+)	+ (–)
55	+	+	+	–	–	–	+	+	+	+
59	+	+	+	–	–	+	+	+	+	+
61	+	+	+	–	+	+	–	+	+	+
65	+ (+)	+ (–)	– (–)	– (–)	– (–)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
67	+	+	–	–	–	+	–	+	+	+
72	+	–	–	+	–	+	–	+	+	+
75	+	–	–	+	–	+	–	+	–	+
79	– (+)	– (–)	– (–)	+ (+)	– (–)	+ (+)	– (–)	+ (–)	– (+)	– (–)
82	–	–	–	+	–	+	–	+	–	–
86	–	–	–	+	–	+	–	+	–	–
89	– (–)	– (–)	– (–)	+ (+)	– (–)	+ (+)	– (–)	+ (+)	– (–)	– (+)
93	+	–	–	–	–	+	–	–	–	–
96	–	–	–	–	–	+	–	–	–	+
100	+	–	–	–	–	+	–	–	–	+
103	+ (+)	+ (+)	– (–)	– (–)	– (–)	+ (–)	+ (+)	– (+)	– (–)	+ (+)
107	–	+	–	–	–	+	+	+	–	+
110	–	+	–	–	–	+	+	–	–	+
114	–	+	–	–	–	+	+	–	–	+
117	– (–)	+ (+)	– (–)	– (–)	– (–)	+ (–)	+ (+)	– (+)	– (–)	+ (+)
121	–	+	–	–	–	+	+	–	–	+
Total[a]	23	34	14	17	1	62	31	53	21	49

[a]Total number of consecutive days, inclusive, for which *E. ewingii* was detected by nested PCR.

Figure 1. Geometric mean titers to *Ehrlichia chaffeensis* and *Ehrlichia canis* on IFA (lines with standard errors) and arithmetic mean peptide values to *Ehrlichia ewingii*/p28 and *Ehrlichia chaffeensis*/VLPT (bars with standard errors) in dogs naturally infested with ticks. Antibodies to *E. canis*/p16 were not detected in any dog.



CHAPTER IV

PERSISTENT *EHRLICHIA EWINGII* INFECTION IN DOGS AFTER NATURAL TICK INFESTATION

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[1]Starkey, L.A., Barrett, A.W., Beall, M.J., Chandrashekar, R., Thatcher, B., Tyrrell, P., Little, S.E., 2015. *Journal of Veterinary Internal Medicine* 29:552-555. Reprinted here with permission of publisher.

ABSTRACT

Background: *Ehrlichia ewingii*, which causes disease in dogs and people, is the most common *Ehrlichia* spp. infecting dogs in the U.S. but little is known about how long *E. ewingii* infection persists in dogs.

Hypothesis/Objectives: To evaluate the persistence of natural infection with *Ehrlichia ewingii* in dogs.

Animals: Four Class A Beagles; no previous exposure to ticks or tick-borne infectious agents.

Methods: Dogs were exposed to ticks by weekly walks through tick habitat in north central Oklahoma; dogs positive for infection with *Ehrlichia* spp. by sequence-confirmed PCR and peptide-specific serology were evaluated for 733 days (D). Whole blood was collected once weekly for PCR, and serum was collected once monthly for detection of antibodies to *E. canis* (peptide p16), *E. chaffeensis* (IFA and VLPT), and *E. ewingii* (peptide p28).

Results: All dogs (4/4) became infected with *Ehrlichia* spp. as evidenced by seroconversion on IFA to *E. chaffeensis* (4/4); PCR detection of *E. ewingii* (4/4) and *E. chaffeensis* (2/4) DNA using both nested and real-time assays; and presence of specific antibodies to *E. ewingii* (4/4) and *E. chaffeensis* (2/4). Infection with *E. chaffeensis* was not detected after D55. Intermittent *E. ewingii* rickettsemia persisted in 3 of 4 dogs for as long as 733 days.

Conclusions and Clinical Importance: Our data demonstrate that dogs infected with *E. ewingii* from tick feeding are capable of maintaining infection with this pathogen long-term, and may serve as a reservoir host for the maintenance of *E. ewingii* in nature.

Keywords: *Amblyomma americanum*, granulocytic ehrlichiosis, reservoir host

Abbreviations:

PCR: polymerase chain reaction

IFA: indirect fluorescence antibody test

VLPT: variable-length PCR target

GMT_{MAX}: maximum geometric mean titer

IgG: immunoglobulin G

INTRODUCTION

Ehrlichia spp. are obligate intracellular bacteria transmitted by ticks that often infect white blood cells of mammals (Rar and Golovljova, 2011). A number of *Ehrlichia* spp. infections have been reported in dogs from the United States, including *E. canis*, *E. chaffeensis*, *E. ewingii*, Panola Mountain *Ehrlichia* sp., and *E. muris* (Little, 2010; Hegarty et al., 2012; Qurollo et al., 2013). *Ehrlichia canis* infection in dogs can cause anorexia, fever, epistaxis, hemorrhage, and sometimes results in death (Little, 2010; Rar and Golovljova, 2011). *Ehrlichia ewingii* also is an important pathogen in dogs. Fever, anorexia, thrombocytopenia, polyarthritis, and central nervous system abnormalities have been associated with *E. ewingii* infection in dogs (Little, 2010; Rar and Golovljova, 2011). Although there is little data to support *E. chaffeensis* causing disease in dogs, this species as well as several other canine *Ehrlichia* spp. are known to cause disease in people (Little, 2010; Nicholson et al., 2010; Rar and Golovljova, 2011).

Ehrlichia ewingii is the most prevalent *Ehrlichia* spp. detected by serology in dogs in the south central and south eastern United States (Beall et al., 2012). Infection is

transmitted by *Amblyomma americanum*, the lone star tick (Little, 2010). Infection with *E. ewingii* can cause clinically relevant disease in dogs, and dogs, in addition to white-tailed deer, also may serve as a reservoir host for this agent (Little et al., 2010; Rar and Golovljova, 2011). Dogs are the primary reservoir host for *E. canis*, and infection can be maintained for several years (Little, 2010; Rar and Golovljova, 2011). There also is potential for dogs to serve as a reservoir host for *E. chaffeensis*, but their role appears to be less important than that of white-tailed deer (Little, 2010; Rar and Golovljova, 2011). To characterize the persistence of infection with *E. ewingii* in dogs after natural tick exposure, we evaluated 4 dogs for 2 years after initial tick exposure.

MATERIALS AND METHODS

Class A Beagle dogs (n=4) infected with *E. ewingii* and *E. chaffeensis* as previously described (Starkey et al., 2014) were used for this study. All research was conducted under an Animal Care and Use Protocol approved by the Institutional Animal Care and Use Committee at Oklahoma State University. Briefly, dogs originally had been infested with ticks on 7 consecutive weekly walks and clinically monitored for evidence of tick-borne infection as previously described (Starkey et al., 2014). Whole blood was collected by jugular venipuncture twice weekly from study day (D) 0 through D121, and weekly from D256 – D733; serum was collected weekly from D0 – D121 and monthly from D256 – D712. Whole blood and serum were stored at –20°C until testing was performed.

Antibodies to *Ehrlichia* spp. were detected using indirect fluorescence antibody (IFA) tests and species-specific peptide ELISA. Sera were tested for antibodies using

commercially available *E. chaffeensis* IFA slides[a] and fluorescein isothiocyanate (FITC)-labeled goat-anti-dog IgG[b] as previously described (Starkey et al., 2014). Sera also were analyzed for presence of antibodies against *E. canis* (p16), *E. chaffeensis* (VLPT), and *E. ewingii* (p28), with results measured by densigraph as previously described (Starkey et al., 2014).

Nested polymerase chain reaction (PCR) assays were performed on DNA extracted from 200 µL of whole blood. To independently confirm the nested PCR results, real-time PCR also was performed on a subset of aliquots of samples collected every 2 weeks from D0 – D121 and every other month from D256 – D712. DNA for nested PCR was extracted using a commercial kit[c] according to the manufacturer's instructions. Extraction of DNA for real-time PCR utilized the High Pure PCR Template Preparation Kit[d] according to manufacturer's instructions. Extracted DNA was stored at –20°C until testing. Nested PCR was employed to amplify species-specific 16S ribosomal DNA fragments using external primers ECC/ECB and internal primers ECA/HE3 (*E. canis),* HE1/HE3 (*E. chaffeensis*), and EE72/HE3 (*E. ewingii*) as previously described, with representative amplicons directly sequenced to confirm identity (Little et al., 2010). Real-time PCR hybridization probe assays were used for detection of the disulfide oxidoreductase gene of *E. chaffeensis* (AF403711) and *E. ewingii* (DQ902688) as previously described (Starkey et al., 2014).

RESULTS

All 4 dogs developed antibodies (inverse titers ≥ 128) on IFA to *E. chaffeensis*; antibodies were first detected by IFA as early as D26 and continued to be detected through the final day of the study in 3 dogs (Figure 1). Maximal inverse titers during the study ranged from 1,024 – 32,768. Near the end of the study, by D712, 2 dogs had titers ≥ 4,096 (Figure 1). Specific antibodies to *E. ewingii* (p28) were absent at D33 for all dogs, detected in 3 dogs by D65, and detected in 1 dog by D89; *E. ewingii* specific antibodies persisted in all 4 dogs through D649 and in 3 dogs through D712. Maximal peptide values ranged from 0.25 – 1.08 as read by a densigraph (Figure 1). Specific antibodies to *E. chaffeensis* (VLPT) were absent at D33 but detected in 2 dogs by D65 and persisted through D712 with maximal peptide values ranging from 0.18 – 0.45 (Figure 1). *Ehrlichia canis* specific antibodies (p16) were not detected in any dog.

Two of 4 dogs had intermittently detectable *E. chaffeensis* DNA using the nested PCR assay on samples collected on D23 – D55 or D30 – D51, respectively, but *E. chaffeensis* DNA was not detected in any dog after D55. Real-time PCR did not detect *E. chaffeensis* DNA in any dog. All 4 dogs had detectable *E. ewingii* DNA by D59 using both PCR assays, with 2 dogs positive as early as D47 with nested PCR and by D51 with real-time PCR. One dog was PCR positive for *E. ewingii* only briefly (D47 – D61). Intermittent *E. ewingii* rickettsemia persisted long-term in 3 dogs. Two dogs were PCR positive D47 – D460 and the third dog was PCR positive D59 – D733. *Ehrlichia canis* DNA was not detected by nested PCR in any dog. Development of clinical illness was not observed in any dog during the course of this study.

DISCUSSION

In the absence of transovarial transmission in ticks, maintenance of *Ehrlichia* spp. in nature requires persistently infected vertebrate hosts as reservoir hosts (Nicholson et al., 2010). The present study showed that some dogs maintain long-term infection with *E. ewingii* after limited exposure to ticks. Three of the 4 dogs we followed maintained infections for 15 months, with 1 dog remaining infected for >2 years. Infection in this 1 dog continued until June 2014, more than 3 years after initial tick exposure (data not shown). Previous work has shown that infection with *E. ewingii* can be detected by PCR intermittently in dogs experimentally infected for at least 5 months after IV inoculation, and DNA also has been amplified from many apparently healthy client-owned or shelter dogs with unknown durations of rickettsemia (Little et al., 2010; Yabsley et al., 2011; Beall et al., 2012). Although many dogs die of serious disease, dogs have been shown to remain infected with *E. canis* for years while maintaining the ability to infect ticks, making them a key reservoir host (Little, 2010; Rar and Golovljova, 2011). The results of the present study suggest a similar situation may occur with *E. ewingii* in which dogs maintain long-term infections, potentially serving as a reservoir host, while also occasionally developing clinical disease associated with the infection. Breed as well as co-infection with multiple tick-borne agents may play a role in persistence of infection. However, results of this study are not consistent in that the 2 *Ehrlichia* spp. co-infected dogs maintained *E. ewingii* infection long-term (460 days) whereas the singly *E. ewingii* infected dogs maintained infection either briefly (up to 61 days) or long-term (>733 days). Moreover, no dog exhibited clinical signs consistent with tick-borne illness.

The persistence of *E. ewingii* infection in the dogs in the present study was documented by both serology and PCR. The inverse titers for the 3 persistently infected dogs exhibited small fluctuations throughout the 2-year study period, remaining within 3-fold from the lowest measured titer (Figure 1). The species-specific average peptide values remained steady for *E. ewingii* (p28) throughout the study whereas the *E. chaffeensis* (VLPT) average peptide values showed an overall gradual decrease during the 2-year study period (Figure 1). Antibody titers to p28 remained stable in 2 dogs over 8 months after the last positive PCR (D460) for *E. ewingii*, and detectable antibodies to VLPT still were present in 2 dogs 22 months after the last positive PCR for *E. chaffeensis*. The *E. chaffeensis* IFA utilized in this study detected antibodies in the sera of 2 dogs consistently PCR-negative for *E. chaffeensis*, suggesting detection of cross-reactive antibodies generated against *E. ewingii.* In the present study, infection with *E. ewingii* was detected in all 4 dogs by both nested and real-time PCR assays, whereas only nested PCR detected *E. chaffeensis* infection in 2 dogs. The reason for the occasional discordant results is not clear, but degradation of DNA could have occurred before sample processing and testing by real-time PCR assays (Allison and Little, 2013). Concurrent use of serologic and molecular diagnostic modalities likely would enhance detection of persistently infected animals.

Almost all of the known *Ehrlichia* spp. that infect dogs, including *E. ewingii* and *E. chaffeensis*, are zoonotic (Nicholson et al., 2010; Rar and Golovljova, 2011). Although most infections occur by tick feeding, transmission of *Ehrlichia* spp. also has been reported through contaminated blood products (Little, 2010). Dogs persistently infected with *E. ewingii* pose a potential infection risk to other animals by transfusion products

and to veterinary staff members that may come into contact with infected blood (Little, 2010). Persistently infected dogs also serve as a source of infection to ticks and may themselves acquire other tick-borne infections, which can lead to more severe disease (Little, 2010). The role that persistent infection with *E. ewingii* plays in the acquisition of and clinical signs associated with additional tick-borne co-infections warrants further exploration.

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Conflict of Interest Declaration: MB, RC, BT, and PT are employees of IDEXX Laboratories, Inc. which manufactures diagnostic assays for Ehrlichia ewingii. In the past 5 years, SL has received research funding, speaker honoraria, and travel reimbursement from IDEXX Laboratories, Inc. LS and AB have no conflicts to report.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

FOOTNOTES

[a] Fuller Laboratories, Fullerton, California

[b] KPL, Gaithersburg, Maryland

[c] Illustra™ blood genomic Prep Mini Spin Kit, GE Healthcare UK Limited, Buckinghamshire, United Kingdom

[d] Roche Applied Science, Indianapolis, Indiana

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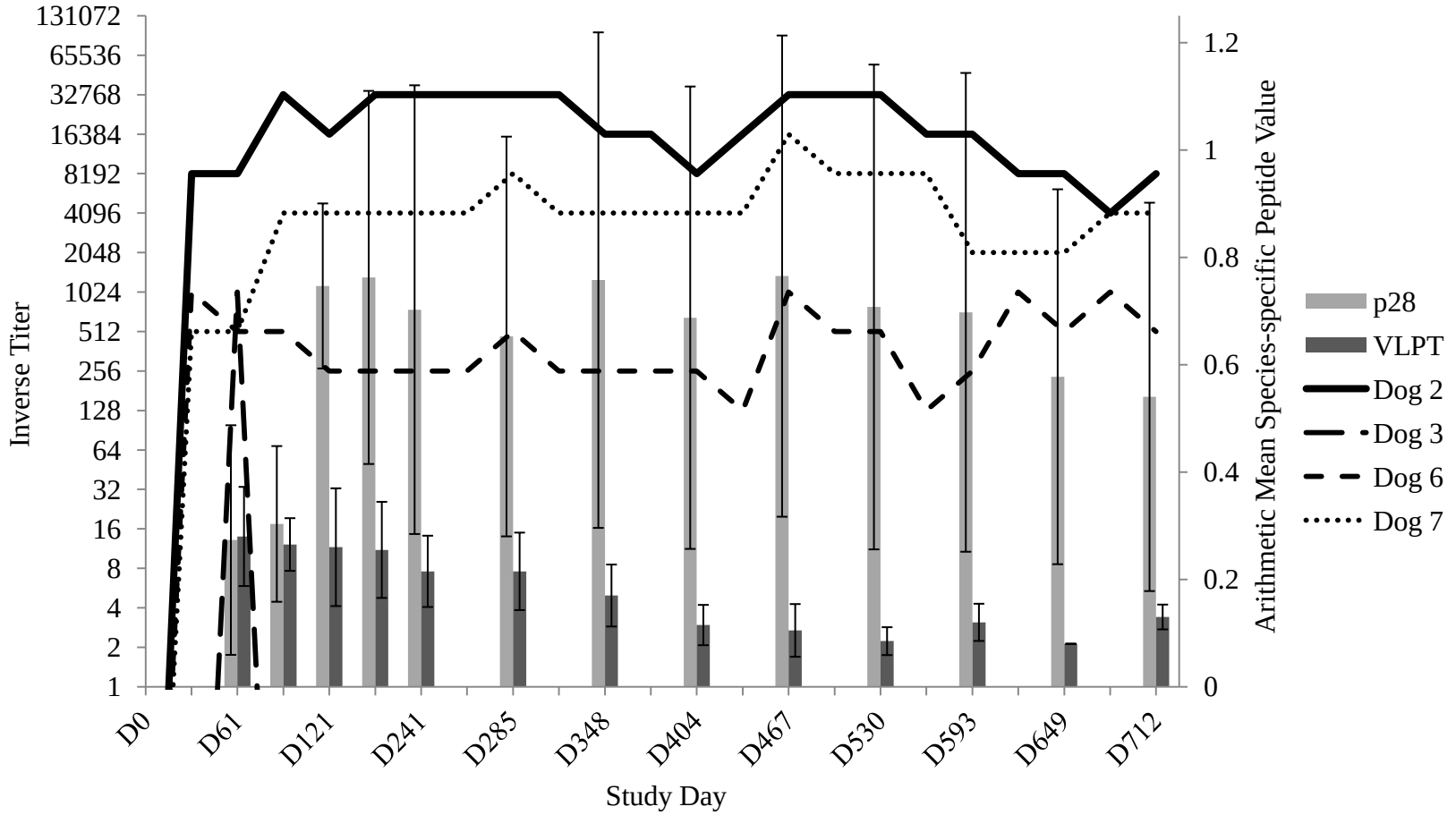
Figure 1. Inverse titers detected by IFA (lines; n=4) and species-specific mean peptide values to *Ehrlichia ewingii*/p28 and *Ehrlichia chaffeensis*/VLPT (bars with standard deviation) in dogs naturally infested with ticks.



CHAPTER V

PILOT TRIAL TO EVALUATE THE EFFECT OF PREVIOUS NATURAL INFECTION WITH *EHRLICHIA CHAFFEENSIS* OR *E. EWINGII* ON CHALLENGE THROUGH TICK FEEDING AND INTRAVENOUS SUB-INOCULATION

Starkey, L.A., Barrett, A.W., Little, S.E.

ABSTRACT

To evaluate the impact of a previous, naturally acquired ehrlichial infection on reinfection, we took four dogs with documented prior *Ehrlichia chaffeensis* or *E. ewingii* infections and walked them through tick habitat once a week for seven weeks. Ticks were identified and blood samples were collected for evaluation by blood smear examination, complete blood count (CBC), indirect fluorescence antibody (IFA) testing, and polymerase chain reaction (PCR) for evidence of reinfection with either *Ehrlichia* spp. Dogs became infested with 50 – 63 adult ticks over the entire infestation period (weekly average tick infestation = 8.4 ± 3.9); 91.9% of adult ticks were *Amblyomma americanum*. Antibodies to *E. chaffeensis* were detected by IFA (inverse titer >64; 512 – 8,192) in 3 of 4 dogs on D0 and titers remained elevated (256 – 16,384), although largely unchanged, through D112; antibodies were not detected in one dog from D0 – D112. Nucleic acid of *E. ewingii* was detected in 1 of 4 dogs intermittently from D0 – D122. None of the other three dogs had detectable DNA of any *Ehrlichia* spp. throughout the study period, and no dog exhibited clinical signs or CBC abnormalities consistent with tick-borne infection. To determine if dogs were resistant to reinfection by other routes, the three dogs that were PCR negative for *Ehrlichia* spp. infection were sub-inoculated intravenously with whole blood containing *E. ewingii* from the single PCR-positive dog on D170 and monitored by blood smear, CBC, IFA, and PCR through D216. Again, no dog developed clinical signs or CBC abnormalities; however, all three sub-inoculated dogs became PCR positive for *E. ewingii* for 10 – 28 days following inoculation. Increases in inverse antibody titer were detected in two dogs, from <64 to 512 and 1024 to 8192, while the titers of the other two dogs remained largely unchanged. Our data suggest that although

dogs previously infected with *Ehrlichia* spp. remain susceptible to reinfection via IV sub-inoculation, previous natural ehrlichial infections acquired from ticks may limit future infections through the same route.

INTRODUCTION

Tick-borne pathogens are present worldwide and cause disease in dogs and a variety of other hosts (McQuiston et al, 1999; Parola and Raoult, 2001; Nicholson et al., 2010; Estrada-Peña and de la Fuente, 2014). *Ehrlichia* spp. are important and common tick-borne pathogens in the United States, particularly for people and dogs living in or traveling to the southeastern quadrant of the nation where *E. chaffeensis* and *E. ewingii* are endemic (Bowman et al., 2009; Beall et al., 2012; Little et al., 2014; Qurollo et al., 2014).

In the absence of a vaccine, the recommended approach for preventing tick-borne infections is to limit exposure to ticks and to use approved acaricidal products (Stafford, 2007; Cisak et al., 2012; Domingos et al., 2013; CDC, 2014). However, it is evident that these methods of preventing infection are insufficient given that dogs and people in endemic areas continue to become infected with *Ehrlichia* spp. Surveys focusing on dogs from states in the southeastern U.S. have shown a high prevalence of *Ehrlichia* spp. infection, with *Ehrlichia* spp. antibodies reported in up to 96.3% of dogs and DNA of *E. chaffeensis* and *E. ewingii* detected in up to 33.3% and 29.6% of dogs, respectively (Dawson et al., 1996; Murphy et al., 1998; Kordick et al., 1999; Liddell et al., 2003; Little et al., 2010; CDC, 2014). Exploration into additional methods for preventing ehrlichial infections is warranted.

Previous work has shown that dogs develop less severe disease and shorter periods of rickettsemia upon reinfection with homologous strains of *E. canis* or *E. ewingii* (Breitschwerdt et al., 1998; Yabsley et al., 2011). Additionally, the use of an attenuated strain of *E. canis* as a vaccine was evaluated in dogs; following challenge with a wild virulent strain of *E. canis* by intravenous inoculation, clinical signs and rickettsial loads were decreased in the dogs that had received the attenuated strain of *E. canis* prior to challenge compared to the dogs that had not received the attenuated strain prior to challenge (Rudoler et al., 2012). In experimentally infected mice, those that received a specific peptide vaccination had lower bacteremia and less severe clinical signs upon challenge compared to non-vaccinated mice (Crocquet-Valdes et al., 2011; Thomas et al., 2011). These studies suggest that while previously infected animals are not completely protected against reinfection, both clinical signs and rickettsemia are reduced. We used a natural tick feeding model and intravenous sub-inoculation to determine if natural canine infections with *E. chaffeensis* and *E. ewingii* resulted in a similar level of protection from reinfection.

MATERIALS AND METHODS

Study Participants and Pre-exposure Antibiotic Therapy

Four class A Beagles (dogs 2, 3, 6, and 7) from a previous study that were infected with *E. chaffeensis* or *E. ewingii* following natural tick exposure (Starkey et al., 2014) were utilized in the present study. Prior to initiation of the present study, all four dogs were monitored for presence of *Ehrlichia* spp. infection by serology and PCR for two years as described previously (Starkey et al., 2015). Infection was confirmed in each

dog at some point in the initial infection trial. Doxycycline hyclate (TEVA Pharmaceutical Industries Ltd., Tikva, Israel) was administered per os (10 mg/kg q 24 hrs) for 28 days 5 months prior to reinfestation to two dogs (dogs 2 and 7) that had been confirmed to be PCR positive for both *E. chaffeensis* and *E. ewingii*. Dog 3 was only briefly PCR positive for *E. ewingii* and was not treated with antibiotics. Dog 6 was intermittently PCR positive for *E. ewingii* throughout the 2 year initial monitoring period and also was not treated.

Tick Exposure and Intravenous Sub-inoculation

To mimic a natural reinfection, dogs were walked through the same tall grass and wooded environment where ticks and *Ehrlichia* spp. infections had been acquired two years prior (Barrett et al., 2014; Starkey et al., 2014). Briefly, dogs were walked in pairs and rotated through 10 plots, spending 10 minutes in each plot (total walk time = 100 minutes) for seven consecutive weeks from April to June, 2013. Between walks dogs were housed indoors to ensure no exposure to ticks other than those acquired from the walks for the entire 216 day study period. Whole body tick counts were performed after each walk as previously described (Little et al., 2007; Barrett et al., 2014); attached adult ticks were morphologically identified to species and allowed to feed to repletion (Keirans and Litwak, 1989). Following the natural infestation, dogs with no PCR evidence of *Ehrlichia* spp. infection were intravenously sub-inoculated on D170 with re-suspended buffy coat from an *E. ewingii* rickettsemic dog (dog 6) as previously described (Yabsley et al., 2011). Briefly, whole blood (35 mL) from dog 6 was aseptically collected via jugular venipuncture into EDTA collection tubes. Blood was immediately centrifuged at 2500 x *g* for 10 minutes and the buffy coat layer and adjacent plasma and red blood cells

were aseptically recovered and re-suspended in filter sterilized 1X phosphate buffered saline (PBS). The total volume was divided equally into three parts (0.8 mL each) and inoculated intravenously into the left cephalic veins of dogs 2, 3, and 7.

Clinical Monitoring and Sample Collection

Dogs were monitored daily (D0 – D55 and D170 – D203) for clinical signs consistent with tick-borne infection (rectal temperature, lethargy, myalgia, ocular discharge, scleral injection, or conjunctivitis). Prior to tick re-exposure and throughout the first four months (D0 – D122) and last two months (D170 – D216) of the study, whole blood (EDTA) was collected twice weekly (D0 – D122 and D170 – D216) and serum was collected weekly (D0 – D112 and D170 – D212). Whole blood and serum samples were stored at –20°C until serology or PCR were performed.

Serology

Antibodies were detected by indirect fluorescence antibody (IFA) testing as previously described (Ristic et al., 1972). Commercially available slides (Fuller Laboratories, Fullerton, California) were used for IFA testing to detect antibodies reactive to *E. chaffeensis* through the use of FITC-labeled goat-anti-dog IgG (KPL, Gaithersburg, Maryland) as previously described (Ristic et al., 1972). Samples were screened at a 1/64 dilution; end-point titers were determined through sequential evaluation of two-fold serial dilutions.

PCR

Nested PCR for detection of *Ehrlichia* spp. DNA was performed as previously described (Little et al., 2010; Starkey et al., 2014). Briefly, DNA was extracted from whole blood according to manufacturer's instructions using the Illustra™ blood genomic

Prep Mini Spin Kit (GE Healthcare UK Limited, Buckinghamshire, UK); amplification of 16S rDNA fragments was achieved through the use of *Ehrlichia* spp. external primers (ECC/ECB) followed by internal species-specific primers (*E. canis*: ECA/HE3; *E. chaffeensis*: HE1/HE3; *E. ewingii*: EE72/HE3). Amplicons were visualized using standard agarose gel electrophoresis with ethidium bromide.

To confirm negative results from the natural re-exposure period, real-time PCR was performed as previously described (Starkey et al., 2014). Briefly, DNA was extracted from whole blood according to manufacturer's instructions using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, Indiana); the disulfide oxidoreductase genes of *E. chaffeensis* (AF403711) *and E. ewingii* (DQ902688) were detected through the use of real-time PCR hybridization probe assays.

CBC and Blood Smear Evaluation

Whole blood was submitted from each dog for complete blood count and routine adjunct blood smear examination (IDEXX Laboratories, Inc., Westbrook, ME) on days –25, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 and to the clinical pathology laboratory service at Oklahoma State University on days 170, 177, 184, 191, and 198.

Statistical Analysis

A Mann-Whitney rank sum test with significance assigned at $P < 0.05$ was employed to compare the total number of ticks acquired by dogs in the current study to the number of ticks acquired by dogs when initial *Ehrlichia* spp. infections were established.

RESULTS

Infestation with ticks was observed in every dog on each walking day (Table 1). The total number of ticks observed on each dog ranged between 58 and 71 (Table 1). Adult ticks present were either *A. americanum* (217 total) or *D. variabilis* (19 total); 28 nymphs were also observed (species not confirmed). Three of four dogs were seropositive to *Ehrlichia* spp. by IFA before tick re-exposure. Initial inverse titers of positive dogs on D0 ranged between 512 and 8192 (Figure 1). Only a slight increase or decrease in monthly titer was observed in any dog following tick re-exposure (D0 – D112), including dog 3, which had no detectable titer throughout the reinfestation period (Figure 1). In three dogs, DNA of *E. chaffeensis* or *E. ewingii* was not detected by either nested or real-time PCR between D0 – D122 (Table 2). Dog 6 was PCR positive for *E. ewingii* on D0 and remained intermittently positive through D122 as detected by both nested and real-time PCR assays (Table 2).

Following IV sub-inoculation, the inverse titer for dog 2 remained largely unchanged from D170 – D212. The inverse titer for dog 7 and 3 increased from 1,024 to 8,192 from D184 – D205, and from less than 64 to 512 from D184 – D198, respectively (Figure 2). Infection with *E. ewingii* was confirmed in all three recipient dogs (dogs 2, 3, and 7) as well as the donor dog (dog 6) by nested PCR following IV sub-inoculation (Table 2). Dog 2 was rickettsemic from D184 – D205, dog 3 from D181 – D209, dog 7 from D181 – D191, and dog 6 on all test dates from D170 – D216 (Table 2).

Clinical signs, significant changes on CBC, or morulae on blood smear were not observed in any dog following tick re-exposure or IV sub-inoculation.

The amount of ticks on dogs in the current study (median = 67.5 ticks) was not significantly different from the amount of ticks on dogs (median = 83 ticks) when initial *Ehrlichia* spp. infections were established (Mann-Whitney U= 2; df = 6; $p = 0.11$).

DISCUSSION

This study is the first to evaluate *Ehrlichia* spp. reinfection in dogs that were naturally infected with *Ehrlichia* spp. by tick feeding. Our results show that following re-exposure to ticks, no dog developed serologic or molecular evidence of reinfection with either *E. chaffeensis* or *E. ewingii*, suggesting immunologic protection from reinfection may have occurred. However, because dogs were exposed in different years, and because negative controls were not walked at the same time as our previously infected dogs, adequate infection may have simply failed to establish.

In comparing the two exposure periods, many variables were consistent including time spent walking through tick habitat, location of tick habitat, time of year, feeding time for ticks (to repletion), and methods used for monitoring both clinical illness and infection. However, some variables differed including average temperature, humidity, and fewer ticks detected on the dogs in the present study. Indeed, lower average daily temperatures in combination with higher humidity and rainfall could have impacted tick questing behavior and thus infestations between the two years (Mesonet, 2015; data not shown).

The average prevalence of infection in ticks may also have differed between the two exposure periods. Unfortunately the prevalence of infection in adult *A. americanum* was not determined in 2011, but in 2013, 5/155 (3.2%) *A. americanum* collected from the

walking site had DNA of *E. ewingii* while none contained DNA of *E. chaffeensis* (data not shown). Additionally, one tick tested positive for DNA of Panola Mountain *Ehrlichia* sp. (PME). This is the first report of PME in questing ticks in Oklahoma although one previous report documented PME in a tick collected from a human in the state (Loftis et al., 2008).

Interestingly, upon *E. ewingii* sub-inoculation, all three inoculated dogs developed detectable rickettsemia albeit in the absence of clinical signs. The two dogs with detectable antibody titers at day of inoculation (D170) had a shorter durations of rickettsemia compared to the dog with no initial titer, although level of titer did not appear to correspond to duration of rickettsemia (Table 2; Figure 2). Two dogs also exhibited an increase in antibody titer following reinfection (Figure 2). The results in the present study are similar to those reported from a previous study evaluating IV challenge in dogs previously infected with *E. ewingii* that showed previously infected dogs became rickettsemic for a similar period of time but did not have clinical signs (Yabsley et al., 2011).

Previous work has shown that up to 39% of dogs in Oklahoma have evidence of past or current infection with *Ehrlichia* spp. (Murphy et al., 1998; Little et al., 2010; Beall et al., 2012; Little et al., 2014; Qurollo et al., 2014). In the absence of tick prevention, the risk of infection to dogs with an *Ehrlichia* spp. can be high; a recent study determined that all (10/10) dogs exposed to tick habitat acquired infection (Starkey et al., 2014). The data from the present study suggest that further research is needed to clarify the role that the components of the immune system play during *Ehrlichia* spp. infection

and reinfection, and that novel methods to prevent tick-borne infection would be a welcome addition to an integrated tick-control program.

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Table 1. Tick infestations on dogs following natural tick re-exposure.

Dog	Total number of attached ticks (% of adults)			
	Amblyomma americanum	***Dermacentor variabilis***	**Nymphs[a]**	**Total**
2	55 (87.3%)	8 (12.7%)	2	65
3	58 (93.5%)	4 (6.5%)	8	70
6	56 (91.8%)	5 (8.2%)	10	71
7	48 (96.0%)	2 (4.0%)	8	58
Total	217 (91.9%)	19 (8.1%)	28	264

[a]Species not confirmed

Table 2. Detection of *Ehrlichia ewingii* by nested (and real-time) PCR in 4 dogs following tick re-exposure and IV sub-inoculation.

Study Day	**Dog number**			
	2	**3**	**6**	**7**
D–25	– (–)	– (–)	+ (+)	– (–)
D–4	–	–	+	–
D0 – D122	– (–)	– (–)	+[a] (+)[b]	– (–)
D170*	−	−	+	−
D174	−	−	+	−
D177	−	−	+	−
D181	−	+	+	+
D184	+	+	+	+
D188	+	+	+	+
D191	+	+	+	+
D195	+	+	+	−
D198	+	+	+	−
D202	+	+	+	−
D205	+	+	+	−
D209	−	+	+	−
D212	−	−	+	−
D216	−	−	+	−
Total[c]	21	28	46	10

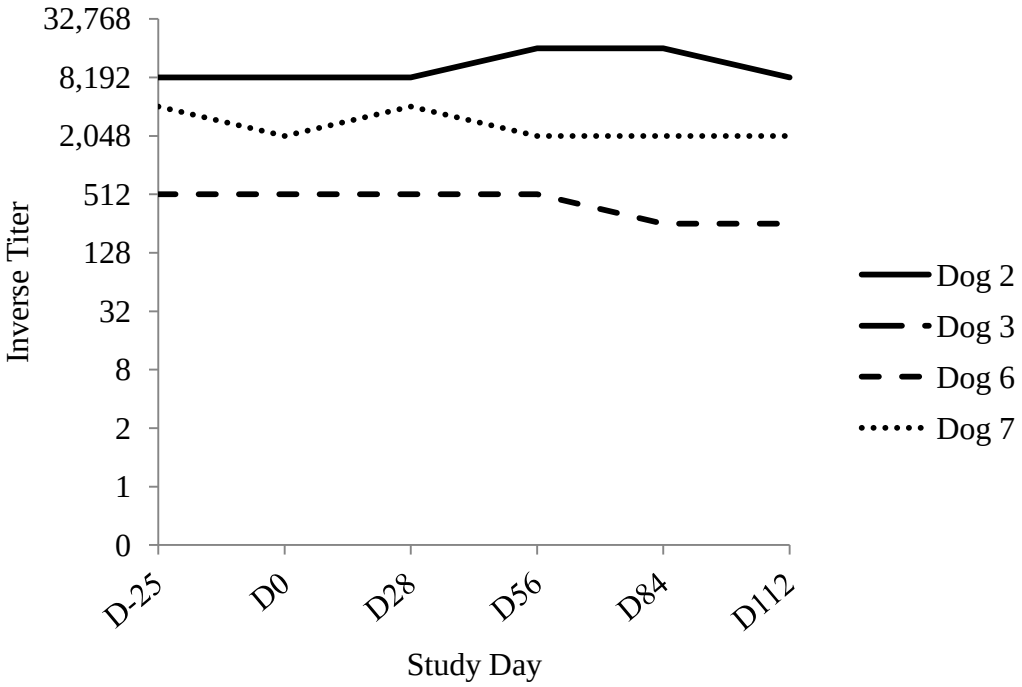
[a]Rickettsemia detected in 29/36 samples throughout this study period following natural tick re-exposure.

[b]Rickettsemia detected in 15/17 samples throughout this study period following natural tick re-exposure.

*Day of IV sub-inoculation: sample collected prior to IV sub-inoculation.

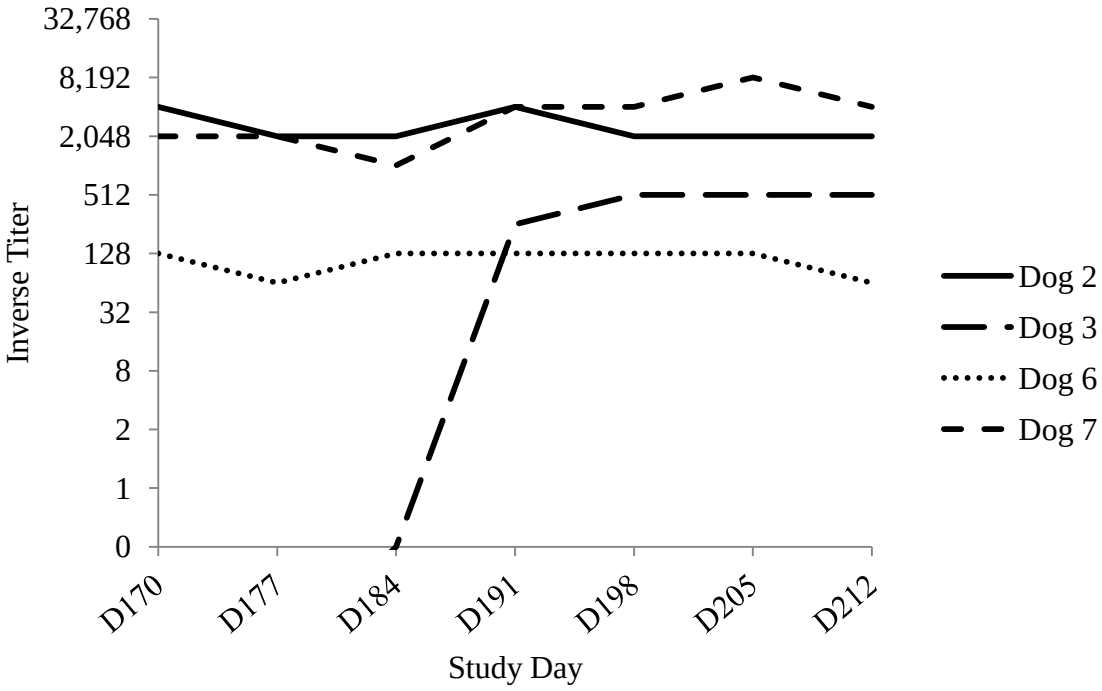
[c]Total number of consecutive days from D170 – D216, inclusive, *E. ewingii* was detected by nested PCR.

Figure 1. Inverse titers detected by IFA in dogs for four months following tick re-exposure.



*Dog 3 did not have a titer from D–25 through D112

Figure 2. Inverse titers detected by IFA in dogs for six weeks following IV sub-inoculation with *E. ewingii*.



CHAPTER VI

PREVALENCE AND IDENTITY OF *EHRLICHIA* SPP. IN DOGS FROM HAITI[1]

[1]Starkey, L.A., Newton, K., Brunker, J., Crowdis, K., Edourad, E.J., Meneus, P., Little, S.E., 2015. To be submitted as part of "Prevalence of vector-borne pathogens in dogs from Haiti" to *Veterinary Parasitology*.

ABSTRACT

Vector-borne pathogens, including the tick transmitted bacteria *Ehrlichia canis*, are of concern to people and dogs on some Caribbean islands, including Haiti, where survey data for *E. canis* and other canine vector-borne infections are lacking. To determine the prevalence of *Ehrlichia* spp. infection in dogs from Haiti, we conducted a molecular and serologic survey of whole blood collected from 210 owned dogs in 2013, 28 (13.3%) of which were infested with *Rhipicephalus sanguineus* ticks at the time of blood collection. No other tick species were identified on these dogs. A commercially available ELISA (SNAP® 4Dx® Plus) detected antibodies of *Ehrlichia* spp. in whole blood samples from 69/210 (32.9%) dogs, and PCR assays detected 16S rDNA fragments of *E. canis* in 15/207 (7.2%) dogs; none of the 207 canine samples tested were PCR-positive for *E. chaffeensis* or *E. ewingii*. Co-infection of *E. canis* with *Anaplasma* spp., *Dirofilaria immitis*, or both was detected by serology in 39/210 (18.6%) dogs. By PCR, co-infections with *Babesia canis vogeli*, *D. immitis*, or *Hepatozoon canis* were detected in 8/207 (3.9%) dogs: The common nature of *E. canis* infections along with the presence of additional vector-borne pathogens in these dogs, some of which are zoonotic, suggests that canine and human health in Haiti would benefit from vector-control programs to prevent infection.

INTRODUCTION

In dogs, just as in people, tick-borne pathogens are a cause of morbidity and mortality. At least five *Ehrlichia* spp. have been reported from dogs throughout the world, all of which are zoonotic: *E. canis, E. chaffeensis, E. ewingii, E. muris,* and Panola Mountain *Ehrlichia* sp. (Donatien and Lestoquard, 1935; Ewing and Buckner, 1965; Maeda et al., 1987; Anderson et al., 1991; Anderson et al., 1992; Dawson and Ewing, 1992; Dawson et al., 1996; Buller et al., 1999; Perez et al., 2006; Reeves et al., 2008; Pritt et al., 2011; Hegarty et al., 2012; Qurollo et al., 2013). Of those *Ehrlichia* spp., *E. canis, E. chaffeensis,* and *E. ewingii* have been reported in dogs from areas outside of the U.S., but only *E. canis* has been reported in dogs from Caribbean islands surveyed to date (Bool and Sutmoller; 1957; Huxsoll et al., 1970; Ndip et al., 2005; Georges et al., 2008; Gutiérrez et al., 2008; Yabsley et al., 2008; Yu et al., 2008; Kelly et al., 2013; Loftis et al., 2013; Qurollo et al., 2014). To our knowledge, there are no reports in the literature regarding the presence of any tick-borne pathogens in dogs from Haiti.

Ehrlichia spp. are vectored by a variety of ticks. *Rhipicephalus sanguineus*, the brown dog tick, is primarily responsible for transmission of *E. canis*, although *Dermacentor variabilis*, the American dog tick, has also been shown to be a competent vector (Groves et al., 1975; Johnson et al., 1998). The primary vector of *E. chaffeensis* and *E. ewingii* is *Amblyomma americanum*, the lone star tick (Anziani et al., 1990; Ewing et al., 1995). Although DNA of *E. chaffeensis* and *E. ewingii* has been detected in other tick species, namely *R. sanguineus* and *D. variabilis*, and *R. sanguineus* has been shown to acquire *E. chaffeensis* from experimentally infected dogs, transmission by these potential vector ticks has not yet been confirmed (Murphy et al., 1998; Steiert and Gilfoy,

2002; Ndip et al., 2007; Fritzen et al., 2011; Stoffel et al., 2014). *Rhipicephalus sanguineus* is reported as the most common tick infesting dogs from the Caribbean islands (L'Hostis et al., 1998; Yabsley et al., 2008; Asgarali et al., 2012; Kelly et al., 2013; Loftis et al., 2013; Crowdis, personal communication).

Clinical signs associated with *Ehrlichia* spp. infections in dogs are usually most severe if infected with *E. canis* (Little, 2010). In acute stages of disease, dogs typically present with a fever and may be lethargic and anorexic; hemogram analysis may reveal a pancytopenia (Wilkins et al., 1967; Huxsoll et al., 1969, 1970; Walker et al., 1970; Huxsoll et al., 1972). Dogs may also develop chronic illness which is usually severe, with renal, ocular, neurologic, and hematologic complications (Mylonakis et al., 2004; Little, 2010). *Ehrlichia ewingii* can also cause disease in dogs, although clinical signs are often less severe than those seen during infection with *E. canis.* Dogs with symptomatic *E. ewingii* infections are usually febrile, thrombocytopenic, and may exhibit polyarthritic lameness (Cowell et al., 1988; Anderson et al., 1992; Goodman et al., 2003; Yabsley et al., 2011). Canine infection with *E. chaffeensis* can be associated with mild clinical illness although most dogs molecularly diagnosed with *E. chaffeensis* infection are asymptomatic (Little, 2010; Dawson and Ewing 1992; Zhang et al., 2003; Starkey et al., 2014).

Canine ehrlichiosis caused by *E. canis* is commonly diagnosed in dogs from the Caribbean, including those presented to veterinary teaching hospitals in the region (Yabsley et al., 2008; Kelly et al., 2013; Loftis et al., 2013). Studies of dogs from the region report seroprevalences of 24.1% to 47.6% while circulating bacterial DNA has been reported from 14.1% to 24.7% of dogs (Georges et al., 2008; Hoff et al., 2008;

Yabsley et al., 2008; Asgarali et al., 2012; Kelly et al., 2013, Loftis et al., 2013; Qurollo et al., 2014). To date, there have been no reports of human infection with *E. canis* from the Caribbean although *R. sanguineus* ticks will feed on people and *E. canis* has been detected in human patients from Venezuela (Venzal et al., 2003; Dantas-Torres et al., 2006; Nicholson et al., 2006; Perez et al., 2006; Otranto et al., 2014; Liyanaarachchi et al., 2015).

Here we report the results of a survey conducted to better understand the ehrlichial agents infected and tick species infesting dogs in Haiti.

MATERIALS AND METHODS

Dogs

From February to April 2013, blood samples were collected from 210 dogs presented to government sponsored vaccination clinics throughout Haiti. Age of each dog was provided by the owner.

Whole Blood Sample Testing

Whole blood (EDTA) samples (n=210) were tested by a commercial lateral flow assay (SNAP® 4Dx® Plus, IDEXX Laboratories, Inc., Westbrook, Maine) designed to detect antibodies generated against *E. canis* and *E. ewingii* (Stillman et al., 2014).

Nested PCR was used to evaluate whole blood samples (n=207) for *Ehrlichia* spp. infection as previously described (Little et al, 2010). Briefly, DNA was extracted from 200 µL of anticoagulated whole blood with a commercial kit (Illustra™ blood genomic Prep Mini Spin Kit, GE Healthcare UK Limited, Buckinghamshire, UK) and then used in nested PCR assays for 16S rDNA fragments as previously described (Little et al., 2010).

Negative water controls were included in each extraction and amplification steps; separate, dedicated laboratory spaces were used from the DNA extraction through purification for sequencing. All amplicons were purified (Wizard PCR Preps, Promega Corporation, Madison, Wisconsin), commercially sequenced (SimpleSeq™, Eurofins MWG Operon Inc., Huntsville, Alabama; Molecular Core Facility at Oklahoma State University, Stillwater, Oklahoma), and resultant sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) and compared with *E. canis* sequences listed in the National Center for Biotechnology Information database.

Tick Collection and Identification

Dogs were examined for the presence of ticks; representative ticks were collected from infested dogs and placed into 70% ethanol and later identified to species by morphological characteristics (Keirans and Litwak, 1989).

Statistical Analysis

Infection with *Ehrlichia* spp. antibodies and infection with *Ehrlichia canis* DNA were compared to age groups (<2, 2 – 3.9, 4 – 6.9, and 7+ years) using Chi-square tests with significance assigned at $p < 0.05$. Age was also compared to *Ehrlichia* spp. antibody status using a Mann-Whitney rank sum test with significance assigned at $p < 0.05$.

RESULTS

Ages of dogs included in this study ranged from 6 months to 13 years (average age = 3.8 ± 2.6 years). Commercial ELISA identified *Ehrlichia* spp. antibodies in 69/210 (32.9%) dogs (Table 1). Serologic evidence of co-exposure or -infection of *E. canis* with at least one additional vector-borne pathogen was seen in 39/210 (18.6%) dogs, including

Anaplasma spp. (13/210; 6.2%), *Dirofilaria immitis* (16/210; 7.6%), and all 3 agents (10/210; 4.8%) (Table 1). *Ehrlichia canis* DNA was detected in 15/207 (7.2%) dogs; evidence of active co-infection with other vector-borne pathogens was identified in 8/207 (3.9%) of dogs, including *Babesia canis vogeli* (1/207; 0.5%), *Dirofilaria immitis* (4/207; 1.9%), and *Hepatozoon canis* (3/207; 1.4%) (data not shown). Sequence of all 15 *E. canis* amplicons were identical to one another and 99.7% identical to previously reported *E. canis* sequences from the U.S. (NR_074283), Italy (GQ857078), and Brazil (KJ995842). Neither *E. chaffeensis* nor *E. ewingii* DNA were detected in any dog.

Tick infestation was identified on 28/210 (13.3%) dogs. All ticks submitted for morphological identification (20 nymphs, 9 male, and 15 female) were morphologically identified as *Rhipicephalus sanguineus* sensu lato.

The prevalence of *Ehrlichia* spp antibodies increased significantly by age (χ^2=12.31; df = 3; p = 0.006), from 12.8% for dogs < 2 years to 31.2%, 39.1%, and 50.0% for dogs 2 – 3.9 years, 4 – 6.9 years, and 7+ years, respectively. However, prevalence of *E. canis* DNA detected by PCR did not differ by age group (χ^2=1.63; df = 3; p = 0.652). The median age of dogs identified as *Ehrlichia* spp. antibody positive was significantly higher (median age = 4 years) than the median age of dogs without *Ehrlichia* spp. antibodies (median age = 3 years; Z=-3.52; df = 208; p < 0.001).

DISCUSSION

This study is the first to document *Ehrlichia canis* infections and *Rhipicephalus sanguineus* infestations in dogs from Haiti. Nearly one-third of dogs tested had detectable antibodies to *Ehrlichia* spp., a prevalence comparable with reports from other islands in

the Caribbean which show seroprevalence of *Ehrlichia* spp. infection ranging between 24% and 48% of tested dogs (Hoff et al., 2008; Yabsley et al., 2008; Asgarali et al., 2012; Kelly et al., 2013, Loftis et al., 2013; Qurollo et al., 2014). The presence of antibodies in these dogs does not necessarily indicate an active, current infection; only 15 of the 69 dogs with antibodies also had detectable DNA of *E. canis* present in their blood to confirm active infection (Baneth et al., 1996; da Costa et al., 2005; Little et al., 2010). It is likely that the remaining antibody-positive dogs were chronically infected with low-level rickettsemia that was below the level of detection for the PCR assay or previously infected with antibodies remaining in circulation; *Ehrlichia canis* DNA was not detected in any seronegative dog. The presence of antibody in dogs with *E. canis* has been documented several years after initial infection (Ewing and Buckner, 1965; Bartsch and Greene, 1996; Harrus et al., 1998). Our finding of fewer PCR-positive dogs in comparison to sero-positive dogs mimics what has been reported in dogs from other Caribbean islands (Yabsley et al., 2008; Kelly et al., 2013; Loftis et al., 2013).

Rhipicephalus sanguineus sensu lato was the only tick identified from any of the dogs examined in this study. Data from other studies of tick infestations on dogs from other Caribbean islands support our finding that *R. sanguineus* is the most common, if not the sole, species of tick infesting dogs in the region (L'Hostis et al., 1998; Yabsley et al., 2008; Asgarali et al., 2012; Kelly et al., 2013; Loftis et al., 2013; Crowdis, personal communication). *Rhipicephalus sanguineus* is now known to be a species complex comprised of as many as 5 distinct species of ticks (Dantas-Torres et al., 2013), and further molecular characterization of the morphotype(s) present in the Caribbean would be of interest. Since the discovery of other *Ehrlichia* spp. DNA in *R. sanguineus* from

other parts of the world, this tick has been implicated as a potential vector for the transmission of *E. chaffeensis* and *E. ewingii*; however, the present study found no molecular evidence of either *E. chaffeensis* or *E. ewingii* in any of the dogs from Haiti, nor has there been any report of a dog infected with either of these *Ehrlichia* spp. from any other island in the Caribbean (Murphy et al., 1998; Ndip et al., 2007; Yabsley et al., 2008; Qurollo et al., 2014; Stoffel et al., 2014).

Routine, consistent use of products that control ticks on dogs and thus may prevent the transmission of tick-borne pathogens is lacking throughout Haiti and on other Caribbean islands (Kelly et al., 2013; Loftis et al., 2013; Crowdis, personal communication). Use in the region is considered further limited by lack of knowledge on the part of pet owners about the risks that ticks pose to pets and people; scarcity of control products; or prohibitive cost (Crowdis, personal communication). Public and pet health may benefit from communicating the health risks associated with ticks to the public through routes such as the established government sponsored rabies vaccination clinics that allowed collection of the samples used in the present paper.

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Table 1. Seroprevalence of *Ehrlichia* spp. and other select vector-borne pathogens in dogs (n=210) from Haiti.

Vector-borne infectious agent	**# dogs positive (%)**
Total *Ehrlichia* spp. antibodies detected	**69/210 (32.9)**
Ehrlichia spp.[b] alone	30/210 (14.3)
Co-infections with *Ehrlichia* spp.	**39/210 (18.6)**
Ehrlichia spp.[b] + *Dirofilaria immitis*[a]	16/210 (7.6)
Ehrlichia spp.[b] + *Anaplasma* spp.[b]	13/210 (6.2)
Ehrlichia spp.[b] + *D. immitis*[a] + *Anaplasma* spp.[b]	10/210 (4.8)

[a]Antigen detected by assay

[b]Antibody detected by assay

CHAPTER VII

CONCLUSIONS

Tick-borne infections of dogs, particularly those caused by *Ehrlichia* spp., are common in the U.S. and other parts of the world (McQuiston et al., 1999; Parola and Raoult, 2001; Ndip et al., 2005; Nicholson et al., 2010; Rani et al., 2011). The two most common *Ehrlichia* spp. infecting dogs from the U.S., *E. chaffeensis* and *E. ewingii*, are vectored by *Amblyomma americanum* (Anziani et al., 1990; Long et al., 2003; Bowman et al., 2009, Beall et al., 2012; Little et al., 2014; Qurollo et al., 2014). The more pathogenic *Ehrlichia* spp. of dogs, *Ehrlichia canis*, is less prevalent in dogs from the U.S., but is present throughout the world, anywhere that *Rhipicephalus sanguineus* ticks are found (Groves et al., 1975; Yabsley et al., 2008; Bowman et al., 2009, Dantas-Torres, 2010; Little, 2010; Beall et al., 2012; Kelly et al., 2013; Loftis et al., 2013; Little et al., 2014; Qurollo et al., 2014). The overarching goals of the research reported in this dissertation were to gain a better understanding of the risk of infection with *Ehrlichia* spp. in dogs exposed to ticks in a natural setting, the duration of those infections, and the potential for reinfection.

Conclusions from four research studies are presented in the following paragraphs:

STUDY 1: CHAPTER III

The goal of the first study was to determine the risk of infection with one or more *Ehrlichia* spp. posed to dogs exposed to ticks in a natural setting where *A. americanum* ticks are common. Current survey data of *Ehrlichia* spp. infections in dogs throughout the U.S. demonstrate that dogs in the southcentral and southeastern states, areas where *A. americanum* is the predominant tick, are more commonly infected with *Ehrlichia* spp. when compared to dogs from other regions of the U.S. (Childs and Paddock, 2003; Bowman et al., 2009; Beall et al., 2012; Little et al., 2014; Qurollo et al., 2014). Following a relatively brief exposure period to various tick habitats in north central Oklahoma, all 10 dogs in this study developed infection with *E. ewingii*; seven dogs developed a co-infection with *E. chaffeensis* as well. Even though infections were subclinical in these dogs, both *E. chaffeensis* and *E. ewingii* have been reported to cause disease in dogs (Cowell et al., 1988; Anderson et al., 1992; Dawson and Ewing, 1992; Goodman et al., 2003; Zhang et al., 2003; Yabsley et al., 2011). These data highlight that the absence of tick-control allows for infection with potentially pathogenic and zoonotic tick-borne agents, and that even brief exposures to ticks can result in infection.

STUDY 2: CHAPTER IV

The second study sought to determine the length of time a dog could remain rickettsemic with *E. chaffeensis* and *E. ewingii* following infection that was acquired through tick feeding over a known infection period. Reports exist of dogs testing positive for *Ehrlichia* spp. infection by both serologic and molecular methods in the absence of any clinical signs, indicating that some of these dogs may be chronically infected (Little

et al., 2010; Kelly et al., 2013; Loftis et al., 2013). Infection with *E. canis* is known to persist in some dogs, yet data are lacking regarding long-term infection of *E. chaffeensis* or *E. ewingii* in dogs following tick feeding (Ewing and Buckner, 1965; Harrus et al., 1998). Experimental studies looking at the longevity of sole infection in dogs with either *E. ewingii* (intravenous injection) or *E. chaffeensis* (subcutaneous injection) revealed that dogs may remain rickettsemic for at least 144 and 117 days, respectively (Zhang et al., 2003; Yabsley et al., 2011). Our data regarding longevity of rickettsemia subsequent to infection acquired via tick feeding revealed that infection with *E. chaffeensis* persisted for no more than 55 days following initial tick exposure in any dog. Conversely, one dog remained rickettsemic with *E. ewingii* for at least 733 days; an additional two dogs were rickettsemic for 460 days each. This is the first report of long-term *E. ewingii* rickettsemia in dogs following infection acquired through tick feeding and contributes to the growing body of research that supports the interpretation that dogs may serve as a reservoir host of *E. ewingii* in nature.

STUDY 3: CHAPTER V

We also sought to evaluate the effect that previous, naturally acquired *Ehrlichia* spp. infections had on the ability of a dog to become reinfected by both tick feeding and IV sub-inoculation. Previous work has shown that dogs and mice that were previously infected with an *Ehrlichia* spp. had less severe clinical signs or decreased bacterial loads when challenged by reinfection (Breitschwerdt et al., 1998; Crocquet-Valdes et al., 2011; Thomas et al., 2011;Yabsley et al., 2011). Our data indicate that reinfection by tick feeding did not occur in dogs infected two years prior with *Ehrlichia* spp. through tick

feeding, but infection with *E. ewingii* via IV sub-inoculation did occur in all three dogs challenged. Although this pilot trial lacked necessary components that would allow full interpretation of the results, such as naïve dogs during the re-exposure walks and larger number of dogs per group, the results suggest that the initial route of infection (i.e. tick feeding) may alter the likelihood of reinfection by the same route. Additional investigations into this concept are needed.

STUDY 4: CHAPTER VI

The aim of the final study was to determine the identity and prevalence of *Ehrlichia* spp. infections in dogs from Haiti, an island where *A. americanum* is absent. *Rhipicephalus sanguineus* ticks are the most common ticks reported infesting dogs from the Caribbean islands. Furthermore, *E. canis*, which is vectored by *R. sanguineus*, is commonly detected in dogs from other Caribbean islands (Groves et al., 1975; L'Hostis et al., 1998; Georges et al., 2008; Hoff et al., 2008; Yabsley et al., 2008; Asgarali et al., 2012; Kelly et al., 2013; Loftis et al., 2013; Qurollo et al., 2014; Crowdis, personal communication). This study is the first to describe the identity and prevalence of vector-borne pathogens in dogs from Haiti. Infection with *E. canis* was detected in 7.2% of dogs by PCR and in 32.9% of dogs by serology. The only ticks identified from dogs in this study were *R. sanguineus*, and 13.3% of dogs were actively infested at time of examination. Our research along with the work of others indicate that vector-borne infections are common in dogs in the Caribbean and that dogs and people on these islands would benefit from the implementation of vector-control programs.

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