UNIVERISTY OF CENTRAL OKLAHOMA

Edmond, Oklahoma

Jackson College of Graduate Studies

Molecular survey of *Ixodes scapularis* associated pathogens from *Odocoileus virginianus* at Lake Arcadia in Edmond, Oklahoma

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements

For the degree of

MASTER OF SCIENCE IN BIOLOGY

By

Russell Smalley IV

2021

Molecular survey of *Ixodes scapularis* associated pathogens from *Odocoileus virginianus* at Lake Arcadia in Edmond, Oklahoma

A THESIS

APPROVED FOR THE DEPARTMENT OF BIOLOGY

May 2021

Robert Brennan Digitally signed by Robert Brennan Date: 2021.05.07 11:59:45 -05'00'

Committee Chairperson

Michelle L. Haynie Digitally signed by Michelle L. Haynie Date: 2021.05.07 11:45:59 -05'00'

Committee Member

Wayne D. Lord Digitally signed by Wayne D. Lord Date: 2021.05.06 10:45:18 -05'00'

Committee Member

Sean Laverty

Committee Member

Table of Contents

ACKNOWLEDGMENTS

I must start by thanking Haris Zafar, John Land, Asma Samour, and Dylan Hance for sacrificing their weekend mornings and nights for an entire semester to help me collect ticks. It was a group effort and I'll always be extremely grateful. I owe so much to the guidance and support of Dr. Robert Brennan. His patience during a quarantine and constant push to further myself have been invaluable. I would like to thank Dr. Lord, Dr. Haynie, and Dr. Laverty for their support, advice, and guidance throughout my entire thesis project. I must thank Dr. Allan for getting me hooked-on biology and encouraging me to get involved with undergraduate research. I would like to thank the University of Central Oklahoma Office of High Impact Practices for funding this project through their RCSA grants. Finally, I have to express my extreme gratitude to my wife Brandee for always being supportive, believing in me, and putting up with my tick talk.

ABSTRACT OF THESIS

University of Central Oklahoma

Edmond, Oklahoma

NAME: Russell Smalley IV

TITLE OF THESIS: Molecular survey of *Ixodes scapularis* associated pathogens from *Odocoileus virginianus* at Lake Arcadia in Edmond, Oklahoma

DIRECTOR OF THESIS: Robert Brennan

PAGES: 62

ABSTRACT:

Odocoileus virginianus (white-tailed deer) is the primary host and vector for *Ixodes scapularis* (deer tick). Most of the research into *Ixodes scapularis* has been geographically restricted to the northeastern United States, with limited interest in Oklahoma until recently as the *Ixodes* populations spreads due to climate change. Ticks serve as a vector for pathogenic bacteria, protozoans, and viruses that pose a significant human health risk. To date, there has been limited research to determine what potential tick-borne pathogens are present in *Ixodes scapularis* in central Oklahoma. Using a one-step multiplex real-time reverse transcription-PCR, *I. scapularis* collected from white-tailed deer were screened for *Anaplasma phagocytophilum, Borrelia burgdorferi, Borrelia miyamotoi, Babesia microti,* and Powassan virus. Ticks (n = 394) were pooled by sex and life-stage into 117 samples. Three pooled samples were positive for *B. miyamotoi* and five pooled samples were positive for Powassan virus. This represents a

prevalence rate of 0.8% and 1.2%, respectively. *Anaplasma phagocytophilum*, *B. burgdorferi*, and *B. microti* were not detected in any samples. This is the first report of *B. miyamotoi* and Powassan virus detection in Oklahoma *I. scapularis* ticks. This demonstrates that *I. scapularis* pathogens are present in Oklahoma and that further surveillance of *I. scapularis* is warranted.

Chapter 1

LITERATURE REVIEW

Ticks (*Ixodida*)

Before discussing tick-borne diseases, a review of what ticks are, their morphology, and life stages is necessary to better understand them as vectors of disease. Ticks belong to the phylum Arthropoda. Arthropods are characterized as invertebrate animals having an exoskeleton, a segmented body, and paired jointed appendages. This phylum contains insects, crustaceans, and many other subphyla, including the subphylum Chelicerata to which ticks belong. Chelicerata contains spiders, scorpions, mites, and ticks. The body of chelicerates contains two tagmata, a prosome and opisthosoma[1]. Some groups have lost a visible distinction between the two. This contrasts with insects, which ticks are often confused with, which have three tagmata. Chelicerata get their name from the unique appendage that exists before the mouth, called the chelicerae. Chelicerae are appendages that have been modified into a system of jaws, these 'jaws' can take on many forms[1]. Within the subphyla is the class Arachnida. Arachnids have 3-segmented chelate chelicerae, their mouthparts or jaws have three segments to them. Arachnids are within the Chelicerata but are further characterized by having eight legs at some stage of their life. Arachnids also lack antennae or wings at any life stage. The Acari subclass have only two body segments, a prosoma and an opisthosoma. These segments are harder to distinguish in this subclass compared to other arachnids. In this group, the chelicerates have evolved into specialized mouth parts for biting and sucking. The superorder Parasitiformes contains the parasitic members of the acari subclass, most notable the tick but also mites. The order Ixodida contains all living and extinct hard ticks dating back to

the Cretaceous period when the earliest fossilized ticks have been discovered in amber[2,3]. All ticks are external parasites, meaning they live by feeding on blood of, primarily, mammals and birds. Ticks have also been known to feed occasionally on reptiles and amphibians when available[4].

There are currently three families of ticks, Ixodidae (hard-bodied ticks), Argasidae (soft bodied ticks), and Nuttalliellidae[2], which contains only one species and is the most basal lineage of all extant ticks. The hard ticks are named for the presence of a scutum, a sclerotized plate on the anterior dorsal side of the tick. In female hard ticks, there is a flexible portion next to the scutum that is referred to as the alloscutum. This allows for the expansion of the body when the tick becomes engorged after and during a blood meal. Males lack this alloscutum because they do not engorge to the extent that females do, instead they have a more rigid scutum called the conscutum. The nymph and adult stages of the tick have a feeding apparatus extension called a gnathosoma, sometimes called the capitulum, which contains the hypostome, chelicerae, and pedipalps. The hypostome is a hard-calcified structure for piercing and anchoring the tick in place during feeding. This structure is so strong that in the attempted removal of a tick from a host, the hypostome often times remains in the host while the remainder of the body is ripped away. The chelicerae are the mouthparts common to all arachnids and act similar to jaws; in ticks they also contain receptors for taste perception and act to pull the tick closer to the host through a breaststroke-like motion[5,6]. Once at tick takes a blood meal, it can then molt into its next life stage.

Lifecycles

Ixodidae all have a similar lifecycle, but as the focus of this work was with *I. scapularis*, I will focus only on the lifecycle *of I. scapularis*. *Ixodes scapularis* has a two-year-long lifecycle that consists of three different life stages marked with unique molts between them; they are the larva, nymph, and adult stages. A blood meal must be taken at each stage before the tick can undergo a molt into the next life stage. For larva, most feeding targets are close-to-ground level mammals like the white-footed mouse (*Peromyscus leucopus*). When screened in the mid to late summer (June through August), more larvae stage ticks are found on this mouse than any other organisms in states with white-footed mice[7]. The high prevalence of larva in the summer is due to eggs hatching in the late spring after temperatures warm. Through the fall and winter, larva will molt if they are able to get a blood meal. Spring is when nymphs arise; they are larger and mobile enough to feed on large mammals like deer and humans when present. Nymph infestations of mice is greatest in the late spring and early summer, this is also when humans are at the greatest risk of infections[7,8]. Due to the small size of the nymph, the slow rate at which they feed, and the anesthetic properties of their saliva, ticks can go unnoticed for days. Nymphs and larva do not have a sex or the ability to sexually reproduce. When nymphs have taken a blood meal, they will molt into adult ticks during the fall. Once ticks have reached adulthood, they reach the sexual stage of their life and as a result will molt into either adult males or adult females. In most species, male ticks must take a blood meal to sexually reproduce, male *I. scapularis* do not[9]. Due to a blood meal being a prerequisite for most ticks to reproduce, reproduction typically occurs on a host. Because a blood meal is not required by male *I. scapularis* ticks, males can and often times do reproduce off a host. Male *I. scapularis* ticks can mate multiple times and

copulation occurs through the male inserting the hypostome and chelicerae into the female genital opening to transfer a spermatophore. A spermatophore is a protein capsule containing sperm that is synthesized and secreted from the male accessory glands within the male tick[10]. Female ticks that have mated and taken a blood meal will then lay eggs that remain dormant through the winter and hatch in the late spring. The ability for ticks to endure harsh winters is key to their survival. Nymphs and adults have been reported to survive temperature ranges between -22℃ to -8℃[11]. There is, however, a direct relationship between the temperature when the female tick lays the eggs and the percent of eggs that survive; temperatures below 24℃ and above 12℃ are acceptable, with 20℃ being the most ideal temperature at which the most eggs are produced and survive to produce larvae[12]. Although ticks of any life stage can survive subzero temperatures, they can only do so for certain periods of time. Long periods of freezing and subzero temperatures, combined with low humidity, lead to almost universal mortality in any *I. scapularis* population[13]. This is the general reason why tick populations are lower or even absent in far northern habitats, higher altitudes, and drier more arid climates.

Climate Change and the Tick Habitat

Climate change is enabling the spread of *I. scapularis* further north into regions of Canada and west into portions of the Great Plains where they were once rare. The number of counties with established populations of *I. scapularis*, six or more individuals or one or more life stages identified in a single year, has more than doubled in the last two decades[14]. North Dakota and Nebraska are an example of states that had no recorded populations of *I.* scapularis two decades ago but now have established populations. The northeastern United States, in particular states like Connecticut, New York, Rhode Island,

Vermont, and New Hampshire have long been considered the center of *I. scapularis* distribution. This focused region has now had to be expanded to include Maine, Pennsylvania, Ohio, West Virginia, Virginia, and North Carolina. Thirty-seven out of 50 states now have established *I. scapularis* tick populations, whereas only 31 states had populations in 1996[11].

As temperatures and climates fluctuate, the tick distribution is expected to expand. In areas with warming trends, the now warmer minimum temperatures will favor the survival of ticks through the winter[11]. The primary reason that *I. scapularis* has been able to extend its geographic range into Canada is due to fewer days with temperatures exceeding the minimum temperature threshold for tick survival. Temperatures that would normally be lethal to ticks now occur for shorter periods of time. As moisture patterns change, ticks can also move into areas that were once too dry for their survival. Much of the Great Plains region, the area west of Mississippi River and east of Rocky Mountains, was generally considered too dry for tick populations to become established, but *Ixodes* has started to move further west into the region[12]. There has been an unfounded consensus in the entomology community that tick populations are confined to the geographic range of particular hosts. With *I. scapularis* being a host generalist however, this is not the case. Beyond the traditional mice and deer that they typically are associated with, they can also feed on birds and lizards[13]. Their generalized host behavior is a factor that favors the expansion of *Ixodes* as climates change.

The tick lifecycle itself might also be altered by climate change. Ticks have a genetic diapause triggered by unfavorable environmental conditions associated with temperature and humidity. When temperatures get too low or too high or humidity

becomes to dry, the metabolic processes of the tick slow and quiescence sets in. The first part of this dormancy is behavioral; ticks will stop host-seeking activities. For female adult *Ixodes*, they will wait to deposit eggs. Temperatures also effect the development rate of the eggs[14]. The tick lifecycle is dependent on the temperature and environment around it. However, it can also adapt to differences in climate as made apparent by the large geographic distribution in which it inhabits, which cover a variety of microclimates.

When addressing the *Ixodes* response to climate change, it is also important to address tick mortality. What conditions, that may or may not be 'normal', can lead to tick mortality? As previously discussed, winter freezes will lead to tick mortality, but the same is true of overheating in late summer. Dehydration can lead to desiccation of ticks; conversely, floods will also lead to tick mortality. The last cause of mortality is due to a tick exhausting all of its metabolic resources in host-seeking activities and failing to find a host. Ticks have a constant battle of trying to find blood meals and to find mates, while also not expending all their energy in order to accomplish both of those tasks. Interestingly, the pathogens they carry seem to have little to no negative impact on their survival and in some cases can even enhance survival. *Anaplasma* for example has been shown to induce expression of a glycoprotein that acts like antifreeze to aid in the survival of *I. scapularis* ticks in the winter[15]. The enhanced survival of the host will increase the chances that the pathogen can survive to transmit to another host.

Pathogen Transmission – Tick to Host

Once bitten by a tick, the tick has to remain unnoticed to allow for a proper blood meal to be consumed. The tick saliva has an arsenal of molecules that block host immune reactions and avert inflammatory responses. The saliva can inhibit IL-12, an interleukin

that aids in the development of naïve T-cells into helper T-cells, and TNF-alpha which triggers inflammatory responses[16,17]. It can also inhibit dendritic cells ability to stimulate helper T-cells production and IL-2 production[16]. One component in the tick saliva that causes these reactions is Prostaglandin E2, however more molecules are also at play. The saliva also has multiple proteins that block the coagulation pathway[18]. The anticoagulant proteins will block clotting at the site of the tick bite, allowing the tick to feed longer. The need to feed longer is also important for the pathogens that need time to migrate from the midgut into the salivary glands for transmission.

When feeding, the tick receives a rush of nutrients from the fresh blood meal. These nutrients not only feed the tick but also the pathogens. It's at this moment the pathogen starts to replicate and enters the ticks gut and then migrates from the gut to the salivary glands where it can then be transmitted to the next host[19]. For *A. phagocytophilum*, the tick protein P11 is used to infect tick haemocytes, which they use for transport to the salivary glands[19]. It is through the saliva of the tick that the pathogen can be transmitted into the capillaries of the new host. The *I. scapularis* tick is known to harbor 91 distinct taxa, 16 of which are pathogens. This includes species of *Borrelia*, *Babesia*, *Anaplasma*, *Ehlirchia*, *Rickettsia*, *Bartonella*, *Theileria* and Flavivirus[20]. For the relevance to this work, we will focus on *Borrelia*, *Babesia*, *Anaplasma*, and Flavivirus all of which are human pathogens that can have a lifelong impact on those are infected by them.

Tick-borne Spirochetes

Spirochetes

Spirochetes are bacteria in the Phylum Spirochaetes and the class Spirochaetea, they are separated from other bacteria due to the unique location of their flagella. The flagellum of a spirochete runs in between the inner and outer membranes of the bacteria, or the periplasmic space. This allows for the classic helical or corkscrew shape of these bacteria and enables a unique locomotive system for them to traverse their environment[21]. With these flagella running through the periplasmic space, it also causes these bacteria to have a greater length to width ratio when compared to other bacteria. The vast majority of known and identified spirochetes are anaerobic free-living bacteria, which are broken into four orders and five families. There are only a few that are known to cause human disease, all of which belong to the Spirochaetaceae family[22].

Spirochaetaceae

The family Spirochaetaceae contains the pathogenic spirochetes that can infect humans and other mammals. Pathogenic spirochetes they have a few characteristics that separate them from other pathogenic bacteria. They are larger than most pathogens, due to a greater length to width ratio. Spirochaetaceae can be up to 50 μm long. Traditional Gram staining techniques often used to differentiate bacteria do not work with spirochetes. *Borrelia burgdorferi* will retain the safranin dye in the outer membrane by default because it's the last dye used, but it's not a true gram-negative bacterium and therefore a gram-stain should not be used as a diagnostic tool. Instead, silver staining can be used, or some form of dark-field microscopy is needed to properly visualize them in a sample. These bacteria are challenging to grow in the laboratory, which makes studying and manipulating them in vitro more difficult than bacteria such as *E. coli*. Organisms that can be cultured in a lab on either agar plates or in media are much easier to

manipulate and study, however, Spirochaetaceae require an animal model for study. Another factor complicating their study is that they have much slower growth rate. Division time for Spirochaetaceae may be anywhere from 24-33 hours for a single division. In comparison, *E. coli* can divide in less than 20 minutes. Other unique features include the lack of toxin production, and as already stated, they retain their flagella internally (endoflagella), whereas most bacteria have extracellular flagella[23].

Borrelia burgdorferi

The most well-studied tick-borne spirochete in North America is *Borrelia burgdorferi*. The sole zoonotic vector of *B. burgdorferi* is *Ixodes* ticks. *Borrelia burgdorferi* circulates between infected vertebrates and uninfected ticks upon taking a blood meal. Once the tick is infected, it can then infect humans, which are a dead-end host, meaning that once infected the pathogen can't be transmitted from the human to another potential host[24]. Once a person is infected, the disease and related symptoms caused by *B. burgdorferi* are referred to as Lyme Disease. Lyme Disease can be persistent and difficult to treat due to various immune evasion strategies the spirochete can employ.

After the tick bite, the innate immune system is able to clear the infection from the dermis but not before *B. burgdorferi* is able to disseminate out of this protective barrier. Once *B. burgdorferi* reaches the lymphatic system or other tissues, it is able to spread through the body[25]. The bacteria from there tends to localize in capillaries and large veins and form stationary adhesions[26]. These adhesions allow the spirochete to avoid any form of immune response. After dissemination, invasion can occur due to the spirochetes ability to adhere to and penetrate tight junctions. In vitro studies have shown

that *B. burgdorferi* can invade multiple cell types without any loss of viability, including fibroblasts, vein endothelial, neuronal cells, and glial cells[25]. Human specific proteases, metaloproteases (MMPs), are often secreted to repair the extra-cellular matrix (ECM) in response to localized inflammation, the kind of inflammation that is often present with infection. *Borrelia burgdorferi* is able to manipulate these MMPs to slow down ECM repair and therefore aid in their invasion of cells[25]. These invasion capabilities could have been an adaptation to avoid immune cells. The invasive abilities are partially due to antigenic variation, which is the ability of *B. burgdorferi* to change its glycoproteins to avoid being detected by the immune system[27]. All of these tactics and methods of dissemination and invasion allow the spirochete to spread to multiple tissue types and eventually results in Lyme disease.

Lyme Disease

Named after Old Lyme, Connecticut, Lyme disease is the most common vector-borne disease in the United States, affecting over 300,000 people annually[28]. *Ixodes scapularis* ticks acquire the causative agent of Lyme disease, *Borrelia burgdorferi*, from feeding on wild animals during the larval stage. The spirochete is maintained within the tick during subsequent molts to be transmitted upon the next feeding. Although *Ixodes* ticks can harbor multiple species of *Borrelia* spirochetes, only *B. burgdorferi* causes Lyme disease. Other *Borrelia* species and the diseases they cause will be discussed later. Once a tick is infected, it must take a blood meal from a human in order to infect them. Only about 2-3% of all persons bitten by *I. scapularis* develop the symptoms associated with Lyme disease and the tick must remain attached between 24 to 48 hours to facilitate pathogen transmission[29].

Lyme Disease: Clinical Presentation

Lyme Disease presents in one of three stages, each with its own distinct symptomology and clinical manifestations. Early localized disease is the first stage in which symptoms develop from 3 to 30 days after the bite of an infected tick. The classic bullseye rash, erythema migrans (EM), is the first sign indicative of a localized cutaneous infection of *B. burgdorferi*. This occurs in 70% to 80% of patients, but *B. burgdorferi* is not the only tick-borne pathogen that can cause erythema migrans; this fact can sometimes complicate diagnosis. The rash itself may appear and feel similar to a sunburn. It can burn or even be hot to the touch and nearby lymphedema may occur. Other localized disease symptoms that may occur with or without erythema migrans include fever, headache, myalgias, and other flulike symptoms. Leukocytosis, leukopenia, elevated erythrocyte sedimentation rate, thrombocytopenia, and liver abnormalities can also be confirmed via laboratory diagnostics during this time of the infection.

Early-disseminated disease, the second stage, is the result of an untreated infection that has begun to spread throughout the body. This typically occurs weeks or months after the initial infected tick bite. Symptoms become more severe and can include cardiac abnormalities, neurological abnormalities such as facial palsy, and multiple erythema migrans lesions. In rare instances, this can develop into meningitis and encephalitis. About 4% to 10% of all Lyme disease patients develop Lyme carditis, which has a favorable prognosis with proper antibiotic treatment[30].

Late-disseminated disease, the third stage, develops months to years after an infected tick bite. Lyme arthritis is the most common symptom. Monoarthritis and oligoarthritis affecting a variety of joints is common. The clinical difference in

symptomology between Lyme arthritis and septic arthritis is the absence of fever and joint effusions[31].

Lyme Disease: Treatment

The most important factor in treating Lyme disease is the awareness of the patient to know that they were bitten by tick. The ability of the patient to communicate to a healthcare professional that their symptoms could potentially be linked to a tick bite can aid in a timely and appropriate treatment and avoid speculative diagnosis. Doxycycline is the antibiotic of choice used in the treatment of Lyme disease. This broad-spectrum antibiotic inhibits protein synthesis within bacteria by binding the 30S ribosomal subunit unique to bacteria. Transfer RNA are blocked from binding to mRNA and therefore polypeptide chains cannot be completed, and bacterial growth is halted[34]. A rare occurrence when treatment begins is a Jarish-Herxheimer-type reaction (JHR). JHR is a rare reaction that occurs within 24 hours of antibiotic treatment and includes shaking, chills, elevated temperature, and an increase or intensification of EM rashes[35]. Symptoms can be even more severe and include acute respiratory distress syndrome, myocardial injury, seizures, and strokes. Research currently indicates this may be caused a non-endotoxin pyrogen and spirochetal lipoproteins that rapidly release upon the death of the spirochete. An outer surface protein of *B. burgdorferi*, A lipoprotein, stimulates cells in culture to upregulate the production of transcription factors for inflammatory cytokines[36]. This reaction typically only occurs in 7% to 30% of patients[35]. If Lyme Carditis occurs, oral doxycycline treatment for 14 to 21 days is typically required. Lyme meningitis can be treated with 14 days of oral doxycycline and corticosteroids to reduce neurological symptoms. Lyme arthritis requires a longer treatment of 28 days of either doxycycline or

cefuroxime axetil, which works to block cross-linking between peptidoglycans in bacterial cell wall synthesis[37]. Treatment often has to be repeated, as pain can persist or re-manifest after the first round of antibiotics. If chronic Lyme arthritis manifests, prolonged antibiotic treatments are not recommended, instead anti-inflammatory drugs and antirheumatic drugs are typically used. Children under 8 years of age that develop Lyme can be given doxycycline as long as the duration of treatment is no longer than 24 days. As with most tick-borne pathogens, tick avoidance is the primary prophylactic action that can help prevent Lyme disease.

Tick-borne Spirochetes: *Borrelia miyamotoi*

Within in the genus *Borrelia* is another tick-borne spirochete, *B. miyamotoi*, that causes a disease similar to Lyme disease but is less severe. *Borrelia miyamotoi* is an emerging pathogen transmitted by *Ixodes* ticks that was first isolated from ticks in 2001[38]. All tick species that have the potential to transmit *B. burgdorferi* also have the potential to transmit *B. miyamotoi*[38]. This includes both species of *Ixodes* ticks in North America. However, the prevalence of *B. miyamotoi* infected ticks is much lower[39]. The current geographic distribution of *B. miyamotoi* is believed to be potentially much larger than *B. burgdorferi* due to the spirochete's ability to transmit transovarially within ticks. For reasons still being investigated, the spirochete is also transmitted faster upon the bite of a tick; both mechanisms could aid in the establishment or rapid spread of the spirochete within a geographic area as it persists within tick lines and is spread to mammals[40]. *Borrelia miyamotoi* infection may be more common than currently acknowledged due to the mild nature of the infection.

Infection with *B. miyamotoi* for the average healthy individual may be completely asymptomatic and self-resolving. For other individuals, it may present as a relapsing fever similar to Lyme disease with symptoms like high fever, myalgia, and arthralgia. In more severe cases, it can lead to thrombocytopenia and leukopenia. If a patient is immunocompromised, infection could progress to meningitis[38]. In general, infection presents as more flu-like than the traditional Lyme disease symptomology, which may be why the infection is under-reported. Part of the asymptomatic nature could be due to a stronger initial immune response by the body.

Studies have demonstrated that *B. miyamotoi* elicit a stronger cytokine response from activated dendritic cells in the skin when compared to *B. burgdorferi*[41]. IL-12, which is a T-cell-stimulating factor that aids in the differentiation of T-cells into Th1 cells, is produced at a much higher concentration when dendritic cells are exposed to *B. miyamotoi*[41]. Overall, the dendritic cells in the skin are able to elicit a stronger T cell response to *B. miyamotoi* than to *B. burgdorferi*, which could potentially explain the lower severity of the disease. This also raises the question as to why the dendritic cells are 'better suited' to handle one spirochete over the other. A longer evolutionary relationship between humans and *B. miyamotoi* may explain the differences in reaction. Conversely, *B. burgdorferi* may just be a more virulent spirochete.

Specific treatment guidelines are still being determined for this spirochete, but in general it is currently treated the same as Lyme disease. The exact antibiotic susceptibility of *B. miyamotoi* is an area of active investigation. Overall, *B. miyamotoi* appears to be highly resistant to amoxicillin. However, in vitro studies have demonstrated susceptibility to doxycycline, azithromycin, and ceftriaxone[42]. This is in line with the

current treatment strategies used; oral doxycycline for 14 to 21 is an effective treatment for both *B. burgdorferi* and *B. miyamotoi*[42].

Anaplasma phagocytophilum

Another tick-borne disease transmitted by *I. scapularis* is Human Granulocytic Anaplasmosis (HGA). *Anaplasma phagocytophilum* is the causative agent on this disease. The endemic areas of HGA overlap with the general distribution of *I. scapularis*[43]. HGA presents as a febrile illness with fever, headache, myalgia, and arthralgia. If a patient is known to have been bitten by a tick, the symptomology can easily be mistaken as Lyme disease with no EM rash. In very rare cases, HGA can progress to meningitis or encephalitis[44]. There seems to be some evidence for lasting immunity once a person clears an initial infection. However, it hasn't been determined yet if this is due to persistent infections in endemic areas or due to memory B and T cells[44]. Doxycycline is the primary antibiotic used to treat anaplasmosis. If *A. phagocytophilum* is the lone cause of disease, then a 10-day treatment with doxycycline is usually sufficient. In the event of a co-infection with *B. burgdorferi*, a 14-day treatment is administered.

Anaplasma phagocytophilum Pathology and Immunology

Anaplasma phagocytophilum primarily infects the neutrophils. Within neutrophils, it can survive and multiple within cytoplasmic vacuoles of polymorphonuclear cells (PMN)[45]. PMN earn their name based on the variety of shapes their nucleus can have. These cells include neutrophils, eosinophils, basophils, and mast cells, all of which *A. phagocytophilum* can infect. *Anaplasma phagocytophilum* is able to survive in these cells by blocking cellular apoptosis through upregulating antiapoptotic-

bcl-2 family member bfl-1 and blocking FAS(CD95)-induced programmed apoptosis. Bcl-2 or B-cell lymphoma 1 is a regulator protein that controls cell death by either inducing it or delaying apoptosis. CD95 is a death receptor on cell surfaces that, when bound to the Fas ligand, initiates apoptosis through the Fas signaling pathway[45]. By disrupting both of these cell death processes, *A. phagocytophilum* is able to survive and thrive within the PMN cells. This bacterium also disrupts the ability of granulocytes to release respiratory bursts by downregulating production of $gp91^{phox}$ and rac2, which are two key proteins in the ability of granulocytes to kill invaders[45].

In an interesting propagation strategy, *A. phagocytophilum* upregulates the expression and production of the chemokine IL-8, which attracts more neutrophils to the infected area for the bacteria to infect[46]. In fact, the amount of IL-8 secreted has been shown to be dependent on the inoculum size of *A. phagocytophilum* in vitro[46]. The bacteria also will upregulate the IL-8 receptor (CXCR2) on the surface of neutrophils. Through both of those actions, *A. phagocytophilum* triggers chemotaxis of neutrophils to the area that it can potentially infect. For most infections however, the initial strong inflammatory reactions are usually sufficient to clear the infection. It is important to keep in mind that tick-borne pathogens do not cause the rapid infections that other bacteria may cause, which can be made apparent when you view this situation from an evolutionary point of view. Tick-borne pathogens like *A. phagocytophilum* need their host to survive. It's the parasite-host relationship that is kept in balance so that both stay alive.

A recent study shed light on how an innate immune response may be responsible for keeping *Anaplasma* in check. Inflammasome is a multiprotein oligomer that works to

activate an inflammatory response. Intracellular pathogens are usually sensed by Nod-like receptors that produce these inflammasome protein units. One example is the NLRC4 Inflammasome, which is produced when NLRs are activated by bacterial type III secretion (T3SS) and/or flagellin. The research has shown that *A. phagocytophilum*, which lacks both the T3SS and flagellin can still cause activation of the NLRC4 inflammasome[47]. The bacteria trigger a novel pathway to activate the inflammasome through eicosanoid prostaglandin E2 and EP3 receptor[47]. Once activated, the NLRC4 inflammasome induces caspase-1 autoproteolysis, which is the process that cleaves proteins to activate both Interleukin 1β, interleukin-18, and initiate cell death[48]. IL-1β and IL-18 are both pro-inflammatory cytokines needed to mount a full immune response against intracellular pathogens like *A. phagocytophilum*. This study highlights how complex the immune response to intracellular pathogens can be and further research into tick-borne pathogens and their immune response is crucial.

Babesia microti

Unlike the other pathogens transmitted by *Ixodes* ticks, *Babesia* is a eukaryotic parasite. *Babesia* is a unicellular apicomplexan which possess an apicoplast. This organelle is crucial to penetrating the host cell. Currently, there are over 100 species in the Genus *Babesia* which includes several threats to mammals. A large portion of *Babesia* research has been devoted to the species that can have an economic impact via infecting livestock. *Babesia bovis* can cause acute disease in cattle and in most cases is fatal, even for healthy cattle[49]. There are three species of *Babesia* that have been identified thus far as causing a malaria-like illness in humans. *Babesia venatorum, B. duncani*, and *B. microti* are all emerging pathogens in the *Babesia* genus that are

transmitted by *Ixodes* ticks[50]. These organisms have global distributions that seems to coincide with the global distribution of different *Ixodes* species*; Ixodes scapularis* in North America, *Ixodes ricinus* in Europe, and *Ixodes persulcatus* in Asia.

Babesia **Lifecycle**

Babesia microti has a two-host life cycle. Traditionally, the primary mammalian host is *P. leucopus*, and the definitive host is *I. scapularis*. While taking a blood meal, an infected *I. scapularis* tick will inoculate sporozoites into the blood stream. These sporozoites will then use their apicoplast to enter erythrocytes and become a trophozoite, or a feeding form of the parasite. Once enough nutrients have been taken from the host, it will undergo asexual budding and become a merozoite. The merozoites eventually lyse the erythrocyte and are now separate gametes, and then can be ingested by another tick taking a blood meal. Once in the gut of a new tick, these gametes will fertilize each other forming an ookinete. The ookinete enters the salivary gland where it develops into the sporozoites once again and is now infective once the tick takes a blood meal. The multiplication of the parasite in red blood cells and the subsequent lysis of them is what results in the manifestation of clinical symptoms of babesiosis. Humans are however a dead-end host unless transmission occurs through blood transfusions, transovarial, or through organ donation. The lifecycle of *B. microti* might look familiar if one has ever studied the lifecycle of malarial diseases. This is because *Babesia* and *Plasmodium* are both in the phylum Apicomplexa.

Babesia is an often-overlooked member of the Apicomplexa, a phylum of eukaryotic parasites. The most commonly known Apicomplexan is *Plasmodium*, the causative agent of malaria, which is transmitted by mosquitoes. Another common

parasite in this group is *Toxoplasma gondii*, the causative agent of Toxoplasmosis, which is usually transmitted through contact with contaminated feline feces. *Babesia*, *Plasmodium*, and *Toxoplasma* share similarities despite the wide range of vectors. To start, they all get their name from the apical organ complex they possess, which is an assembly of secretory organelles and structural features that it requires and uses for host cell invasion. The invasion process involves the exocytosis of three different organelles; micronemes, rhoptries, and the dense granules[51]. The invasion begins when the apical complex comes into physical contact with a potential host cell and the parasite. The micronemes contain protein motifs that have a binding affinity to hepatocytes, i.e. are used to bind to red blood cells and therefore enter the cell. Rhoptries are then released and aid in the formation of the vacuole around the apicomplexan. Finally, the dense granules are secreted to steal purines from the host cell in order for the parasite to reproduce. Even with all of these in common, the genome sequence of *B. microti* did reveal some substantial differences.

The genome size is the first factor separating *B. microti* from the other Apicomplexa. *Babesia microti* has only three chromosomes that encode approximately 3,500 proteins[52]. The overall genome is approximately 6.5 Mbp, which makes it the smallest Apicomplexan genome. It has been suggested that the small size of the genome might have occurred through reduction as the parasites host become more specific to only mammals. In other words, the large diversion from the expected Apicomplexan genome might be due to its specific nature; as it evolved to infect only the erythrocytes of mammals unnecessary genes were lost. Despite sharing the genus name with *B. bovis*, the genome analysis of *B. microti* actually puts it within its own clade in the phylum of

Apicomplexa. The genomic information also demonstrated the high dependence on the host for survival. *B. microti* has no genes for β-oxidation or hemoglobin degradation. This calls into question exactly how the parasite survives and what exactly it utilizes within red blood cells. Proteomic analysis reveals that *B. microti* has genes for phospholipid synthesis and the genes essential for glycolysis[53]. Because *B. microti* has no β-oxidation genes, which is the process by which fatty acids are catabolized, it must not rely on fatty acids for energy production via the electron transport chain. It appears that *B. microti* can only utilize glycolysis for energy production. Using this knowledge of *B. microti* metabolism, potential targets for anti-parasitic medications can be developed.

For most people, an infection with any *Babesia* spp. is self-limiting and largely asymptomatic. For those who are immunocompromised, elderly, or those who are splenectomised, symptomology can be much more severe. Symptoms can include hemolytic anemia, renal failure, hepatomegaly, splenomegaly, and can even be fatal. The most common source of *Babesia* infection is ticks. Other sources include blood transfusion and transplacental exchange[54]. The asymptomatic nature of *Babesia* infections presents a potentially deadly situation for those needing blood transfusions. The first line of defense against *B. microti* is the spleen. After being bitten by an infected tick, the parasite will eventually wind up in the marginal zone of the spleen which contains neutrophils, macrophages, and dendritic cells that recognize and phagocytize them as they get trapped in the sieve that is the red pulp of the spleen. The T-cells within the white pulp of the spleen secrete IFN-γ, an inducer of class II major histocompatibility complex, which activates macrophages through antigen presentation and simultaneously activates B-cells to produce and secret *B. microti* specific antibodies[55]. The dendritic cells

in the spleen are responsible for presenting antigen to the T-cells for activation and therefore activation of B-cells. Also aiding in this process is that *Babesia* infected red blood cells pass more slowly through white and red pulp[56]. Similar to malaria, antibody interference seems to be crucial to clearing *Babesia*, especially in those who are immunocompromised. When an antibody can bind to the pathogen and block entry into new red blood cells, this acts to enhance opsonization and ultimately clears the parasite through macrophages, NK cells, and complement pathways[57]. On the other hand, persistence of *Babesia* parasites is still possible in healthy adults through several evasive strategies *Babesia* employs. One way is the inherent protection that is inferred if the pathogen can successfully enter a red blood cell. *Babesia*-induced adhesion molecules will also cause red blood cells to attach to the surface of the vascular endothelium, preventing clearance in the spleen[58]. Antibodies against these adhesion proteins have been detected in humans, however, there exists variability in the protein coding regions that produce these adhesions. Ultimately, multiple forms of adhesions proteins may be produced to evade antibodies.

Babesia Diagnosis and Treatment

To properly diagnosis babesiosis, the disease caused by an infection with *B. microti*, you first must be able to identify the symptoms. The typical manifestations of babesiosis include fever, anemia, thrombocytopenia, elevated lactate dehydrogenase (a marker of erythrocyte damage), and hyperbilirubinemia (elevated bilirubin, also due to hemolysis of red blood cells)[59]. If symptomology matches up a known tick bite, the potential for a tick bite, or the patient lives in an endemic area, the diagnosis can proceed. The fastest, simplest, and most cost-effective diagnostic tool is a Giemsa or Wright's thin

stain of the patients' blood. The Wright's stain and Giemsa stain consist of two dyes; eosin dye, which stains red blood cells pink/red, and methylene blue, which stains the various forms of the parasite blue. This allows for easy visualization of trophozoites within red blood cells. The *B. microti* trophozoites will appear as rings with one dot inside a red blood cell. If asexual reproduction has taken place, you can visualize the four merozoites that arrange in a tetrad. If a red blood cell has ruptured therefore freeing the merozoites, free merozoites can be visualized attached to the outer surface of red blood cells[60]. Polymerase Chain Reaction (PCR) is a viable option if the patient is in the early stages of infection. *Babesia microti* DNA can be detected in a patient's serum months after the infection has been cleared, the same is true for antibody or immunofluorescent assays[60].

In the rare incident that an asymptomatic patient is identified as having a *B. microti* infection, no treatment is recommended; instead, the course of action is to let the immune system clear the parasite. For symptomatic suffers of babesiosis, the treatment is a seven to ten-day course of oral azithromycin and atovaquone[60,61]. If the infection is severe, it is recommended to treat with intravenous clindamycin along with oral quinine or atovaquone[61,62]. For immunocompromised patients or those suffering from relapsing babesiosis, a higher dose oral azithromycin is recommended.

Powassan Virus

Powassan virus (POWV) is a novel tick-borne encephalitic virus (TBEV) from the family Flaviviridae. There are many different families of viruses that ticks can carry including Asfarvivirdae, Bunyaviridae, Orthomyxoviridae, Reoviridae, and Rhabdoviridae[63]. Many of these viruses remain unclassified and are simply identified as

tick-borne encephalitis virus. Powassan has been grouped in the serocomplex of the tick-borne encephalitic flaviviruses. The neglected nature of Powassan is due to the low prevalence, unfortunately though, its prevalence is rising[64]. In 2010, only eight cases of Powassan disease were reported in the United States, but by 2019 that number had increased to 37 cases a year[65]. As far as research has demonstrated, Powassan virus is spread exclusively by *I. scapularis* ticks. Therefore, the incidence of the disease appears to be limited to those areas with a population of *I. scapularis* ticks. With the spread of *Ixodes* ticks due to climate change, the potential for spread of Powassan virus or other TBEV should be taken seriously. With a fatality rate over 30% and no treatment, proactive surveillance of Powassan should be performed.

Flaviviridae

Powassan virus belongs to the viral family Flavivirdae (yellow virus in Latin), which obtained its name from yellow fever virus. The hosts for these viruses are mammals, including humans. Arthropods act as vectors[66]. Notable members of this family include Hepatitis C and Dengue virus. All members of this group are enveloped with an RNA-based genome. The virion (individual viral particle) is a spherical lipid envelope with two or three capsid proteins and can range in size from 40-60 nm. The genome is a positive sense RNA strand approximately 9.0-13 kb in length[66]. The genome is one continuously read open reading frame (ORF) and translation of the ORF is cap dependent. A 5' cap allows for ribosomal binding and therefore translation. The viral genome is used to directly produce viral proteins. Replication of these viruses takes place in the cytoplasm; the viruses use the endoplasmic reticulum to assemble virions that

are secreted through the vesicle transport system. Therefore, the lipid envelope is actually acquired from the endoplasmic reticulum of the host mammalian cell. Within the family Flaviviridae is the genus Flavivirus, which contains over 50 arthropod-borne viruses.

Flavivirus

Viruses in the genus flaviviruses are either mosquito-borne or tick-borne viruses. The diseases they cause can vary from a mild flu-like illness to encephalitis or hemorrhagic fever. Some of the more familiar names in this genus include West Nile, Zika, Dengue, and Powassan virus[67]. The flaviviruses are commonly divided into two groups; those that are tick-borne and those that are mosquito-borne. The tick-borne flaviviruses are all closely related and belong to one viral complex. The mosquito-borne flaviviruses display more diversity and distinction amongst them. The split between the two groups occurred around 40,000 years ago; which was 60,000 years after the proposed appearance of the common ancestor of all the flaviviruses[68]. Interestingly enough, multi-host flaviviruses exist in mosquito-borne flavivirus but only single host flaviviruses exist within the tick-borne flaviviruses, which is likely due to the difference in diversity[68]. Again, the diseases caused by mosquito-borne flaviviruses can range from hemorrhagic to severe neurological disease, but for tick-borne flaviviruses, thus far, the disease caused seems to be limited to encephalitis or neurological symptoms. There are several classic mosquito-borne flaviviruses to discuss, but as the focus of this paper is that of tick-borne pathogens, I will focus on the tick-borne flaviviruses.

Tick-Borne Encephalitis

TBE or tick-borne encephalitis is the general term for the disease caused by the various flaviviruses transmitted by *Ixodes* ticks. It was first identified by Soviet scientists in what is now Russia as a severe neurological disease that was afflicting forest workers[68]. A less severe disease was then discovered to be linked to unpasteurized milk from infected animals. Both were linked to *Ixodes persulcatus*, the 'European' species of *Ixodes*. The general symptomology associated with TBE usually manifests rapidly. What starts as a high fever and headache can quickly progress to paralysis and is fatal in 20-30% of cases[69]. The patients that survive usually have severe neurological complications. The currently understood life cycle of TBE viruses is perpetuated between *Ixodes* ticks and rodents. As with most zoonotic diseases, the pathogen enters the animal through the bite of tick and then uninfected ticks pick up the pathogen by feeding on infected animals. With flaviviruses, Powassan included, the viruses can be spread through transovarial transmission which further enhances persistence in the population[70].

Powassan virus is the only tick-borne encephalitic flavivirus currently known to be in North America[71]. The name comes from the town of Powassan, Ontario, where in 1958 a boy died of encephalitis. From the autopsy of the brain tissue, Powassan virus was identified[72]. Over the past decade, there has been an increase in Powassan virus due to various factors associated with the spread of *Ixodes* ticks and human interactions with them. Sporadic surveillance studies have been conducted since that 1958 discovery to test *Ixodes* ticks and potential mammalian hosts for POWV. The virus is considered to have two hot spots, one being the north eastern portion of the United States extending into Nova Scotia, Canada, down to Virginia[71]. The second hot spot is the northern tier of the central United states which includes Minnesota, Michigan, and Wisconsin. It has been

detected in states much farther from the epicenters such as Colorado and California, presumably due to one of the primary hosts, the white-tailed deer (*Odocoileus virginianus*)[71]. White-tailed deer can harbor a flavivirus that is extremely similar, known as deer tick virus (DTV), named from the *I. scapularis* ticks from which it was isolated. Powassan virus and deer tick virus have an 84% nucleotide sequence identity match and 94% amino acid identity[71]. Both viruses are maintained and perpetuated in an environment by *I. scapularis* and mammal hosts, typically *Odocoileus virginianus* and *Peromyscus leucopus;* however, *Tamiasciurus hudsonicus* (American red squirrel) and *Tamias amoenus* (Yellow-pine chipmunk) also act as mammalian reservoirs for POWV. *Ixodes scapularis* is the only tick known to be a vector for POWV and DTV. Ticks in the genus *Dermacentor* are a suspected vector of POWV, but this has not yet been confirmed[72]. *Ixodes scapularis* ticks, through their questing behavior, do not preferentially feed on any one mammal species, they will feed on the first mammal they encounter. Other *Ixodes* species, such as *I. cookie*, rarely feed on humans as they rarely exhibit questing behavior. Their feeding is limited to groundhogs[74]. It's worth noting that *P. leucopus* can survive experimental infection with Powassan virus with no signs of the disease and even intracranially injected virus only causes mild inflammation compared to what presents in human infections[75]. Because of the natural behavior of *I. scapularis* to be a generalist in terms of targets for feeding, it serves as a potential public health risk as a spreader of Powassan virus to humans.

Powassan Virus Disease

The neurologic disease caused by Powassan virus is similar to what is caused by a typical tick-borne encephalitic virus. Once infected, disease progression typically follows

a 5-week course. One week after a being bitten by an infected tick, a febrile illness sets in, as does viremia, usually associated with fever, sore throat, headache, and disorientation. A few weeks to a month after infection, encephalitic disease sets in with more severe symptoms including vomiting, respiratory distress, speech difficulties, paralysis, and seizures[76]. Past this point, severe and lifelong complications will exist, including memory problems, muscle wasting, chronic headaches, and partial paralysis or hemiplegia. All of this is caused by viral replication in neuronal cells. In the cases in which autopsies were performed, necrosis of the brain has been observed as well was neuronal cell death in the brainstem, cerebellum, and thalamus[77]. Animal model testing demonstrates that neurons and glial cells, non-neuronal cells of the CNS, are the primary targets of Powassan virus for replication and subsequent destruction upon lysis[77]. Because of neuronal death in locations like the brainstem, it is clear why paralysis and partial paralysis is a common effect of Powassan virus. The severity of these disease symptoms and the rapid onset can act to complicate diagnosis and treatment.

Powassan Virus Diagnosis and Treatment

To meet the clinical standards for diagnosis of Powassan virus, the patient must have a fever greater that 38℃ (100.4^{o}F), signs of central nervous systems or peripheral nervous system dysfunction, and one or more laboratory-based criteria. Detection of Powassan virus antigen or nucleic acid in blood or cerebrospinal fluid or Powassan virus specific IgM antibodies, while also testing negative for IgM for other arthropod-borne viruses, will suffice as laboratory-based confirmation of Powassan virus. Issues can arise in diagnosis if the patient has already entered the encephalitic stage of the disease, which is when most patients are hospitalized. By that point in the disease, Powassan RNA will

no longer be detectable in blood or cerebrospinal fluid[71]. Therefore, if Powassan is suspected and the patient is already having severe symptoms, IgM antibody testing is the recommended course of action. Once diagnosed, there is unfortunately no specific therapy to treat Powassan virus. Once diagnosed, treatment usually focuses on reducing inflammation. High-dose corticosteroids have been used successfully with patients that have survived[71]. Several treatments have been attempted unsuccessfully. For example, intravenous immunoglobulin was attempted, the patient survived but with severe neurological impairment[78]. Currently, treatment is relegated to supportive care to help the patient stay hydrated, support breathing if needed, and reducing swelling/inflammation of the brain. There is no vaccine currently approved for Powassan virus, however, promising work is being done. Work at the University of Washington at St. Louis has demonstrated that a mRNA vaccine can induce immunity in mice. The mRNA is enclosed in a lipid-nanoparticle and the mRNA codes for two structural proteins of the virus, proteins prM and E. Once this mRNA is translated, they act as antigens for helper T-cells and ultimately neutralizing antibodies against these proteins are produced[79]. After one dose, the mice were protected from a lethal challenge dose of Powassan virus. The mRNA vaccine also produced cross-neutralizing antibodies, antibodies that bind to antigens from other tick-borne flaviviruses. To date, this is the most promising study available on a potential vaccine for Powassan virus. As previously stated, with no vaccine or treatment available, proper surveillance is crucial to follow the spread of Powassan virus as *I. scapularis* expands its habitat with climate change.

CHAPTER 2

Molecular survey of *Ixodes scapularis* associated pathogens from *Odocoileus virginianus* at Lake Arcadia in Edmond, Oklahoma

INTRODUCTION

Odocoileus virginianus (white-tailed deer) have a large geographic distribution, extending from the 60th parallel of Canada to Panama, which is more extensive than any other big game mammal on the North American Continent[80]. The primary host of *Ixodes scapularis* is *O. virginianus*. *Ixodes scapularis* is also known to be the primary vector of Lyme disease to humans in North America[81]. Some of the pathogens known to be vectored by *I. scapularis* include *Borrelia burgdorferi, Borrelia miyamotoi, Anaplasma phagocytophilum, Babesia microti*, and Powassan virus. Each of these pathogens has been documented to cause human illness, including cases in Oklahoma[73,84,85,86,87,88,89]. Lyme disease has now been reported in 49.2% of American counties, which is a 44.7% increase since 1998 when records started[81]. As of 2015, 46.8% of Oklahoma counties have reported *Ixodes* populations; this also includes reported cases of Lyme disease in 22 counties, including Oklahoma County[81,82]. With the changes in climate, the expansion of human activities, and the already large distribution of white-tailed deer, human exposures to *I. scapularis* is on the rise[83]. The behavior of *I. scapularis* also makes collecting questing ticks difficult by trapping. Studies of Oklahoma *Ixodes* have shown that collecting ticks in the evenings directly from hosts provides the best opportunity to collect ticks[81].

Due to the expansion of the *I. scapularis* geographic range, a better understanding of pathogen prevalence within feeding ticks Oklahoma is needed. Information on the pathogen prevalence carried by *I. scapularis* in central Oklahoma is lacking, creating a knowledge gap with important public health implications because this is the most populated region of the state. The goal of my research is to determine the prevalence of tick-borne pathogens in *I. scapularis* collected from host-harvested *Odocoileus virginianus*. The hypothesis is that the prevalence of *Borrelia* species will be higher than the prevalence of *Babesia* and Powassan virus.

MATERIALS AND METHODS

Collection

With permission from the Arcadia Lake Wildlife management, ticks were collected from 25 deer during bow hunting season, October 1st through December 30th, 2018. This collection was done during times of peak adult tick activity[81]. Samples were collected by grasping the ticks as close to the surface of the skin as possible and pulling upwards with steady and even pressure. It is important to keep mouthparts intact for identification. The head, ears, chin, chest, and axillae of the deer were examined for ticks. The fur was carefully pulled back to visually examine each deer and take an estimate of the total tick load of the deer. Ticks were placed in a vial containing 70% isopropyl alcohol. Vials were kept in a cooler on ice. The date, sex, weight, and the approximate age of deer, zone harvested, and time of day harvested were recorded. Ticks were transported to the lab at UCO for identification and stored at -80^{o}C until nucleic acid extraction.

Identification

Tick species were identified as *Ixodes scapularis* based on mouthparts, body markings, and other features as laid out in previous research[90,91]. Life stage and sex was also determined.

Tick Homogenization and DNA/RNA Extraction

Ixodes scapularis ticks were pooled by sex and life stage based on the deer they were sampled from. Furthermore, engorged females were pooled separately from non-engorged females. The ticks were placed in a screw cap lysis tube with sterile bashing beads and DNA/RNA shield from ZYMO Research to ensure stability of genetic material and homogenized on a bead beater[92]. DNA and RNA was extracted and purified using the Quick-DNA/RNA Pathogen Miniprep protocol from ZYMO Research[92].

Multiplex Real-Time Reverse Transcription-PCR

Each pathogen target was selected for by a unique gene target as previously described[93]. Primers for each gene target and probes are shown in Table 1. A one-step real-time reverse transcription-PCR (RT-qPCR) previously described was used to detect pathogen targets[93]. Following the Invitrogen RNA UltraSense protocol; 2.5 µl of the enzyme mix, 10 µl of the 5X reaction mix, 1 µl of both forward and reverse primers for all 5 targets, 1 µl of each target probe, 10 µl of the sample, and 12.5 µl of DPEC-treated water was used for a total reaction volume of 50 µl. The reverse transcription step was performed at 55°C for 15 min, followed by incubation at 95°C for 10 min. The PCR consisted of 40 cycles (95°C for 15 s and 60°C for 30 s). The RT-qPCR was performed on a Bio-Rad Bio cfx96 real time system C1000 touch thermal cycler.

Table 1: Pathogens and Target Genes with primer and probe sequences

Pathogen	Disease	Gene Target	Forward and Reverse Primers	Probe
Borrelia burgdorferi	Lyme Disease	ospA	CCTTCAAGTACTCCAGATCCATTG AACAAAGACGGCAAGTACGATC	6-FAM-CAACAGTAGACAAGCTTGA-IBFQ
Babesia microti	Babesiosis	Cox1	CATCATGCCAGGCCTGTTTG GAAGAAACCACAAGAGCAAATGC	YAK-TACTACCCATACTGGTCGGTGCTCC-IBFQ
Anaplasma phagocytophilum	Anaplasmosis	16s rRNA	GGCATGTAGGCGGTTCGGT CACTAGGAATTCCGCTATCCTCTCC	TAMRA-GCCAGGGCTTAACCCTGGAGCT-IBRQ
Borrelia miyamotoi	Lyme	Flab	AGCACAAGCTTCATGGACATTGA GAGCTGCTTGAGCACCTTCTC	TxR-TGTGGGTGCAAATCAGGATGAAGCA-IBRQ
Powassan Virus	Encephalitis	3'-UTR	GTGATGTGGCAGCGCACC CTGCGTCGGGAGCGACCA	Cy5-CCTACTGCGGCAGCACACACAGTG-IBRQ

Traditional PCR using a Bio-Rad C1000 thermal cycler was performed on each sample that was sample positive by RT-qPCR. The presence of a single amplicon was confirmed by gel electrophoresis. The identity of each amplicon was determined by Sanger sequencing through ETON-Biosciences, California. Sequences were compared using NCBI Blast for percent matches to known target sequences.

Results:

Ticks (n = 394) were pooled into 117 total samples. Of these samples, 3 positives for *B. miyamotoi* and 5 positives Powassan virus were detected. This represents a prevalence rate of 0.8% and 1.2%, respectively. *Anaplasma phagocytophilum*, *B. burgdorferi*, and *B. microti* were not detected in any samples. All 5 positive Powassan samples were collected from the same deer. *Borrelia miyamotoi* positive sample pools consisted of males and nymphs. The Powassan positive sample pools consisted of engorged females, non-engorged females, males, and nymphs.

Discussion:

Tick-borne diseases continue to spread westward from the northeast epicenters due to climate change and are predicted to increase establishment in new areas[94]. The absence of *B. burgdorferi* is supported by previous research in Oklahoma[81]. *Borrelia miyamotoi* is commonly found with *B. burgdorferi* and can be maintained in the same mammalian hosts[39]. The presence of one species in Oklahoma potentially demonstrates that these two spirochetes are being maintained in the local populations either in *O. virginianus* and/or other mammals. Research into what potential pathogens *P. leucopus* . is carrying in central Oklahoma in comparison to other states in more endemic areas might help shed light on this. Other potential animals in Oklahoma that have been known to be reservoirs of *B. burgdorferi* include *Turdus migratorius* (American Robins), *Tamias striatus* (chipmunks), *Blarina brevicauda* (short-tailed shrews), and *Sciurus carolinensis* (eastern gray squirrel)[95,96]. *Ixodes scapularis* ticks collected from *O. virginianus* has demonstrated that *B. miyamotoi* can persist in the tick population through vertical transmission[97]. Antibody testing for *B. burgdorferi* in Oklahoma from white-tailed deer could be validated by research demonstrating the presence of *B. burgdorferi* antibodies in white-tailed deer in Texas[98]. Multiple studies have demonstrated the presence of *B. burgdorferi* in white-tailed deer and *I. scapularis* in Texas at low prevalence rates[99,100,101]. In Missouri, *B. burgdorferi* was successfully isolated and cultivated from *Ixodes* ticks found on *Sylvilagus floridanus* (cottontail rabbits)[102]. These two neighboring states identifying *B. burgdorferi* in *I. scapularis* lends support to the presence of the closely related *B. miyamotoi* in *I. scapularis* ticks in Oklahoma.

The incidence of *B. microti* induced babesiosis has increased in the United States over the last decade[55]. Co-infection with *B. microti* and *B. burgdorferi* is also common,

demonstrating the ability of these two pathogens to persist in the same tick[104]. Models have shown that the presence *of B. burgdorferi* can actually aid in the establishment of *B. microti*[105]. *Babesia* spp. has been identified in Oklahoma *Ursus americanus* (black bears) on which *I. scapularis* was also present [106]. This study was unable to detect *B. microti* from *I. scapularis*, demonstrating that this *I. scapularis* associated pathogen has not spread to Oklahoma with the expansion of the deer tick population.

The presence of Powassan virus in central Oklahoma represents a jump in terms of the previously known geographical range of states with known *I. scapularis* positive results or cases of Powassan encephalitis. The large geographic range of white-tailed deer and the spread of *I. scapularis* ticks in the United States is thought to potentially explain the increase in POWV cases[108]. North Carolina and North Dakota have had positives case of Powassan encephalitis since 2000, demonstrating the spread from its origin in Ontario, Canada[109]. POWV infected white-tailed deer have been documented before in previous studies, again highlighting the potential role of reservoir host testing along with vector testing[108]. No cases of Powassan encephalitis have been reported in Oklahoma, so the presence of this flavivirus demonstrates the need to study potential differences in the reservoir hosts in endemic vs. non-endemic areas and to conduct further surveillance of the *I. scapularis* population.

The absence of *A. phagocytophilum* could be due to the incidence rate in *I. scapularis* ticks in central Oklahoma being extremely low. The amount of ticks acquired in this study might not have been sufficient to detect *A. phagocytophilum*. Previous studies of *I. scapularis* in Oklahoma have also demonstrated an absence of *A. phagocytophilum*[81]. The prevalence of *A. phagocytophilum* is increasing and with it,

HGA in the United states[44]. Studies have demonstrated that white-tailed deer are suitable natural reservoirs for *A. phagocytophilum*[109]. As *I. scapularis* populations continue to spread, proactive surveillance of these pathogens, including *A. phagocytophilum*, is still recommended.

Conclusion:

As *I. scapularis* continues to spread south and west from the northeastern United States, so too should the research into their associated pathogens. This study demonstrates the presence of *I. scapularis* associated pathogens that were not previously reported in Oklahoma. Proactive surveillance of *I. scapularis* pathogens are recommended as this tick continues to grow and expand its range, with climate change potentially increasing their prevalence rates in the state and increasing the potential for human exposure to these pathogens.

Chapter 3

SUMMARY AND CONCLUSIONS

Most research into *I. scapularis* and their pathogens has been geographically restricted to areas where the diseases they vector are more prevalent. With climate change and the expansion of the *I. scapularis* range, the lack of surveillance of *I. scapularis* pathogens in Oklahoma has left a gap in the knowledge that could have an impact on human health. To investigate the pathogens that *I. scapularis* ticks were potentially harboring, *I. scapularis* ticks were collected from their primary host, *Odocoileus virginianus*. These deer ticks were pooled by sex and life stage, homogenized, DNA/RNA was extracted, and a one-step reverse transcriptase qPCR reaction was used to screen for five of the most human relevant pathogens transmitted by *I. scapularis.* Of these samples, 3 positives for *B. miyamotoi* and 5 positives Powassan virus were detected. This represents a prevalence rate of 0.8% and 1.2%, respectively. *Anaplasma phagocytophilum, B. burgdorferi*, and *B. microti* were not detected in any samples

In the larger context of *I. scapularis* research conducted in Oklahoma, this study is in agreement with other studies on the absence of *A. phagocytophilum* and *B. burgdorferi* in *I. scapularis* ticks in the state[81]. Further investigation into what pathogens the vertebrate reservoirs in Oklahoma harbor could also be insightful. Starting with the preferred mammalian hosts of *I. scapularis,* like the white-tailed deer and white-footed mouse, antibody testing could aid in this goal. Other mammals in Oklahoma known to be fed upon by *I. scapularis* and known to serve as reservoirs for *I. scapularis* associated pathogens could also be antibody tested for these five pathogens. Mammals such as chipmunks, short-tailed shrews, and eastern gray squirrels would fit these criteria. Lizards

and skinks also have a role in future *I. scapularis* research in Oklahoma. Research has demonstrated that tick-host associations shift from mammals in the north to reptiles in the south[110]. Southern deer ticks show strong selective attachment to lizards which are inefficient reservoirs for *B. burgdorferi*. In the northeast, *I. scapularis* feed mostly on mammals that are efficient reservoirs for *B. burgdorferi*, allowing the tick to become infected and allow for potential transmission to humans[110]. Furthermore, continued surveillance of *I. scapularis* themselves is warranted, whether field collected or harvested from vertebrate hosts. Continued research into the spread of *I. scapularis* in Oklahoma at the county scale could help in potential target sites for trapping and harvesting.

There are additional pathogens that *I. scapularis* can transmit to humans and some they are known to harbor, but human transmission has not yet been established. This includes *Bartonella henselae*, the causative agent of cat scratch disease, and *Bartonella quintana*, the causative agent of trench fever, both of which are referred to as bartonellosis. Bartonellosis has recently been re-categorized as an emerging zoonotic disease that *Ixodes* ticks can transmit[111]. Bartonellosis symptoms can range from skin lesions to acute sepsis[111]. Although this gram-negative intracellular bacterium is usually considered an opportunistic pathogen, the range of disease it can cause makes it medically important to investigate. *Babesia odocoilei* is eukaryotic parasite, in the same genus as *Babesia microti*, that *I. scapularis* ticks transmit that causes cervid babesiosis and it is not fully understood if it affects humans or not[112]. South Bay virus is a novel bunyavirus, an order of RNA viruses found in arthropods, that have been discovered in *I. scapularis* with currently unknown distributions and its impact on humans is currently unidentified[87]. The pathogens listed above have not been investigated in *I. scapularis*

ticks in Oklahoma. With current molecular testing lessening the workload, screening *Ixodes* ticks for potential pathogens has become a manageable task that should be viewed as a necessity as deer tick populations increase and expand.

It has also been determined that some of the non-pathogenic bacteria and microbes in ticks can affect vector-competency and favorable influence tick physiology[87]. Research has shown that there are many bacteria that ticks inherit maternally that aid in their survival and potentially aid in the survival and competency of tick-borne pathogens. Some of these bacteria include *Coxiella, Rickettsiella, Arseonphonus, Cardinium, Spiroplasma,* and *Midichloria*[113]. The presence or absence of these in Oklahoma *I. scapularis* ticks could help explain the differences in pathogen prevalence compared to other regions.

This study has demonstrated that *I. scapularis* pathogens have spread into Oklahoma with the expansion of *I. scapularis* ticks. The presence of pathogens not previously reported in Oklahoma, like *B. miyamotoi* and Powassan, demonstrate that *I. scapularis* and its associated pathogens are spreading into new regions of the United States. It also highlights the need for additional pathogen surveys of *I. scapularis* ticks, the reservoirs hosts, and other tick-microbiome factors in Oklahoma. With the limited research on Oklahoma deer ticks, this study provides novel insight into the pathogens present in Oklahoma *Ixodes* ticks. This information can be used to better educate the public about the potential tick-borne diseases in Oklahoma.

Work Cited:

1.Dunlop, Jason A. et al. (2017). Segmentation and tagmosis in Chelicerata. Arthropod Structure & Development :*46(3), 395-418*.

2.Barker, S. C. et al. (2004). Systematics and evolution of ticks with a list of valid genus and species names. *Parasitology, 129(7), S15–S36.*

3.Poinar, George Jr et al. “A new genus of hard ticks in Cretaceous Burmese amber (Acari: Ixodida: Ixodidae).” *Systematic parasitology* vol. 54,3 (2003): 199-205. doi:10.1023/a:1022689325158

4.Apperson, C S et al. “Relative utilization of reptiles and rodents as hosts by immature Ixodes scapularis (Acari: Ixodidae) in the coastal plain of North Carolina, USA.” *Experimental & applied acarology* vol. 17,10 (1993): 719-31. doi:10.1007/BF00051830

5.Sara Fernandes Soares et al. *Study on cheliceral sensilla of the brown dog tick Rhipicephalus sanguineus (Latreille, 1806) (Acari: Ixodidae) involved in taste perception of phagostimulants*. Acta Tropica Volume 126, Issue 1, April 2013, Pages 75-83. https://doi.org/10.1016/j.actatropica.2013.01.006

6.Dania Richter et al. *How ticks get under your skin: insertion mechanics of the feeding apparatus of Ixodes ricinus ticks*. Proc. R. Soc. B 2013 .No volume or page number provided 28020131758 http://doi.org/10.1098/rspb.2013.1758

7.Mannelli, Alessandro, et al. *Influence of Season and Habitat on Ixodes Scapularis Infestation on White-Footed Mice in Northwestern Illinois*. The Journal of Parasitology, vol. 80, no. 6, 1994, pp. 1038–1042. JSTOR, www.jstor.org/stable/3283457.

8.*Lifecycle of Blacklegged Ticks*, Centers for Disease Control and Prevention, Division of Vector-Borne Diseases November 2011

9.Kocan, Katherine M et al. *"Insights into the development of Ixodes scapularis: a resource for research on a medically important tick species."* Parasites & vectors vol. 8 592. 14 Nov. 2015, doi:10.1186/s13071-015-1185-7

10.Marc J. Klowden *"Encyclopedia of Insects (Second Edition)"* 2009, Pages 940-941 Academic Press. https://doi.org/10.1016/B978-0-12-374144-8.00249-6

11.Rebecca J. Eisen et al. *County-Scale Distribution of Ixodes scapularis and Ixodes pacificus (Acari: Ixodidae) in the Continental United States*. Journal of Medical Entomology, 53(2), 2016, 349–386 doi: 10.1093/jme/tjv237

12.Burks, C.S et al. (1996), *The role of direct chilling injury and inoculative freezing in cold tolerance of Amblyomma americanum, Dermacentor variabilis and Ixodes scapularis*. Physiological Entomology, 21: 44-50. doi:10.1111/j.1365-3032.1996.tb00833.x

13.N. H. Ogden et al. *Investigation of Relationships Between Temperature and Developmental Rates of Tick Ixodes scapularis (Acari: Ixodidae) in the Laboratory and Field* , Journal of Medical Entomology, Volume 41, Issue 4, 1 July 2004, Pages 622–633, https://doi.org/10.1603/0022-2585-41.4.622

14.Rebecca J. Eisen at al. *Linkages of Weather and Climate With Ixodes scapularis and Ixodes pacificus (Acari: Ixodidae), Enzootic Transmission of Borrelia burgdorferi , and Lyme Disease in North America* , Journal of Medical Entomology, Volume 53, Issue 2, March 2016, Pages 250–261, https://doi.org/10.1093/jme/tjv199

15.Neelakanta, Girish et al. “*Anaplasma phagocytophilum* induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold.” *The Journal of clinical investigation* vol. 120,9 (2010): 3179-90. doi:10.1172/JCI42868

16.Sá-Nunes, Anderson et al. “Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in Ixodes scapularis saliva.” *Journal of immunology (Baltimore, Md. : 1950)* vol. 179,3 (2007): 1497-505. doi:10.4049/jimmunol.179.3.1497

17.Hsieh CS et al. "Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages". *Science*. **260** (5107): 1993. doi:10.1126/science.8097338. PMID 8097338

18.Narasimhan, S et al. “A novel family of anticoagulants from the saliva of Ixodes scapularis.” *Insect molecular biology* vol. 11,6 (2002): 641-50. doi:10.1046/j.1365-2583.2002.00375.x

19.Liu, Lei et al. “*Ixodes scapularis* salivary gland protein P11 facilitates migration of *Anaplasma phagocytophilum* from the tick gut to salivary glands.” *EMBO reports* vol. 12,11 1196-203. 28 Oct. 2011, doi:10.1038/embor.2011.177

20.Nelder, Mark P et al. “Human pathogens associated with the blacklegged tick *Ixodes scapularis*: a systematic review.” *Parasites & vectors* vol. 9 265. 5 May. 2016, doi:10.1186/s13071-016-1529-y

21.Limberger RJ. The periplasmic flagellum of spirochetes. J Mol Microbiol Biotechnol. 2004;7(1-2):30-40. doi: 10.1159/000077867. PMID: 15170401.

22.Gupta RS, Mahmood S, Adeolu M (2013). "A phylogenomic and molecular signature based approach for characterization of the phylum Spirochaetes and its major clades: proposal for a taxonomic revision of the phylum". Front Microbiol. 4 (217): 217

23.Ali Karami et al. Spirochaetaceae Phylum. The Prokaryotes – Actinobacteria, 2014 Incomplete citation?

24.Radolf JD et al. "Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes". Nature Reviews. Microbiology 2012. 10 (2): 87–99.

25.Hyde, Jenny A. "*Borrelia burgdorferi* Keeps Moving and Carries on: A Review of Borrelial Dissemination and Invasion." Frontiers in immunology vol. 8 114. 21 Feb. 2017, doi:10.3389/fimmu.2017.00114

26.Moriarty TJ et al. *"Real-time high resolution 3D imaging of the Lyme disease spirochete adhering to and escaping from the vasculature of a living host".* PLoS Pathog(2008) 4:e1000090. doi:10.1371/journal.ppat.1000090

27.Weis, Janet. *"Critical Needs and Gaps in Understanding Prevention, Amelioration, and Resolution of Lyme and Other Tick-Borne Diseases: The Short-Term and Long-Term Outcomes: Workshop Report"*. The National Academies: 97–101 2011.

28.Dennis DT et al. *Reported distribution of Ixodes scapularis and in Ixodes pacificus (Acari: Ixodidae) in the United States*. J Med Entomol. 1998; 35:629–638. [PubMed: 9775584]

29.LoGiudice K, et al. *The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk*. Proc Natl Acad Sci USA. 2003; 100:567–571. [PubMed: 12525705]

30.Hu LT. *Lyme Disease*. Ann Intern Med. 2016;164(9):ITC65-ITC80. doi:10.7326/AITC201605030

31.Carriveau A, Poole H, Thomas A. *Lyme Disease*. Nurs Clin North Am. 2019;54(2):261-275. doi:10.1016/j.cnur.2019.02.003

32.Scheffold, Norbert et al. "*Lyme carditis--diagnosis, treatment and prognosis.*" Deutsches Arzteblatt international vol. 112,12 (2015): 202-8. doi:10.3238/arztebl.2015.0202

33.Sheila L. Arvikar et al. Diagnosis and Treatment of Lyme Arthritis. Infectious Disease Clinics of North America, Volume 29, Issue 2, June 2015, Pages 269-280

34.Maaland, Marit Gaastra et al. "*Pharmacodynamics of doxycycline and tetracycline against Staphylococcus pseudintermedius: proposal of canine-specific breakpoints for doxycycline*." Journal of clinical microbiology vol. 51,11 (2013): 3547-54. doi:10.1128/JCM.01498-13

35.Butler, Thomas. "*The Jarisch-Herxheimer Reaction After Antibiotic Treatment of Spirochetal Infections: A Review of Recent Cases and Our Understanding of Pathogenesis.*" The American journal of tropical medicine and hygiene vol. 96,1 (2017): 46-52. doi:10.4269/ajtmh.16-0434

36.Bulut, Y et al. "*Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A*

lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling." Journal of immunology (Baltimore, Md.: 1950) vol. 167,2 (2001): 987-94. doi:10.4049/jimmunol.167.2.987

37.Bui T, Preuss CV (2020). Cephalosporins. StatPearls. Incomplete citation?

38.Wormser et al. "Borrelia miyamotoi: An Emerging Tick-Borne Pathogen" The American Journal of Medicine 2018 Incomplete citation – no volume or page numbers

39.Alan G. Barbour et al. "*Niche Partitioning of Borrelia burgdorferi and Borrelia miyamotoi in the Same Tick Vector and Mammalian Reservoir Species*". The American Journal of Tropical Medicine and Hygiene, Volume 81, Issue 6, 1 Dec 2009, p. 1120 – 1131

40.Breuner NE et al. "*Transmission of Borrelia miyamotoi sensu lato relapsing fever group spirochetes in relation to the duration of attachment by Ixodes scapularis nymphs*". Ticks Tick Borne Dis. 2017;8(5):677-681.

41.Mason, Lauren M K et al. "*Borrelia miyamotoi* Activates Human Dendritic Cells and Elicits T Cell Responses." *Journal of immunology (Baltimore, Md.: 1950)* vol. 204,2 (2020): 386-393. doi:10.4049/jimmunol.1801589

42.Koetsveld, Joris et al. "*In Vitro* Antimicrobial Susceptibility of Clinical Isolates of *Borrelia miyamotoi*." *Antimicrobial agents and chemotherapy* vol. 62,7 e00419-18. 26 Jun. 2018, doi:10.1128/AAC.00419-18

43.Bakken JS, Dumler JS. Human granulocytic anaplasmosis. Infect Dis Clin North Am. 2015;29(2):341-355. doi:10.1016/j.idc.2015.02.007

44.Ismail N, McBride JW. Tick-Borne Emerging Infections: Ehrlichiosis and Anaplasmosis. Clin Lab Med. 2017;37(2):317-340. doi:10.1016/j.cll.2017.01.006

45.Harald Wajant. The Fas Signaling Pathway: More Than a Paradigm. Science 31 May 2002: Vol. 296, Issue 5573, pp. 1635-1636 DOI: 10.1126/science.1071553

46.Mustafa Akkoyunlu et. Al. *Exploitation of Interleukin-8-Induced Neutrophil Chemotaxis by the Agent of Human Granulocytic Ehrlichiosis*. Infection and Immunity Sep 2001, 69 (9) 5577-5588; DOI: 10.1128/IAI.69.9.5577-5588.2001

47.Wang X, et al. *The Prostaglandin E2-EP3 Receptor Axis Regulates Anaplasma phagocytophilum-Mediated NLRC4 Inflammasome Activation* (2016) PLOS Pathogens 12(8): e1005803. https://doi.org/10.1371/journal.ppat.1005803

48.Jorgensen I, Miao EA (May 2015). "Pyroptotic cell death defends against intracellular pathogens". Immunological Reviews. 265 (1): 130–42. doi:10.1111/imr.12287

49.Wendy C Brown et al. “Immune Control of *Babesia bovis* infection”. Veterinary Parasitology Volume 138, Issues 1–2, 31 May 2006, Pages 75-87

50.Young, Kaitlin M et al. “Zoonotic Babesia: A scoping review of the global evidence.” PloS one vol. 14,12 e0226781. 30 Dec. 2019, doi:10.1371/journal.pone.0226781

51.J.F Dubremetz et al. “Invited review Apical organelles and host-cell invasion by Apicomplexa” International Journal for Parasitology Volume 28, Issue 7, 1 July 1998, Pages 1007-1013

52.Emmanuel Cornillot et al. *"Sequencing of the smallest Apicomplexan genome from the human pathogen Babesia microti"*, Nucleic Acids Research, Volume 40, Issue 18, 1 October 2012, Pages 9102–9114, https://doi.org/10.1093/nar/gks700

53.Ruben Magni et al. Analysis of the *Babesia microti* proteome in infected red blood cells by a combination of nanotechnology and mass spectrometry. International Journal for Parasitology Volume 49, Issue 2, February 2019, Pages 139-144

54.Centers for Disease Control and Prevention (CDC). Babesiosis surveillance - 18 States, 2011. MMWR Morb Mortal Wkly Rep. 2012 Jul 13;61(27):505-9. PMID: 22785341.

55.Evan M. Bloch et al. "Persistence of *Babesia microti* Infection in Humans" Pathogens 2019, 8(3), 102; https://doi.org/10.3390/pathogens8030102

56.Vannier, E.; Krause, P.J. Human Babesiosis. N. Engl. J. Med.2012,66, 2397–2407

57.Krause, P.J et al. *"Persistent and relapsing babesiosis in immunocompromised patients"*. Clin. Infect. Dis.2008,46, 370–37

58.Allred, D.R. Babesiosis: Persistence in the face of adversity. *Trends Parasitol*.2003,19, 51–55.

59.Edgar Sanchez et al. Diagnosis, Treatment, and Prevention of Lyme Disease, Human Granulocytic Anaplasmosis, and Babesiosis. JAMA. 2016;315(16):1767-1777. doi:10.1001/jama.2016.2884.

60.Wormser GP, Dattwyler RJ, Shapiro ED, et al. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis. 2006;43(9):1089-1134.

61.Krause PJ, Lepore T, Sikand VK, et al. Atovaquone and azithromycin for the treatment of babesiosis. N Engl J Med. 2000;343(20):1454-1458.

62.Wormser GP, Dattwyler RJ, Shapiro ED, et al. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis. 2006;43(9):1089-1134.

63.Kemenesi G, Bányai K. 2019. Tick-borne flaviviruses, with a focus on Powassan virus. Clin Microbiol Rev 32:e00106-17.

64.Hermance, Meghan E, and Saravanan Thangamani. “Powassan Virus: An Emerging Arbovirus of Public Health Concern in North America.” Vector borne and zoonotic diseases (Larchmont, N.Y.) vol. 17,7 (2017): 453-462. doi:10.1089/vbz.2017.2110

65.ArboNET, Arboviral Diseases Branch, Centers for Disease Control and Prevention. 2019. https://www.cdc.gov/powassan/statistics.html

66.Simmonds, Peter et al. “ICTV Virus Taxonomy Profile: Flaviviridae.” *The Journal of general virology* vol. 98,1 (2017): 2-3. doi:10.1099/jgv.0.000672

67.Holbrook MR. Historical Perspectives on Flavivirus Research. Viruses. 2017;9(5):97. Published 2017 Apr 30. doi:10.3390/v9050097

68.Pettersson JH, Fiz-Palacios O. “Dating the origin of the genus Flavivirus in the light of Beringian biogeography.” *J Gen Virol*. 2014 Sep; 95(Pt 9):1969-1982.

69.Růžek D, et al. Tick-borne encephalitis: pathogenesis and clinical implications. *Travel Med Infect Dis*. 2010 Jul; 8(4):223-32.

70.Slovak M., et al. Survival dynamics of tick-borne encephalitis virus in *Ixodes ricinus* ticks. Ticks Tick-Borne Dis. 2014;5:962–969. doi: 10.1016/j.ttbdis.2014.07.019.

71.Meghan E. Hermance, Saravanan Thangamani. “Powassan Virus: An Emerging Arbovirus of Public Health Concernin North America”. Vector-Borne and Zoonotic Diseases, Volume 17, Number 7, 2017. No page numbers

72.McLean DM, Donahue W. Powassan virus: Isolation of virus from a fatal case of encephalitis. Can Med Assoc J 1959; 80:708–711.

73.Doug E. Brackney et al. *Short Report: Stable Prevalence of Powassan Virus in Ixodes scapularis in a Northern Wisconsin Focus*. Am. J. Trop. Med. Hyg., 79(6), 2008, pp. 971–973.

74.Ko RC. Biology of *Ixodes cookei* Packard (Ixodidae) of groundhogs (Erxleben). Can J Zool 1972; 50:433–436.

75.Mlera L, Meade-White K, Saturday G, Scott D, et al. *Modeling Powassan virus infection in Peromyscus leucopus, a natural hos*t. PLoS Negl Trop Dis 2017; 11:e0005346.

76.Tavakoli N, Wang H, Dupuis M, Hull R, et al. *Fatal case of Deer tick virus encephalitis*. N Engl J Med 2009; 60:2099–2107.

77.Frolova MP, Isachkova LM, Shestopalova NM, Pogodina VV. *Experimental encephalitis in monkeys caused by the Powassan virus*. Neurosci Behav Physiol 1985; 15:62–69.

78.Piantadosi A, Rubin DB, McQuillen DP, Hsu L, et al. *Emerging Cases of Powassan Virus Encephalitis in New England: Clinical Presentation, Imaging, and Review of the Literature*. Clin Infect Dis 2016; 62:707–713.

79.VanBlargan, Laura A et al. "*An mRNA Vaccine Protects Mice against Multiple Tick-Transmitted Flavivirus Infections*." Cell reports vol. 25,12 (2018): 3382-3392.e3. doi:10.1016/j.celrep.2018.11.082

80.Taylor, Walter P. *The white-tailed deer of North America*. Ecology and conservation of wild herbivores in temperate countries. Part II. Western Hemisphere (1961) page 203-220.

81.Trisha R. Dubie et al. *Questing behavior and analysis of tick-borne bacteria in Ixodes scapularis (Acari: Ixodidae) in Oklahoma*. Journal of Medical Entomology, 55(6), 2018, 1569–1574 doi: 10.1093/jme/tjy133 6 August 2018.

82.Reiner, K L et al. "The descriptive epidemiology of Lyme disease in Oklahoma." *The Journal of the Oklahoma State Medical Association* vol. 84,10 (1991): 503-9.

83.Eisen, Rebecca J, and Lars Eisen. "The Blacklegged Tick, *Ixodes scapularis*: An Increasing Public Health Concern." *Trends in parasitology* vol. 34,4 (2018): 295-309. doi:10.1016/j.pt.2017.12.006

84.Jason R. Duell et al. *Prevalence and Species of Ticks on Horses in Central Oklahoma,* Journal of Medical Entomology, Volume 50, Issue 6, 1 November 2013, Pages 1330–1333, https://doi.org/10.1603/ME13117.

85.Mason V. Reichard et al. *Inoculation of White-Tailed Deer (Odocoileus virginianus) with Ap-V1 Or NY-18 Strains of Anaplasma phagocytophilum and Microscopic Demonstration of Ap-V1 In Ixodes scapularis Adults that Acquired Infection from Deer as Nymphs*. Vector-Borne and Zoonotic Diseases. Oct 2009.ahead of printhttp://doi.org/10.1089/vbz.2008.0106 This should have a journal and page number now if it was published in 2009

86.Antonia Dibernardo et al. *The prevalence of Borrelia miyamotoi infection, and co-infections with other Borrelia spp. In Ixodes scapularis ticks collected in Canada*. Parasites & Vectors2014,7:183http://www.parasitesandvectors.com/content/7/1/18. Journal and page numbers?

87.Shaun T. Cross et al. *Co-Infection Patterns in Individual Ixodes scapularis Ticks Reveal Associations between Viral, Eukaryotic and Bacterial Microorganisms*. Viruses 2018, 10, 388; doi:10.3390/v10070388

88.Hinten S et al. 2008. *Increased recognition of Powassan encephalitis in the United States, 1999–2005*. Vector Borne Zoonotic Dis 2008 8:6, 733-740. doi:10.1089/vbz.2008.0022.

89.Sarah E Mays et al. *Prevalence of five tick-borne bacterial genera in adult Ixodes scapularis removed from white-tailed deer in western Tennessee*. Mayset al. Parasites & Vectors

2014,7:473http://www.parasitesandvectors.com/content/7/1/473. Journal and page numbers?

90.James E. Keirans, Lance A Durden. *Illustrated Key to Nymphs of the Tick Genus Amblyoma (Acari: Ixodidae) Found in the United States*. J. Med. Entomol 35(4) 489-495 1998.

91.James E. Keirans, Taine R. Litwak. *Pictorial Key to the Adults of Hard Ticks, Family Ixodidae (Ixodia: Ixodoidea), East of the Mississippi River*. J. Med Entomol 26(5); 435-448 (1989).

92.ZYMO Research. Quick-DNA/RNA™PathogenMiniprep. https://www.zymoresearch.com/collections/quick-dna-rna-pathogen-kits/products/quick-dna-rna-pathogen-miniprep

93.Rafal Tokarz et al. *Detection of Anaplasma phagocytophilum, Babesia microti, Borrelia burgdorferi, Borrelia miyamotoi, and Powassan Virus in Ticks by a Multiplex Real-Time Reverse Transcription-PCR Assay*. March/April 2017 Volume 2 Issue 2 mSphere. Page numbers?

94.Gardner AM, Pawlikowski NC, Hamer SA, et al. Landscape features predict the current and forecast the future geographic spread of Lyme disease. Proc Biol Sci. 2020;287(1941):20202278.

95.Richter D, Spielman A, Komar N, et al. Competence of American Robins as Reservoir Hosts for Lyme Disease Spirochetes. Emerging Infectious Diseases. 2000;6(2):133-138. doi:10.3201/eid0602.000205.

96.Salkeld DJ, Leonhard S, Girard YA, et al. Identifying the reservoir hosts of the Lyme disease spirochete Borrelia burgdorferi in California: the role of the

western gray squirrel (Sciurus griseus). Am J Trop Med Hyg. 2008;79(4):535-540.

97.Han S, Lubelczyk C, Hickling GJ, Belperron AA, Bockenstedt LK, Tsao JI. Vertical transmission rates of *Borrelia miyamotoi* in *Ixodes scapularis* collected from white-tailed deer [published correction appears in Ticks Tick Borne Dis. 2019 May 22;:]. Ticks Tick Borne Dis. 2019;10(3):682-689.

98.Adetunji SA, Krecek RC, Castellanos G, et al. Seroprevalence of *Borrelia burgdorferi* antibodies in white-tailed deer from Texas. Int J Parasitol Parasites Wildl. 2016;5(2):168-174. Published 2016 Jun 13.

99.Rawlings JA, Teltow GJ. Prevalence of *Borrelia* (Spirochaetaceae) spirochetes in Texas ticks. J Med Entomol. 1994 Mar;31(2):297-301.

100.Feria-Arroyo TP, Castro-Arellano I, Gordillo-Perez G, et al. Implications of climate change on the distribution of the tick vector Ixodes scapularis and risk for Lyme disease in the Texas-Mexico transboundary region. Parasit Vectors. 2014;7:199. Published 2014 Apr 25.

101.Mitchell EA, Williamson PC, Billingsley PM, Seals JP, Ferguson EE, Allen MS. Frequency and Distribution of Rickettsiae, Borreliae, and Ehrlichiae Detected in Human-Parasitizing Ticks, Texas, USA. Emerg Infect Dis. 2016;22(2):312-315.

102.Oliver JH Jr, Kollars TM Jr, Chandler FW Jr, James AM, Masters EJ, Lane RS, Huey LO. First isolation and cultivation of *Borrelia burgdorferi* sensu lato from Missouri. J Clin Microbiol. 1998 Jan;36(1):1-5.

103.Kocan AA, Mukolwe SW, Murphy GL, Barker RW, Kocan KM. Isolation of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) from *Ixodes scapularis* and *Dermacentor albipictus* ticks (Acari: Ixodidae) in Oklahoma. J Med Entomol. 1992 Jul;29(4):630-3.

104.Parveen N, Bhanot P. *Babesia microti-Borrelia Burgdorferi* Coinfection. Pathogens. 2019;8(3):117. Published 2019 Jul 31.

105.Dunn JM, Krause PJ, Davis S, et al. *Borrelia burgdorferi* promotes the establishment of *Babesia microti* in the northeastern United States. PLoS One. 2014;9(12):e115494. Published 2014 Dec 29.

106.Skinner D, Mitcham JR, Starkey LA, Noden BH, Fairbanks WS, Little SE. Prevalence of *Babesia* Spp., *Ehrlichia* Spp., And Tick Infestations in Oklahoma Black Bears (*Ursus Americanus*). J Wildl Dis. 2017;53(4):781-787.

107.Campagnolo ER, Tewari D, Farone TS, Livengood JL, Mason KL. Evidence of Powassan/deer tick virus in adult black-legged ticks (*Ixodes scapularis*) recovered from hunter-harvested white-tailed deer (*Odocoileus virginianus*) in Pennsylvania: A public health perspective. Zoonoses Public Health. 2018;65(5):589-594.

108.Campbell O, Krause PJ. The emergence of human Powassan virus infection in North America. *Ticks Tick Borne Dis*. 2020;11(6):101540.

109.Dugan VG, Yabsley MJ, Tate CM, et al. Evaluation of white-tailed deer (*Odocoileus virginianus*) as natural sentinels for *Anaplasma phagocytophilum*. Vector Borne Zoonotic Dis. 2006;6(2):192-207.

110. Ginsberg HS, Hickling GJ, Burke RL, Ogden NH, Beati L, LeBrun RA, et al. (2021) Why Lyme disease is common in the northern US, but rare in the south: The roles of host choice, host-seeking behavior, and tick density. PLoS Biol 19(1):e3001066. doi:10.1371/journal.pbio.3001066

111. Maggi RG, Toliver M, Richardson T, Mather T, Breitschwerdt EB. Regional prevalences of *Borrelia burgdorferi*, *Borrelia bissettiae*, and *Bartonella henselae* in *Ixodes affinis*, *Ixodes pacificus* and *Ixodes scapularis* in the USA. Ticks Tick Borne Dis. 2019;10(2):360-364. doi:10.1016/j.ttbdis.2018.11.015

112. Milnes EL, Thornton G, Léveillé AN, et al. *Babesia odocoilei* and zoonotic pathogens identified from Ixodes scapularis ticks in southern Ontario, Canada. Ticks Tick Borne Dis. 2019;10(3):670-676. doi:10.1016/j.ttbdis.2019.02.016

113. Bonnet, Sarah I et al. "The Tick Microbiome: Why Non-pathogenic Microorganisms Matter in Tick Biology and Pathogen Transmission." Frontiers in cellular and infection microbiology vol. 7 236. 8 Jun. 2017, doi:10.3389/fcimb.2017.00236

Appendix

Deer Collection Data

Date	Deer #	Time	Sex	Weight	Approximate age	Zone Harvested
10/04/18	1	9:41 AM	Female	64 lbs	5.5	2
10/05/18	2	10:12 AM	Female	82 lbs	2.5	22
10/05/18	3	8:35 PM	Male	140 lbs	3.5	10
10/06/18	4	8:57 AM	Female	72 lbs	3.5	4
10/06/18	5	7:26 PM	Female	84 lbs	3.5	25
10/07/18	6	8:45 PM	Female	86 lbs	3.5	24
10/12/18	7	11:40 AM	Female	40 lbs	0.5	22
10/12/18	8	9:10 PM	Female	90 lbs	2	6
10/13/18	9	8:15 AM	Male	112 lbs	2.5	24
10/20/18	10	8:20 PM	Female	85 lbs		20
10/21/18	11	8:25	Female	108 lbs	5.5	17
10/21/18	12	9:40 AM	Male	95 lbs	2.5	24
10/21/18	13	9:50 AM	Male	59 lbs	1.5	20
10/22/18	14	9:00 AM	Male	71 lbs	1.5	14
10/26/18	15	1:00 PM	Male	126 lbs	2.5	2
10/27/18	16	11:12 AM	Male	86 lbs	2.5	21
10/28/18	18	1:30 PM	Male	168 lbs	4.5	24
11/1/18	19	8:20 PM	Male	160 lbs		23
11/2/18	23	8:00 PM	Male	164 lbs		10
11/3/18	24	10:00 AM	Female	80 lbs	3	21
11/3/18	25	8:30 PM	Female	102 lbs		17
11/9/18	28	11:00 AM	Female	94 lbs		10
11/9/18	29	5:40 PM	Male	38 lbs	0.5	11
11/10/18	30	10:50	Female	80 lbs	4.5	4
11/15/18	31		Male	82 lbs	1.5	9

Total Tick Collection Data

Tick Species	Amount	Precent (%)
Ixodes scapularis	394	86.98
Amblyomma americanum	33	7.28
Amblyomma maculatum	13	2.87
Rhipicephalus sanguineus	13	2.87

***Ixodes scapularis* Collection Data**

Life Stage	Amount	Percent (%)
Adult Female (Engorged)	96	24.3
Adult Female (non-engorged)	59	15.0
Adult Males	175	44.4
Nymphs	64	16.2