The canine host serving as a sentinel species for tick-borne diseases caused by *Anaplasma, Ehrlichia* and *Borrelia* pathogens impacting human health in the USA

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Abstract

Tick-borne diseases continue to threaten the health of people, dogs, and agricultural animals. In the USA, human Lyme disease, caused by *Borrelia burgdorferi*, has the highest incidence, followed by diseases resulting from *Ehrlichia* and *Anaplasma* species. We investigated the prevalence of these diseases in dogs as the same pathogens are also known to cause infections in the human host. Blood samples collected from clinically suspected dogs from across the USA were assessed for antibodies for four different tick-borne pathogens. Molecular detection and culture recovery methods were also performed to detect the presence of the pathogen. A total of 1,340 samples were assessed for *A. phagocytophilum*, two *Ehrlichia* species: *E. canis* and *E. chaffeensis*, and *B. burgdorferi*. Positive samples included 286 (21.3%) for *A. phagocytophilum*, 228 (17.01%) for *E. chaffeensis*, 233(17.3%) for *E. canis*, and 366 (27.2%) for *B. burgdorferi*. Co-infection of *A. phagocytophilum* with *E. canis* and *E. chaffeensis* was observed in 64 and 65 dogs, respectively, whereas with *B. burgdorferi*, we observed in 76 dogs. Similarly, 34 dogs had antibodies for *Ehrlichia* species and *B. burgdorferi.* Two hundred and four dogs tested positive for the two *Ehrlichia* species, while eight dogs were positive for all three genera pathogens. Quantitative real-time Reverse Transcriptase PCR (qRT-PCR) was performed on 171 samples were randomly selected to assess the presence of *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii*. Culture recovery experiments performed for *Ehrlichia* species on 66 IFA positive serum/blood did not result in any positives, and similarly, all 171 samples tested negative by molecular methods targeted to detect the bacterial DNA.

We observed a significant overlap in the geographical distribution of the samples that tested positive for the pathogens belonging to all three diseases in dogs. These data are similar to the CDC-reported human prevalence data for tick-borne diseases for anaplasmosis, ehrlichiosis, and borreliosis. Our data suggest that tick-borne diseases in dogs closely resemble the prevalence data documented for humans. Thus, monitoring canine infections has important implications in serving as the sentinel species for human tick-borne diseases as well as aiding in improving the companion animal health.

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Dedication

I dedicate my work to my beloved parents, my brother and my grandparents.

Chapter 1 - Literature and Significance

Infectious Diseases

Infectious diseases are a leading cause of mortality and morbidity worldwide in the past and present. Infectious diseases have a significant impact on the health of humans and animals. Infectious disease contributes to one-third of the human deaths worldwide. Vector-borne infections caused by bacteria, parasites, or viruses account for more than 17% of the infectious diseases (https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases). More recently, emerging tick-borne diseases are increasing, and they are identified as the second leading cause in impacting human and animal health (Anderson & Magnarelli, 2008).

Ectoparasites

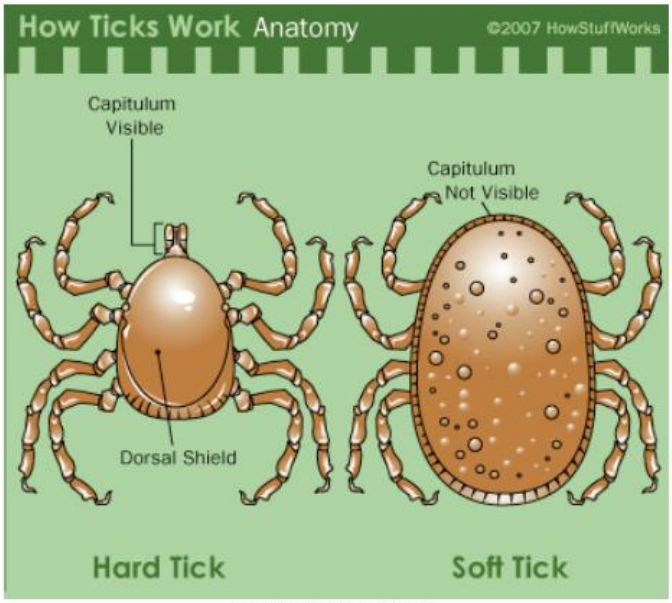
Ectoparasites are organisms that reside on the outer surface of the skin or in the skin's superficial layers. The ectoparasites feed on animals and humans for a bloodmeal. The two main categories of ectoparasites are insects and arachnids (Levinson et al., 2020). The blood-sucking arthropods, such as mosquitoes, significantly impact humans and animals by causing one of the deadliest diseases, such as malaria. Ticks are the second major ectoparasites which are vectors for various bacterial, viral, and parasitic disease-causing agents impacting animal and human health (Sonenshine & Roe, 2013)

Ticks as ectoparasites

Ticks are obligate blood-sucking arthropod vectors transmitting infectious agents to animals and humans. Ticks are the small arthropods belonging to the subphylum *Chelicerata*, class *Arachnida*, subclass *Acari*, the *acarine* order, and suborder *Ixodida*. *Ixodida* ticks are

classified into three families, namely, *Ixodidae, Argasidae,* and *Nuttalliedae* (Nicholson et al., 2019). There are two types of ticks, hard ticks and soft ticks, which are commonly referred to as *Ixodidae* and *Argasidae,* respectively. The hard ticks consist of the tough sclerotized plate, called scutum, on the dorsal body surface (Sonenshine & Roe, 2013) . The soft ticks have a pseudoscutum that lacks the smooth appearance of the scutum (Sonenshine & Roe, 2013) (Figure 1.1). The soft ticks include the genus *Argas, Carios, Ornithodoros,* and *Otobius*. The ticks belonging to these genera primarily parasitize the avian species (Nicholson et al., 2019). One exception is the genus *Ornithodoros* which also affects mammalian species and acts as a vector of relapsing fever spirochetes to animals and humans (Nicholson et al., 2019).

The hard ticks are commonly found in the tropical and sub-tropical regions (Nuttall, 1905). The range of the hard ticks has been expanding in recent years due to human activities and climatic changes. The hard ticks transmit various pathogens and impact the health of diverse animal populations and people (Liu & Bonnet, 2014). They are also responsible for causing various livestock diseases impacting the economy of many countries around the world. The hard ticks transmit several pathogens to the animal and human host through the wildlife reservoir host (Dantas-Torres et al., 2012; Liu & Bonnet, 2014; Sonenshine & Roe, 2013). The species of hard ticks impacting animal and human health belong to the genus *Ixodes, Amblyomma, Dermacentor,* and *Rhipicephalus.* Ticks belonging to these genera are distributed worldwide and transmit various infectious agents (Nicholson et al., 2019).



Hard tick vs. soft tick

Figure 1.1: Classification of ticks

https://repellosa.com/helpful-hints/how-ticks-work/

The life cycle of ticks

The life cycle of a hard tick consists of four developmental life stages: egg, larva, nymph, and adult (Walker, 1998) (Figure 1.2). Female hard ticks lay enormous numbers of eggs following mating with a male after a blood meal. The eggs hatch to become larvae characterized by the presence of six legs. The larvae usually feed on small vertebrate hosts such as mice. Following completing the bloodmeal, the larvae detach and drop from the host and molt to the nymphal stage characterized by the presence of eight legs. Nymphs usually remain dormant in the winter and start feeding in the spring (Sonenshine & Roe, 2013) . Following acquiring a blood meal from another host, such as mammals including humans, birds, or from reptiles, nymphs transform to the adult stage as males or females. The adult ticks will feed typically on mammals. The timeline required for completing all the developmental stages ranges from less than a year to three years (Sonenshine & Roe, 2013) . The ticks usually prefer to feed on

different hosts at each stage, but some tick species, like the brown dog tick, feed on the same host during all life stages (https://www.cdc.gov/ticks/life_cycle_and_hosts.html).

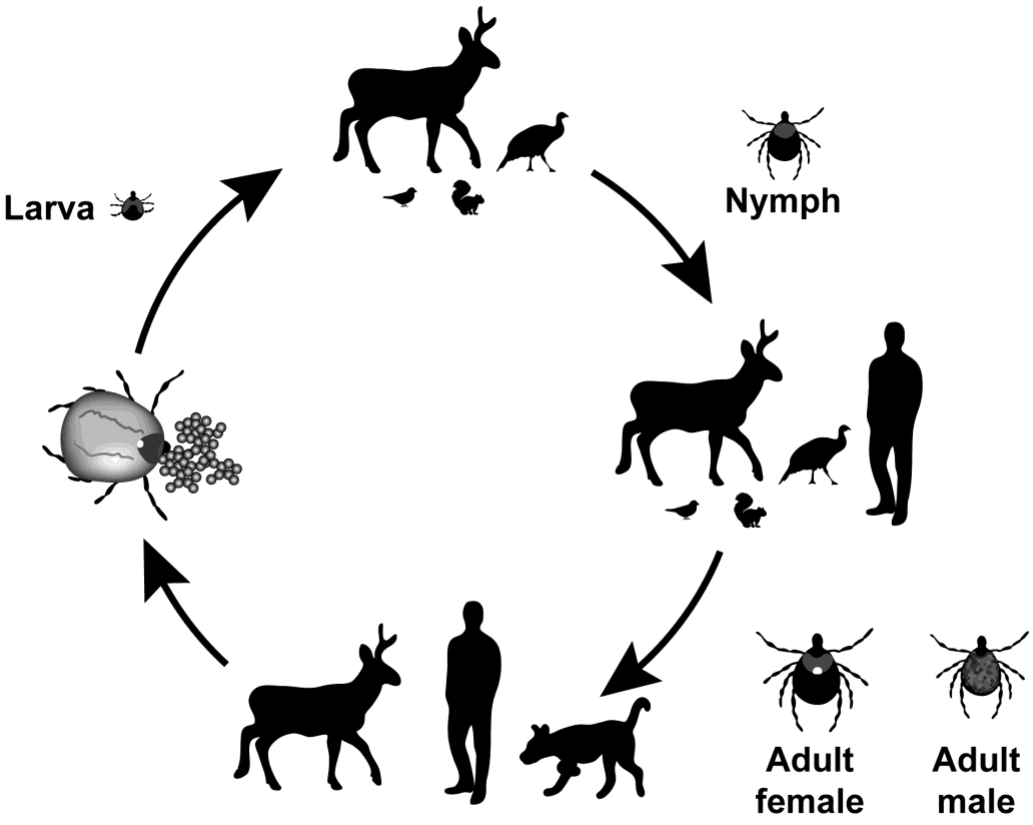


Figure 1.2: Life cycle of an *Amblyomma americanum* tick
(Figure created by Dr. Roman Ganta)

Tick-borne diseases caused by hard ticks

Tick-borne diseases (TBDs) caused by hard ticks have long been known as a major concern in impacting the health of companion and livestock animals, while such diseases were not considered as a public health concern until the mid 1970s. Lyme disease (LD) is the first identified human tick-borne disease caused by *Borrelia burgdorferi* in the United States and several other *Borrelia* species throughout the world (Burgdorfer et al., 1982).

LD is the most commonly reported vector-borne disease in North America, Europe, and parts of Asia. The disease is frequently reported in the north-eastern and north-central regions of the USA (Bacon et al., 2008). The etiologic agents for LD in Europe are *B. garinii* and *B. afzelii*, and in Asia, it is *B. garinii* (Bush & Vazquez-Pertejo, 2018; Marques, 2010). The primary vectors for LD are *Ixodes* species; *I. scapularis* and *I. pacificus* in the United States, *I. ricinus* in Europe, and *I. persulcatus* in Asia.

The second major human tick-borne diseases are caused by rickettsial pathogens belong to the families *Anaplasmataceae* and *Rickettsiaceae* in the USA. *Anaplasmataceae* pathogens include *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Ehrlichia muris* sub species *eauclairensis*. Rickettsiaceae pathogens include *Rickettsia rickettsii*, *Rickettsia parkeri* and *Rickettsia conorii* in the USA.

A. phagocytophilum causes granulocytic anaplasmosis, which was discovered in 1992 in the United States (Chen et al., 1994). The bacterium infects granulocytes, particularly neutrophils, and the pathogen infections are frequently reported from the north-eastern and upper midwestern states(Madison-Antenucci et al., 2020). *A. phagocytophilum* is transmitted by the *Ixodes* species ticks in the United States, Europe, and Asia (Woldehiwet, 2010).

Ehrlichia chaffeensis and *E. ewingii* are transmitted by *Amblyomma americanum* tick and are the causative agents of monocytic ehrlichiosis and ewingii ehrlichiosis in people, respectively. *E. chaffeensis* was reported for the first time in 1986, while *E. ewingii* was discovered in 1999 in the United States (Paddock & Childs, 2003) (Anderson et al., 1992). *E. chaffeensis* infects monocytes and *E. ewingii* infects neutrophils. Infections with these pathogens are frequently reported in the south-eastern and south-central United States from the east coast extending westward to Texas (https://www.cdc.gov/ehrlichiosis/stats/index.html). *Ehrlichia*

muris sub species *eauclairensis* causes ehrlichiosis in humans and transmitted by *I. scapularis*. The disease was first discovered in 2009 and is commonly reported in residents of Wisconsin and Minnesota (Pritt et al., 2017; Pritt et al., 2011).

R. rickettsii is the etiologic agent for the Rocky Mountain Spotted Fever (RMSF). It was reported about a century ago and is responsible for causing highly fatal disease in people (Gottlieb et al., 2018). *R. rickettsii* was initially identified as transmitted by *Dermacentor* species ticks; *D. variabilis* and *D. andersonii.* Subsequently, this organism was also vectored by *A. americanum* and *Rhipicephalus sanguineus* ticks. RMSF was discovered in the Rocky Mountains but is now endemic in the South-eastern, Pacific, and Western United States (Biggs et al., 2016) and also reported in other countries of North and South Americas. *R. rickettsii* infects the endothelial cells and multiplies rapidly, disseminating into the bloodstream (Gottlieb et al., 2018).

Diseases caused by *Ixodes* and *Amblyomma* species ticks

Ixodes and *Amblyomma* species transmit a wide range of pathogens worldwide, affecting the health of humans and animals. Some of the important bacterial diseases caused by pathogens transmitted by *Ixodes* species ticks are LD and Human Granulocytic Anaplasmosis (HGA). Several *Borrelia* species pathogens are responsible for the LD, while *Anaplasma phagocytophilum* causes HGA. Granulocytic ehrlichiosis in people referred as Human Ewingii Ehrlichiosis and Human Monocytic Ehrlichiosis are caused by the *Amblyomma americanum* transmitted pathogens, *Ehrlichia ewingii* and *Ehrlichia chaffeensis,* respectively. Similar diseases are also well-documented in several livestock animals and dogs. A brief summary about the pathogens and the diseases is presented below.

Lyme Disease

Lyme disease (LD), also referred as the Lyme Borreliosis (LB), is caused by the spirochete bacterium, *B. burgdorferi* and by several related bacteria (https://www.cdc.gov/lyme/index.html#:~:text=Lyme%20disease%20is%20caused%20by,skin%20rash%20called%20erythema%20migrans) (Bush & Vazquez-Pertejo, 2018; Littman et al., 2018; Uesaka et al., 2016). LD is identified as the most widely documented tick-borne disease impacting humans in the US and many parts of the world. It is also frequently reported in dogs, horses, cats and cattle. The history of LD began in 1975 in Connecticut when many residents experienced arthritis-like symptoms. (https://portal.ct.gov/DPH/Epidemiology-and-Emerging-Infections/A-Brief-History-of-Lyme-Disease-in-Connecticut). The causative agent was discovered in 1982 from infected *Ixodes* species ticks (Burgdorfer et al., 1982).

LD is transmitted by *Ixodes* species ticks to both humans and animals. *I. scapularis* acts as the primary vector in the upper Midwestern and north-eastern parts of the USA, and *I. pacificus* is the vector for the pathogen in the western part of the country. The etiologic agents for LD in Europe are *B. garinii*, *B. afzelii,* and *B. garinii* in Asia (Bush & Vazquez-Pertejo, 2018; Marques, 2010). The primary vectors for LD in Europe and Asia are *I. ricinus* and *I. persulcatus*, respectively. The black-legged tick *(I. scapularis)* feeds on numerous wild rodents, especially white-footed mice and chipmunks, lizards, ground foraging birds, sheep, deer (Parola & Raoult, 2001). The main reservoir of *B. burgdorferi* in the North-eastern United States is the *Peromyscus leucopus* (white-footed mice), but several birds are also identified as good reservoirs of *B. burgdorferi* (Bb) (Parola & Raoult, 2001).

LD is endemic in the Northeast and Northern and Midwestern regions of the United States, while it is widespread in Europe, Asia, and Japan (Jaenson & Talleklint, 1992; Oda et al.,

2017). The emergence of LD is observed in Canada in 2004 due to the expansion of *I. scapularis* into eastern and central Canada (Wikel, 2018). The possible expansion might be due to the migratory birds (Steere et al., 2016). In 2010, LD cases of 22,561 are reported to CDC and this number is increased to 33,666 in 2018 (https://www.cdc.gov/lyme/datasurveillance/recent-surveillance-data.html). These numbers do not reflect the actual cases due to undiagnosed cases, inconsistency in reporting, and empirical treatment (Moore et al., 2016). The actual number of new cases reported annually is estimated to be 300,000 (Steere et al., 2016).

The clinical picture of LD consists of early localized, early disseminated, and late disease. Symptoms like fever, chills, headache, fatigue, muscle and joint ache, and erythema migrans (EM) rashes are evident in the early stages (Moore et al., 2016). The onset of EM is reported in 70 to 80% of people and occurs at the site of the tick bite within 3 to 30 days. The early disseminated stage occurs within days to weeks after the initial symptoms, and patients continue to experience the above-mentioned clinical signs (Madison-Antenucci et al., 2020). More severe symptoms like neurological manifestations, including cranial nerve palsies, meningitis, radiculopathy, and rarely carditis, are also reported in many patients. Intermittent or persistent arthritis in one or more joints is the most common in late-stage LD.

Laboratory diagnosis is based on serological testing because *Borrelia* species are absent in the blood during acute infection (Movilla et al., 2016). Serological testing is based on Standard two-tier testing (STTT), in which an initial enzyme immunoassay is followed by Western blot (Movilla et al., 2016). The two types of EIA include the whole cell sonicate and the more specific C6 peptide ELISA (Sanchez et al., 2016). The C6 EIA is more specific because it utilizes the *Borrelia*-specific peptides to reduce the cross-reactivity to antigens from related species (Madison-Antenucci et al., 2020).

Antibiotics like Doxycycline, amoxicillin, cefuroxime, and azithromycin are recommended depending on various factors such as the age of a patient, side effects, allergy, and clinical disease manifestations (Sanchez et al., 2016). Currently, there are no vaccines available, although previously a vaccine named, LYMERix was offered for some time before being discontinued. Valneva and Pfizer have developed a vaccine candidate, VLA15, which targets Outer surface protein A (OspA) of *Borrelia* are currently in Phase 2 human trials (https://www.cdc.gov/lyme/prev/vaccine.html#:~:text=A%20vaccine%20for%20Lyme%20disease,this%20vaccine%20decreases%20over%20time)

***Ehrlichia* species of importance to human and animal health**

The *Ehrlichia* species pathogens which are important to human, companion and livestock health are,

- *Ehrlichia chaffeensis*
- *Ehrlichia canis*
- *Ehrlichia ewingii*
- *Ehrlichia ruminantium*
- *Ehrlichia muris eauclairensis*

Ehrlichia chaffeensis

E. chaffeensis is transmitted by the Lone star tick (*Amblyomma americanum*). It is maintained in white-tailed deer and so this animal is considered as the reservoir host (Paddock & Childs, 2003). This bacterium infects polymorphonuclear leukocytes, preferentially monocytes and macrophages, and it causes monocytic ehrlichiosis in humans and animals. The bacterium infects people of all ages although immunocompromised people are affected severely (Paddock

et al., 2001). This pathogen is discovered from a blood smear of severely ill human patient with multiple tick bites in 1986 (Paddock & Childs, 2003).

Ehrlichiosis is regarded as a significant public health problem in 1990. (Eng et al., 1990) . The *E. chaffeensis* vector tick, *A. americanum*, is identified as important due to its expanding geographic distribution, increasing population density, and its role as the vector of several other pathogens (Wikel, 2018). This tick is widely distributed from south-eastern United states to west-central Texas and north to Iowa, but the geographic distribution is expanding into the Mid-Atlantic states, New England, and Maine (Keirans & Lacombe, 1998). The reported *E. chaffeensis* infections have steadily increased over the years. The case fatality rate is 3% due to the severity of the disease, particularly if the patient is immunocompromised (Dumler et al., 2007). *E. chaffeensis* can also be transmitted through blood transfusion and organ transplantation from donors to recipients (Sachdev et al., 2014). *E.chaffeensis* is frequently reported from 35 states as of 2015 (Adams et al., 2017).

E. chaffeensis exists in two morphological forms, dense-cored cells (DC) and reticulate cells (RC). Both forms can reside within the phagocytic vacuoles (morulae) of an infected monocyte or macrophage, while DC form can also be found in the blood (Zhang et al., 2007). The developmental cycle of *E. chaffeensis* begins with DC form gaining entry into a monocyte or macrophage, which transforms to the replicating RC form. The RC form multiplies by binary fission for approximately 48 h and matures to RC form after about 72 h post infection. The DCs are released by host cell lysis or exocytosis to begin a new cycle of infection (Zhang et al., 2007).

The general clinical features experienced in patients after exposure to infected ticks are malaise, low-back pain, gastrointestinal symptoms, or sudden onset of fever (>39°C), usually are

observed in about 7-10 days following the pathogen transmission from an infected tick (Rikihisa, 2010). However, as the disease progresses, people experience fever, headache, myalgias, nausea, arthralgias, and malaise. Some of the hematological abnormalities observed are thrombocytopenia, anemia, and mild elevation of hepatic transaminase (Rikihisa, 2010).

Diagnosis of HME is usually based on confirmatory testing by several laboratory methods. The most widely available diagnostic method is serological testing which detects and measures the antibodies. Additional diagnostic tests include indirect immunofluorescence assay, Western blot, PCR, ELISA, and peripheral blood smear examination. Treatment for HME is usually based on tetracycline derivates such as Doxycycline (Rikihisa, 2010). Rifampicin is prescribed to some patients where doxycycline treatment may not be an option, such as in the case of pregnant women and children (Branger et al., 2004).

Ehrlichia canis

Ehrlichia canis causes canine monocytic ehrlichiosis (CME), which is a potentially fatal tick-borne ehrlichial infection. *E. canis* infection is reported initially in 1935 in Algeria. The disease is now frequently reported throughout the world with the exception of Australia and New Zealand . (Harrus et al., 1999). Although less frequent, *E. canis* infections are also reported in people (Perez et al., 2006) (Conrad, 1989). The primary vector for E. canis is Rhipicephalus sanguineus (brown dog tick) (Mylonakis et al., 2019). The domestic dog serves as a primary host for all the life stages of R. sanguineus. The geographical distribution of the brown dog tick is widespread globally (Diaz et al., 2018). Similar to E. chaffeensis, E. canis also infects monocytes and macrophages (Cohn, 2003). *E. canis* infections in dogs may induce fever, depression, lethargy, anorexia, generalized lymph adenomegaly, splenomegaly, bleeding tendencies, and ocular abnormalities (Mylonakis et al., 2019). CME is diagnosed by

hematological and serological analysis, such as by Indirect Immunofluorescence assay (IFA) and ELISA, culture recovery, and by PCR (Harrus & Waner, 2011). The IFA test is considered a gold standard for detecting *E. canis* infections (McBride et al., 2003). The CME is treated with tetracycline derivatives, such as Doxycycline. Infection, however, can persist throughout the life of an animal (Wen et al., 1997)

Ehrlichia ewingii

E. ewingii primarily infects neutrophils and causes canine granulocytic ehrlichiosis (Madison-Antenucci et al., 2020). This pathogen is also known to cause infections in people and causing the disease, human Ewingii ehrlichiosis (Ewing et al., 1971). This pathogen is reported in 1971 from a dog in Arkansas as homologous to *E. canis* (Ewing et al., 1971). Molecular evidence defined it as a new *Ehrlichia* species in 1992 (Anderson et al., 1992). *E. ewingii* causes acute disease in dogs and the signs may include fever, lethargy, anorexia, and neutrophilic arthritis. Neurological symptoms like tremor, anisocoria, tremors, and a head tilt have also been described in naturally infected dogs (Goodman et al., 2003). However, the symptoms are milder than CME (Goldman et al., 1998) but might exhibit severe outcomes if they are co-infected with *E.canis* (William L. Nicholson, 2019).

HEE infections in people are documented for the first time in 1999 in four human patients from Missouri with clinical signs included fever, headache, thrombocytopenia, and with or without leukopenia (Buller et al., 1999). Human cases are primarily reported from Missouri, although 10 other states also documented the infections (Chen et al., 1994) (Buller et al., 1999). A recent study in the USA, based on the serological test results collected over 16 years, found that canine *Ehrlichia* spp seroprevalence is increasing in both endemic and non-endemic areas of the USA (Gettings et al., 2020). The clinical symptoms of humans resemble that of HME. There

have been no fatal cases reported due to *E. ewingii* because of the less severity compared to *E. chaffeensis*. *E. ewingii* can be rarely transmitted through transfusion, but one case was reported, which is attributed to platelet transfusion in 2011 (Regan et al., 2013). HEE became a reportable disease to CDC in the US in 2008 (Biggs et al., 2016). Similar to *E. chaffeensis*, this pathogen is also transmitted by *A. americanum* and white-tailed deer acting as the reservoir host, and dogs may also serve as reservoirs of infection (Liddell et al., 2003). *E. ewingii* infections are more frequently documented from south-central and south-eastern parts of the United States, where *A. americanum* has the widespread distribution. *E. ewingii* is also reported from Africa and Brazil (Ndip et al., 2005; Oliveira et al., 2009).

HEE can be diagnosed through real-time PCR, Giemsa-stained peripheral blood smear, and by indirect IFA test. *E. ewingii* may be visualized in neutrophils of the peripheral blood smears during acute phage of infection, although it cannot be distinguishable from A. phagocytophilum infection. In serological assays, antibodies to *E. ewingii* may cross-react with E. chaffeensis, making the diagnosis difficult (Paddock et al., 2001). HEE infection is treated with doxycycline for 5 to 7 days (Buller et al., 1999).

Ehrlichia ruminantium

E. ruminantium is the causative agent for Heartwater, an economically important disease in ruminants impacting throughout sub-Saharan Africa and parts of the Caribbean. This disease is reported from both domestic and wild ruminants, such as cattle, sheep, goats and antelopes, elephants and deer. *E. ruminantium* is transmitted by several *Amblyomma* species ticks. The major transmitting vectors, however, are *A. variegatum* (tropical bont tick) and *A. hebraeum* (Parola et al., 1999). *A. variegatum*, originated from Africa, is also well-established in several Caribbean islands (Allan et al., 1998) (Pegram & Eddy, 2002). Heartwater is also reported from

three Caribbean islands; Guadeloupe, Marie Galante and Antigua (Uilenberg et al., 1984). This pathogen remains a continuous threat to the livestock industry in Northern, Central and South American countries (Deem, 1998). The heartwater pathogen primarily infects vascular endothelial cells and the infection can reach the brains of infected animals causing the neurological diseases. The clinical manifestations of the heartwater include high fever, development of central nervous system signs, may become disoriented and show signs of the motor disorder including abnormal walking, muscle twitching, and trembling. The disease severity may vary in different host species and breeds (Cowdry, 1925) (Uilenberg et al., 1984). The fatalities in cattle are usually associated with severe encephalitis and from the destruction of vascular endothelium. The commonly used diagnostic method for animals died of heartwater is based on the necropsy analysis combined with brain smear examination for the identification of the organisms. Diagnostic methods may also include PCR, Western blot, IFA and ELISA. Oxytetracycline or doxycycline is effective in treating the early stages of the disease, but the animals remain carriers for a long time (Peek & Divers, 2018).

Ehrlichia muris subsp eauclairensis

Ehrlichia muris eauclairensis is a recently discovered causative agent of ehrlichiosis in humans. This bacterium is discovered in 2009 from four patients from Wisconsin and Minnesota having symptoms similar to other ehrlichiosis (Pritt et al., 2011). The bacterial DNA is detected in 17 *I. scapularis* ticks collected from Minnesota and Wisconsin. This new Ehrlichia species, was initially named as the *E.muris* like agent (EMLA) because its high genetic homology to *E. muris* and later renamed as *Ehrlichia muris* eauclairensis (Castillo et al., 2015) (Pritt et al., 2017)

I. scapularis is identified as the primary transmitting vector for this pathogen (Pritt et al., 2011), while the white–footed mice (*Peromyscus leucopus*) are considered as the reservoir host

(Lynn et al., 2017). Infections with this pathogen are reported only from Wisconsin and Minnesota, even though the distribution of the tick is known to exist most of north-eastern part of the USA. A recent study assessing 75,077 patient samples collected between 2007 – 2013 revealed positives in 69 patients, and the majority of the patients come from Minnesota and Wisconsin (Johnson et al., 2015). The common symptoms observed in the patients with this pathogen infection may include fever, malaise, thrombocytopenia, and lymphopenia. The infections are treated with doxycycline (Pritt et al., 2011).

***Anaplasma* species of importance to human and animal health**

The *Anaplasma* species pathogens which are important to human, companion and livestock health are,

- *Anaplasma phagocytophilum*
- *Anaplasma platys*
- *Anaplasma marginale*

Anaplasma phagocytophilum

A. phagocytophilum is an obligate intracellular tick-borne pathogen which causes infections in horses, dogs, cattle and people. In people, the clinical disease is referred as human granulocytic anaplasmosis (HGA). This bacterium primarily infects granulocytes (Carrade et al., 2009). *A. phagocytophilum* first discovered in leukocytes of sheep in Scotland in 1910. It is referred to as tick-borne fever in Europe with documented cases reported in sheep, cattle, goats, and deer (Foggie, 1951; Woldehiwet, 1983). Infections in horses and dogs are identified in California in 1969 and 1982, respectively (Madewell & Gribble, 1982; Madigan & Gribble, 1987). The first human case in the United States is reported in 1992 (Chen et al., 1994). *A. phagocytophilum* infections are also documented from Europe (Matei et al., 2019) (Woldehiwet,

2010) . The highest seroprevalence in people is identified in the upper midwestern, north-eastern, and western parts of the US, possibly due to the high *I. scapularis* tick distribution (Bowman et al., 2009).

Ixodes species are the vectors for *A. phagocytophilum* in the US, Europe, and Asia. *I. scapularis* (black-legged tick) and *I. pacificus* (Western black-legged tick) serve as the vector in the US, while *I. ricinus* (Castor bean tick) and *I. persculatus* (taiga tick) are regarded the vectors in Europe and Asia, respectively (Thomas et al., 2009) The white-footed mice (*Peromyscus leucopus*) and eastern chipmunks are likely acting as reservoir hosts in midwestern and eastern US, dusky-footed woodrats, grey squirrels, and chipmunks in the western states of the country. Dogs and humans serve as accidental hosts for this pathogen (Carrade et al., 2009).

Human granulocytic anaplasmosis became a reportable disease in 2000. Aged persons and immunocompromised people are more susceptible to acquire the disease and are more likely to have a life-threatening complication and more likely to die (Dahlgren et al., 2015). Anaplasmosis cases are more frequently reported from upper midwestern and the north-eastern United States. The number of anaplasmosis cases increased steadily from 348 cases in 2000 to 5,762 cases in 2017 (https://www.cdc.gov/anaplasmosis/stats/index.html)

The disease presents as an acute illness accompanied by anorexia, lethargy, fever, lameness, thrombocytopenia, and doxycycline is prescribed for treating granulocytic anaplasmosis (Yancey et al., 2018). Hematological abnormalities in patients may include thrombocytopenia, leukopenia, anemia, and elevated hepatic transaminase levels (Dumler et al., 2005). The diagnosis of the infections are mostly by serological testing, peripheral blood smear examination, PCR, immunohistochemistry, and at times by culture isolation (Guzman et al., 2021). Vaccine for *A. phagocytophilum* is not yet available (Stuen et al., 2013)

Anaplasma marginale

A. marginale is the causative agent for bovine anaplasmosis, which is an economically important disease of the cattle. Bovine anaplasmosis is prevalent in tropical and subtropical regions worldwide, including South and Central America, the United States, southern Europe, Africa, Asia, and Australia (https://www.merckvetmanual.com/circulatory-system/blood-parasites/anaplasmosis?query=bovine%20anaplasmosis). It is also endemic in Mexico, Central, South America, and in the Caribbean (Kocan & de la Fuente, 2003). Wild ruminants such as Elk, water buffalo, pronghorn, bighorn, sheep, deer, and antelopes are also affected by *A. marginale* (Kuttler, 1984). *A. marginale* replicates inside the erythrocytes, which leads to the loss of RBCs (Kocan et al., 2004). Transmission of the pathogen occurs either by ticks or by transfer of infected erythrocytes to susceptible cattle mechanically from biting flies or by contaminated fomites including needles or surgical instruments, or by transplacental transmission across the placenta of the offspring (Yunik et al., 2016) (Kocan et al., 2004) . The biological tick vector for *A. marginale* are *Dercamentor* species, such as *D. andersoni* (Rocky Mountain wood tick), *D. variabilis* (American Dog Tick), *D. occidentalis* (Pacific Coast Tick), and *D. albipictus* (Winter or Moose Tick) (Kocan et al., 2004). Clinical signs include anemia, fever, weight loss, lethargy, death, icterus without hemoglobinemia, and hemoglobinuria (Kocan et al., 2003).

Bovine anaplasmosis impacts cattle of all ages, although calves are considered less susceptible to the clinical disease. Cattle infected with *A. marginale* remain as infected carriers for the remainder of their lives, irrespective of whether they develop or not develop clinical disease (Aubry & Geale, 2011). Diagnosis is typically based on the blood smear preparation from clinically infected animals during the acute phase of the infection and then finding

inclusions within erythrocytes following polychromatic staining (Aubry & Geale, 2011). Diagnosis in carrier animals is performed by serological or molecular techniques, such as the PCR and real-time PCR.

The preventive measures include maintenance of the pathogen-free herds is challenging, although few measures are considered, such as the vector control, infection and treatment methods in endemic regions, and minimize importation of animals from the disease endemic regions (Aubry & Geale, 2011). Preimmunization of the cattle with *A .centrale* is followed to prevent the severity of highly virulent *A. marginale* species is also used in certain parts of the world, such as in the southern parts of Africa (Brizuela et al., 1998).

Anaplasma platys

A. platys causes canine cyclic thrombocytopenia (CCT) in dogs which infects platelets and forms basophilic intracellular morulae (Harvey et al., 1978). The causative agent is first reported in dogs from Florida in 1978 (Harvey et al., 1978). This pathogen is widely distributed in America, Africa, Asia, the middle east, Southern Europe, and Australia. (Matei et al., 2016) (De Tommasi et al., 2014) . The competent vector for *A. platys* is yet to be defined, although *Rhipicephalus sanguineus* is regarded as the vector (De Tommasi et al., 2014). The first human case with *A. platys* was described in 2014 when two women from Venezuela are presented with chronic, non-specific clinical signs and exposure to *R. sanguineus* (Arraga-Alvarado et al., 2014). Dogs can be asymptomatic or exhibit mild symptoms but can be fatal due to severe thrombocytopenia and subsequent potential for hemorrhaging (Gaunt et al., 2010; Lanza-Perea et al., 2014). The disease can be diagnosed with an IFA test, staining blood smear analysis to find inclusions within the platelets, and by PCR (Lara et al., 2020). This pathogen infections also respond to doxycycline treatment (Lara et al., 2020).

Impact of tick-borne diseases

Tick-borne diseases have always been a major concern in impacting the health of several livestock and companion animals. Tick-borne diseases are also emerging as the major concern for human health since the last three to four decades in the USA and many parts of the world. The infections are responsible for causing significant economic losses resulting from the loss of primarily milk and meat production. Tick-borne diseases that contribute to the economic losses in livestock animals are anaplasmosis, babesiosis, heartwater, and theileriosis worldwide (Rios, 2018). These diseases cause decreased appetite, reduced milk production, lower weight gain, loss of body condition, reproductive effects, abortions, lower pregnancy and birth rate, death in some animals. *Rhipicephalus microplus* causes the greatest economic impact in cattle population due to its worldwide distribution (Rios, 2018). The cost of the treatment, expenses to control the tick burden, trade restrictions of animals between areas and countries contribute to the economic losses (Rajput et al., 2006). The cattle industry losses worldwide are estimated as $13.9 – 18.7 billion per year (Rios, 2018).

Small ruminants like sheep, goats, and lamb also act as essential meat and milk sources in different countries. These ruminants play a crucial role in the income earned from skins, wool (Rios, 2018). Ticks cause intensive lameness in goats and substantial financial losses in the livestock industry by causing damages to the leather and skin of sheep, goats, and cattle. The total annual loss of small ruminants due to tick-borne diseases are estimated at around 70 million USD (Yin & Luo, 2007) .

Pigs are also susceptible to tick infestation, and the main economic impact is due to African Swine fever (Zimmerman et al., 2010). There are not many cases of tick infestations in birds due to the modern production systems. The two important ticks in poultry are *Argas*

persicus and *Argas radius. A. persicus* affects ducks, poultry, turkeys, pigeons, and canaries (Rios, 2018). Ticks also infest horses to a certain extent; *Dermacentor, Ixodes,* and *Amblyomma* species are the most common ticks in the horses. The diseases cause the restriction of international mobilization of horses, preventing their participation in sporting events (Jongejan & Uilenberg, 2004).

Companion animals, in particular dogs, suffer the consequences of tick-borne diseases. *Babesiosis* and *Ehrlichiosis* are the most important, with *Ehrlichia canis* being frequently fatal (Jongejan & Uilenberg, 2004). Tick-borne zoonotic diseases are becoming more common in the world's temperate regions, posing an ever-increasing public health threat (Jongejan & Uilenberg, 2004). The abundance of white-tailed deer has increased in the United States and much of Western Europe, consistent with a correlation in the tick density. An increase in recreational activities in rural-tick-infested areas has increased the number of humans bitten by the ticks (Jongejan & Uilenberg, 2004). Lyme disease is one of the most commonly reported diseases in humans, and the costs for medical care, surveillance, and laboratory diagnosis are very high. As one of the world's largest and fastest-growing sectors, international human travel has a significant impact on health care (Jongejan & Uilenberg, 2004).

In the previous pages, a brief overview has been provided about various pathogens and how they impact humans and animals; however, understanding the importance of these pathogens remains still very limited and much more research needs to be undertaken in order to define disease pathogenesis, host responses and the persistence of these pathogens. Despite several advances, significant gaps of knowledge exist in knowing how tick-borne bacterial

pathogens can overcome the host immunity and persist inside them. Such lack of knowledge also creates a barrier in developing vaccines.

Some research progress has been made in understanding the immunity using animal infection studies for pathogens of importance to human health. However, most of the research has been focused on diseases such as HME and HGA and significantly less research is focused on HEE (Madison-Antenucci et al., 2020). No fatalities are documented for HEE compared to 3% and 1.2% fatality rates reported for HME and HGA, respectively (Bakken & Dumler, 2015; Ismail et al., 2010).

Our research group has described the differential expression of *E. chaffeensis* as one of the essential survival mechanisms in the vertebrate and the tick host (Singu et al., 2005; Singu et al., 2006). In recent years, our group has developed ways to successfully create mutations in the *E. chaffeensis* genome to identify genes crucial for the bacterial persistence *in vivo* (Cheng et al., 2013; Wang et al., 2020; Wang et al., 2017). The ability of mutants to block an active infection in naïve deer and dogs has been aiding in identifying genes critical for the pathogen's persistent infection (Jaworski et al., 2017). This research also paved the way for the development of an efficacious live-attenuated vaccine that elicits pathogen-specific CD4+ T-cell immunity (McGill et al., 2016; Nair et al., 2015).

Other researchers have also investigated potential vaccine candidates for HME and HGA. One of them identified an Entry-Triggering Protein of *Ehrlichia* (EtpE) as the first ehrlichial protein vaccine against HME (Budachetri et al., 2020). (Naimi et al., 2020) have demonstrated that immunization against *A. phagocytophilum* invasion protein A (AipA) and *A. phagocytophilum* surface protein (Asp14) binding domains elicit protective immune responses when assessed in the mouse model.

Besides vaccine development, researchers are also investigating how the rickettsial bacteria evade the host immune response and persist inside the host. One of the possible ways described by (Yan et al., 2018) is that *E. chaffeensis* secretes its effector protein (Etf-2) into the host-cell cytoplasm and avoids the fusion with host cell lysosome, which is an important innate immune defense. The other proposed mechanism is that *E. chaffeensis* uses its surface invasion, the Entry-triggering protein of *Ehrlichia* (EtpE-C), to bind to the mammalian DNAse and blocks the generation of reactive oxygen species (ROS) generation by host monocytes and macrophages. This was demonstrated to be a unique mechanism by which *E. chaffeensis* hijacks phagocyte NADPH oxidase (Mohan Kumar et al., 2013; Teymournejad & Rikihisa, 2020). A more recent study has described the ability of *E. chaffeensis* to acquire the host membrane lipids for its survival (Lin et al., 2020).

One of the mechanisms by which *A. phagocytophilum* evades host response is by inhibiting superoxide generation by human neutrophils (Mott & Rikihisa, 2000). *A. phagocytophilum* and *E. chaffeensis* are cholesterol-dependent pathogens. *A. phagocytophilum* exploits the host low-density lipoprotein (LDL) uptake pathway during the infection to recover host cholesterol (Xiong et al., 2009). A recent study by (Green et al., 2020) described that *A. phagocytophilum* secretes surface protein 14 (Asp14) which binds to host cell surface protein disulfide isomerase (PDI) for the successful infection. The above summarized findings provide insight on the mechanisms of bacterial evasion and persistence in the mammalian host. *E. chaffeensis* and *A. phagocytophilum* have also been shown to be transmitted through blood transfusion and organ transplantation. This is a rare occurrence, but it can cause morbidity and mortality either if the diagnosis is delayed or if the donor is immunocompromised (Fine et al., 2016; Proctor & Leiby, 2015; Sachdev et al., 2014).

These research investigations help to define the methods of control and prevention, including the development of an effective vaccine. Despite all this progress, we have many research gaps to be filled, including the lack of defined methods of monitoring the disease spread and risk of infections to people occurring from a tick transmission and via blood and organ transfers. In this study, a survey was performed that focused on infections in the canine host, and this was compared with human infection data with a goal to determine if the canine host can serve as the sentinel species for tick-borne diseases caused by *Anaplasma*, *Ehrlichia*, and *Borrelia* pathogens impacting human health in the USA. The importance of this study is discussed in more detail in Chapter 2.

Chapter 2 - Scope of the thesis

An overview of tick-borne pathogens impacting humans and animals have been provided in the review of literature. The primary tick–transmitted pathogens affecting humans in the USA are *Anaplasma, Borrelia,* and *Ehrlichia* species pathogens. These pathogens have a broad host range, and so, they cause infection in humans, dogs, cattle, horses, and cats (Stuen et al., 2013) (Tilly et al., 2008) (https://www.cfsph.iastate.edu/Factsheets/pdfs/ehrlichiosis.pdf).

It is also evident that many pathogens causing infections in people are previously documented in several other species, most notably in dogs (Buller et al., 1999; Dumler et al., 2005; Harvey et al., 1978; Perez et al., 2006).

The focus of this research is mainly on *Anaplasma, Borrelia,* and *Ehrlichia* species pathogens. Lyme disease (LD) caused by *B. burgdorferi* is the most commonly reported in humans (https://www.cdc.gov/lyme/stats/humancases.html), dogs, and to a lesser extent in horses, cattle, and cats (Popovic et al., 1993).

Likewise, more particularly *E. chaffeensis, E.canis, E.ewingii, A.phagocytophilum,* and *A.platys* are very frequently reported in humans and dogs (Rikihisa, 2010) (Dumler et al., 2005) (Conrad, 1989) (Harris et al., 2016) (Arraga-Alvarado et al., 2014) (Harvey et al., 1978). These pathogens infect humans and dogs in a very similar manner, and this might be due to the ticks feeding on a broad range of hosts. *A. americanum,* the vector for *E. chaffeensis* and *E. ewingii,* is a very aggressive tick feeding on humans, dogs, and other small mammals (https://www.cdc.gov/ticks/tickbornediseases/tickID.html) (Childs & Paddock, 2003).

Similarly, *Ixodes* species are the vectors for *A. phagocytophilum* and *B. burgdorferi* and are aggressive in acquiring bloodmeals from humans and similarly from dogs

(https://www.cdc.gov/ticks/tickbornediseases/tickID.html)
(https://capcvet.org/guidelines/ixodes-scapularis-and-ixodes-pacificus/).

It is unclear how much of the documented data predict the risk of pathogen spread from one host species to another and the infection status. Such data will be valuable in defining the disease prevalence, transmission, and describing control methods. Surveillance studies have been carried out in both dogs and humans, but the investigations of human cases are very limited. Human cases are often reported to CDC because disease documentation is required for reportable diseases. However, only a fraction of cases is reported (https://www.cdc.gov/ticks/tickbornediseases/tickID.html).

Several serological studies have described the infection prevalence of various *Anaplasmataceae* pathogens in the USA and worldwide. (Bowman et al., 2009) assessed the prevalence and geographic distribution of *Dirofilaria immitis, B. burgdorferi, E. canis,* and *A. phagocytophilum* in dogs in the USA based on a serological survey found that at least one agent was prevalent in each state. Overall, the study demonstrated the prevalence of these pathogen infections over a wide geographic region.

Another study in Canada evaluated the prevalence of *Anaplasma* spp., *B. burgdorferi, D. immitis, Ehrlichia* spp., in dogs from 2008 – 2015 using SNAP ® 4Dx ® test and observed a significant increase in the seroprevalence for *B. burgdorferi* and *Ehrlichia* spp. (Evason et al., 2019).

A recent study in 2021 by (Little et al., 2021) evaluated the seroprevalence of the four commonly reported vector-borne pathogens in dogs in the USA from 2013 to 2019. The study reported the increased seroprevalence of *Ehrlichia* spp. in every region but particularly in the Southeastern regions. Geographic expansion of *A. phagocytophilum* and *B. burgdorferi* was also

apparent in the endemic areas. Similar serological surveys have been carried out from different parts of the world, demonstrating the expansion of these tick-borne pathogens (Ebani, 2019; Ebani et al., 2014; Movilla et al., 2016).

To our knowledge, we did not find any studies describing the infection prevalence with a direct comparison between humans and dogs. We reasoned that a direct comparison would help us understand the prevalence and assess the geographical areas and people at risk. It will be beneficial to monitor the infections in an animal that serves as a good sentinel. We reasoned that such species could be dogs because they are very similar to humans in acquiring all those pathogens.

Chapter 3 - Materials and Methods

Sample collection

Canine whole blood or serum samples were received from various veterinary hospitals across the United States. Samples were received as 0.5 ml of serum or 1 ml of whole blood. Clinicians were approached for sending the samples which were suspected of tick-borne illness. No details were available regarding the clinical status of the dog. The samples were tested for the presence of antibodies against *Borrelia burgdorferi* using canine-specific Lyme ELISA and an indirect immunofluorescence assay (IFA) for *A. phagocytophilum*, *E. canis,* and *E. chaffeensis*. The samples were received from veterinary hospitals through overnight shipping on ice. Plasma was obtained from whole blood by centrifuging at 4000 g (Eppendorf, Enfield, CT) for 5 min on the same day of arrival. The sample tubes were then marked with sample number, the date of collection, and stored at -20^{0}C until further analysis.

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was performed on serum or plasma samples using canine specific C6 peptide as antigen. Serum/plasma samples obtained from clinically suspected dogs were assessed for the presence of *B. burgdorferi*-specific canine antibodies according to the protocol from Zoetis with minor modifications. Briefly, 96-well C8 maxisorp Nunc Immuno module ELISA plates (Thermo Fisher Scientific, Waltham, MA) were coated with *Borrelia* C6 peptide at a concentration of 5 µg/mL in 0.1 M carbonate buffer (pH-9.2) and incubated for one h at room temperature. 300 µL of blocking buffer containing 5% non-fat dry milk was added to the wells and incubated for one h at room temperature to reduce the background interference. For determining the concentration of C6-peptide specific canine IgGs, dilutions of standard serum (Zoetis, Kalamazoo, MI) and serum /plasma samples from dogs were diluted to 1:250 in

blocking buffer. The concentrations of specific antibodies in the standard serum were expressed as Lyme equivalents. A range of 0 to 200 standard serum were included in each plate along with samples and the blank. The diluted serum/plasma samples and the standard serum was added to duplicate antigen-coated wells and incubated for one h at room temperature. The wells were washed three times with 200ul of wash buffer containing 50 mM Tris-HCl pH 8.0 and 0.05% w/v CHAPS (Sigma Aldrich, Saint Louis, MO) followed by incubation with horseradish peroxidase (HRP) conjugated goat anti-dog IgG h+l (ICL, Portland, OR) at a dilution of 1:4000 for one hour. Following the incubation with secondary antibody, the walls were washed with 200ul of wash buffer and the TMB (3,3',5,5'- tetramethylbenzidine) (EMD Millipore Corp, Billerica, MA) substrate was added to the wells. The color development was measured within 8 min at 650 nm using ELISA reader (). The standard curve was plotted with measured OD values for the standard serum versus corresponding concentrations in Lyme equivalents. The concentration of C6-peptide specific canine antibodies in the samples were determined by linear regression analysis and were expressed as Lyme equivalents. If the concentration of specific antibodies in the sample value was below 20 Lyme equivalents, the dog was considered negative; if the concentration was in the range of 20-40 Lyme equivalents, the dog was considered low-positive, and if the concentration of antibodies was greater than 40, the dog was considered high-positive.

Immunofluorescence assay (IFA)

Canine sera or plasma samples were assessed for the presence of *Ehrlichia* and *Anaplasma* antibodies by performing IFAs according to pathogen-specific assays using canine IgG Antibody kits (Fuller Laboratories, Fullerton, CA). Serum/plasma samples were diluted at 1:80,1:160 and 1:320 in 0.05 M PBS (pH 7.2) to test for the presence of antibodies against *E.*

chaffeensis, *E. canis*, and *A. phagocytophilum*. Positive controls containing reactive canine serum were diluted to 1:160. The antigen slides were purchased from Fuller Laboratories, and 10 µL of positive control, sample, and negative control was added to the wells on the pathogen-specific antigen slides and incubated in the humidified chamber for 30 min at 37^0C. The slides were washed with 0.05 M PBS three times by pouring the PBS on to the slides with the wash bottle. Ten µL of the secondary antibody containing affinity purified FITC conjugated rabbit anti-dog IgG was added to all the wells on the slide and incubated for 30 min, followed by washing for three times. Ten µL of mounting medium containing 50% glycerol was added and covered with a coverslip. The slides were then viewed under the fluorescence microscope. The presence of antibodies was confirmed by apple-green, fluorescent inclusions (morulae) in the cytoplasm of infected cells.

RNA isolation

RNA was isolated from serum/plasma samples for molecular analysis using TRIzol ™ LS Reagent (Invitrogen Life Technologies, Carlsbad, CA). Two hundred fifty µl of serum/plasma each was mixed with 750 µl of Trizol LS reagent and was stored at -80^0C until further use. To isolate RNA, frozen samples were defrosted and to each tube, 200 µl of chloroform was added and incubated for 2-3 min at room temperature. The samples were then centrifuged for 15 min at 12,000 g at 4^0C, and the aqueous phase was transferred to a new tube. Then, 0.5 mL of isopropanol was added to the supernatant and incubated for 10 min at room temperature followed by centrifugation for 10 min at 12,000 g at 4^0C. The supernatant was discarded, and the pellet was resuspended in 1 mL of 75% ethanol and centrifuged for 5 min at 12,000 g at 4^0C. The supernatant was then discarded, and the RNA pellet was air-dried for 5-10

min, followed by resuspending it in 50 µl of RNase -free water. The RNA samples were transferred to -80^{0}C freezer until further use.

Real-Time Quantitative RT-PCR

The presence of pathogen-specific RNAs in the above isolated RNAs were assessed by real-time quantitative reverse transcriptase PCR (qRT-PCR) using gene-specific TaqMan assay as per the previously described method (Sirigireddy & Ganta, 2005) with a few minor modifications outlined in (Tables 3.1 and 3.2). A negative-control reaction included a no-template PCR. Similarly, a positive-control reaction included the known *A. phagocytophilum, E. chaffeensis, E. canis* genomic DNA as the template and plasmids for *A. platys* and *E. ewingii.* qRT-PCR assays were performed using the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The PCR analysis was carried out in a 25 µl reaction mixture using Superscript ™ III platinum ™ Taq Mix (Life Technologies, Carlsbad, CA), by following the temperature cycles; reverse transcription step at 48^{0}C for 30 min, then the initial denaturation step for 3 min at 95°C, followed by 40 cycles of denaturation for 15 sec at 95°C, annealing at 50°C for 30 sec and extension at 60°C for 1 min. The reaction setup details were included in Table 3.1.

Table 3.1: Primers used in qRT-PCR for the detection of *Anaplasma* and *Ehrlichia* species

Primers	Sequence	Temperature (°C)
Ehrlichia/Anaplasma TaqMan forward primer	5' ctcagaacgaacgctgg	54.6
Ehrlichia TaqMan reverse primer	5'catttctaatggctattcc	51.5
Anaplasma TaqMan reverse primer	5'catttctagtggctatccc	53

Table 3.2: Probes used in qRT-PCR for the dtection of *Anaplasma* and *Ehrlichia* species

Probes	Sequence	Temperature (°C)
E. chaffeensis	5'TET/cttataacctttggttataaataattgttag/ BQH2*	55.6
E. canis	5'YAKtatagcctctggctataggaaattgttag/ BQH2*	60.7
E. ewingii	5'FAMctaaatagtctctgactatttagatagttgttag/BQH2*	59.5
A. phagocytophilum	5'TET/cggatttttgtcgtagcttgctatgat/ BQH2*	60.1
A. platys	5'FAM/ttgctataaagaataattagtggcagacg/ BQH2*	58.3

Table 3.3: qRT-PCR reaction mix

Component	Volume
Superscript ™ RT/Platinum ™ Taq Mix	0.5 µL
2X Reaction Mix	12.5 µL
Forward Primer, 10µM	0.5 µL
Reverse Primer, 10µM	0.5 µL
Fluorogenic Probe, 10µM	0.5 µL
Template	5 µL
Nuclease free water	5.5 µL

Detection of *E. chaffeensis* and *E. canis* by culture recovery method

Canine blood samples were centrifuged (Eppendorf, Enfield, CT, USA) at 4000 g for 5 min. Plasma from each blood sample was transferred into a fresh 1.5 ml Eppendorf tube and stored for serological testing. About 1 ml of the buffy coat was transferred to a sterile 15 ml Falcon tube containing 10 ml red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) and mixed well until complete lysis of erythrocytes. The samples were then centrifuged at 4,000 *g* for 5 min, and the supernatants were discarded. The buffy coat from each sample was then resuspended in 200 µL of sterile 1X PBS. Assessment of *E. canis* or *E. chaffeensis* infection was done by adding 100 µL each of cell suspension into a 12-well sterile culture plate containing 1 ml of DH82 (Canine Macrophage cell line; ATCC # CRL-10389) cell culture having about 80% confluence. The cultures were grown according to (Cheng & Ganta, 2008). Briefly, DH82 cells were initially cultured in a tissue culture flask in MEM (Modified Eagle Media) containing 35 ml of 7.5% fetal bovine serum and 6ml of 200mM L-Glutamine and

grown in 37^0C with 5% CO_2 until the cells become confluent (i.e., until they reach the cell count of 10^5 - 10^6 per ml of media). Once the cells become confluent, DH82 cells were resuspended and transferred 1 ml each per well of 12 well plates with fresh media and mixed with 0.1 ml each of buffy coat cell suspensions. Infection was monitored twice a week by microscopically examining the Hema3-stained slides for 10 weeks to determine if a sample tested positive or negative for *E. chaffeensis* and *E. canis* infection (Nair et al., 2015).

Chapter 4 - Results

Prevalence of Pathogens

A total of 1,340 canine blood/serum samples received during March 2018-March 2020 were used to perform IFA for monitoring *Anaplasma* and *Ehrlichia* species antibodies and ELISA to monitor *B. burgdorferi* antibodies. Three hundred sixty-five samples (27.2%) tested positive for the presence of antibodies against *B. burgdorferi*, while 268 samples tested positive for *A. phagocytophilum*, 228 samples (17.01%) for *E. chaffeensis*, and 223 samples (17.3%) for *Ehrlichia canis* (Tables 4.1 and 4.2)

Table 4.1: Number of canine samples tested positive for antibodies against *Anaplasma, Borrelia,* and *Ehrlichia* species, United States

Type of assay	Pathogen	Number of samples tested	Number of positive samples	(%) positivity
IFA	*A. phagocytophilum*	*1340*	*286*	*21.3*
IFA	*E. chaffeensis*	*1340*	*228*	*17.01*
IFA	*E. canis*	*1340*	*233*	*17.3*
ELISA	*B. burgdorferi*	*1340*	*365*	*27.2*

Table 4.2: Number of dogs tested positive for the antibodies to *B. burgdorferi, A. phagocytophilum, E. chaffeensis* and *E. canis* by states

Region	Samples Tested	*A. phagocytophilum*	*B. burgdorferi*	*E. canis*	*E. chaffeensis*
Alabama	1	1/1	0	0	0
Alaska	1	0/1	0	0	0
Arizona	7	5/7	2	1	1
Arkansas	81	9/81	5	55	53
California	86	28/86	8	17	16
Colorado	11	1/11	2	0	1
Connecticut	8	8/8	1	0	1
Delaware	1	0/1	0	1	1
Florida	65	14/65	18	9	12
Georgia	25	2/25	3	14	15
Hawaii	0	0	0	0	0
Idaho	2	0/2	0	0	2
Illinois	60	11/60	19	5	4
Indiana	92	11/92	33	17	14
Iowa	38	7/38	6	6	6
Kansas	16	2/16	2	7	8
Kentucky	5	2/5	2	1	2
Louisiana	2	0/2	0	0	0
Maine	2	1/2	2	1	0

Maryland	10	2/10	4	1	1
Massachusetts	1	0/1	1	0	0
Michigan	87	14/87	21	4	6
Minnesota	50	20/50	16	4	4
Mississippi	1	0/1	0	1	0
Missouri	28	3/28	1	7	9
Montana	0	0	0	0	0
Nebraska	2	0/2	0	2	2
Nevada	12	2/12	1	0	0
New Hampshire	4	3/4	1	0	0
New Jersey	75	21/75	14	6	6
New Mexico	9	2/9	3	2	2
New York	83	13/83	28	5	4
North Carolina	40	4/40	11	10	9
North Dakota	4	1/4	3	0	0
Ohio	79	11/79	32	11	10
Oklahoma	0	0	0	0	0
Oregon	4	1/4	1	0	0
Pennsylvania	55	25/55	24	2	1
Rhode Island	0	0	0	0	0

South Carolina	5	0/5	1	1	2
South Dakota	0	0	0	0	0
Tennessee	1	0/1	0	0	0
Texas	68	17/68	8	17	17
Utah	1	1/1	0	0	0
Vermont	11	4/11	7	0	0
Virginia	50	8/50	12	11	10
Washington	20	4/20	5	7	7
West Virginia	49	5/49	34	4	2
Wisconsin	88	26/88	34	3	2
Wyoming	0	0	0	0	0

Co-infection of Pathogens

Among the 1,340 samples, the highest co-infection was observed with *B. burgdorferi* and *A. phagocytophilum* for 76 samples (5.6%). The second highest co-infection was observed for *A. phagocytophilum* with *E. chaffeensis* for 65 samples (4.8%), and 64 samples (4.7%) with *E. canis.* Co-infection of *B. burgdorferi* with *E. chaffeensis* and *E. canis* was also observed in 34 samples (2.5%). Samples were also found to be co-infected with three and four pathogens. Eleven samples (0.8%) were identified as co-infected with *B. burgdorferi, A. phagocytophilum,* and *E. chaffeensis,* and nine samples (0.6%) for *B. burgdorferi, A. phagocytophilum,* and *E. canis.* Eight samples (0.5%) were found to be co-infected with all four pathogens. Table 4.3 summarizes these co-infections.

Table 4.3: Co-infection of samples with *Anaplasma, Borrelia,* and *Ehrlichia* species

Co-infection	Number of co-infected samples	Positivity (%)
B. burgdorferi +A. phagocytophilum	76	5.6
B. burgdorferi +E. chaffeensis	34	2.5
B. burgdorferi +E. canis	34	2.5
A. phagocytophilum +E. chaffeensis	65	4.8
A. phagocytophilum +E. canis	64	4.7
B. burgdorferi + A. phagocytophilum +E. chaffeensis	11	0.8
B. burgdorferi + A. phagocytophilum +E. canis	9	0.6
B. burgdorferi + A. phagocytophilum +E. chaffeensis +E. canis	8	0.59
E. chaffeensis only	27	2
E. canis only	31	2.3

Borrelia burgdorferi

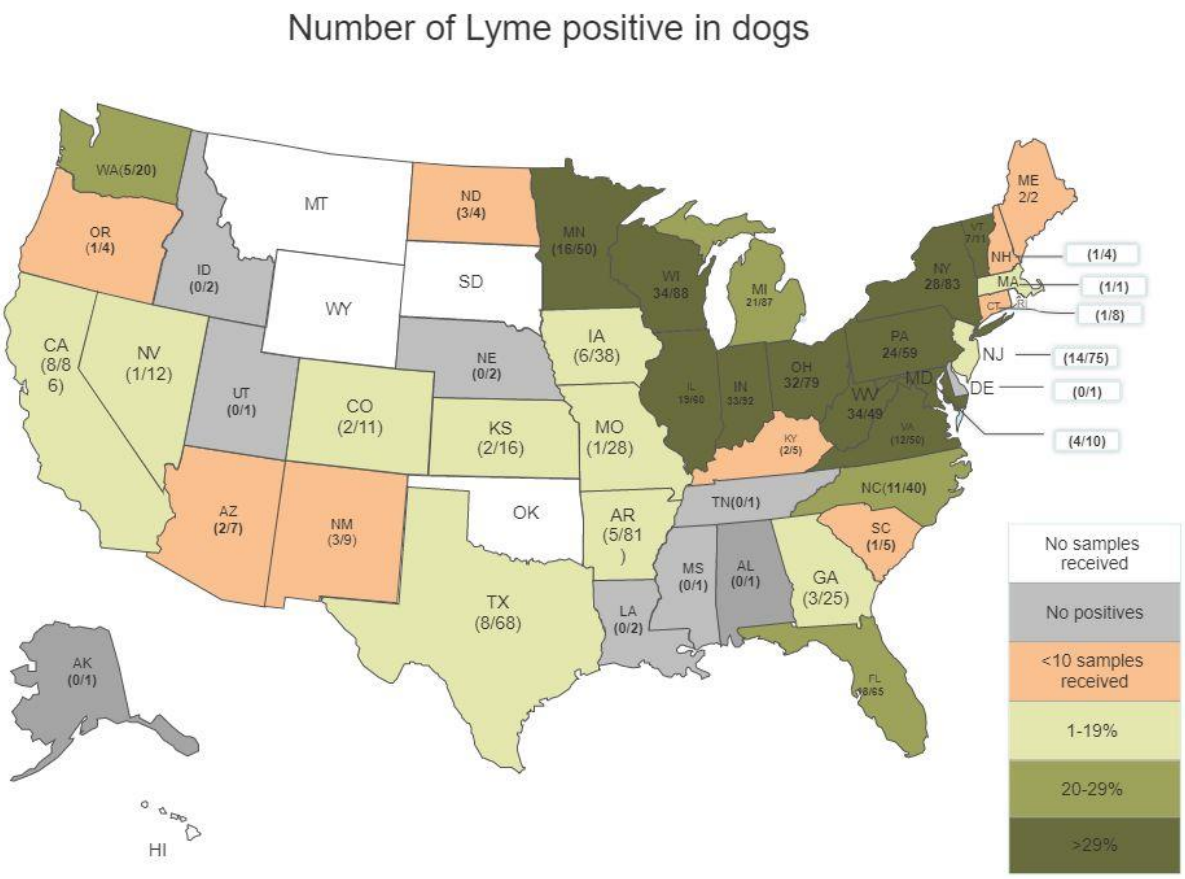


Figure 4.1: Number of Lyme positive in dogs, United States, 2018-2020

Canine blood/serum samples that were assessed were recovered from 44 states, and samples from 36 states tested positive for the presence of *B. burgdorferi*. The majority of test positive samples (>29%) were observed from Minnesota, Wisconsin, Illinois, Indiana, Ohio, Pennsylvania, West Virginia, New York, Vermont, Maryland, and Virginia. Five states (North Dakota, Arizona, New Mexico, Kentucky, and Maine) also had higher sample positives (>29%), but samples from these states were less than ten. The cut off was considered as minimum of ten samples received from each state for drawing a meaningful conclusion. States with no evidence of test positives were also observed. These states include Idaho, Utah, Nebraska, Tennessee,

Louisiana, Mississippi, Alabama, Alaska, and Delaware. The samples were not received from Montana, Wyoming, South Dakota, Oklahoma, Hawaii, and Rhode Island (Figure 4.1).

Anaplasma phagocytophilum

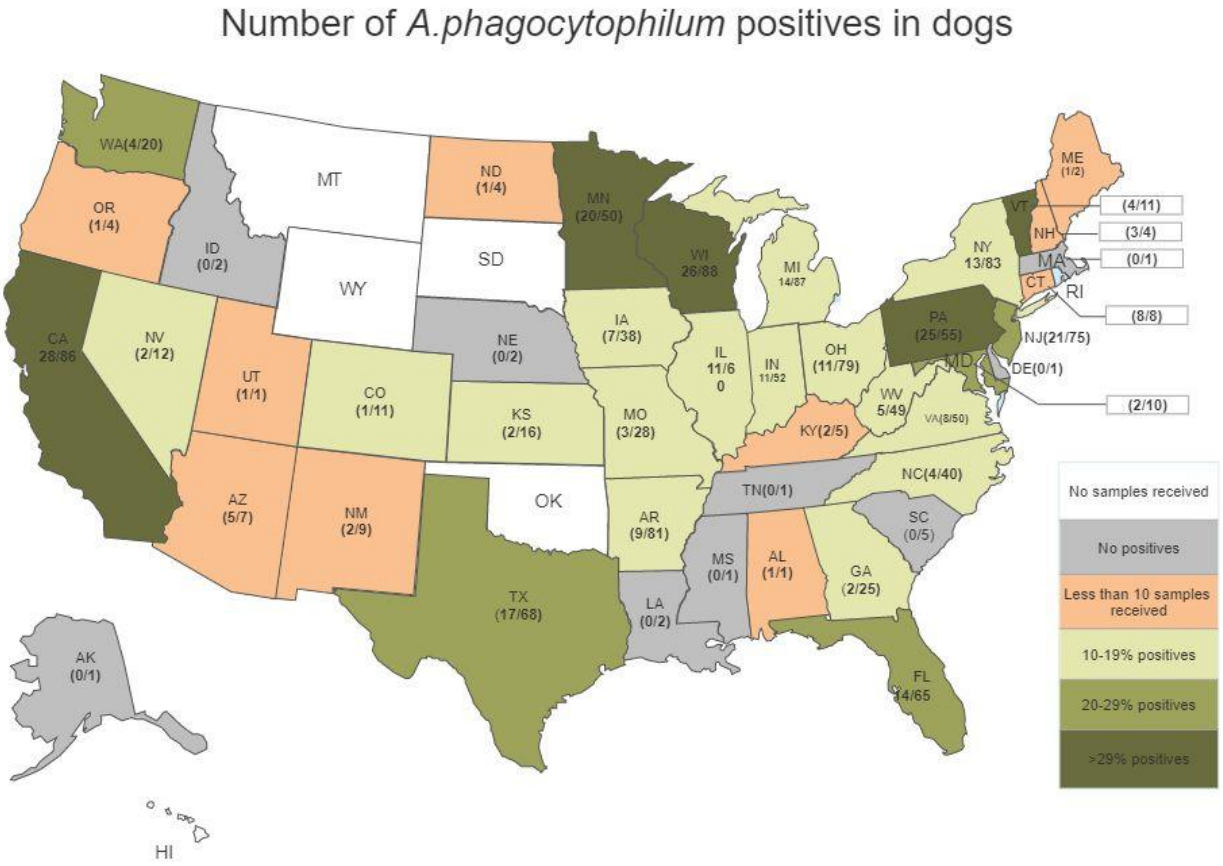


Figure 4.2: Number of *A. phagocytophilum* positives in dogs, United States, 2018-2020

A. phagocytophilum positives were represented from 35 states. The majority of the positives (>29%) were from Minnesota, Wisconsin, Pennsylvania, Vermont, and California. Eight other states (Utah, Arizona, Alabama, Kentucky, Connecticut, New Hampshire, and Maine) also had higher seroprevalence (>29%), but the samples represented less than ten samples. States with no evidence of positives include Idaho, Nebraska, Tennessee, Louisiana, Mississippi, South Carolina, Alaska, Massachusetts, and Delaware. The samples were not

received from the following states: Montana, Wyoming, South Dakota, Oklahoma, Hawaii, and Rhode Island (Figure 4.2).

Ehrlichia canis

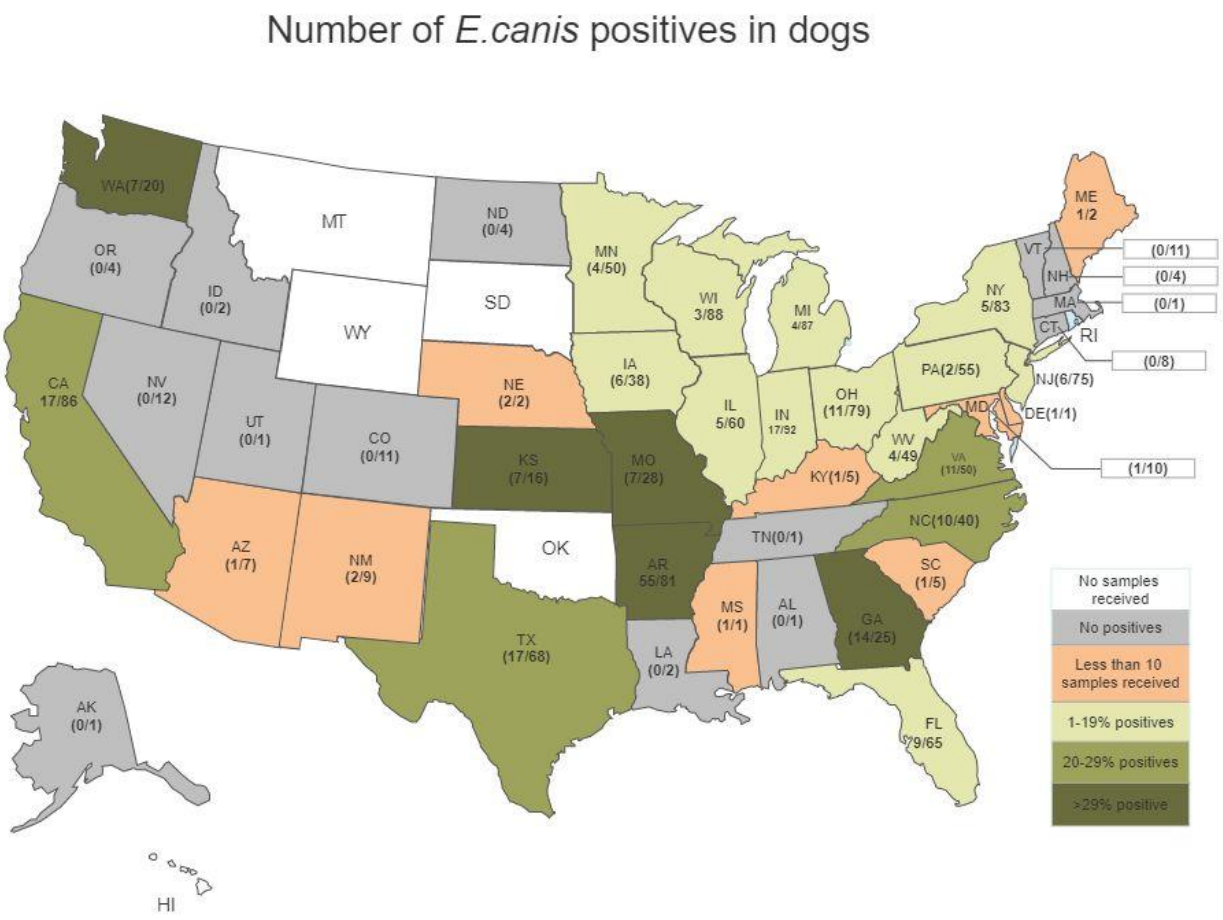


Figure 4.3: Number of E. canis positives in dogs, United States, 2018-2020

Canine blood/serum samples were also tested for the presence of antibodies to *E. canis*. We observed positives in samples collected from 30 of the 44 states. The majority of the positives (>29%) were from Washington, Kansas, Missouri, Arkansas, and Georgia. Three states; Nebraska, Mississippi, and Maine, also had higher sero positives (>29%), but the sample numbers were less than ten per state. Samples from twelve states had 10-19% positives for *E.*

canis. The states with no evidence of positives include Oregon, Idaho, Nevada, Utah, Colorado, North Dakota, Tennessee, Louisiana, Alabama, Alaska, Massachusetts, New Hampshire, Vermont, and Connecticut. The samples were not received from Montana, Wyoming, South Dakota, Oklahoma, Hawaii, and Rhode Island (Figure 4.3).

Ehrlichia chaffeensis

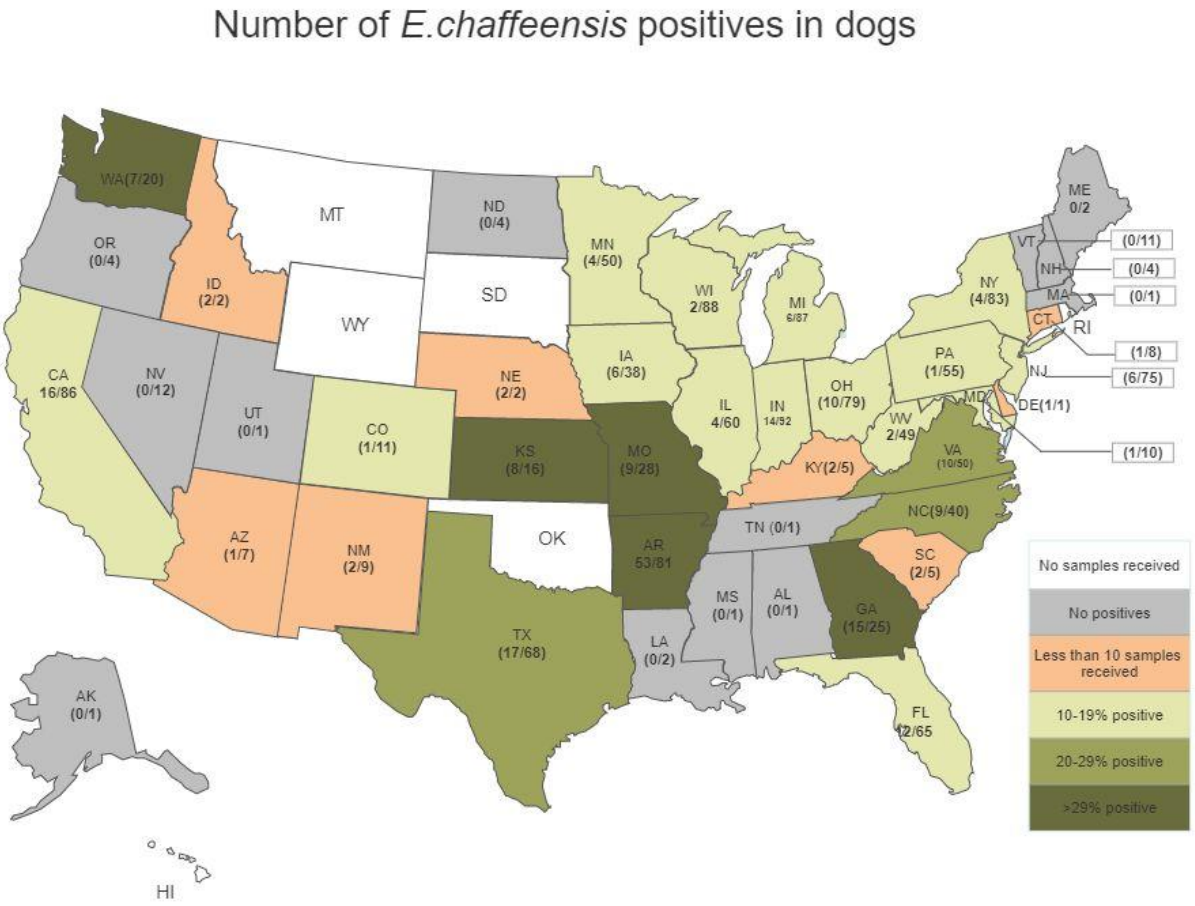


Figure 4.4: Number of *E. chaffeensis* positives in dogs, United States, 2018-2020

Canine samples were similarly tested for the presence of antibodies to *E. chaffeensis*, and samples from 31 states tested positive. The majority of the positives (>29%) were from Washington, Kansas, Missouri, Arkansas, and Georgia and although four other states (Idaho, Nebraska, Kentucky, South Carolina) had a higher positive rate (>29%), sample numbers from these states were less than ten. Samples from fourteen states had 10-19% *E. chaffeensis positives.* No evidence of positives was observed in Oregon, Nevada, Utah, North Dakota, Tennessee, Louisiana, Mississippi, Alabama, Alaska, Massachusetts, New Hampshire, Vermont, and Maine. The canine samples were not received from Montana, Wyoming, South Dakota, Oklahoma, Hawaii, and Rhode Island (Figure 4.4).

Quantitative real-time Reverse Transcriptase PCR (qRT-PCR)

Pathogen-specific qRT-PCR using TaqMan probes and primers was performed on 85 randomly selected RNA samples isolated from canine serum samples for the detection of *A. phagocytophilum, A. platys, E. canis, E. chaffeensis,* and *E. ewingii.* The qRT-PCR assay was first performed with positive controls using designed specific primers and probes to ensure that the primers and probes worked well (Figure 4.5). The positive controls used in the experiment are genomic DNAs obtained from *in vitro* cultures for *E. chaffeensis, E. canis*, and *A. phagocytophilum*, while positive control plasmids were used for *E. ewingii* and *A. platys.* The reactions with no templates added served as negative controls. We found no evidence for the presence of RNA in all samples tested (Figure 4.6).

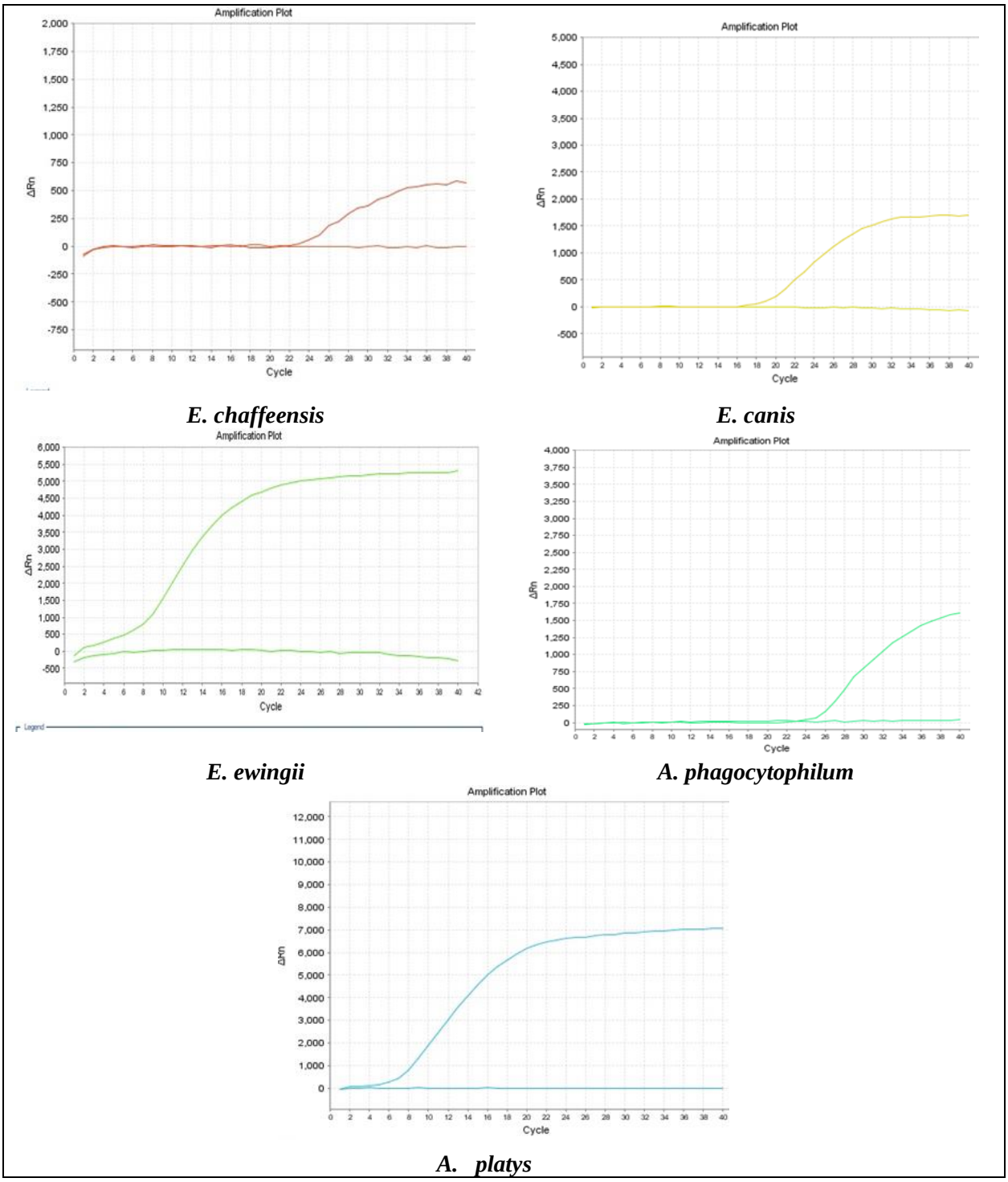


Figure 4.5: qRT-PCR for positive controls with pathogen-specific probes

qRT-PCR was performed with known positive controls for a) E. chaffeensis with JOE probe b) E. canis with Yak probe c) E. ewingii with FAM probe d) A. phagocytophilum with TET probe e) A. platys with FAM probe. The sigmoidal and horizontal line represent positive and negative control, respectively.

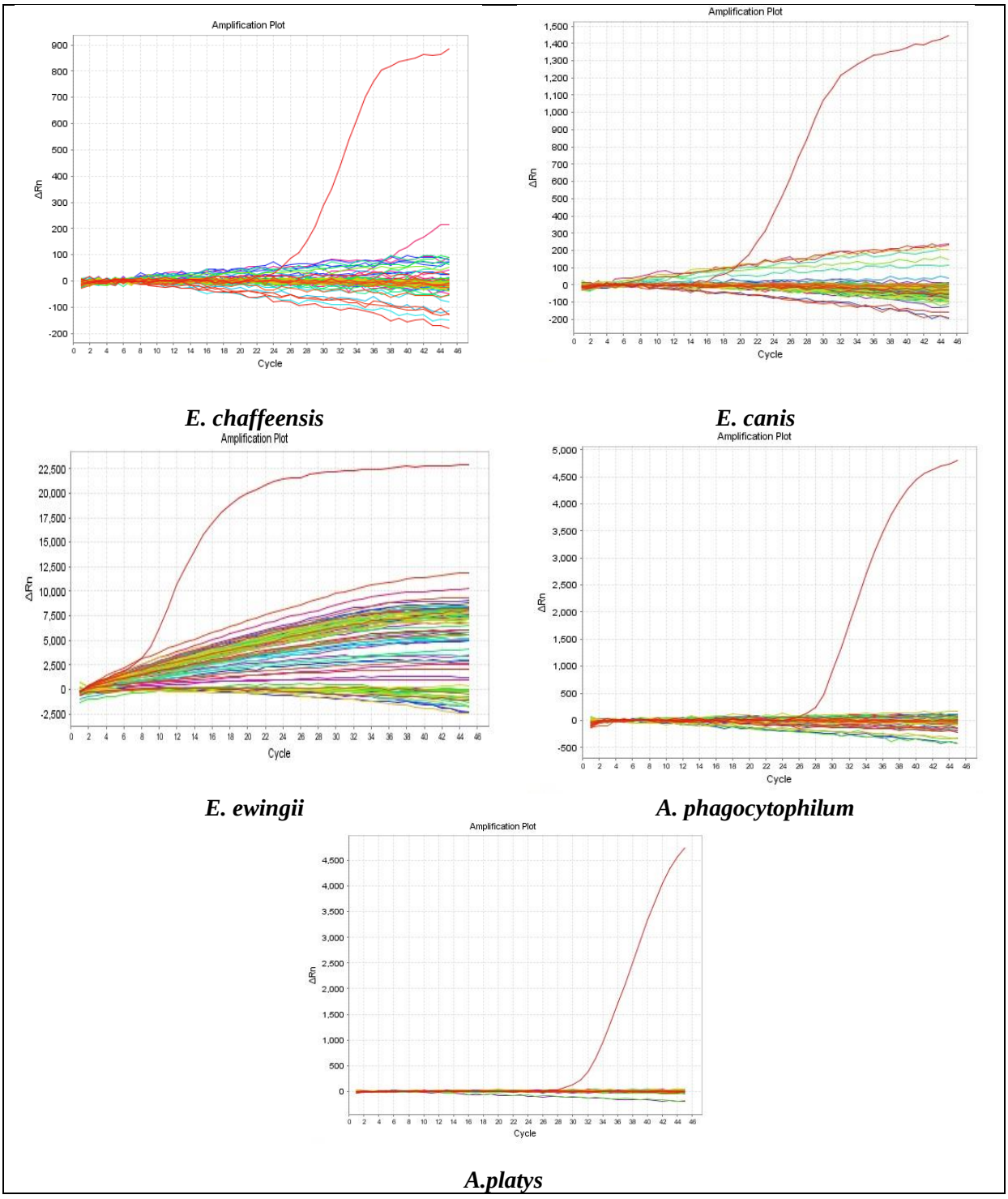


Figure 4.6: qRT-PCR for RNA samples with pathogen-specific probes

qRT-PCR was performed with RNA isolated from canine samples with specific primers and probes a) Detection of E. chaffeensis with JOE probe b) E. canis with Yak probe c) E. ewingii with FAM probe d) *A. phagocytophilum* with TET probe e) *A. platys* with FAM probe. The curve represents the positive control, and the straight line represents the negative control.

Detection of Ehrlichia canis and Ehrlichia chaffeensis in culture

Buffy coats from 66 samples used in DH82 and monitored for 8-10 weeks for the infection. We did not find any evidence for the presence of infection *in* the culture.

Chapter 5 - Discussion

We assessed 1,340 canine blood/serum samples for the presence of antibodies for four major vector-borne disease pathogens in dogs presented to veterinary hospitals from 44 states of the USA. The highest prevalence (>29% of samples positive) was observed for Lyme borreliosis. The positives were more frequently observed in most of the northeastern, mid-Atlantic, and north-central USA. The second highest seroprevalence (20-29% of samples positives) was observed in Minnesota, Wisconsin, Illinois, Indiana, Ohio, Pennsylvania, New York, Vermont, West Virginia, Virginia, and Maryland. *Ixodes* species ticks transmitting this pathogen is previously reported as more frequent in these states (Mead, 2015). We then compared the canine sample serological data with the human cases reported to the CDC in 2018. Our data for canine Lyme borreliosis sample positives were very similar to those observed and documented for the prevalence of ticks and human infections with *B. burgdorferi* (Figure 5.1).

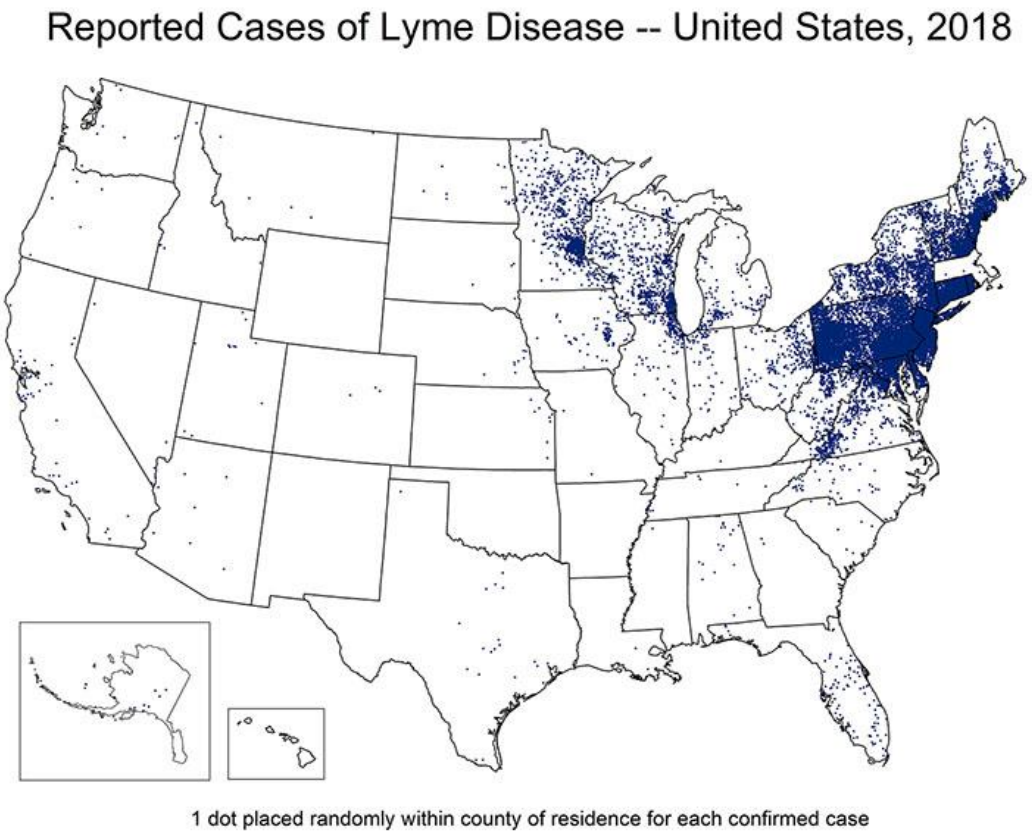


Figure 5.1: CDC surveillance data for reported human cases of LD in the United States in 2018

https://www.cdc.gov/lyme/datasurveillance/index.html

In the United States, *I. scapularis* and *I. pacificus* are identified as the transmitting vectors for *A. phagocytophilum* (Caulfield & Pritt, 2015). The highest percent of canine sample positives (29%) for this pathogen were observed in Minnesota, Wisconsin, Vermont, and Pennsylvania, followed by Washington, Texas, Florida, and Maryland had high seroprevalence in the range (20-29%) (Fig. 4.2). The majority of the samples collected from other states also had a high positive rate (10-19%) for *A. phagocytophilum.* The canine data for *A. phagocytophilum,* was also similar to the annually reported incidence for human anaplasmosis (Figure 5.2).

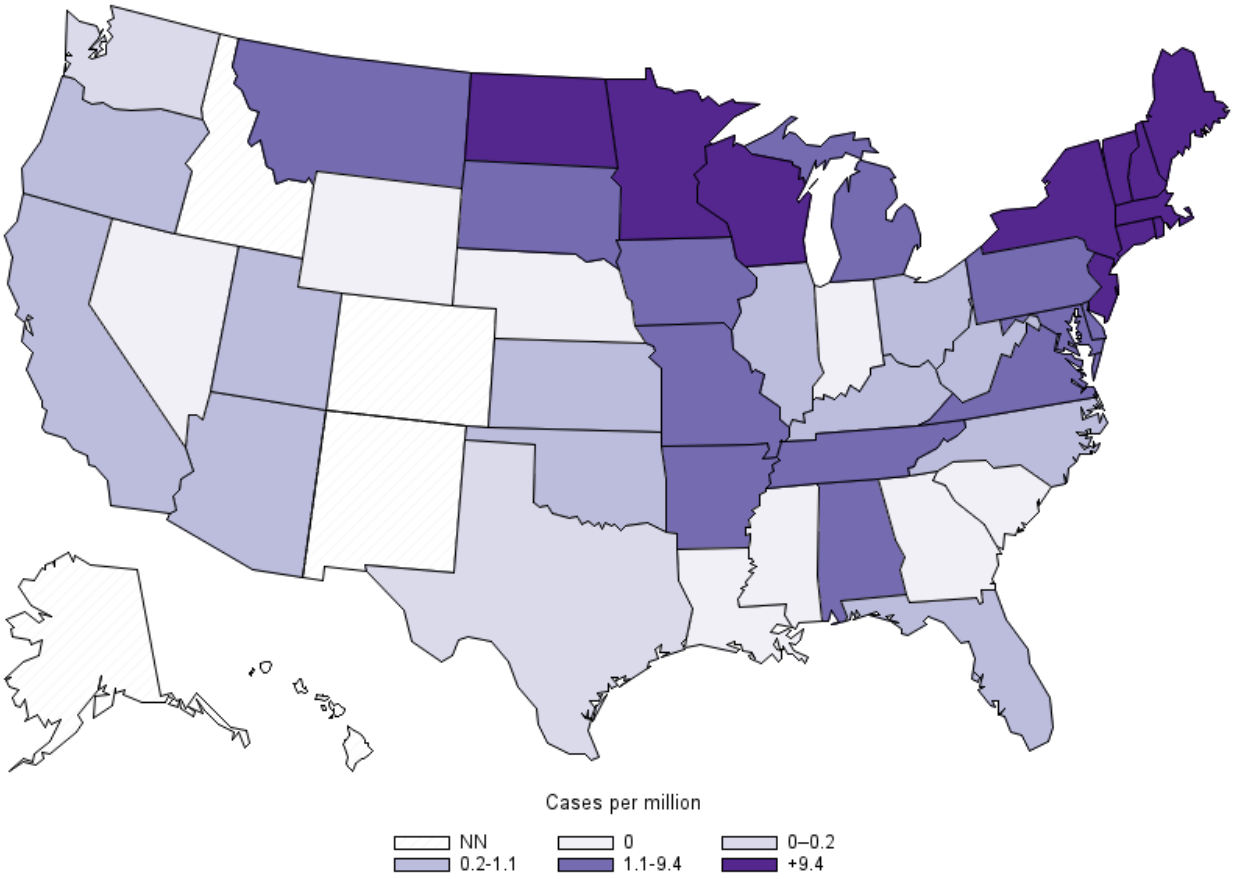


Figure 5.2: Annual reported incidence (per million population) for anaplasmosis – United States, 2018

https://www.cdc.gov/anaplasmosis/stats/index.html

While we cannot rule out the serological cross reactions in some of the positives due to antigenic cross-reaction of *A. phagocytophilum* with *A. platys. R. sanguineus,* also known as the brown dog tick, is considered as the transmitting vector for *A. platys,* and this tick is widely distributed throughout the US (Bowman et al., 2009). Co-infection of *I. scapularis* ticks and dogs with *B. burgdorferi* and *A. phagocytophilum* has been previously reported (Beall et al., 2008; Lee et al., 2014). Our study identified 76 canine samples co-infected with *B. burgdorferi* and *A. phagocytophilum* which is expected because both the pathogens share common tick vectors.

E. canis seroreactive dogs were the highest (>29% of samples positive) in Kansas, Missouri, Arkansas, Georgia, and Washington, while California, Texas, North Carolina, and Virginia had 20-29% of samples positives. A lower percent of positives (10-19%) were observed in the majority of northeastern, mid-Atlantic states and Florida. Unlike other pathogens having variations in positives with a great deal of correlation specific to geographic regions, *E. canis* positives were observed from all states with more or less in similar numbers. This observation was not surprising since the tick vector for this pathogen is primarily associated with dogs and is an indoor tick known to be present nationwide (Bowman et al., 2009). Since *E. canis* is primarily the canine pathogen (Dantas-Torres, 2010), we do not have any human data to compare although few human cases are described *E. canis (Perez et al., 2006)*. Our data are similar to previously documented canine samples (Bowman et al., 2009) (Figure 5.3).

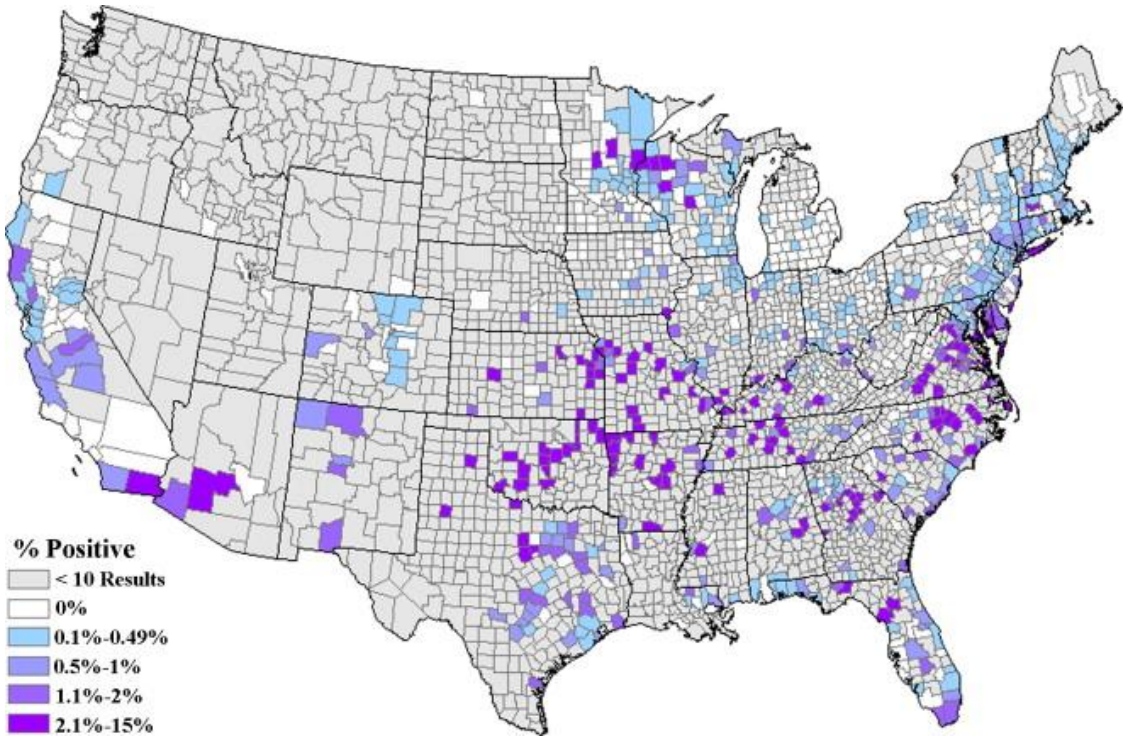


Figure 5.3: Evidence of antibodies to E. canis in dogs in the United States. (Bowman et al., 2009)

E. chaffeensis transmitted by *A. americanum* tick is more prevalent in southern regions and the eastern USA (CDC, 2017). We observed the highest positives (>29%) in samples collected from Kansas, Missouri, Arkansas, Washington, and Georgia. The majority of the northeastern, mid-Atlantic states also had positives ranging from 10 to 19%. Human documented ehrlichiosis cases are similar to our estimated prevalence data from the canine sample analysis (Figure 5.4). Some of the *Ehrlichia* positives may be co-infected with *E. canis* and *E. ewingii* due to antigenic cross-reaction.

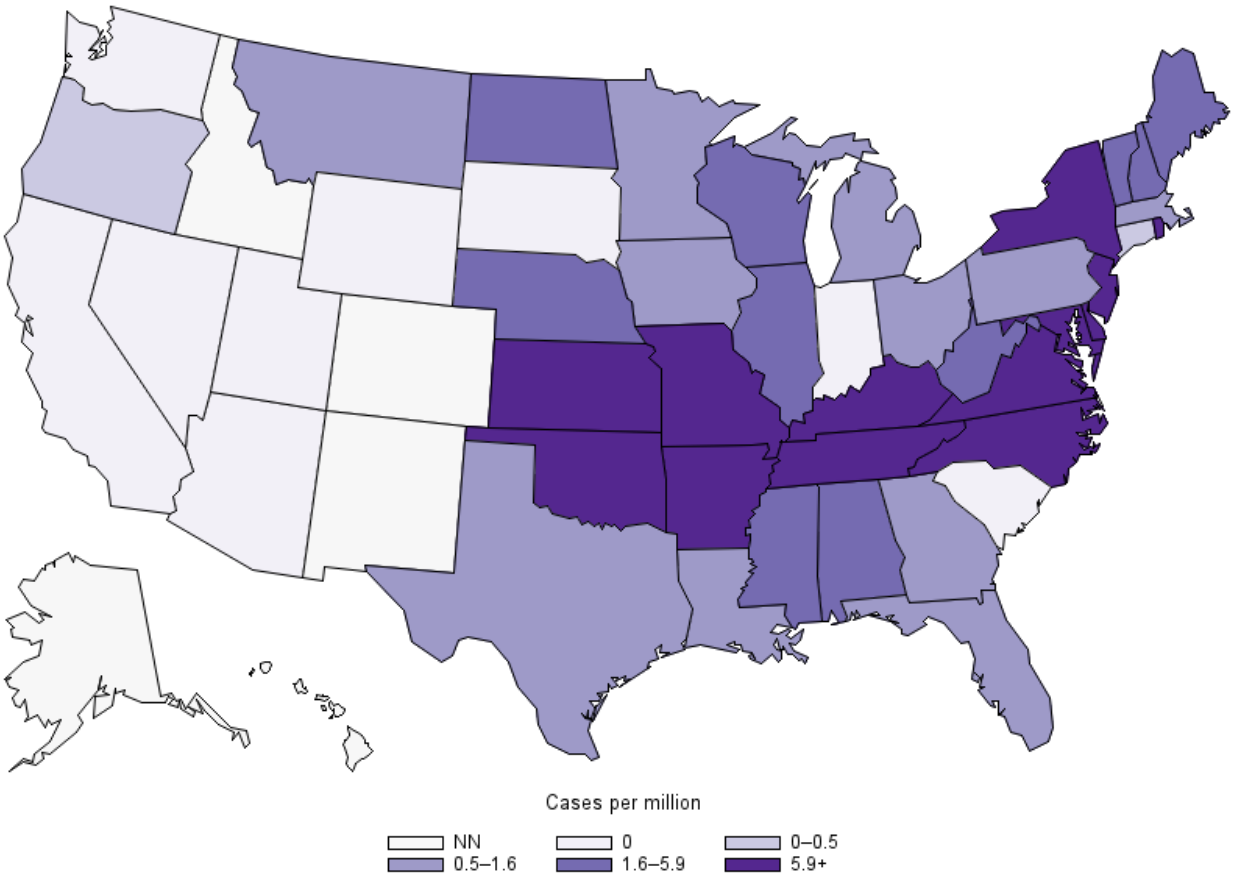


Figure 5.4: Annual reported incidence (per million population) for ehrlichiosis – United States, 2018.

https://www.cdc.gov/ehrlichiosis/stats/index.html

Co-infection of *E. chaffeensis* and *E. canis* was observed in 204 dogs and this large number might be due to the serological cross reactivity from IFA. Co-infection of dogs with *Anaplasma* species, *B. burgdorferi* and *Ehrlichia* species are reported previously by a study in Canada (Evason et al., 2019). Our study identified eight dogs as co-infection positives for *A. phagocytophilum, B. burgdorferi, E. canis,* and *E. chaffeensis.* To our knowledge, this is the first study that identified co-infection with all four pathogens.

We also performed Taq Man-based real-time quantitative Reverse transcriptase PCR (qRT-PCR) to detect *Anaplasmataceae* pathogens; *A. phagocytophilum, A. platys, E. canis, E. chaffeensis,* and *E. ewingii.* We also attempted to recover culture positives for *E. chaffeensis* and *E. canis* in DH-82 culture. However, we found no evidence of either PCR or culture positives. We reasoned that this might be due to the improper handling of blood samples collected and processed, such as the delay in shipments and storage, shipment conditions. Also, the majority of the samples may also represent persistent infected canine samples with a very low rate of actively circulating bacteria.

Our canine serological positive data comparison with the CDC-documented human data suggested that there is a great deal of correlation of test positives of canine cases with human cases. Thus, dogs can serve as excellent sentinels for the tick-borne diseases caused by *Anaplasma, Borrelia,* and *Ehrlichia* species pathogens.

Efforts will be further undertaken by investigating if the samples may test positive when assessed by DNA.

Bibliography

Adams, D. A., Thomas, K. R., Jajosky, R. A., Foster, L., Baroi, G., Sharp, P., Onweh, D. H., Schley, A. W., Anderson, W. J., & Nationally Notifiable Infectious Conditions, G. (2017). Summary of Notifiable Infectious Diseases and Conditions - United States, 2015. *MMWR Morb Mortal Wkly Rep*, *64*(53), 1-143. https://doi.org/10.15585/mmwr.mm6453a1

Allan, S. A., Barre, N., Sonenshine, D. E., & Burridge, M. J. (1998). Efficacy of tags impregnated with pheromone and acaricide for control of Amblyomma variegatum. *Med Vet Entomol*, *12*(2), 141-150. https://doi.org/10.1046/j.1365-2915.1998.00080.x

Anderson, B. E., Greene, C. E., Jones, D. C., & Dawson, J. E. (1992). Ehrlichia ewingii sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. *Int J Syst Bacteriol*, *42*(2), 299-302. https://doi.org/10.1099/00207713-42-2-299

Anderson, J. F., & Magnarelli, L. A. (2008). Biology of ticks. *Infect Dis Clin North Am*, *22*(2), 195-215, v. https://doi.org/10.1016/j.idc.2007.12.006

Arraga-Alvarado, C. M., Qurollo, B. A., Parra, O. C., Berrueta, M. A., Hegarty, B. C., & Breitschwerdt, E. B. (2014). Case report: Molecular evidence of Anaplasma platys infection in two women from Venezuela. *Am J Trop Med Hyg*, *91*(6), 1161-1165. https://doi.org/10.4269/ajtmh.14-0372

Aubry, P., & Geale, D. W. (2011). A review of bovine anaplasmosis. *Transbound Emerg Dis*, *58*(1), 1-30. https://doi.org/10.1111/j.1865-1682.2010.01173.x

Bacon, R. M., Kugeler, K. J., Mead, P. S., Centers for Disease, C., & Prevention. (2008). Surveillance for Lyme disease--United States, 1992-2006. *MMWR Surveill Summ*, *57*(10), 1-9. https://www.ncbi.nlm.nih.gov/pubmed/18830214

Bakken, J. S., & Dumler, J. S. (2015). Human granulocytic anaplasmosis. *Infect Dis Clin North Am*, *29*(2), 341-355. https://doi.org/10.1016/j.idc.2015.02.007

Beall, M. J., Chandrashekar, R., Eberts, M. D., Cyr, K. E., Diniz, P. P., Mainville, C., Hegarty, B. C., Crawford, J. M., & Breitschwerdt, E. B. (2008). Serological and molecular prevalence of Borrelia burgdorferi, Anaplasma phagocytophilum, and Ehrlichia species in dogs from Minnesota. *Vector Borne Zoonotic Dis*, *8*(4), 455-464. https://doi.org/10.1089/vbz.2007.0236

Biggs, H. M., Behravesh, C. B., Bradley, K. K., Dahlgren, F. S., Drexler, N. A., Dumler, J. S., Folk, S. M., Kato, C. Y., Lash, R. R., Levin, M. L., Massung, R. F., Nadelman, R. B.,

Nicholson, W. L., Paddock, C. D., Pritt, B. S., & Traeger, M. S. (2016). Diagnosis and Management of Tickborne Rickettsial Diseases: Rocky Mountain Spotted Fever and Other Spotted Fever Group Rickettsioses, Ehrlichioses, and Anaplasmosis - United States. *MMWR Recomm Rep*, *65*(2), 1-44. https://doi.org/10.15585/mmwr.rr6502a1

Bowman, D., Little, S. E., Lorentzen, L., Shields, J., Sullivan, M. P., & Carlin, E. P. (2009). Prevalence and geographic distribution of Dirofilaria immitis, Borrelia burgdorferi, Ehrlichia canis, and Anaplasma phagocytophilum in dogs in the United States: results of a national clinic-based serologic survey. *Vet Parasitol*, *160*(1-2), 138-148. https://doi.org/10.1016/j.vetpar.2008.10.093

Branger, S., Rolain, J. M., & Raoult, D. (2004). Evaluation of antibiotic susceptibilities of Ehrlichia canis, Ehrlichia chaffeensis, and Anaplasma phagocytophilum by real-time PCR. *Antimicrob Agents Chemother*, *48*(12), 4822-4828. https://doi.org/10.1128/AAC.48.12.4822-4828.2004

Brizuela, C. M., Ortellado, C. A., Sanabria, E., Torres, O., & Ortigosa, D. (1998). The safety and efficacy of Australian tick-borne disease vaccine strains in cattle in Paraguay. *Vet Parasitol*, *76*(1-2), 27-41. https://doi.org/10.1016/s0304-4017(97)00047-2

Budachetri, K., Teymournejad, O., Lin, M., Yan, Q., Mestres-Villanueva, M., Brock, G. N., & Rikihisa, Y. (2020). An Entry-Triggering Protein of Ehrlichia Is a New Vaccine Candidate against Tick-Borne Human Monocytic Ehrlichiosis. *mBio*, *11*(4). https://doi.org/10.1128/mBio.00895-20

Buller, R. S., Arens, M., Hmiel, S. P., Paddock, C. D., Sumner, J. W., Rikhisa, Y., Unver, A., Gaudreault-Keener, M., Manian, F. A., Liddell, A. M., Schmulewitz, N., & Storch, G. A. (1999). Ehrlichia ewingii, a newly recognized agent of human ehrlichiosis. *N Engl J Med*, *341*(3), 148-155. https://doi.org/10.1056/NEJM199907153410303

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., & Davis, J. P. (1982). Lyme disease-a tick-borne spirochetosis? *Science*, *216*(4552), 1317-1319. https://doi.org/10.1126/science.7043737

Bush, L. M., & Vazquez-Pertejo, M. T. (2018). Tick borne illness-Lyme disease. *Dis Mon*, *64*(5), 195-212. https://doi.org/10.1016/j.disamonth.2018.01.007

Carrade, D. D., Foley, J. E., Borjesson, D. L., & Sykes, J. E. (2009). Canine granulocytic anaplasmosis: a review. *J Vet Intern Med*, *23*(6), 1129-1141. https://doi.org/10.1111/j.1939-1676.2009.0384.x

Castillo, C. G., Eremeeva, M. E., Paskewitz, S. M., Sloan, L. M., Lee, X., Irwin, W. E., Tonsberg, S., & Pritt, B. S. (2015). Detection of human pathogenic Ehrlichia muris-like agent in Peromyscus leucopus. *Ticks Tick Borne Dis*, *6*(2), 155-157. https://doi.org/10.1016/j.ttbdis.2014.11.006

Caulfield, A. J., & Pritt, B. S. (2015). Lyme Disease Coinfections in the United States. *Clin Lab Med*, *35*(4), 827-846. https://doi.org/10.1016/j.cll.2015.07.006

Chen, S. M., Dumler, J. S., Bakken, J. S., & Walker, D. H. (1994). Identification of a granulocytotropic Ehrlichia species as the etiologic agent of human disease. *J Clin Microbiol*, *32*(3), 589-595. https://doi.org/10.1128/JCM.32.3.589-595.1994

Cheng, C., & Ganta, R. R. (2008). Laboratory maintenance of Ehrlichia chaffeensis and Ehrlichia canis and recovery of organisms for molecular biology and proteomics studies. *Curr Protoc Microbiol*, *Chapter 3*, Unit 3A.1. https://doi.org/10.1002/9780471729259.mc03a01s9

Cheng, C., Nair, A. D., Indukuri, V. V., Gong, S., Felsheim, R. F., Jaworski, D., Munderloh, U. G., & Ganta, R. R. (2013). Targeted and random mutagenesis of Ehrlichia chaffeensis for the identification of genes required for in vivo infection. *PLoS Pathog*, *9*(2), e1003171. https://doi.org/10.1371/journal.ppat.1003171

Childs, J. E., & Paddock, C. D. (2003). The ascendancy of Amblyomma americanum as a vector of pathogens affecting humans in the United States. *Annu Rev Entomol*, *48*, 307-337. https://doi.org/10.1146/annurev.ento.48.091801.112728

Cohn, L. A. (2003). Ehrlichiosis and related infections. *Vet Clin North Am Small Anim Pract*, *33*(4), 863-884. https://doi.org/10.1016/s0195-5616(03)00031-7

Conrad, M. E. (1989). Ehrlichia canis: a tick-borne rickettsial-like infection in humans living in the southeastern United States. *Am J Med Sci*, *297*(1), 35-37. https://doi.org/10.1097/00000441-198901000-00008

Cowdry, E. V. (1925). Studies on the Etiology of Heartwater : I. Observation of a Rickettsia, Rickettsia Ruminantium (N. Sp.), in the Tissues of Infected Animals. *J Exp Med*, *42*(2), 231-252. https://doi.org/10.1084/jem.42.2.231

Dahlgren, F. S., Heitman, K. N., Drexler, N. A., Massung, R. F., & Behravesh, C. B. (2015). Human granulocytic anaplasmosis in the United States from 2008 to 2012: a summary of national surveillance data. *Am J Trop Med Hyg*, *93*(1), 66-72. https://doi.org/10.4269/ajtmh.15-0122

Daniel E.Sonenshine , R. M. R. (1991). Overview: Ticks, People and Animals. In *Biology of Ticks* (Vol. 1). Oxford University Press. https://doi.org/https://doi.org/10.1017/S0007485300042668

Dantas-Torres, F. (2010). Biology and ecology of the brown dog tick, Rhipicephalus sanguineus. *Parasit Vectors*, *3*, 26. https://doi.org/10.1186/1756-3305-3-26

Dantas-Torres, F., Chomel, B. B., & Otranto, D. (2012). Ticks and tick-borne diseases: a One Health perspective. *Trends Parasitol*, *28*(10), 437-446. https://doi.org/10.1016/j.pt.2012.07.003

De Tommasi, A. S., Baneth, G., Breitschwerdt, E. B., Stanneck, D., Dantas-Torres, F., Otranto, D., & de Caprariis, D. (2014). Anaplasma platys in bone marrow megakaryocytes of young dogs. *J Clin Microbiol*, *52*(6), 2231-2234. https://doi.org/10.1128/JCM.00395-14

Deem, S. L. (1998). A review of heartwater and the threat of introduction of Cowdria ruminantium and Amblyomma spp. ticks to the American mainland. *J Zoo Wildl Med*, *29*(2), 109-113. https://www.ncbi.nlm.nih.gov/pubmed/9732023

Diaz, F. E., Martinez-Valdebenito, C., Lopez, J., Weitzel, T., & Abarca, K. (2018). Geographical distribution and phylogenetic analysis of Rhipicephalus sanguineus sensu lato in northern and central Chile. *Ticks Tick Borne Dis*, *9*(4), 792-797. https://doi.org/10.1016/j.ttbdis.2018.03.004

Dumler, J. S., Choi, K. S., Garcia-Garcia, J. C., Barat, N. S., Scorpio, D. G., Garyu, J. W., Grab, D. J., & Bakken, J. S. (2005). Human granulocytic anaplasmosis and Anaplasma phagocytophilum. *Emerg Infect Dis*, *11*(12), 1828-1834. https://doi.org/10.3201/eid1112.050898

Dumler, J. S., Madigan, J. E., Pusterla, N., & Bakken, J. S. (2007). Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. *Clin Infect Dis*, *45 Suppl 1*, S45-51. https://doi.org/10.1086/518146

Ebani, V. V. (2019). Serological Survey of Ehrlichia canis and Anaplasma phagocytophilum in Dogs from Central Italy: An Update (2013(-)2017). *Pathogens*, *8*(1). https://doi.org/10.3390/pathogens8010003

Ebani, V. V., Bertelloni, F., Torracca, B., & Cerri, D. (2014). Serological survey of Borrelia burgdorferi sensu lato, Anaplasma phagocytophilum, and Ehrlichia canis infections in rural and urban dogs in Central Italy. *Ann Agric Environ Med*, *21*(4), 671-675. https://doi.org/10.5604/12321966.1129912

Eng, T. R., Harkess, J. R., Fishbein, D. B., Dawson, J. E., Greene, C. N., Redus, M. A., & Satalowich, F. T. (1990). Epidemiologic, clinical, and laboratory findings of human ehrlichiosis in the United States, 1988. *JAMA*, *264*(17), 2251-2258. https://www.ncbi.nlm.nih.gov/pubmed/2214103

Evason, M., Stull, J. W., Pearl, D. L., Peregrine, A. S., Jardine, C., Buch, J. S., Lailer, Z., O'Connor, T., Chandrashekar, R., & Weese, J. S. (2019). Prevalence of Borrelia burgdorferi, Anaplasma spp., Ehrlichia spp. and Dirofilaria immitis in Canadian dogs, 2008 to 2015: a repeat cross-sectional study. *Parasit Vectors*, *12*(1), 64. https://doi.org/10.1186/s13071-019-3299-9

Ewing, S. A., Roberson, W. R., Buckner, R. G., & Hayat, C. S. (1971). A new strain of Ehrlichia canis. *J Am Vet Med Assoc*, *159*(12), 1771-1774. https://www.ncbi.nlm.nih.gov/pubmed/5128792

Fine, A. B., Sweeney, J. D., Nixon, C. P., & Knoll, B. M. (2016). Transfusion-transmitted anaplasmosis from a leukoreduced platelet pool. *Transfusion*, *56*(3), 699-704. https://doi.org/10.1111/trf.13392

Foggie, A. (1951). Studies on the infectious agent of tick-borne fever in sheep. *J Pathol Bacteriol*, *63*(1), 1-15. https://doi.org/10.1002/path.1700630103

Gaunt, S., Beall, M., Stillman, B., Lorentzen, L., Diniz, P., Chandrashekar, R., & Breitschwerdt, E. (2010). Experimental infection and co-infection of dogs with Anaplasma platys and Ehrlichia canis: hematologic, serologic and molecular findings. *Parasit Vectors, 3*(1), 33. https://doi.org/10.1186/1756-3305-3-33

Gettings, J. R., Self, S. C. W., McMahan, C. S., Brown, D. A., Nordone, S. K., & Yabsley, M. J. (2020). Local and regional temporal trends (2013-2019) of canine Ehrlichia spp. seroprevalence in the USA. *Parasit Vectors*, *13*(1), 153. https://doi.org/10.1186/s13071-020-04022-4

Goldman, E. E., Breitschwerdt, E. B., Grindem, C. B., Hegarty, B. C., Walls, J. J., & Dumler, J. S. (1998). Granulocytic ehrlichiosis in dogs from North Carolina and Virginia. *J Vet Intern Med*, *12*(2), 61-70. https://doi.org/10.1111/j.1939-1676.1998.tb02096.x

Goodman, R. A., Hawkins, E. C., Olby, N. J., Grindem, C. B., Hegarty, B., & Breitschwerdt, E. B. (2003). Molecular identification of Ehrlichia ewingii infection in dogs: 15 cases (1997-2001). *J Am Vet Med Assoc*, *222*(8), 1102-1107. https://doi.org/10.2460/javma.2003.222.1102

Gottlieb, M., Long, B., & Koyfman, A. (2018). The Evaluation and Management of Rocky Mountain Spotted Fever in the Emergency Department: a Review of the Literature. *J Emerg Med*, *55*(1), 42-50. https://doi.org/10.1016/j.jemermed.2018.02.043

Green, R. S., Naimi, W. A., Oliver, L. D., Jr., O'Bier, N., Cho, J., Conrad, D. H., Martin, R. K., Marconi, R. T., & Carlyon, J. A. (2020). Binding of Host Cell Surface Protein Disulfide Isomerase by Anaplasma phagocytophilum Asp14 Enables Pathogen Infection. *mBio*, *11*(1). https://doi.org/10.1128/mBio.03141-19

Guzman, N., Yarrarapu, S. N. S., & Beidas, S. O. (2021). Anaplasma Phagocytophilum. In *StatPearls*. https://www.ncbi.nlm.nih.gov/pubmed/30020713

Harris, R. M., Couturier, B. A., Sample, S. C., Coulter, K. S., Casey, K. K., & Schlaberg, R. (2016). Expanded Geographic Distribution and Clinical Characteristics of Ehrlichia ewingii Infections, United States. *Emerg Infect Dis*, *22*(5), 862-865. https://doi.org/10.3201/eid2205.152009

Harrus, S., & Waner, T. (2011). Diagnosis of canine monocytotropic ehrlichiosis (Ehrlichia canis): an overview. *Vet J*, *187*(3), 292-296. https://doi.org/10.1016/j.tvjl.2010.02.001

Harrus, S., Waner, T., Bark, H., Jongejan, F., & Cornelissen, A. W. (1999). Recent advances in determining the pathogenesis of canine monocytic ehrlichiosis. *J Clin Microbiol*, *37*(9), 2745-2749. https://doi.org/10.1128/JCM.37.9.2745-2749.1999

Harvey, J. W., Simpson, C. F., & Gaskin, J. M. (1978). Cyclic thrombocytopenia induced by a Rickettsia-like agent in dogs. *J Infect Dis*, *137*(2), 182-188. https://doi.org/10.1093/infdis/137.2.182

Ismail, N., Bloch, K. C., & McBride, J. W. (2010). Human ehrlichiosis and anaplasmosis. *Clin Lab Med*, *30*(1), 261-292. https://doi.org/10.1016/j.cll.2009.10.004

Jaenson, T. G., & Talleklint, L. (1992). Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete. *J Med Entomol*, *29*(5), 813-817. https://doi.org/10.1093/jmedent/29.5.813

Jaworski, D. C., Cheng, C., Nair, A. D. S., & Ganta, R. R. (2017). Amblyomma americanum ticks infected with in vitro cultured wild-type and mutants of Ehrlichia chaffeensis are competent to produce infection in naive deer and dogs. *Ticks Tick Borne Dis*, *8*(1), 60-64. https://doi.org/10.1016/j.ttbdis.2016.09.017

Johnson, D. K., Schiffman, E. K., Davis, J. P., Neitzel, D. F., Sloan, L. M., Nicholson, W. L., Fritsche, T. R., Steward, C. R., Ray, J. A., Miller, T. K., Feist, M. A., Uphoff, T. S., Franson, J. J., Livermore, A. L., Deedon, A. K., Theel, E. S., & Pritt, B. S. (2015). Human Infection with Ehrlichia muris-like Pathogen, United States, 2007-2013(1). *Emerg Infect Dis*, *21*(10), 1794-1799. https://doi.org/10.3201/eid2110.150143

Jongejan, F., & Uilenberg, G. (2004). The global importance of ticks. *Parasitology*, *129 Suppl*, S3-14. https://doi.org/10.1017/s0031182004005967

Keirans, J. E., & Lacombe, E. H. (1998). First records of Amblyomma americanum, Ixodes (Ixodes) dentatus, and Ixodes (Ceratixodes) uriae (Acari: Ixodidae) from Maine. *J Parasitol*, *84*(3), 629-631. https://www.ncbi.nlm.nih.gov/pubmed/9645873

Kocan, K. M., & de la Fuente, J. (2003). Co-feeding studies of ticks infected with Anaplasma marginale. *Vet Parasitol*, *112*(4), 295-305. https://doi.org/10.1016/s0304-4017(03)00018-9

Kocan, K. M., de la Fuente, J., Blouin, E. F., & Garcia-Garcia, J. C. (2004). Anaplasma marginale (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. *Parasitology*, *129 Suppl*, S285-300. https://doi.org/10.1017/s0031182003004700

Kocan, K. M., de la Fuente, J., Guglielmone, A. A., & Melendez, R. D. (2003). Antigens and alternatives for control of Anaplasma marginale infection in cattle. *Clin Microbiol Rev*, *16*(4), 698-712. https://doi.org/10.1128/cmr.16.4.698-712.2003

Kuttler, K. L. (1984). Anaplasma infections in wild and domestic ruminants: a review. *J Wildl Dis*, *20*(1), 12-20. https://doi.org/10.7589/0090-3558-20.1.12

Lanza-Perea, M., Zieger, U., Qurollo, B. A., Hegarty, B. C., Pultorak, E. L., Kumthekar, S., Bruhl-Day, R., & Breitschwerdt, E. B. (2014). Intraoperative bleeding in dogs from Grenada seroreactive to Anaplasma platys and Ehrlichia canis. *J Vet Intern Med*, *28*(6), 1702-1707. https://doi.org/10.1111/jvim.12442

Lara, B., Conan, A., Thrall, M. A., Ketzis, J. K., Branford, G. C., & Rajeev, S. (2020). Serologic and Molecular Diagnosis of Anaplasma platys and Ehrlichia canis Infection in Dogs in an Endemic Region. *Pathogens*, *9*(6). https://doi.org/10.3390/pathogens9060488

Lee, X., Coyle, D. R., Johnson, D. K., Murphy, M. W., McGeehin, M. A., Murphy, R. J., Raffa, K. F., & Paskewitz, S. M. (2014). Prevalence of Borrelia burgdorferi and Anaplasma phagocytophilum in Ixodes scapularis (Acari: Ixodidae) nymphs collected in managed red pine forests in Wisconsin. *J Med Entomol*, *51*(3), 694-701. https://doi.org/10.1603/me13140

Levinson, W., Hong, C.-P., Joyce, A. E., Nussbaum, J., & Schwartz, B. (2020). Ectoparasites That Cause Human Disease. In *Review of Medical Microbiology and Immunology* (16 ed., pp. 864). McGraw-Hill Professional Publishing.

Liddell, A. M., Stockham, S. L., Scott, M. A., Sumner, J. W., Paddock, C. D., Gaudreault-Keener, M., Arens, M. Q., & Storch, G. A. (2003). Predominance of Ehrlichia ewingii in Missouri dogs. *J Clin Microbiol*, *41*(10), 4617-4622. https://doi.org/10.1128/jcm.41.10.4617-4622.2003

Lin, M., Grandinetti, G., Hartnell, L. M., Bliss, D., Subramaniam, S., & Rikihisa, Y. (2020). Host membrane lipids are trafficked to membranes of intravacuolar bacterium Ehrlichia chaffeensis. *Proc Natl Acad Sci U S A*, *117*(14), 8032-8043. https://doi.org/10.1073/pnas.1921619117

Little, S., Braff, J., Place, J., Buch, J., Dewage, B. G., Knupp, A., & Beall, M. (2021). Canine infection with Dirofilaria immitis, Borrelia burgdorferi, Anaplasma spp., and Ehrlichia spp. in the United States, 2013-2019. *Parasit Vectors*, *14*(1), 10. https://doi.org/10.1186/s13071-020-04514-3

Littman, M. P., Gerber, B., Goldstein, R. E., Labato, M. A., Lappin, M. R., & Moore, G. E. (2018). ACVIM consensus update on Lyme borreliosis in dogs and cats. *J Vet Intern Med*, *32*(3), 887-903. https://doi.org/10.1111/jvim.15085

Liu, X. Y., & Bonnet, S. I. (2014). Hard tick factors implicated in pathogen transmission. *PLoS Negl Trop Dis*, *8*(1), e2566. https://doi.org/10.1371/journal.pntd.0002566

Lynn, G. E., Oliver, J. D., Cornax, I., O'Sullivan, M. G., & Munderloh, U. G. (2017). Experimental evaluation of Peromyscus leucopus as a reservoir host of the Ehrlichia muris-like agent. *Parasit Vectors*, *10*(1), 48. https://doi.org/10.1186/s13071-017-1980-4

Madewell, B. R., & Gribble, D. H. (1982). Infection in two dogs with an agent resembling Ehrlichia equi. *J Am Vet Med Assoc*, *180*(5), 512-514. https://www.ncbi.nlm.nih.gov/pubmed/7061334

Madigan, J. E., & Gribble, D. (1987). Equine ehrlichiosis in northern California: 49 cases (1968-1981). *J Am Vet Med Assoc*, *190*(4), 445-448. https://www.ncbi.nlm.nih.gov/pubmed/3558086

Madison-Antenucci, S., Kramer, L. D., Gebhardt, L. L., & Kauffman, E. (2020). Emerging Tick-Borne Diseases. *Clin Microbiol Rev*, *33*(2). https://doi.org/10.1128/CMR.00083-18

Marques, A. R. (2010). Lyme disease: a review. *Curr Allergy Asthma Rep*, *10*(1), 13-20. https://doi.org/10.1007/s11882-009-0077-3

Matei, I. A., D'Amico, G., Yao, P. K., Ionica, A. M., Kanyari, P. W., Daskalaki, A. A., Dumitrache, M. O., Sandor, A. D., Gherman, C. M., Qablan, M., Modry, D., & Mihalca, A. D. (2016). Molecular detection of Anaplasma platys infection in free-roaming dogs and ticks from Kenya and Ivory Coast. *Parasit Vectors*, *9*, 157. https://doi.org/10.1186/s13071-016-1443-3

Matei, I. A., Estrada-Pena, A., Cutler, S. J., Vayssier-Taussat, M., Varela-Castro, L., Potkonjak, A., Zeller, H., & Mihalca, A. D. (2019). A review on the eco-epidemiology and clinical management of human granulocytic anaplasmosis and its agent in Europe. *Parasit Vectors*, *12*(1), 599. https://doi.org/10.1186/s13071-019-3852-6

McBride, J. W., Corstvet, R. E., Gaunt, S. D., Boudreaux, C., Guedry, T., & Walker, D. H. (2003). Kinetics of antibody response to Ehrlichia canis immunoreactive proteins. *Infect Immun*, *71*(5), 2516-2524. https://doi.org/10.1128/iai.71.5.2516-2524.2003

McGill, J. L., Nair, A. D., Cheng, C., Rusk, R. A., Jaworski, D. C., & Ganta, R. R. (2016). Vaccination with an Attenuated Mutant of Ehrlichia chaffeensis Induces Pathogen-Specific CD4+ T Cell Immunity and Protection from Tick-Transmitted Wild-Type Challenge in the Canine Host. *PLoS One*, *11*(2), e0148229. https://doi.org/10.1371/journal.pone.0148229

Mead, P. S. (2015). Epidemiology of Lyme disease. *Infect Dis Clin North Am*, *29*(2), 187-210. https://doi.org/10.1016/j.idc.2015.02.010

Mohan Kumar, D., Yamaguchi, M., Miura, K., Lin, M., Los, M., Coy, J. F., & Rikihisa, Y. (2013). Ehrlichia chaffeensis uses its surface protein EtpE to bind GPI-anchored protein DNase X and trigger entry into mammalian cells. *PLoS Pathog*, *9*(10), e1003666. https://doi.org/10.1371/journal.ppat.1003666

Moore, A., Nelson, C., Molins, C., Mead, P., & Schriefer, M. (2016). Current Guidelines, Common Clinical Pitfalls, and Future Directions for Laboratory Diagnosis of Lyme Disease, United States. *Emerg Infect Dis*, *22*(7). https://doi.org/10.3201/eid2207.151694

Mott, J., & Rikihisa, Y. (2000). Human granulocytic ehrlichiosis agent inhibits superoxide anion generation by human neutrophils. *Infect Immun*, *68*(12), 6697-6703. https://doi.org/10.1128/iai.68.12.6697-6703.2000

Movilla, R., Garcia, C., Siebert, S., & Roura, X. (2016). Countrywide serological evaluation of canine prevalence for Anaplasma spp., Borrelia burgdorferi (sensu lato), Dirofilaria immitis and Ehrlichia canis in Mexico. *Parasit Vectors*, *9*(1), 421. https://doi.org/10.1186/s13071-016-1686-z

Mylonakis, M. E., Harrus, S., & Breitschwerdt, E. B. (2019). An update on the treatment of canine monocytic ehrlichiosis (Ehrlichia canis). *Vet J*, *246*, 45-53. https://doi.org/10.1016/j.tvjl.2019.01.015

Naimi, W. A., Gumpf, J. J., Green, R. S., Izac, J. R., Zellner, M. P., Conrad, D. H., Marconi, R. T., Martin, R. K., & Carlyon, J. A. (2020). Immunization against Anaplasma phagocytophilum Adhesin Binding Domains Confers Protection against Infection in the Mouse Model. *Infect Immun*, *88*(10). https://doi.org/10.1128/IAI.00106-20

Nair, A. D., Cheng, C., Jaworski, D. C., Ganta, S., Sanderson, M. W., & Ganta, R. R. (2015). Attenuated Mutants of Ehrlichia chaffeensis Induce Protection against Wild-Type Infection Challenge in the Reservoir Host and in an Incidental Host. *Infect Immun*, *83*(7), 2827-2835. https://doi.org/10.1128/iai.00487-15

Ndip, L. M., Ndip, R. N., Esemu, S. N., Dickmu, V. L., Fokam, E. B., Walker, D. H., & McBride, J. W. (2005). Ehrlichial infection in Cameroonian canines by Ehrlichia canis and Ehrlichia ewingii. *Vet Microbiol*, *111*(1-2), 59-66. https://doi.org/10.1016/j.vetmic.2005.08.010

Nicholson, L. W., Sonenshine, E. D., Noden, H. B., & Brown, N. R. (2019). Ticks (Ixodida). In *Medical and Veterinary Entomology* (Vol. Third Edition, pp. 603-672). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-814043-7.00027-3

Nuttall, G. H. F. (1905). Ticks and Tick-Transmitted Diseases. *Trans Epidemiol Soc Lond*, *24*, 12-32. https://www.ncbi.nlm.nih.gov/pubmed/29419268

Oda, R., Kutsuna, S., Sekikawa, Y., Hongo, I., Sato, K., Ohnishi, M., & Kawabata, H. (2017). The first case of imported Borrelia miyamotoi disease concurrent with Lyme disease. *J Infect Chemother*, *23*(5), 333-335. https://doi.org/10.1016/j.jiac.2016.12.015

Oliveira, L. S., Oliveira, K. A., Mourao, L. C., Pescatore, A. M., Almeida, M. R., Conceicao, L. G., Galvao, M. A., & Mafra, C. (2009). First report of Ehrlichia ewingii detected by molecular investigation in dogs from Brazil. *Clin Microbiol Infect*, *15 Suppl 2*, 55-56. https://doi.org/10.1111/j.1469-0691.2008.02635.x

Paddock, C. D., & Childs, J. E. (2003). Ehrlichia chaffeensis: a prototypical emerging pathogen. *Clin Microbiol Rev*, *16*(1), 37-64. https://doi.org/10.1128/cmr.16.1.37-64.2003

Paddock, C. D., Folk, S. M., Shore, G. M., Machado, L. J., Huycke, M. M., Slater, L. N., Liddell, A. M., Buller, R. S., Storch, G. A., Monson, T. P., Rimland, D., Sumner, J. W., Singleton, J., Bloch, K. C., Tang, Y. W., Standaert, S. M., & Childs, J. E. (2001). Infections with Ehrlichia chaffeensis and Ehrlichia ewingii in persons coinfected with human immunodeficiency virus. *Clin Infect Dis*, *33*(9), 1586-1594. https://doi.org/10.1086/323981

Parola, P., & Raoult, D. (2001). Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin Infect Dis*, *32*(6), 897-928. https://doi.org/10.1086/319347

Parola, P., Vestris, G., Martinez, D., Brochier, B., Roux, V., & Raoult, D. (1999). Tick-borne rickettiosis in Guadeloupe, the French West Indies: isolation of Rickettsia africae from Amblyomma variegatum ticks and serosurvey in humans, cattle, and goats. *Am J Trop Med Hyg*, *60*(6), 888-893. https://doi.org/10.4269/ajtmh.1999.60.888

Peek, F. S., & Divers, J. T. (2018). Rebhun's Diseases of Dairy Cattle. In *Rebhun's Diseases of Dairy Cattle* (3 ed.). Saunders.

Pegram, R. G., & Eddy, C. (2002). Progress towards the eradication of Amblyomma variegatum from the Caribbean. *Exp Appl Acarol*, *28*(1-4), 273-281. https://doi.org/10.1023/a:1025375206346

Perez, M., Bodor, M., Zhang, C., Xiong, Q., & Rikihisa, Y. (2006). Human infection with Ehrlichia canis accompanied by clinical signs in Venezuela. *Ann N Y Acad Sci*, *1078*, 110-117. https://doi.org/10.1196/annals.1374.016

Popovic, N., Djuricic, B., & Valcic, M. (1993). [The importance of Lyme borreliosis in veterinary medicine]. *Glas Srp Akad Nauka Med*(43), 277-285. https://www.ncbi.nlm.nih.gov/pubmed/8262422 (Problem i znacaj lajm borelioze u veterinarskoj medicini.)

Pritt, B. S., Allerdice, M. E. J., Sloan, L. M., Paddock, C. D., Munderloh, U. G., Rikihisa, Y., Tajima, T., Paskewitz, S. M., Neitzel, D. F., Hoang Johnson, D. K., Schiffman, E., Davis, J. P., Goldsmith, C. S., Nelson, C. M., & Karpathy, S. E. (2017). Proposal to reclassify Ehrlichia muris as Ehrlichia muris subsp. muris subsp. nov. and description of Ehrlichia muris subsp. eauclairensis subsp. nov., a newly recognized tick-borne pathogen of humans. *Int J Syst Evol Microbiol*, *67*(7), 2121-2126. https://doi.org/10.1099/ijsem.0.001896

Pritt, B. S., Sloan, L. M., Johnson, D. K., Munderloh, U. G., Paskewitz, S. M., McElroy, K. M., McFadden, J. D., Binnicker, M. J., Neitzel, D. F., Liu, G., Nicholson, W. L., Nelson, C. M., Franson, J. J., Martin, S. A., Cunningham, S. A., Steward, C. R., Bogumill, K., Bjorgaard, M. E., Davis, J. P., McQuiston, J. H., Warshauer, D. M., Wilhelm, M. P., Patel, R., Trivedi, V. A., & Eremeeva, M. E. (2011). Emergence of a new pathogenic Ehrlichia species, Wisconsin and Minnesota, 2009. *N Engl J Med*, *365*(5), 422-429. https://doi.org/10.1056/NEJMoa1010493

Proctor, M. C., & Leiby, D. A. (2015). Do leukoreduction filters passively reduce the transmission risk of human granulocytic anaplasmosis? *Transfusion*, *55*(6), 1242-1248. https://doi.org/10.1111/trf.12976

Rajput, Z. I., Hu, S. H., Chen, W. J., Arijo, A. G., & Xiao, C. W. (2006). Importance of ticks and their chemical and immunological control in livestock. *J Zhejiang Univ Sci B*, *7*(11), 912-921. https://doi.org/10.1631/jzus.2006.B0912

Regan, J., Matthias, J., Green-Murphy, A., Stanek, D., Bertholf, M., Pritt, B. S., Sloan, L. M., Kelly, A. J., Singleton, J., McQuiston, J. H., Hocevar, S. N., & Whittle, J. P. (2013). A

confirmed Ehrlichia ewingii infection likely acquired through platelet transfusion. *Clin Infect Dis*, *56*(12), e105-107. https://doi.org/10.1093/cid/cit177

Rikihisa, Y. (2010). Anaplasma phagocytophilum and Ehrlichia chaffeensis: subversive manipulators of host cells. *Nat Rev Microbiol*, *8*(5), 328-339. https://doi.org/10.1038/nrmicro2318

Rios, O. J. B. H. a. C. G.-. (2018). Economic and Health Impact of the Ticks in Production Animals. In M. A. a. P. K. Perera (Ed.), *Ticks and Tick-Borne Pathogens*. Intechopen. https://doi.org/10.5772/intechopen.81167

Sachdev, S. H., Joshi, V., Cox, E. R., Amoroso, A., & Palekar, S. (2014). Severe life-threatening Ehrlichia chaffeensis infections transmitted through solid organ transplantation. *Transpl Infect Dis*, *16*(1), 119-124. https://doi.org/10.1111/tid.12172

Sanchez, E., Vannier, E., Wormser, G. P., & Hu, L. T. (2016). Diagnosis, Treatment, and Prevention of Lyme Disease, Human Granulocytic Anaplasmosis, and Babesiosis: A Review. *JAMA*, *315*(16), 1767-1777. https://doi.org/10.1001/jama.2016.2884

Singu, V., Liu, H., Cheng, C., & Ganta, R. R. (2005). Ehrlichia chaffeensis expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. *Infect Immun*, *73*(1), 79-87. https://doi.org/10.1128/IAI.73.1.79-87.2005

Singu, V., Peddireddi, L., Sirigireddy, K. R., Cheng, C., Munderloh, U., & Ganta, R. R. (2006). Unique macrophage and tick cell-specific protein expression from the p28/p30-outer membrane protein multigene locus in Ehrlichia chaffeensis and Ehrlichia canis. *Cell Microbiol*, *8*(9), 1475-1487. https://doi.org/10.1111/j.1462-5822.2006.00727.x

Sirigireddy, K. R., & Ganta, R. R. (2005). Multiplex detection of Ehrlichia and Anaplasma species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J Mol Diagn*, *7*(2), 308-316. https://doi.org/10.1016/s1525-1578(10)60559-4

Sonenshine, D. E., & Roe, R. M. (2013). *Biology of Ticks Volume 1*. Oxford University Press, Incorporated. http://ebookcentral.proquest.com/lib/ksu/detail.action?docID=1538390

Steere, A. C., Strle, F., Wormser, G. P., Hu, L. T., Branda, J. A., Hovius, J. W., Li, X., & Mead, P. S. (2016). Lyme borreliosis. *Nat Rev Dis Primers*, *2*, 16090. https://doi.org/10.1038/nrdp.2016.90

Stuen, S., Granquist, E. G., & Silaghi, C. (2013). Anaplasma phagocytophilum--a widespread multi-host pathogen with highly adaptive strategies. *Front Cell Infect Microbiol*, *3*, 31. https://doi.org/10.3389/fcimb.2013.00031

Teymournejad, O., & Rikihisa, Y. (2020). Ehrlichia chaffeensis Uses an Invasin To Suppress Reactive Oxygen Species Generation by Macrophages via CD147-Dependent Inhibition of Vav1 To Block Rac1 Activation. *mBio*, *11*(2). https://doi.org/10.1128/mBio.00267-20

Thomas, R. J., Dumler, J. S., & Carlyon, J. A. (2009). Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and Ehrlichia ewingii ehrlichiosis. *Expert Rev Anti Infect Ther*, *7*(6), 709-722. https://doi.org/10.1586/eri.09.44

Tilly, K., Rosa, P. A., & Stewart, P. E. (2008). Biology of infection with Borrelia burgdorferi. *Infect Dis Clin North Am*, *22*(2), 217-234, v. https://doi.org/10.1016/j.idc.2007.12.013

Uesaka, K., Maezawa, M., & Inokuma, H. (2016). Serological survey of Borrelia infection of dogs in Sapporo, Japan, where Borrelia garinii infection was previously detected. *J Vet Med Sci*, *78*(3), 463-465. https://doi.org/10.1292/jvms.15-0392

Uilenberg, G., Barre, N. C., E.;, Burridge, M. J., & Garris, G. I. (1984). Heartwater in the Caribbean. In *Preventive Veterinary Medicine* (Vol. 2, pp. 255-267). Elsevier. https://doi.org/https://doi.org/10.1016/0167-5877(84)90068-0

Walker, D. H. (1998). Tick-transmitted infectious diseases in the United States. *Annu Rev Public Health*, *19*, 237-269. https://doi.org/10.1146/annurev.publhealth.19.1.237

Wang, Y., Nair, A. D. S., Alhassan, A., Jaworski, D. C., Liu, H., Trinkl, K., Hove, P., Ganta, C. K., Burkhardt, N., Munderloh, U. G., & Ganta, R. R. (2020). Multiple Ehrlichia chaffeensis Genes Critical for Its Persistent Infection in a Vertebrate Host Are Identified by Random Mutagenesis Coupled with In Vivo Infection Assessment. *Infect Immun*, *88*(10). https://doi.org/10.1128/IAI.00316-20

Wang, Y., Wei, L., Liu, H., Cheng, C., & Ganta, R. R. (2017). A genetic system for targeted mutations to disrupt and restore genes in the obligate bacterium, Ehrlichia chaffeensis. *Sci Rep*, *7*(1), 15801. https://doi.org/10.1038/s41598-017-16023-y

Wen, B., Rikihisa, Y., Mott, J. M., Greene, R., Kim, H. Y., Zhi, N., Couto, G. C., Unver, A., & Bartsch, R. (1997). Comparison of nested PCR with immunofluorescent-antibody assay for detection of Ehrlichia canis infection in dogs treated with doxycycline. *J Clin Microbiol*, *35*(7), 1852-1855. https://doi.org/10.1128/JCM.35.7.1852-1855.1997

Wikel, S. K. (2018). Ticks and Tick-Borne Infections: Complex Ecology, Agents, and Host Interactions. *Vet Sci*, *5*(2). https://doi.org/10.3390/vetsci5020060

Woldehiwet, Z. (1983). Tick-borne fever: a review. *Vet Res Commun*, *6*(3), 163-175. https://doi.org/10.1007/BF02214910

Woldehiwet, Z. (2010). The natural history of Anaplasma phagocytophilum. *Vet Parasitol*, *167*(2-4), 108-122. https://doi.org/10.1016/j.vetpar.2009.09.013

Xiong, Q., Lin, M., & Rikihisa, Y. (2009). Cholesterol-dependent anaplasma phagocytophilum exploits the low-density lipoprotein uptake pathway. *PLoS Pathog*, *5*(3), e1000329. https://doi.org/10.1371/journal.ppat.1000329

Yan, Q., Lin, M., Huang, W., Teymournejad, O., Johnson, J. M., Hays, F. A., Liang, Z., Li, G., & Rikihisa, Y. (2018). Ehrlichia type IV secretion system effector Etf-2 binds to active

RAB5 and delays endosome maturation. *Proc Natl Acad Sci U S A*, *115*(38), E8977-E8986. https://doi.org/10.1073/pnas.1806904115

Yancey, C. B., Diniz, P., Breitschwerdt, E. B., Hegarty, B. C., Wiesen, C., & Qurollo, B. A. (2018). Doxycycline treatment efficacy in dogs with naturally occurring Anaplasma phagocytophilum infection. *J Small Anim Pract*, *59*(5), 286-293. https://doi.org/10.1111/jsap.12799

Yin, H., & Luo, J. (2007). Ticks of small ruminants in China. *Parasitol Res*, *101 Suppl 2*, S187-189. https://doi.org/10.1007/s00436-007-0688-3

Yunik, M. E., Galloway, T. D., & Lindsay, L. R. (2016). Active surveillance of Anaplasma marginale in populations of arthropod vectors (Acari: Ixodidae; Diptera: Tabanidae) during and after an outbreak of bovine anaplasmosis in southern Manitoba, Canada. *Can J Vet Res*, *80*(2), 171-174. https://www.ncbi.nlm.nih.gov/pubmed/27127345

Zhang, J. Z., Popov, V. L., Gao, S., Walker, D. H., & Yu, X. J. (2007). The developmental cycle of Ehrlichia chaffeensis in vertebrate cells. *Cell Microbiol*, *9*(3), 610-618. https://doi.org/10.1111/j.1462-5822.2006.00812.x

Zimmerman, J. F., Karrriker, A. L., Ramirez, A., Schwartz, J. K., Stevenson, W. G., & Zhang, J. (2010). *Diseases of Swine* (10 ed.). wiley-Blackwell.