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Modeling human exposure to Babesia spp. utilizing a hunting dog cohort in the United States

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MODELING HUMAN EXPOSURE TO *BABESIA* SPP. UTİLIZING A HUNTING DOG COHORT IN THE UNITED STATES

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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Abstract

Studies have shown that *Babesia microti* coinfection has been found in approximately 25% of Lyme patients. With the range of *B. microti* increasing along with the spread of Lyme disease the incidence and prevalence will increase. At the greatest risk with a 20% fatality rate in cases of severe babesiosis are immunocompromised patients, the elderly, and splenectomized patients. Despite Babesia being best studied in the U.S., there are many underlying, understudied risk factors that contribute to the spread of disease. Serial sampling of tick-borne infections in human is discouraged and therefore indicator species such as domesticated animal are useful in furthering knowledge of related variables to exposure. Dogs in particular have been shown to be reliable models for many infectious disease and we hypothesize the same holds true for babesiosis. To achieve this , we performed an analysis of a nested case-control study within a cohort of hunting dogs from a *Leishmania* vaccine trial were tested for tick-borne diseases at two time points, enrollment in vaccine trial and 6 months later, during active tick-disease season. Exposure to *Babesia* spp. was defined as either positive enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) tests. Multiple logistic regression models were constructed from data of both time points using demographic and biological factors related to disease transmission. Of 214 dogs, 56 had *Babesia* exposure, with a prevalence rate of 26.2%. At enrollment the model showed a significance of age and exposure to *Anaplasma platys*. At the 6 month time point, 29 dogs were lost to follow up leaving a total of 185 dogs sampled at month 6 with 63 testing positive for *Babesia* spp. The cumulative incidence rate for the 6 month interval was 34.1%. No variables were found to be significant in the month 6 model.

Despite differences in *Babesia* that infect humans and dogs, the hunting dog cohort reflects a model that validates the environmental exposures, coinfections, and demographic variables that affect transmission of the pathogen. Results at the enrollment time point show that coinfection with *Anaplasma platys* has an odds ratio of 16.5 (95% CI 1.62,168.5) which is vectored by the same tick as *B. conradae*, *B. canis vogeli*, and *B. gibsoni*. Furthermore, findings of this study cast doubt on the ability of *Ixodes scapularis* to act as capable vector for canine-infecting *Babesia* species.

Public Abstract

Babesiosis is a disease caused by parasites of *Babesia* species that is spread through ticks. Babesiosis can affect humans and many other mammals all over the world. In the United States, babesiosis is mainly caused by *Babesia microti* with additional species of *Babesia* infecting dogs. Dogs have long been known to be a good indicator species for human tick-borne infection due to the shared spaces they have with humans and their tendency to explore and pick up diseases in the environment. This study used a group of hunting dogs to determine the presence of *Babesia* infection. The goal was to determine a predictive model for human infection. Infection was defined as having a positive antibody test or molecular test for *Babesia* species in blood samples. Blood samples were taken at two time points, tested, and compared. Statistical methods were used to analyze the results of the tests and compare them with variables such as region, gender, age, and other diseases the dogs were exposed to. Of 214 dogs, 56 had *Babesia* infections, with a prevalence rate of 26.2%. At the first time point the model showed age and infection with *Anaplasma platys* as being significant. At the 2nd time point, 29 dogs were lost to follow up, leaving a total of 185 dogs sampled with 63 testing positive for *Babesia* spp. The rate for the 2nd time point was 34.1%. No variables were found to be significant in the model for the 2nd time point. Despite differences in *Babesia* that infect humans and dogs, the hunting dog cohort reflects a model that validates the environmental exposures, coinfections, and demographic variables that affect transmission of the pathogen. Furthermore, findings of this study cast doubt on the ability of *Ixodes scapularis* to act as capable vector for canine-infecting *Babesia* species.

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Introduction

Babesiosis is a disease found in mammalian and some avian species with a worldwide distribution.[1, 2] Babesiosis is caused by intraerythrocytic protozoa belonging to the phylum Apicomplexa which includes other parasitic protist species such as *Plasmodium*, *Cryptosporidium*, and *Toxoplasma*.[1, 2] *Babesia* was first described in 1888 by Dr. Victor Babes in cattle in Romania.[3] Five years later in the United States, Smith and Kilbourne described a similar intraerythrocytic parasite found in cattle with Texas Cattle Fever.[3] Historically, babesiosis has been of main concern to the cattle industry, particularly in tropical climates, where *B. bovis, B. divergens* and *B. bigemina* have been responsible for severe disease commonly known as red water fever and resulting in significant financial loss.[3] In humans, babesiosis as a zoonotic disease was first confirmed in 1957 in a 33 year old Yugoslavian splenectomized cattle herder who was found to be infected with *B. bovis*.[2, 4] In the United States, the first case of babesiosis was identified in 1966 in California in a splenectomized patient who had received multiple blood transfusions.[4] Three years later, in a New Jersey hospital, a second confirmed case was identified in a patient that was found to have malaria-like parasites on a blood smear, later identified as *Babesia*.[5] Since then human babesiosis has been found to be caused primarily by four different species: *B. microti*, *B. divergens*, *B. duncani* and *B. venatorum* with *B. microti* having the highest rate of infection in the United States.[1-3]

Human babesiosis is considered a rare, emerging zoonotic disease found on all continents save Antarctica.[2, 3, 6] The transmission cycle for all species of zoonotic *Babesia* spp. are not fully understood, with *B. microti* in the U.S. and *B. divergens* in

Europe being the most thoroughly studied.[1-3] In species with known transmission cycles, small rodents serve as reservoir host species. Transmission occurs via *Ixodidae* family ticks primarily in the nymph stage of the tick's lifecycle although transmission can occur through the bite of adult ticks.[7, 8] In the Midwest and Eastern US where. *B. microti* predominates, the white-footed mouse (*Peromyscus leucopus*) is the primary host species and the black-legged tick or brown deer tick (*Ixodes scapularis*) acts as the vector.[2, 6] Both reservoir host and tick vectors are also known to carry *Borrelia burgdorferi,* the causative agent of Lyme disease.[6, 9] Humans, like dogs, are a dead-end host for *Babesia* spp.[1, 2] Infection is asymptomatic or mildly symptomatic in the majority of those exposed. Severe disease is more likely in patients who are immunocompromised, elderly, and particularly if asplenic.[2, 3, 10, 11] *Babesia* spp. have also been found to be transmitted vertically and via blood transfusion.[10-12] According to the American Red Cross, *B. microti* is the most commonly transmitted blood-borne parasite. At this time there are no FDA approved tests to screen the blood supply for *Babesia*.[12, 13] Transmission through organ and tissue transplantation has been suggested as a possible pathway but to what extent this happens remains controversial.[12, 14] Unlike vector-transmitted *Babesia* which has a seasonality, transfusion transmission can occur year round.[11, 12] Since the first cases were reported in the 1960's, as more information has been gained regarding coinfection, severity of disease in at-risk patients, geographic spread of host and vector, and increased prevalence, the scientific and medical communities have taken more interest in this neglected zoonotic disease.[1-3, 6, 8]

Canine babesiosis is caused by a wider array of species that are more diverse and often more difficult to characterize than known zoonotic species.[15-17] The primary species of concern for canine disease in North America are *B. canis vogeli*, *B. conradae*, and *B. gibsoni*. [1, 15, 17] Occasional detection of *B. microti*-like infections have been found in foxes in North Carolina.[18] Interestingly, *B. microti*-like infections have also been found in domestic dogs in Spain.[17] It is important to note that *B. microti* has multiple serovars and to date none have been documented as infectious to both humans and canids.[6, 17-19] However, much is still unknown about the role bridge hosts, such as domestic dogs and wild canids, play in the transmission cycle of most *Babesia* spp and whether dogs may serve to bring additional ticks into areas for human exposure.[6, 18] [20]Domesticated species, particularly companion animals, have shown to be a source of infection to humans for many zoonoses.[6, 21] Given the close relationship and shared environments of humans and domestic dogs, modeling of infectious disease in dogs as predictors of human disease has proven effective.[6, 21-23] The hypothesis of this study is that a cohort of hunting dogs will be an effective predictive model to evaluate longitudinally for human exposure to *Babesia*.

Epidemiology

Zoonotic *Babesia* spp can be found across the Americas, Europe, Africa, and Asia with pathogen species, host, and vector varying depending on locale.[1, 3, 6, 17, 24] North America and Europe have been more widely studied with regional reporting of disease in South America, Africa, Asia, and Australia.[1, 3, 25-27] In tropical climates, symptoms of *Babesia* can present as more deleterious disease which is one of the likely explanations why *Babesia* surveillance is absent or low.[1, 3, 25-27]

North and South America

In January 2011, *Babesia* became a reportable disease to the Centers for Disease Control and Prevention (CDC) with 18 states participating. Health departments in those states reported 1124 confirmed or suspected cases with 97% (1097) of the reports coming from 7 endemic states: Minnesota, Wisconsin, Connecticut, Massachusetts, New Jersey, New York, and Rhode Island.[12, 28] Ten cases of transfusion-related transmission was reported and one case of vertical transmission reported.[12, 28] Infecting *Babesia* species was identified on 429 of the cases and all were found to be *B. microti,* the primary species found in the endemic regions of the Midwest and New England.[2, 28, 29] The most recent statistics from 2014 show 30 states reported 1744 cases with 1636 (93.8%) cases reported from the initial 7 endemic states.[30] Incidence rates which were calculated based on the populations of the reporting states averaged 0.8 cases/100,000 from 2011-2014 (Appendix 1).[12, 30]

Babesia microti is the primary species responsible for human babesiosis in North America. The geographic range of the disease is limited to the range of the black-legged tick (*I. scapularis*) and the white-footed mouse (*P. leucopus*).[1, 2, 6] Endemic areas include the Midwestern U.S., northeastern U.S., and central Canada.[2, 6, 31] Coinfection with *B. burgdorferi* is common due to the shared vector and host. Coinfection has been detected in up to 19% of black-legged tick nymphs and adults in regions endemic to both *B. burgdorferi* and *B. microti*.[8, 32] *Babesia microti* coinfection in ticks is low due to factors of reduced fitness versus *B. burgdorferi* and low parasite levels found in over-wintering nymphs[32]. In white-footed mice, the rate of co-infection has been shown to vary between 12%-42% likely due to multiple exposures to feeding

ticks.[32, 33] Human studies have shown regional variability with rates of exposure between 40%-50% with the highest rates in central New England where both diseases have presumptively been endemic for the longest periods of time.[8, 32] In New York State, a study published in 2016 has shown that there was a prevalence of 26.9% amongst patients testing positive for Lyme disease to also have babesiosis.[7] Conversely, due to the higher rates of Lyme disease than babesiosis, approximately two-thirds of babesiosis patients have been found to also have Lyme disease.[32] Coinfection with *Anaplasma* spp. has also been observed with a disease rate of approximately 13% in endemic regions of the U.S.[32] Co-infection with *Ehrlichia*, while probable due to the shared vector, has been poorly studied due to rarity of occurrence and the limited geographic range in which *Babesia* and *Ehrlichia* species overlap in the Midwestern U.S.[34] Co-infection of *B. burgdorferi* and *Ehrlichia* spp has a rate of approximately 7% thus extrapolating a rate for co-infection with *Babesia* is difficult.[34]

Other species of *Babesia* have been found in western and southern states but occurrence of these infections are less frequent and current studies suggest the pathogens have a lower presence in the environment.[1, 2, 35] In the early 1990's, three cases of babesiosis were diagnosed, two via blood transfusion and one presumed tick vectored. Upon serological testing, all samples were found to be novel species of *Babesia* and referred to as WA1. Further phylogenetic analysis determined the samples were most closely related to *B. divergens* which is a zoonotic bovine species of *Babesia* endemic to Europe.[35] In 2002 another case of *Babesia* was diagnosed in an asplenic patient who presumably contracted the pathogen through a tick vector. Serological and phylogenetic analysis found a *B. divergens*-like parasite. The results were compared to

the WA1 samples from earlier cases and found to be unrelated.[35] Several dozen more cases of WA1 and CA-type infections have been found along the west coast and through phylogenetic analysis have been grouped together into a new species, called *B. duncani*[35]. The tick vector was believed to be *I. pacificus* with an unknown primary host species.[36, 37] Further cases of *B. divergens*-like babesiosis have occurred in asplenic patients in Missouri and Kentucky without apparent connection between cases.[1, 2]

Babesiosis in South America has been under studied. Studies that have been conducted show diversity of species and prevalence. In Colombia, *B. bovis* and *B. bigemina* were found in a cross-sectional study of 194 male subjects.[38] Prevalence of exposure using IFA testing was found to be 3.6% (7) overall with an adjusted prevalence of 7.9% in cattle ranchers.[38] Similarly in Bolivia, a study of 271 people from two rural communities found a prevalence of 3.3% (9) reactive for *B. microti* antigens using IFAT. An additional 85 people were tested and found to have a total seroprevalence of 45.7% (34).[26] The findings of both studies suggest that babesiosis is more common in South America than anticipated and that further research needs to be conducted to determine range, vectors, and hosts of disease.[26, 38]

Europe

Human babesiosis in Europe is far less prevalent than the U.S. and is caused mostly by *B. divergens* through the tick vector, *Ixodes ricinus*.[2, 39, 40] *Babesia microti* has also been documented but is not as common and is associated with blood-transfusion transmitted disease.[10, 39] Since the 1950's, approximately 50 cases of babesiosis have been reported across Europe.[41] Half of those cases were reported in the United Kingdom and France with sporadic cases reported in the rest of the continent.[2, 41]

Infection is similar to other parts of the world following a seasonal emergence of the disease based on rainfall levels that influence the life cycle of the vector.[2, 39]

Africa

Cases of suspected babesiosis have been regionally reported across the continent mainly from Egypt and South Africa [1, 27, 32]. Multiple species have been found including *B. divergens, B. bigemina, and B. bovis.* [27, 42] A 2016 study of malaria in the Democratic Republic of the Congo (DNC) suggested a possible co-infection link between *B. microti* and *Plasmodium falciparum* with 19 of the 306 children in the study showing *B. microti* on blood smears.[25] In Kenya, wild baboons and African Green Monkeys were found to be infected with *B. microti*.[42] Several serotypes of *B. microti* exist and the study did not test beyond basic species identity.[42] Non-human primates have not been implicated as a host species for *Babesia* spp. in Africa, but the findings from Kenya and DNC warrants further investigation. Overall, *Babesia* spp. infection in Africa has been poorly studied due to the presence of more highly virulent diseases that present similarly symptomatically or are of greater concern.[25, 27, 42]

Asia

The study of *Babesia* spp. in Asia has occurred mostly in the People's Republic of China and Japan.[1, 2, 24] Multiple economic, political, health, and education issues have made the prevalence of babesiosis hard to determine.[1, 2, 24] The pathogens found have been *B. microti* and *B. divergens* carried by *Ixodes persulcatus* with animal reservoirs suggested to range from rodents to small primates.[1, 24] Increased awareness has led to increased diagnosis but due to similarity of symptoms to other diseases, it is still largely undiagnosed or misdiagnosed.[24]

Australia

The first reported case of babesiosis in Australia occurred in 2010 in a motor vehicle accident patient whose condition declined over several months for unknown reasons until his death. Post-mortem testing of blood revealed presence of *B. microti*.[43] Phylogenetic analysis traced the pathogen back to U.S. endemic species. Having no indication of infection from travel or other reason related to import of ticks from the U.S. it was determined the pathogen was obtained domestically despite no known competent vector or host.[43] In the years following, two additional cases of babesiosis were diagnosed with no travel histories and the parasites were identified as *B. microti* and *B. duncani*.[6] Historically babesiosis was considered an issue specific to cattle ranching but the emergence of disease in humans suggests zoonotic *Babesia* has geographically spread to Australia.[43, 44]

Transmission cycle

Vector lifecycle

Vector-borne transmission of *Babesia* spp. to humans has only been confirmed for *Ixodidae* family ticks. The species of *Ixodes* likely varies by continent and area, with the parasite-vector-host transmission cycle unknown for many locales.[1, 2, 6] For *I. scapularis,* the vector for *B. microti* in North America, the two-year cycle begins when female ticks lay eggs in the spring. After hatching into the larval stage late summer, larvae ingest *B. microti* when taking a blood meal from a rodent host, most commonly *P. leucopus*.[1, 2, 6, 45] Larvae molt into nymphs the following spring and feed on mice, humans or other mammals whereupon the parasite is transmitted (Figure 2).[2, 45, 46] While nymphs and adult ticks carry transmissible stages of the parasite, it is commonly

spread to humans during the nymphal stage likely due to increased contact with hosts, increased seasonal activity of vector and hosts, and the small size of the nymph causes it to be unnoticed while feeding.[1, 2, 45, 47] After the nymph molts to the adult state, it feeds on white-tailed deer (*Odocoileus virginianus*) that have not been found to acquire disease but may serve as reservoirs.[1, 2] The following spring, female ticks lay eggs but since *B. microti* does not transmit transovarially, eggs are not infected.[45, 47]

Parasite lifecycle

The lifecycle of *Babesia* spp. is complex having a sexual reproductive stage in the tick vector and an asexual reproductive stage in the host (Figure 1).[45] Initially, male and female gametes are ingested by the larval stage of the tick vector from the white-footed mouse host. Gametes undergo fertilization in the tick gut, forming ookinetes and migrate through the gut epithelium. Ookinetes then move to the salivary glands and become sporoblasts that are dormant until the larva molts into the nymph.[45, 48] Sporoblasts are activated and release several thousand sporozoites that enter the salivary ducts.[2, 45, 48] When a nymph takes a blood meal, sporozoites are introduced to the host at the injection site then move into the host's blood stream.[45, 49] *Babesia* spp are obligate intraerythrocytic parasites and cannot complete their host life cycle in non-erythrocytic host cells.[2, 10, 11, 48, 49] Sporozoites directly invade erythrocytes and undergo asexual reproduction, known as merogony.[48, 49] The resulting trophozoites undergo two rounds of binary fission/budding forming merozoites in a tetrad form.[45, 49] This tetrad form known as a "Maltese cross" is a diagnostic characteristic of *Babesia* spp. under microscopy.[1, 45, 49] In the definitive host, some merozoites do not multiply and become gametocytes that will be ingested by a feeding tick and continue the sexual productive

stage of the life cycle.[49] In humans, a dead-end host, gametocytes are not formed.[1, 12, 49] The erythrocyte eventually lyses as the merozoites bud and split apart, entering into the blood stream, able to attach to and invade another erythrocyte.[49] The invasion of erythrocytes occurs via merozoite surface and internal structures highly conserved throughout apicomplexans and very well described in *Toxoplasma gondii*.[49] Merozoites have a cytoskeleton structure known as a pellicular complex and three types of apical organelles: micronemes, rhoptries, and dense granules/spherical bodies.[49] Upon initial contact with the erythrocyte, microneme proteins are released for attachment to the membrane.[49] Rhoptry proteins are then excreted to form a tight junction to the erythrocyte's membrane to allow for merozoite movement inside the cell in a parasitophorous vacuole.[49] Finally, the contents of the spherical body are released and the merozoite[49] can begin budding.

Transfusion-Transmitted *Babesia* (TTB)

Transfusion-transmitted *Babesia* also been found to occur due to the intraerythrocytic nature of the pathogen.[10-12] A 2008 study by the U.S. Food and Drug Administration determined that *Babesia* spp. were the most commonly transmitted pathogens from blood transfusions in the United States with most of the cases being *B. microti*.[12, 13] Prevalence of TTB varies widely with geography with one study in Connecticut showing 1 case/601 transfused red blood cells (RBCs) and a later studying showing 1 case/100,000 units RBCs transfused.[10] Nationally the American Red Cross found an approximate prevalence of 1 case/one million RBC units.[11] Alarmingly, of those that experience TTB, the mortality rate is about 20%.[11] Explanations for the high mortality rate are likely due to underlying illnesses or comorbidities prior to transfusion that allowed for the development of severe babesiosis.[10, 11] Currently, *Babesia* is not

tested for following blood donation but the American Red Cross is conducting a study to determine an effective screening test for *Babesia* spp.[13]

Diagnosis and Treatment

Misdiagnosis

Clinically, human babesiosis presents similarly to many diseases. In some countries where the prevalence of severe diseases, such as malaria, are common, babesiosis is often misdiagnosed.[24, 25, 27] In regions where *Babesia* shares a common tick vector with a more prevalent pathogen, babesiosis may be overlooked due to patient-reported vector exposure or lack of information.[7, 32-34] In parts of the U.S. where *B. burgdorferi* and *B. microti* overlap, this is often the case.[32, 34]

Clinical presentation

Healthy patients with *Babesia* infection will be asymptomatic and the parasite will be cleared by the host immune system.[1, 6, 45] Patients with mild disease present with symptoms of fever, malaise, weakness, sweats, and chills.[10-12] Severe cases of babesiosis present with hemolytic anemia, high fever, myalgia, and worsened symptoms of the mild form.[10-12, 14] Due to the general nature of mild disease symptoms, factors such as seasonality, travel, and age should be considered when making a diagnosis.[32, 45]

Diagnosis

Early stages of infection can have low levels of parasitemia, making diagnosis based on microscopy difficult with few parasites seen on blood smear.[1, 41] In countries with endemic malaria, rarity of babesiosis and symptomatic similarity of *Babesia* spp. to *Plasmodium* spp can make diagnosis difficult.[24, 26, 27, 38, 50] Under microscopy, the

distinguishing characteristic of *Babesia* spp is the “Maltese cross” form of the merozoite, but is rarely observed and requires examining of several slides of Giemsa stain blood smears to be detected.[1, 10, 11] The most sensitive and specific test for *Babesia* is through PCR, amplifying the 18s rRNA gene which is indicative of *Babesia* spp.[1, 10] Immunofluorescence assays and ELISA tests are additional diagnostics available that are commonly used.[2, 24, 41]

Treatment

Treatment depends on species of *Babesia* the patient is infected with and severity of disease.[1, 41] Mild disease from *Babesia divergens* and *B. venatorum* is successfully treated with a 7-10 day course of clindamycin.[1, 41] Infection with *B. duncani* is treated with clindamycin along with quinine or quinidine.[1, 41] Treatment of *B. microti* involves using atovaquine and azithromycin but treatment with clindamycin and quinine is also appropriate.[1, 41] Severe cases of disease require intravenous treatment with clindamycin with quinine in addition to blood transfusion to reduce parasite load and treat anemia.[1] Drug resistance has been found with *B. microti*, particularly in immunocompromised patients.[1] However, given the rarity of severe disease, more research is needed to assess the effectiveness of long-term anti-babesial therapies versus compounds that have been shown to directly affect the DNA and RNA synthesis in certain species of *Babesia*.[1]

Methods

Design

Nested case-control in a cohort of American Foxhounds enrolled in a *Leishmania* vaccine trial.

Study Population

Dogs for this study come from a cohort of American Foxhounds enrolled in a *Leishmania* vaccine trial at the Center for Emerging Infectious Disease-Petersen Lab at the University of Iowa. In 2007, the Petersen Lab began at Iowa State University before relocating to The University of Iowa in 2013. The Petersen lab is primarily involved in passive surveillance of *Leishmania* in domestic dogs in the U.S. through diagnostic testing. In 2016 a *Leishmania* vaccine trial was started in American Foxhounds belonging to 10 hunts associated with the Masters of Foxhound Association (MFHA). Approximately six hundred foxhounds were enrolled in the vaccine trial from Western, Midwestern, Eastern, and Southern regions of the U.S.

Whole blood samples from all dogs being considered for enrollment in the vaccine trial were tested in the Petersen Lab at enrollment using the IDEXX SNAP 4Dx test for *Ehrlichia* spp., *B. burgdorferi*, and *Anaplasma* spp. antibodies along with *Dirofilaria* antigens. Samples that tested positive were matched on sex and region with two non-reactive controls. Samples were sent to IDEXX for further testing. Proprietary ELISA tests were performed to identify individual species of *Anaplasma, Ehrlichia, Borrelia*, and *Babesia*. PCR was also performed to detect species specific antigens for *Rickettsia, T. cruzi, Bartonella, Hematozoon, Candidatus and Mycoplasma.* Results were reported back to the Petersen lab. Repeated samples were sent at month 6 to IDEXX and all tests were performed on the new time point samples. A third sample set was sent for analysis after month 9, in November.

Power Calculation

A 2010 canine *Babesia* study showed a 10.6% frequency in positive samples submitted from 22 states. Since PCR detects current infections, the prevalence of exposures would likely be higher. For this reason, an estimate of 25% will be used for detection of exposure to *Babesia* spp. Using an online sample size program at: https://www.stat.ubc.ca/~rollin/stats/ssize/b2.html, a power of 0.70 is achieved to obtain a clinically relevant prevalence of 0.25 using a two-sided t-test, $\alpha=0.05$ and a sample size of 92 for each group. Initially, 214 samples were sent to IDEXX for further testing. These samples became the sample set for this study. Twenty-nine dogs were lost to follow-up prior to the month 6 samples being taken and the power calculation was based on this sample size (n=185) to be shared for both time points. The power calculation using 185 would also power the initial calculations of prevalence at enrollment using the full nested cohort of 214 dogs.

Analysis

Specific Aim I: Determine the prevalence at enrollment and cumulative incidence at month 6 of *Babesia* exposure within the *Leishmania* vaccine trial cohort samples that were sent to IDEXX for tick-borne disease testing.

Analysis Method:

Prevalence will be calculated at enrollment with the number of dogs testing positive for *Babesia* spp. divided by the total number of dogs tested at that time point (n=214). Dogs that were positive for PCR or had positive reactivity on canine vector assay will be considered cases for exposure to *Babesia* spp. Month six data will be used to calculate cumulative incidence for the 6 month period between enrollment and

the 6 month time point. Univariate analyses was performed to compare prevalence by gender and region using a two-sample t-test.

Specific Aim II: Determine the variables that are related to *Babesia* infection including coinfections to other tick-borne diseases (*B. burgdorferi, Anaplasma* spp*., Ehrlichia* spp*. A. phagocytophilum, A. platys, E. canis, E. chaffeensis, and E. ewingii*).

Analysis Method:

Descriptive statistics for *Babesia* positive and negative samples was reported at each time point. Proportions were reported for categorical sex, region, and co-infections, and mean with Interquartile Range (IQR) was calculated for the continuous variable, age. A Chi-square test was used to determine the p-values for categorical variables region, sex, and coinfections. Due to the binary nature of the outcome variable, *Babesia* exposure, multiple logistic regression was performed to evaluate how the aforementioned variables are related to *Babesia* exposure at each time point. Stepwise variable selection was used to select only the most important variables in predicting *Babesia* exposure.

Results

Satisfying part of Aim 1, to prevalence of *Babesia* spp. exposed dogs was calculated at enrollment. Fifty-six samples were found to be positive for *Babesia* spp. of the 214 samples sent to IDEXX. Prevalence was calculated as 56/214 * 100 = 26.2%. Prevalence calculation was performed to determine a baseline understanding of the commonality of an infection within the cohort.

The second objective for Aim 1 was to calculate the cumulative incidence of *Babesia* spp exposure at month 6. Twenty-nine dogs from the larger *Leishmania* vaccine trail were lost to follow-up, with 185 dogs remaining at month six. Because re-exposure can occur and detectible levels of antibodies diminish, any positive test for *Babesia* spp at month 6 was considered a new exposure. Sixty-three dogs tested positive and incidence was calculated as 63/185 * 100= 34.1%. Cumulative incidence was measured to determine new cases amongst the dogs at risk over the six month time point.

Univariate analysis using SAS statistical software was performed for variables for enrollment and month 6. The sample had an equal numbers of males and females at enrollment. At month six, slightly more males (M=19.5%) than females (M=14.5%) were reactive for *Babesia* spp (p=0.257). Mean age was significantly different for exposed dogs (M= 4.71 years at enrollment and 4.03 at month six) versus unexposed dogs (M= 3.83 at enrollment and M=3.86 at month 6; p<0.001 at both times) as seen in Table 1. *Babesia* by region was higher in the East at 13% and was at 3.75% for the Midwest and South, and 5.5% for the West. At month 6 all regions saw an increase in *Babesia* with East 15%, Midwest 8.5%, South 5%, and West 5.5% (Table 1). Coinfections had a wide range of prevalence with *B. burgdorferi* having the highest at 8%, *A. phagocytophilum* 5.5%, *A. platys* 3%, *E. canis* 0.5%, *E. ewingii* 1.5%, *E. chaffeensis* 1%, *Anaplasma* spp. 4.75%, and *Ehrlichia* spp. 1% at enrollment. Cumulative incidence increased between winter and summer time points for most coinfections save *B. burgdorferi* 7.5% and *A. platys* 2.75% (Table 1). Incidence increases were seen in *A. phagocytophilum* 6.5%, *E.*

canis 2.0%, *E. ewingii* 4.5%, *E. chaffeensis* 1.75%, *Anaplasma* spp. 5%, and *Ehrlichia* spp. 5.5% (Table1).

Further univariate analysis was performed by stratifying coinfections by region at both time points. Many regions did not have coinfections so an odds ratio was not able to be calculated. Of those regions that had coinfections at enrollment, *A. phagocytophilum* in the East with an OR of 2.87 (95% CI 1.10, 7.44), p=0.0275 and *A. platys* in the West with an OR 16.5 (95% CI 1.62, 168.5), p=0.0129 (Table 2). At 6 months, no coinfections were statistically significant (Table 3).

To complete Aim 2, stepwise multiple logistic regression was used. The selected model for enrollment included only age and *A. platys* for predicting *Babesia* spp exposure (Table 4). The model for enrollment resulted in an equation of *Babesia* spp. exposure= -1.8444 + 0.1673 (age) + 1.2110 (*A. platys*). [OJJ1]For each year increase in age, there is a 0.1673 greater likelihood of exposure to *Babesia* spp. with an odds ratio of 1.182, (95% CI 1.037, 1.348). Furthermore, if there is exposure to *A. platys* there is a 1.2110 increase in likelihood of *Babesia* spp. exposure with an odds ratio of 3.360, (95% CI 1.103, 10.238). At 6 months, no variables were shown to be significant in predicting *Babesia* spp. exposure (Table 5).

Characteristic	Enrollment *Babesia* spp.			Month 6 *Babesia* spp.		
	Positive n=56	Negative n=158	p	Positive n=63	Negative n=122	p
Sex, n (%) Male Female	29 (13.5) 27 (12.5)	80 (37.5) 78 (36.5)	0.8821	36 (19.5) 27 (14.5)	59 (32) 63 (34)	0.2574
Mean age (IQR), years	**4.71 (3-6)**	**3.83 (2-5)**	**<0.001**	**4.03 (2-6)**	**3.86 (2-5)**	**<0.001**
Region, n (%) East Midwest South West	28 (13) 8 (3.75) 8 (3.75) 12 (5.5)	76 (35.5) 32 (15) 16 (7.5) 34 (16)	0.6924	28 (15) 16 (8.5) 9 (5) 10 (5.5)	59 (32) 19 (10) 12 (6.5) 32 (17.5)	0.1761
Coinfections, n (%) *B. burgdorferi* ***A. phagocytophilum*** *A. platys* *E. canis* *E. ewingii* *E. chaffeensis* *Anaplasma spp* *Ehrlichia spp*	17 (8) **12 (5.5)** 7 (3) 1 (0.5) 3 (1.5) 2 (1) 10 (4.75) 2 (1)	35 (16) **17 (8)** 7 (3) 3 (1.5) 10 (4.75) 4 (2) 17 (8) 19 (9)	0.2186 0.0055 0.2395 1.0 1.0 0.6532 0.1693 0.0726	14 (7.5) 12 (6.5) 5 (2.75) 4 (2) 8 (4.5) 3 (1.75) 9 (5) 5 (2.75)	25 (13.5) 18 (9.75) 8 (4.5) 5 (2.75) 9 (5) 6 (3.25) 15 (8) 10 (5.5)	0.7845 0.4528 0.7658 0.4925 0.2351 1.0 0.7026 0.9511

Table 1: Descriptive statistics of study population in accordance with *Babesia* spp. exposure.

Enrollment	East		Midwest		South		West	
Co-infection	**OR (95%CL)**	**p**	**OR (95%CL)**	**p**	**OR (95%CL)**	**p**	**OR (95%CL)**	**p**
B. burgdorferi	1.92 (0.80,4.63)	0.1421	1.19 (0.20,7.25)	1.0	1.0 (0.08,13.02)	1.0	-	-
A. phagocyto-philum	**2.87 (1.10,7.44)**	0.0272	0.78 (0.66,0.93)	1.0	-	-	-	-
A. platys	4.44 (0.70,28.12)	1.0	-	-	-	-	**16.5 (1.62,168.5)**	**0.0129**
E. canis	-	-	0.79 (0.68,0.93)	1.0	2.14 (0.12,39.47)	1.0	-	-
E. chaffeensis	-	-	2.14 (0.12,39.47)	1.0	-	-	-	-
E. ewingii	0.90 (0.09,9.04)	1.0	-	-	2.33 (0.26,20.66)	0.58	-	-
Anaplasma spp.	2.10 (0.79,5.60)	0.1348	-	-	-	-	3.00 (0.17,52.10)	
Ehrlichia spp.	-	-	-	-	1.44 (0.19,11.04)	0.7223	-	-

Table 2: Odds Ratios for *Babesia* Coinfection by Region at Enrollment.

6 Months	East		Midwest		South		West	
Co-infection	**OR (95% CL)**	**p**	**OR (95% CL)**	**p**	**OR (95% CL)**	***p***	**OR (95% CL)**	**p**
B. burgdorferi	0.93 (0.37, 2.38)	0.89	1.96 (0.28, 13.51)	0.6418	1.38 (0.07, 25.4)	1.0	-	-
A. phagocyto-philum	1.17 (0.44, 3.09)	0.7513	-	-	-	-	3.44 (0.20, 60.72)	0.4239
A. platys	1.06 (0.18, 6.15)	1.0	1.21 (0.15, 9.76)	1.0	1.38 (0.07, 25.43)	1.0	-	-
E. canis	-	-	-	-	-	-	-	-
E. chaffeensis	-	-	0.87 (0.16, 4.60)	1.0	-	-	-	-
E. ewingii	-	-	0.63 (0.15, 2.52)	0.51	3.14 (0.24, 41.41)	0.5534	-	-
Anaplasma spp.	1.30 (0.29, 5.85)	0.7085	1.96 (0.28, 13.51)	0.6418	0.86 (0.11, 6.62)	1.0	0.60 (0.06, 5.84)	1.0
Ehrlichia spp.	-	-	0.63 (0.15, 2.52)	0.5079	-	-	-	-

Table 3: Odds Ratios for *Babesia* Coinfections by Region at 6 Months.

Parameter	**Estimate**	**Standard Error**	**P value**	**Odds Ratio (95%CI)**
Intercept	-1.8444	0.3447	<0.001	--
Age	0.1673	0.0670	0.0125	1.182 (1.037,1.348)
Anaplasma platys	1.2119	0.5684	0.0330	3.360 (1.103,10.238)

Table 4: Results from *Babesia* spp Exposure Logistic Regression Model at Enrollment.

Discussion

Previous studies from around the world have shown that prevalence of canine *Babesia* infection has ranged from 1.1%-29% depending on region and sample population.[17, 51, 52] For this study, prevalence[PC2] of exposure at enrollment was calculated as 26.2%. The level initially set to determine if the level of exposure was significant was set at 25% based on a prior study showing a prevalence of 10.6% based on PCR alone. This study used PCR and ELISA to test for *Babesia* spp., so a higher prevalence would be expected due to testing for current, recent, and past exposure.

Univariate analysis produced interesting descriptive data for the study. For both time points, males and females were evenly distributed with a slightly higher number of males showing exposure at month 6 (Table 1). Explanation for this may be from females being held back from hunts due to having puppies, thus decreasing exposure opportunities. Of the 29 dogs that were lost to follow up, 15 of them were females (Figure 2). Loss to follow up (LTF) was due to a variety of reasons, most often due to death or being traded to other hunts. Hunts in the East showed the highest proportion of LTF at 16.3%, with the remaining hunts having lower LTFs of Midwest 10.0%, South 12.5%, and West 9.7% (Figure 4). Due to the greater number of hunts proportionally in the East, it follows that those hunts would have higher percentages of LTF.

Proportionally, exposures were higher in the Eastern region. Foxhunting was first established on the east coast during colonial times, it follows that more hunts exist in the East. Of the 214 dogs that were part of this study, approximately half of them were from the East. Approximately 20% of hunts were in the Midwest and West, with the remaining 10% in the South. At month 6 the percentage of exposures increased in all

regions. In the East, the number of exposures remained steady at 28 but the increase in percentage was due to the higher number of dogs lost to follow up. A higher increase was found in the Midwest with little loss to follow up. An increase in approximately 5% could be attributed to a number of factors, such as higher rainfall in the spring that resulted in increased tick levels. The South and West saw smaller increases in exposure. Stratified by region, two coinfections showed relevance. *Anaplasma phagocytophilum* in the East showed an odd ratio of 2.87 (95% CL 1.10, 7.44) (Table 2). Due to the difference in tick vector the explanation for this deserves more consideration. The other significant coinfection, *A. platys* in the West (Table 2), showed a heightened risk with an OR of 16.5 (95% CL 1.62, 168.5). This result makes biological sense because the tick vector for canine *Babesia* spp. (*Rhipicephalus sanguineus*) does not compete with the vectors for the other coinfections (*I. scapularis* and *Amblyomma americanum*). Month six stratification did not show any statistically significant results.

The regression model for enrollment showed significance for age and *A. platys*. Month 6 did not show any significant variables in predicting exposure. For enrollment, the significance of age could be due to the nature of the lives of foxhounds. Training is started at a young age for hunting and they are worked hard, going on a large number of hunts each year. Dogs that are older could have a host of comorbidities including coinfections not included in this study, such as *Leishmania*, and physical issues which could have contributed to early deaths. Therefore older dogs may have been lost to follow-up at month 6 that were more prone to carrying *Babesia* and other coinfections.

Polymerase Chain Reaction results for this study produced one positive sample, showing *B. conradae* in an Eastern hunt. Infection with *B. conradae* has been found to

be rare, with cases initially reported in the 1990's in California in eleven dogs and has been sporadically reported since in western states.[53, 54] Phylogenetic analysis determined *B. conradae* to belong to a clade of Babesia found in the western U.S.[53] The presence of a case of *B. conradae* in the eastern U.S. presents an interesting finding. However, further investigation into the dog's history would need to be performed before hypothesizing an explanation.

Limitations and Biases

Limitations and biases of this study were largely related to aspects of the study sample and testing methods. Of the over 600 dogs enrolled in the *Leishmania* vaccine trial, 214 blood and serum samples were sent to IDEXX for tick-vectored disease testing. A larger sample size would provide a higher power, where this study had a power of 0.70. Additional limitation was that only one of the samples was PCR positive, possibly due to the limited time points involved in the sampling and analysis. Exposure was limited to the number of dogs that were seropositive for *Babesia* spp. and no species specific information was available besides the one PCR case. [PC3]PCR testing itself presents a bias due to needing a minimum level of parasites for detection. For *Babesia*, a minimum level of 2.4 parasites/µL is needed for the most sensitive real time PCR.[55] Due to the typically low parasite loads in asymptomatic hosts, concentration of parasites may have been too low for detection. Antibody titer from an exposure prior to enrollment may have resulted in a reactive sample at month 6 without a subsequent exposure after enrollment. This would skew the result of the month 6 incidence, reporting more new exposures than actually occurred[PC4]. This presents a possible scenario of chronic low levels of parasitemia that are too low for detection by PCR, which could be evidenced by the lone case of PCR-detected *B. conradae* at

enrollment. These existing infections paired with antibody response to new exposures during prime exposure season during the six months between enrollment and month 6 may result in a higher incidence rate at month six.

Effect modification in this study could have potentially come from the use of tick control, whether through collars, medications, or topical treatments. Hunts were found to employ a variety of tick control methods of varying effectiveness and intent of use. Due to the high levels of seropositivity found for several pathogens, the effect of tick control does not appear to have a significant effect on preventing exposure.

Conclusion

Modeling for human babesiosis had results different than initially hypothesized, but the reason why is what makes the results intriguing. The tick vectors for *B. microti* (Figure 4) and canine *Babesia* spp. (Figure 5) are known to be different but coinfection with *Borrelia burgdorferi* has been speculated by many studies to be significant in dogs as well as humans. The results of this study indicate through the lack of associate between *Babesia* exposure and *Borrelia* that the competency of *R. sanguineus* as a vector for Lyme disease appears to be unlikely.

Blood and serum samples were selected from those that were reactive on the IDEXX 4Dx SNAP test. The surprising results of the IDEXX testing was the high levels of *Babesia* spp. present in the samples. These results surpassed levels that were previously found in other studies. Pairing these findings with that of the co-infections founds present in the samples (Figures 6, 7), this indicates the potential for this cohort

of hunting dogs as a good cohort to study for environmental exposures to infectious disease.

Future studies utilizing this cohort should focus on testing all remaining dogs for *Babesia* and coinfections to obtain a more accurate picture of infection in this cohort. Taking samples from regular seasonal time points for a period of years would provide a more complete picture of the disease. More sampling would also increase the potential for detection by PCR. An intriguing finding in this study was the one PCR positive case at enrollment of *B. conradae*, which has previously been found only in California, but was from an Eastern hunt. Hunting dogs are frequently traded, and that may have been the case with this dog. However the dog in question was 6 years old and likely was not a recent trade due to age.

Outside of this study, the abundance of avenues of study for *Babesia* has been apparent. Areas such as Africa, Asia, and South America have little information available and would benefit from prevalence and phylogenetic studies to determine levels of infection and pathogens identity and diversity. On a local and regional level, the presence of *Babesia* is largely unknown outside of surveillance of vectors and hosts. Significance of Lyme disease in the Midwest overshadows most other tick-borne diseases and there is growing evidence to support claims that co-infection with *Babesia* can produce more severe states of disease. This claim also has been proposed for diseases in other parts of the world, particularly regarding co-infection with *Plasmodium*.

Babesiosis is arguably a disease of growing concern. Due to its worldwide presence, the possibility of *Babesia* spp. to present health concerns for at-risk populations and particularly within the blood supply presents a real danger to public

health. Little is known about this often overlooked protozoan, and in many parts of the world, information is non-existent beyond a few reports of disease discovered by accident. Estimates of prevalence of babesiosis are hard to determine due to the underdiagnosis/misdiagnosis of disease and a lack of awareness, but most experts agree that it is likely one of the most common blood parasites behind trypanosomes. Continued study and education regarding the pathogen will be a necessary component to more fully elucidate the impact of *Babesia*.

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Figures

Figure 1: Lifecycle of B. microti within vector and host. https://www.cdc.gov/parasites/babesiosis/biology.html

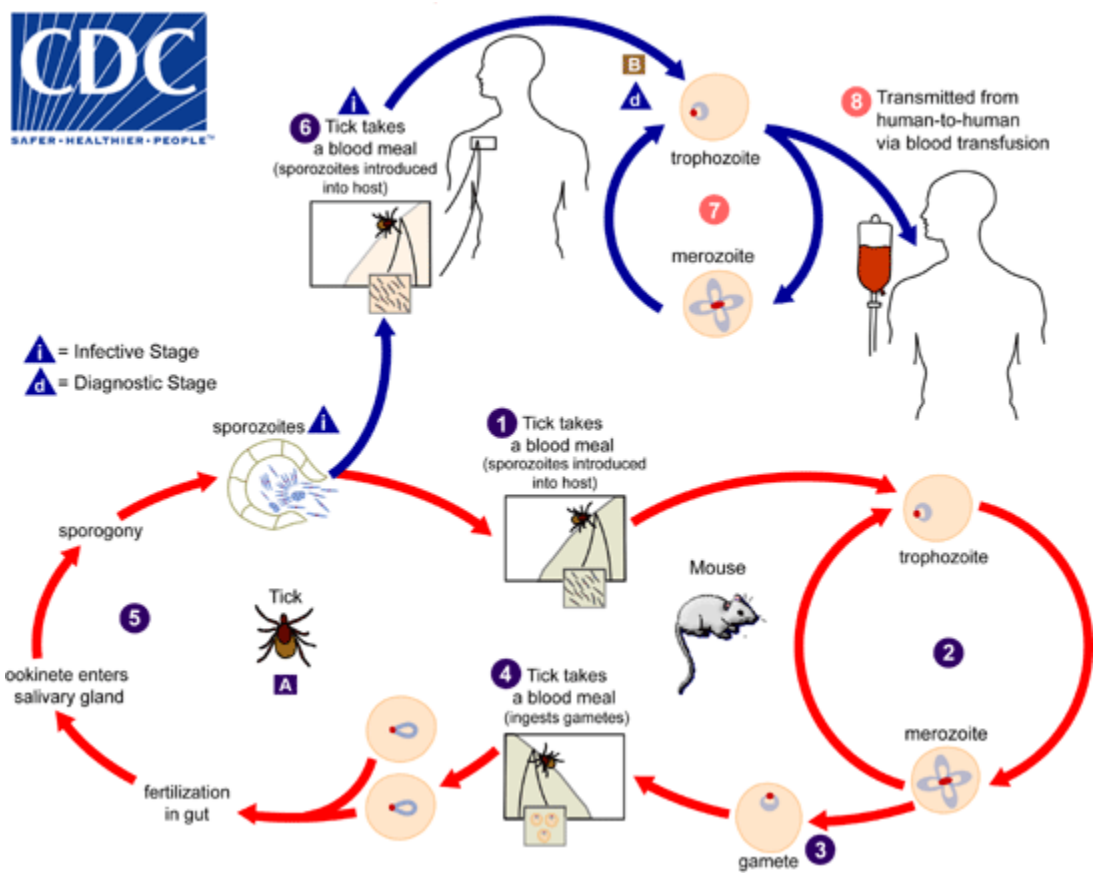


Figure 2: Month six loss to follow-up by sex.

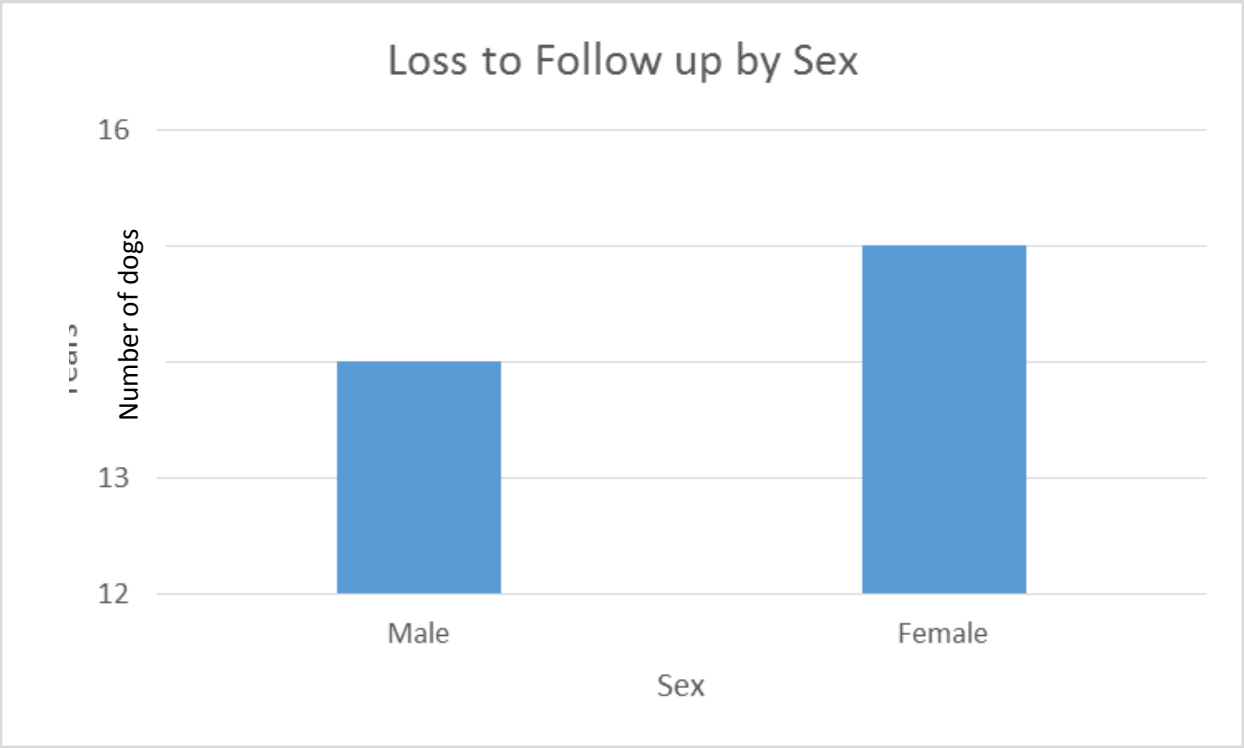


Figure 3: Month six loss to followup by region.

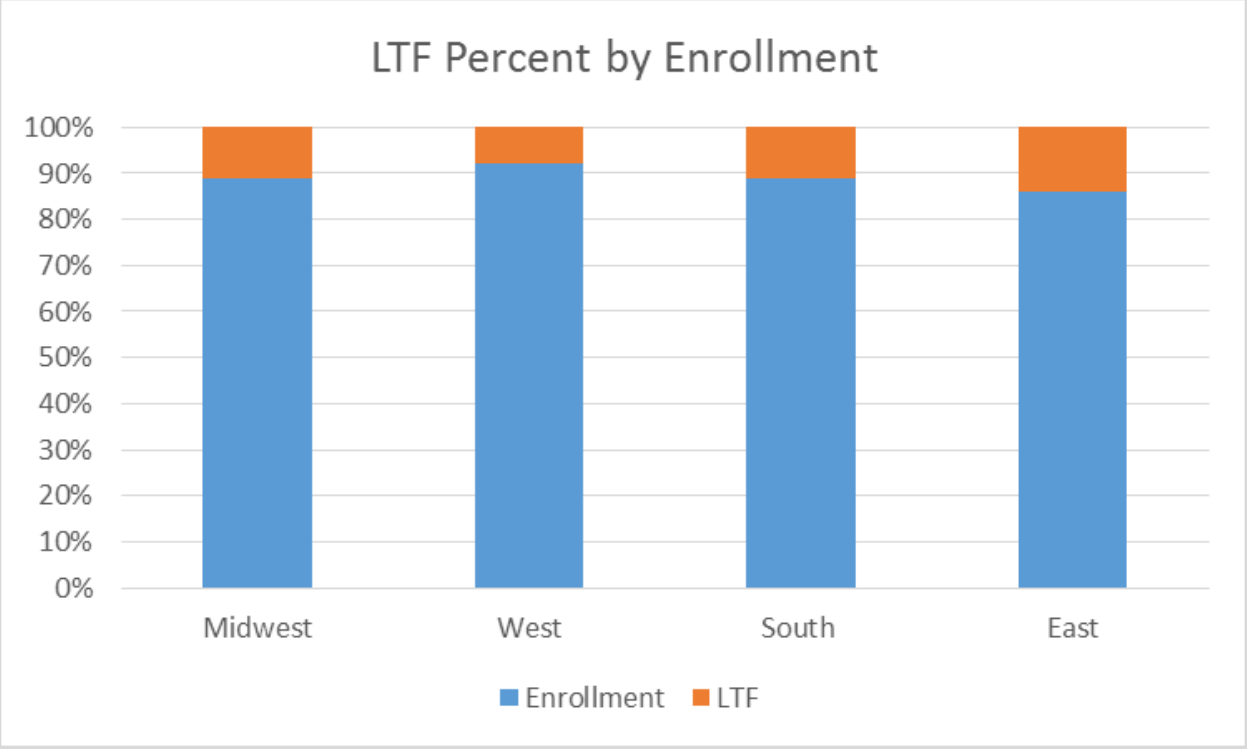


Figure 4: Loss to follow-up as a proportion of enrollment

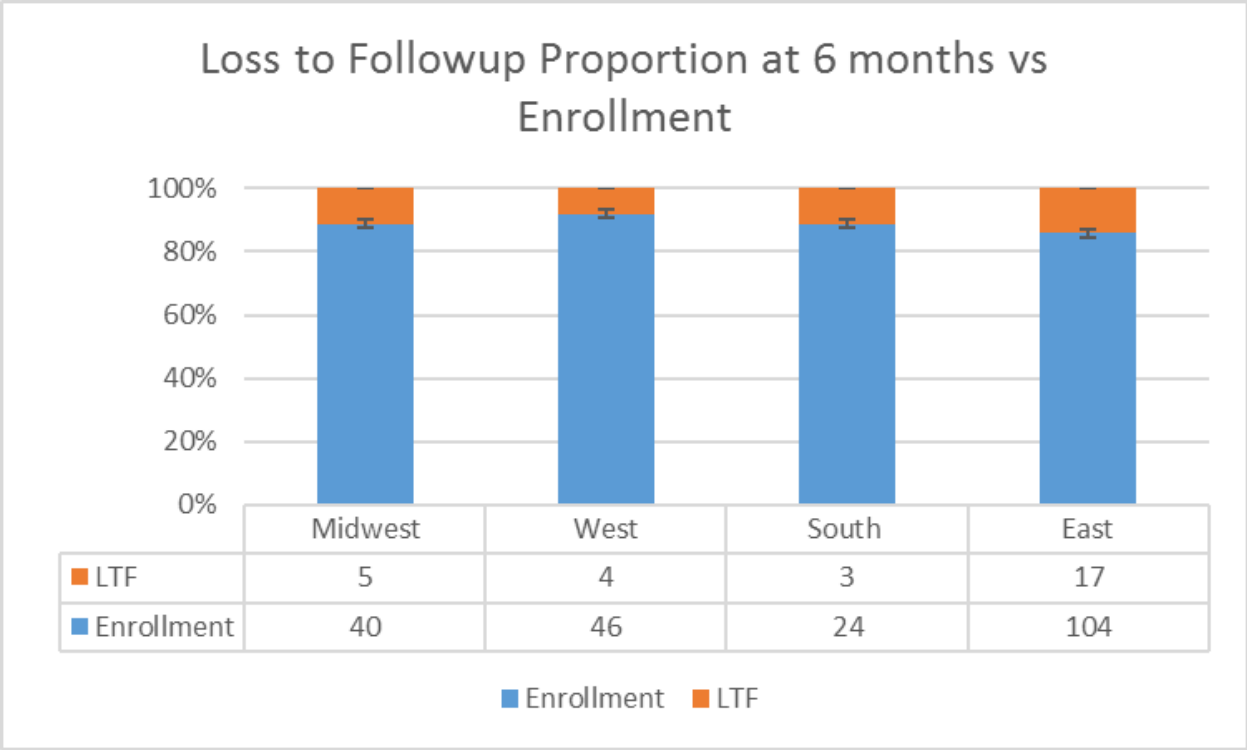


Figure 5: *Ixodes scapularis* range in U.S. (https://www.cdc.gov/ticks/geographic_distribution.html)

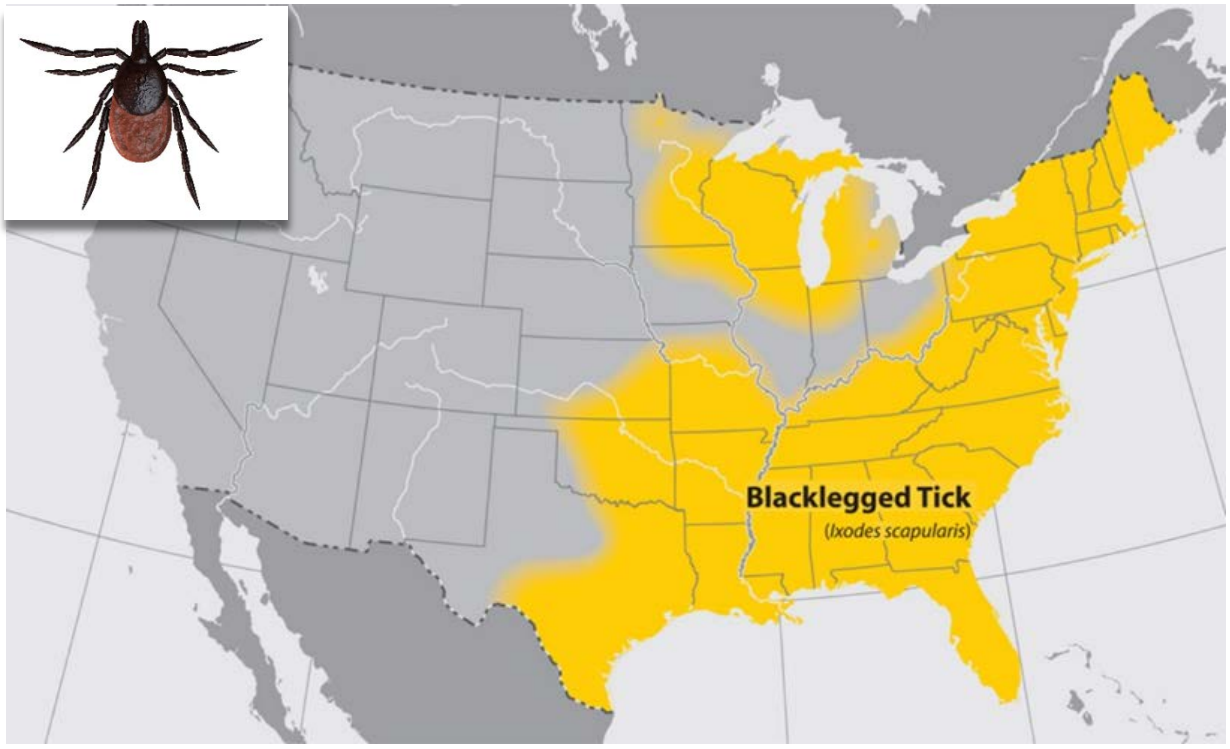


Figure 6: *Rhipicephalus sanguineus* range in U.S. (https://www.cdc.gov/ticks/geographic_distribution.html)

Figure 7: Distribution of co-infections by region at enrollment (https://www.cdc.gov/ticks/geographic_distribution.html)

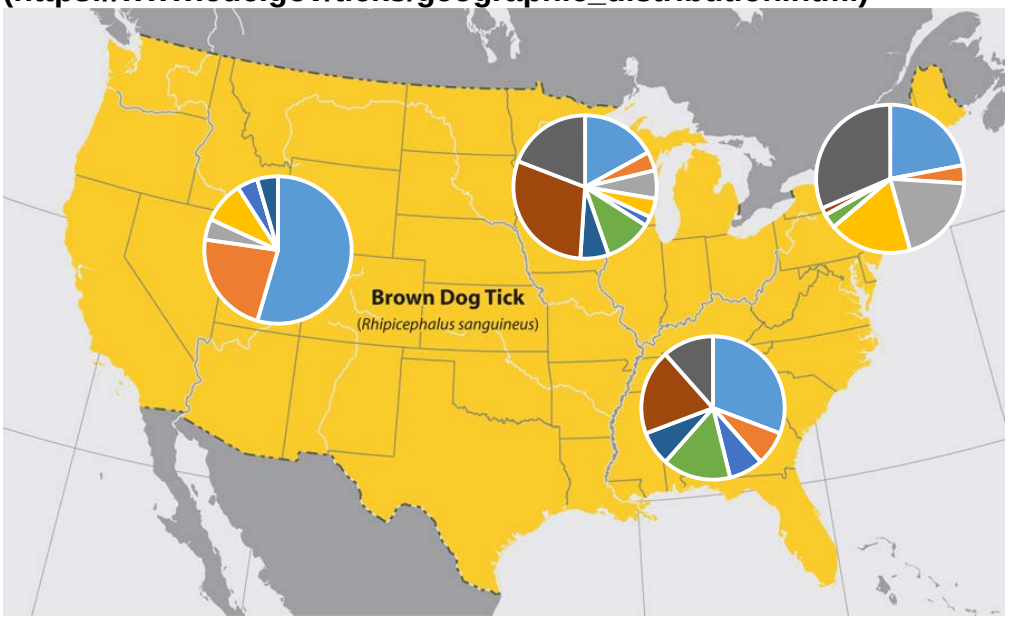
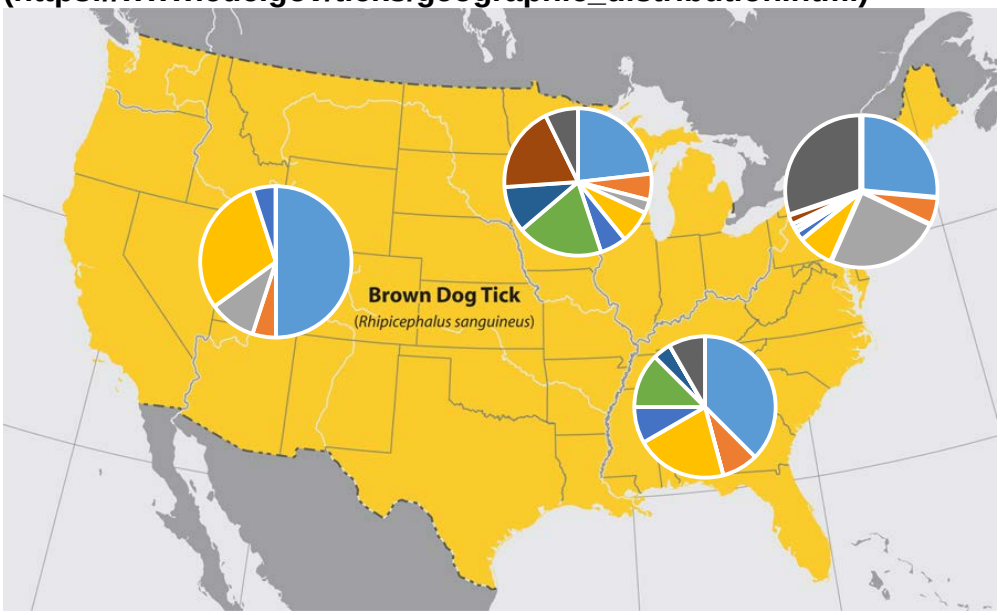


Figure 8: Distribution of co-infections by region at month 6. (https://www.cdc.gov/ticks/geographic_distribution.html)



Appendix

Appendix 1. Babesia reporting, U.S. state-level cases and incidence rates/100,000 population by year. https://www.cdc.gov/parasites/babesiosis/data-statistics/index.html

State	2011		2012		2013		2014	
	Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate
Alabama	1	<0.1	0	0.0	0	0.0	1	<0.1
Alaska	-	-	-	-	-	-	-	-
Arizona	-	-	-	-	-	-	-	-
Arkansas	-	-	-	-	-	-	-	-
California	1	<0.1	0	0.0	2	0.2	1	0.1
Colorado	-	-	-	-	-	-	-	-
Connecticut	74	2.1	123	3.4	268	7.5	205	5.7
Delaware	1	0.1	0	0.0	2	0.2	1	0.1
District of Columbia	-	-	-	-	-	-	-	-
Florida	-	-	-	-	-	-	-	-
Georgia	-	-	-	-	-	-	-	-
Hawaii	-	-	-	-	-	-	-	-
Idaho	-	-	-	-	-	-	-	-
Illinois	-	-	-	-	-	-	-	-
Indiana	0	0.0	1	<0.1	1	<0.1	0	0.0
Iowa	-	-	-	-	-	-	-	-
Kansas	-	-	-	-	-	-	-	-
Kentucky	-	-	-	-	-	-	-	-
Louisiana	-	-	-	-	2	<0.1	0	0.0
Maine	9	0.7	10	0.8	36	2.7	42	3.2
Maryland	4	0.1	3	0.1	9	0.2	2	<0.1
Massachusetts	208	3.1	261	3.9	417	6.2	537	8.0
Michigan	0	0.0	0	0.0	2	<0.1	2	<0.1

Minnesota	73	1.4	41	0.8	64	1.2	49	0.9
Mississippi	-	-	-	-	-	-	-	-
Missouri	-	-	-	-	-	-	-	-
Montana	-	-	-	-	-	-	0	0.0
Nebraska	0	0.0	1	0.1	1	0.1	0	0.0
Nevada	-	-	-	-	-	-	-	-
New Hampshire	13	1.0	19	1.4	22	1.7	42	3.2
New Jersey	166	1.9	92	1.0	171	1.9	159	1.8
New Mexico	-	-	-	-	-	-	-	-
New York	418	2.1	253	1.3	534	2.7	471	2.4
North Carolina	-	-	-	-	-	-	-	-
North Dakota	1	0.1	0	0.0	1	0.1	0	0.0
Ohio	-	-	-	-	-	-	1	<0.1
Oklahoma	-	-	-	-	-	-	-	-
Oregon	1	<0.1	0	0.0	0	0.0	1	<0.1
Pennsylvania	-	-	-	-	-	-	-	-
Rhode Island	73	6.9	56	5.3	142	13.5	172	16.3
South Carolina	-	-	-	-	1	<0.1	3	0.1
South Dakota	-	-	-	-	1	<0.1	1	0.1
Tennessee	1	<0.1	0	0.0	0	0.0	0	0.0
Texas	-	-	-	-	1	<0.1	1	<0.1
Utah	-	-	-	-	-	-	0	0.0
Vermont	2	0.3	2	0.3	6	10	3	0.5
Virginia	-	-	-	-	-	-	-	-
Washington	0	0.0	0	0.0	1	<0.1	4	0.1
West Virginia	-	-	-	-	0	0.0	0	0.0
Wisconsin	80	1.4	45	0.8	76	1.3	43	0.7
Wyoming	0	0.0	0	0.0	0	0.0	0	0.0
Total	1,126	0.8	911	0.6	1,761	1.0	1,744	0.8